

BIOCHEMICAL GENETICS OF OROSOMUCOID

CENTRE FOR NEWFOUNDLAND STUDIES

**TOTAL OF 10 PAGES ONLY
MAY BE XEROXED**

(Without Author's Permission)

ANN MARIE F. BUTLER



BIOCHEMICAL GENETICS OF OROSOMUCOID

BY

©Ann Marie F. Butler, B.Sc.

A thesis submitted to the School of Graduate
Studies in partial fulfillment of the
requirements for the degree of
Master of Science

Faculty of Medicine
Memorial University of Newfoundland

August 1986

St. John's

Newfoundland

Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

L'autorisation a été accordée à la Bibliothèque nationale du Canada de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur (titulaire du droit d'auteur) se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation écrite.

ISBN 0-315-36973-6

ABSTRACT

This investigation demonstrates that electrophoretically cryptic genetic variation involving methionine residues, can be resolved using site-specific cleavage of the polypeptide chain with cyanogen bromide, followed by SDS-polyacrylamide electrophoresis of the fragments. Orosomucoid, also called α_1 -acid glycoprotein, was used as the model to test this approach because the proportion of methionine residues has been reported to be less than unity in this protein, evidence that can be interpreted as due to heterogeneity. A method to purify the model protein, orosomucoid, from as little as 0.5 ml of serum was developed, as a first step in this investigation. Genetic analysis of familial and population material confirmed a polymorphism, Val/Met, at residue 156.

ACKNOWLEDGMENTS

I extend my heartfelt thanks to my supervisor, Dr. Ronald Payne, who directed me through the paths of this endeavor with his patient teaching, continued support, and advice, so graciously given.

My thanks also to the members of my supervisory committee, Dr. James Orr and Dr. William Davidson, for reading my thesis.

I can not forget the expert technical assistance I received from Carol Ann Kirkland.

Finally, to David, thanks for understanding.

Table of Contents

INTRODUCTION	1
Objectives	1
History	1
Physical and chemical properties	3
Electrophoretic mobility	3
Isoelectric and isoionic points	3
Molecular weight	4
Solubility	6
Denaturation	7
Immunochemical properties	7
Metabolism	8
Peptide structure	8
Amino acid sequence	9
Amino acid substitutions	13
Homology	14
Disulfide bonds	14
Carbohydrate structure	16
Sialic acid deficient orosomucoid	22
Hormone effects on carbohydrates	22
Polymorphism and variants	23
Definition of terms	23
Polymorphism	23
Variants	24
A membrane form of orosomucoid	24
Biological role of orosomucoid	25
Summary of methods of isolation and purification	27
Source	27
Precipitation procedures	28
Precipitation with ammonium sulfate	28
Precipitation with ethanol at low temperatures	29
Ion-exchange chromatography	29
Pseudo-affinity chromatography	31
MATERIALS AND METHODS	32
I. DEVELOPMENT OF A METHOD	
Source	34
Ammonium sulfate precipitation	34
Materials	34
Method	34

Ethanol precipitation	35
Materials	35
Methods	36
Trichloroacetic acid precipitation	36
Materials	36
Method	36
Polyacrylamide gel electrophoresis	37
Materials	37
Method	38
Preparation of gels	38
Samples	39
Electrophoresis	39
Staining	39
Results of 7.5% polyacrylamide gel electrophoresis	39
Purification step	42
N,N'-diallyl-tartardiamide (DATD)-substituted polyacrylamide gels	42
Materials	43
Method	43
Solubilizing the gel	43
Results	44
Anion-exchange chromatography (DEAE-Sephadex A50)	44
Materials	44
Method	45
Preparation of resin	45
Samples	45
Chromatography	45
Results	46
Cation chromatography (Amberlite IRC-50)	51
Materials	51
Methods	52
Samples	52
Chromatography	52
Results	55
Seromuroid determination	55
Materials	58
Method	58
Comment on column chromatographic methods	59
II. DEVELOPMENT OF A PRACTICAL TWO-STEP ISOLATION METHOD	60
Reactive Blue 2-Sephadex CL-6B Chromatography	60
Materials	60
Method	61

Preparation of trichloroacetic acid soluble fraction	62
Materials	62
Method	62
Quality and Yield	65
Reduction and S-carboxymethylation	65
Materials	68
Method	68
Cyanogen bromide treatment of reduced-carboxymethylated orosomuroid	69
Materials	69
Method	69
Molecular weight of cyanogen bromide fragments	70
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	74
Materials	74
Methods	76
Preparation of 15% acrylamide gel	76
Samples	76
Electrophoresis	77
Staining	77
Results	78
Population and familial samples	78
RESULTS	81
Isolation of orosomuroid	81
Allele frequency	81
Mendelian inheritance	81
DISCUSSION	85
REFERENCES	91
APPENDIX	104

List of Tables

Table 1: Molecular Weight Estimations of Human Plasma Orosomucoid	5
Table 2: 156 ^{Met,Val} Frequency in 49 random samples.	82
Table 3: Inheritance of the 156 ^{Val,Met} alleles in 25 mating pairs.	83

List of Figures

Figure 1:	The amino acid sequence of orosomucoid.	10
Figure 2:	Schematic model of orosomucoid.	15
Figure 3:	Orosomucoid oligosaccharide structure.	18
Figure 4:	Electrophoretic patterns of serum precipitated by different techniques.	40
Figure 5:	Absorbance (280nm) of DEAE-Sephadex column eluate.	47
Figure 6:	Electrophoretic analysis of DEAE-sephadex A50 column eluate.	49
Figure 7:	Absorbance (280nm) of Amberlite IRC-50 column eluate.	53
Figure 8:	Electrophoretic analysis of Amberlite IRC-50 column eluate.	56
Figure 9:	Absorbance (280nm) of the Reactive Blue 2-Sepharose CL-6B column eluate and absorbance (540nm) of the "seromucoid" component.	63
Figure 10:	Electrophoretic analysis of orosomucoid preparation.	66
Figure 11:	Molecular weight estimation of CNBr fragments.	72
Figure 12:	Electrophoretic patterns of orosomucoid CNBr fragments in SDS-polyacrylamide gels.	79

INTRODUCTION

Objectives

The objectives of this project were to develop a method to isolate orosomucoid cleanly from as little as 500 λ of serum, to cleave the isolated protein with cyanogen bromide, to study the inheritance of the alleles conditioning the Val/Met variation at position 156, and to determine the frequencies of these alleles in the general population. All these objectives have been met, and are discussed in this thesis.

History

A soluble carbohydrate-rich material in serum deproteinated by heat was first reported in 1892 by Freund (cited in Rimington, 1940). The presence of nitrogen in this preparation was overlooked and Freund called the preparation "Tiergummi". This same serum fraction was later investigated by Zanetti (cited in Rimington, 1940) and because it resembled ovomucoid (both have a high carbohydrate content) the term "seromucoid" was given to this fraction.

A protein was isolated from this heterogeneous serum fraction by ammonium sulfate precipitation (Winzler et al., 1948; Weimer et al., 1950) and was called orosomucoid, from the Greek *oros*, serum.

Electrophoresis at pH 4-4.5 reveals orosomucoid as the major component in the seromucoid fraction. Under these conditions orosomucoid

migrates towards the positive electrode (Winzler et al., 1948).

About the same time as ammonium sulfate precipitation was used to isolate orosomucoid Schmid (1950) independently isolated a glycoprotein, by fractionating Cohn's fraction VI with ethanol precipitation, and named it α_1 -acid glycoprotein because of its low isoelectric point.

Orosomucoid and α_1 -acid glycoprotein are the same protein (Popenoe, 1955). In addition to α_1 -acid glycoprotein, orosomucoid has also been called the MP-1 component (Mehl et al., 1949) and α_1 -acid serumucoid (Schmid, 1953). The name orosomucoid will be employed exclusively in this report.

Orosomucoid is a glycoprotein: proteins in this class contain by definition greater than 1% carbohydrate and vigorous hydrolysis is required to cleave the carbohydrate group from the protein (Winzler, 1960). Glycoproteins containing 30-50% carbohydrate (orosomucoid contains approximately 45%) are grouped together under the term "mucoid" (Winzler, 1960). These "mucoid" proteins characteristically demonstrate high solubility and stability due to the presence of the carbohydrate, which bears multiple anionic residues (N-acetylneuraminic or sialic acids). These carbohydrate chains may be linked to the peptide N-acetylglycosidically, from N-acetylglycosamine to asparagine, or alternatively linked O-acetylglycosidically to the hydroxyl groups of serine or threonine. Mild borohydride treatment of the glycopeptide in alkali conditions cleaves the O-glycosidically linked carbohydrates, but more drastic treatment is required to split the N-glycosidically linked

carbohydrate from the protein (Winzler, 1960).

Orosomuroid is classified as one of the "acute phase proteins" because its serum concentration is greatly increased in various pathological conditions, in acute inflammation, in trauma, and even in pregnancy.

Physical and chemical properties

Electrophoretic mobility

Electrophoresis at pH 8.6 in a non-restricting matrix (paper or agarose) is the standard condition used to define plasma protein mobilities. The electrophoretic mobility of orosomuroid at pH 8.6 is strongly anodal, thus classifying orosomuroid as an α_1 globulin (Winzler, 1960). The electrophoretic mobility changes to that of a β_1 globulin when orosomuroid is desialized (Krotoski and Weimer, 1966; Schmid et al., 1967). Weimer and Rice (1957) first demonstrated this phenomenon of decreased electrophoretic mobility after desialization by electrophoresis of orosomuroid previously exposed to 0.6N perchloric acid at room temperature.

Isoelectric and isoelectric points

The reported isoelectric point of orosomuroid varies. A value as low as pH 1.8 was reported by Weimer et al. (1950) but Schmid (1950), using a different buffer system, determined the isoelectric point of this protein to be pH 2.7. Because the type and concentration of anions present in the buffer system will influence the observed isoelectric point (Schmid, 1975) it would have been more appropriate to discuss the isoelectric point, a constant,

4

for this protein. The isoelectric point is the pH measured in water and in the absence of other solutes, at which the net charge of the protein is zero. The isoelectric point of native orosomucoid is pH 3.53 (Schmid et al., 1962).

Popenoe and Drew (1957) attribute low isoelectric points to the sialic acids present in the protein and removal of the sialic acids increased the isoelectric point to pH 4.5 in sodium acetate buffer. Schmid et al. (1967) determined the isoelectric point of desialized orosomucoid to be pH 5.4.

Molecular weight

One of the first estimations of the molecular weight of orosomucoid was made by Smith et al. (1950) as 44,100 daltons. More recent molecular weight estimations (Table 1) give a value closer to 40,000 daltons. The higher molecular weight determination may be due to partial denaturation of the protein (Jeanloz, 1972).

The molecular weight of the polypeptide chain was calculated by Schmid et al. (1973), from the amino acid composition of this protein to be 21,270 daltons. Schmid (1975) assumed that the carbohydrate and polypeptide units account for approximately 45% and 55% of the molecular weight, respectively, and estimated the molecular weight of orosomucoid from the molecular weight of the peptide to be 39,500 daltons. This value agrees with the lower estimations of molecular weight reported in Table 1. The assumed molecular weight of orosomucoid for this report is 40,000 daltons.

Table 1: Molecular Weight Estimations of Human Plasma
Orosomucoid

Method of estimation	Molecular weight (daltons)	Reference
Sedimentation-diffusion	44,100	Smith et al. (1950)
Sedimentation-viscosity	43,000	Bezkorovainy (1965)
Disc electrophoresis	38,800	Kalous (1965)
Sedimentation-diffusion	33,000	Kawasaki et al. (1966)
Sedimentation-diffusion	40,000	Schultze and Heremans (1955)
Sedimentation equilibrium	40,000	Li and Li (1970)
Sedimentation equilibrium	39,000	Kawahara et al. (1973)
Amino acid sequence	39,500	Schmid et al. (1973)
Sedimentation diffusion	44,680	Oss and Bronson (1974)
Sedimentation diffusion	40,800	Charlwood et al. (1976)

Solubility

Orosomuroid is the most soluble of the plasma proteins. This high solubility has been attributed, in part, to the net negative charge at physiological pH and the presence of hydrophilic residues (Schmid, 1975). Although release of sialic acid occurs at elevated temperatures, and at pH lower than 4.0, this protein remains soluble in boiling water. Orosomuroid is a true globular protein. At low ionic strengths the solubility increases with increasing concentrations of sodium chloride and solubility is a minimum at the isoelectric point.

Orosomuroid is also soluble in 0.6M perchloric acid, 0.2M sulfosalicylic acid, 0.3M trichloroacetic acid and ammonium sulfate concentrations below 70% saturation (Schmid, 1975). Rimington (1940) erroneously reported that 0.3M trichloroacetic acid would precipitate orosomuroid.

The solubility of orosomuroid in water-ethanol mixtures was investigated by Schmid (1953). The addition of heavy metals was required to precipitate the protein in low concentrations of ethanol. Orosomuroid in 75% ethanol at pH 3.5 will precipitate (Schmid, 1953). In addition to insolubility in saturated ammonium sulfate or zinc sulfate, orosomuroid is precipitated by saturated solutions of sodium phosphate at pH 3.0 to pH 7.0 (Schmid, 1953) and by acidified phosphotungstic acid (Weimer et al., 1950). Phosphotungstic acid is the most effective precipitating agent for orosomuroid.

Denaturation.

Orosomucoid can be denatured by boiling in distilled water, or by 10M LiBr, 10M urea or 5M guanidine HCl. A viscous mixture results when orosomucoid in the presence of 30% ethanol and 30% ether is left at room temperature, but in all cases the denatured orosomucoid remains soluble (Schmid, 1975).

Immunochemical properties

Although orosomucoid is a weak antigen in most systems, antibodies against orosomucoid have been produced. Using whole serum, human orosomucoid was shown to be slightly antigenic in rabbits (Jager, 1953). The antigenicity of human orosomucoid in rabbits can be increased when orosomucoid is treated with neuraminidase (Athineos et al., 1962). Why the antigenicity increases with removal of sialic acid from the native protein is not known, but sialic acid is not important to the interaction of orosomucoid and a specific antibody (Athineos et al., 1962).

Antibodies to human orosomucoid have also been prepared in chickens (Stilberg et al., 1955). These antibodies, which have been used to determine orosomucoid quantitatively in both normal and pathological blood, demonstrate that approximately 10% of the total serum glycoprotein is orosomucoid (Stilberg et al., 1955); Barker and Whitehead (1963) have shown that the antigenic site of orosomucoid is in the polypeptide chain and not the carbohydrate portion of the protein. Exposure of orosomucoid to solutions of dilute acids at room temperature or elevated temperatures has little effect on in vitro antigenicity (Krotoski

and Weimer, 1966).

Metabolism

Saricone (1963) demonstrated that the mammalian liver synthesizes both the carbohydrate and polypeptide units of this protein. While the peptide chain is still on the ribosome the transfer of an N-acetylglucosaminyl residue to an asparaginyl residue of the nascent polypeptide chain initiates the carbohydrate synthesis. Additional monosaccharides are added as the protein passes through the smooth and rough endoplasmic reticulum and the final transfer of sialyl residues occurs in the Golgi complex (Jamieson and Ashton, 1973). Removal of terminal sialic acids clears orosomucoid from the circulation by facilitating binding to a receptor protein on liver cell plasma membranes (Morell et al., 1971). ¹³¹I labelling of orosomucoid demonstrated that the half-life of the native protein is 5.5 days (Winzler, 1965) while desialized orosomucoid has a half-life of only about two minutes (Morell et al., 1971).

Peptide structure

Orosomucoid is a single polypeptide chain consisting of 181 residues (Schmid et al., 1973). Support for the presence of a single polypeptide chain came from gel electrophoresis and immunochemistry (Krotoski and Weimer, 1966) as well as the presence of one mole of amino-terminal and one mole of carboxyl-terminal amino acids per one mole of protein. The amino-terminal is not free and could not be detected by dinitrophenylation (Schmid, 1954). Pyrrolidonecarboxylic acid, one mole per mole of protein

was later identified as the amino terminal amino acid (Ikenaka et al., 1966). This finding was confirmed by Doolittle (1972). There is one mole of carboxyl terminal serine per mole of protein (Schmid et al., 1959).

Amino acid sequence

The complete amino acid sequence of orosomuroid has been determined by Schmid and his collaborators (Ikenaka et al., 1972; Schmid et al., 1973) and is given in Figure 1. The amino acid sequence of residues 1 through 111 was reported by Ikenaka et al. (1972) and the amino acid sequence of the remaining residues, 112 through 181, was reported by (Schmid et al., 1973). Native orosomuroid was treated with cyanogen bromide (CNBr) desialized, and the fragments generated were subjected to tryptic cleavage, or alternatively chymotryptic digestion. Prior to the report of the complete amino acid composition of this protein, partial amino acid sequences had been reported with some discrepancies (Charlwood et al., 1976; Goa, 1961; Marshall and Porath, 1968; Kitamura and Yamashina, 1972).

The amino acid composition reported by Schmid et al. (1973) agrees closely with the partial sequence determined by Kitamura and Yamashina (1972).

The differences between investigators in the determination of amino acid composition of orosomuroid reported have been attributed to multiple amino acid substitutions in this protein. The different molecular weight values assumed for this protein by different investigators, (40,000 - 44,000 daltons), the different techniques used to analyze the amino acid

Figure 1: The amino acid sequence of orosomucoid.

The amino-terminal amino acid is a pyrrolidonecarboxyl (PAC) residue. Asterisks mark sites of cyanogen bromide cleavage of the methionyl residue bonds (111 and 156). Circles mark the attachment sites of the carbohydrate groups to the Asn residues (15, 38, 54, 75, and 85).

Adapted from Schmid et al. (1973).

PCA-Ile-Pro-Leu-Cys-Ala-Asn-Leu-Val-Pro-Val-Pro-Ile-Thr-Asn-
15

Ala-Thr-Leu-Asp-Arg-Ile-Thr-Gly-Lys-Trp-Phe-Tyr-Ile-Ala-Ser-
Gln 30

Ala-Phe-Arg-Asn-Glu-Glu-Tyr-Asn-Lys-Ser-Val-Glu-Glu-Ile-Gln-
Ala 45

Ala-Thr-Phe-Phe-Tyr-Phe-Thr-Pro-Asn-Lys-Thr-Glu-Asp-Thr-Ile-
Ala 60

Phe-Leu-Arg-Glu-Tyr-Gln-Thr-Arg-Gln-Asp-Gln-Cys-Ile-Tyr-Asn-
Phe 75

Thr Thr-Tyr-Leu-Asn-Val-Gln-Arg-Glu-Asn-Gly-Thr-Ile-Ser-Arg-
Ser Ser Val 90

Thr Val-Gly-Gly-Gln-Glu-His-Val-Ala-His-Leu-Leu-Ile-Leu-Arg-
Glu Arg Phe 105

Asp-Thr-Lys-Thr-Leu*Phe Gly Ser Tyr Leu Asp-Asp-Glu-Lys-
Tyr Leu Ala Phe Asp Val Asn 120

Asn-Trp-Gly-Leu-Ser-Phe-Tyr-Ala-Asp-Lys-Pro-Glu-Thr-Thr-Lys-
Val 135

Glu-Gln-Leu-Gly-Glu-Phe-Tyr-Glu-Ala-Leu-Asp-Cys-Leu-Cys-Ile-
Arg 150

Pro-Arg-Ser-Asp-Val-Met*-Tyr-Thr-Asp-Trp-Lys-Lys-Asp-Cys-Glu-
Lys Val 165

Pro-Leu-Glu-Lys-Gln-His-Glu-Lys-Arg-Lys-Gln-Glu-Glu-Gly-Glu-
180

Ser-COOH

composition and the different degrees of homogeneity of the protein preparations may also attribute to the discrepancies (Schmid, 1975).

CNBr selectively cleaves the methionyl peptide bond. Analysis of a pooled source of reduced carboxymethylated orosomuroid treated with CNBr revealed three new amino-terminal amino acids, and four fragments, CNBr-I, CNBr-II, CNBr-III and CNBr-IV. The new amino-terminal amino acids, leucine, phenylalanine and tyrosine were obtained in unequal molar yields (Schmid et al., 1973). Previous reports disagreed on the methionine content of orosomuroid and one or two methionine residues had been detected in the protein. Schmid et al. (1968), reported a value of 1.3 moles of methionine per mole of orosomuroid in a pooled source.

The discovery of the three new amino-terminal amino acids present in unequal molar amounts was explained when the amino acid sequence of the CNBr fragments was examined. The formation of CNBr-III and CNBr-IV which are included in CNBr-II is the result of an amino acid substitution of methionine for valine at position 156. Approximately one-quarter of the peptide chains of pooled orosomuroid have this additional methionine (Emura et al., 1971).

The CNBr-I fragment is the amino-terminal sequence and extends from residues 1 to 111. CNBr-II, CNBr-III and CNBr-IV are the carboxyl terminal fragments. CNBr-II extends from residues 112 to 181 (Ikenaka et al., 1972). CNBr-III and CNBr-IV extend from residues 112 to 156 and residues 157 to 181, respectively (Emura et al., 1971).

Amino acid substitutions

Twenty-one of the 181 residues of a pooled source of orosomucoid have amino acid substitutions (Figure 1). Ten amino acid substitutions were detected in the carboxyl-terminal CNBr fragment (Emura et al., 1971) and 11 amino acid substitutions were reported in the amino-terminal CNBr fragment (Schmid et al., 1973). CNBr-III contains all 10 amino acid substitutions contained in the carboxyl-terminal fragment and is referred to as the variable region. CNBr-IV, containing no amino acid substitutions, is referred to as the constant region of the carboxyl-terminal fragment (Emura et al., 1971).

At no position were more than two amino acids detected, even though pooled material was used for these studies. Single point mutations can explain all but two of these replacements, at positions 32 and 110 (Schmid, 1975). Glycine, histidine, proline and tryptophan are not substituted, consistent with the low mutability rates for these amino acids yet arginine, leucine, phenylalanine and tyrosine, all associated with even lower mutability rates, are sites of substitutions (Schmid, 1975).

The polypeptide chain has an asymmetric distribution of hydrophilic and hydrophobic residues. The amino-terminal half is relatively hydrophobic but the five carbohydrate groups attached in this region makes this section hydrophilic (Schmid et al., 1977).

Homology

Orosomuroid was the first reported single-chain protein with sequence similarities to other plasma proteins. The multiple amino acid substitutions of orosomuroid are also common in the immunoglobulins. A segment of orosomuroid (residues 112 to 132) has been found to be highly homologous with a section of the α chain of haptoglobin (Emura et al., 1971). Residues 1 to 34 possess an homology with certain L and H chains of IgG (Schmid et al., 1973). Another segment (residues 77 to 125) has a high degree of homology with an IgG H chain (Schmid et al., 1973). It has been suggested that orosomuroid probably evolved in whole or in part from a common ancestral immunoglobulin (Emura et al., 1971; Ikenaka et al., 1972; Schmid et al., 1973).

More recently, sequence similarity between the C-terminal half of human epidermal growth factor binding domain and orosomuroid has been reported (Toh et al., 1985).

Disulfide bonds

Schmid and his collaborators (1974) have determined the position of the disulfide bonds present in orosomuroid. The half-cystine residue 5 is linked to the half-cystine residue 147 and the half-cystine residue 72 is linked to the half-cystine residue 164. The two disulfide bonds of orosomuroid are partially buried (Schmid et al., 1976). A schematic diagram of orosomuroid is given in Figure 2.

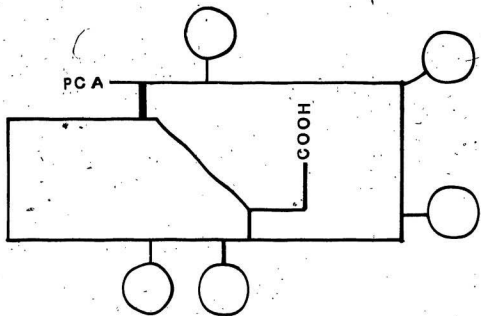


Figure 2: Schematic model of orosomucoid.

The continuous line represents the polypeptide moiety of orosomucoid with the two disulfide bonds (between residues 5 and 147 and between residues 72 and 164). Circles represent the five carbohydrate groups attached to asparagine residues 15, 38, 54, 75, and 85. Redrawn from Schmid et al. (1974).

Carbohydrate structure

Orosomuroid has the highest carbohydrate content of the human serum proteins. There are five glycosylation sites in orosomuroid and the five carbohydrate units are all covalently linked through N-acetyl glucosamine to asparagine residues in the hydrophobic half of the polypeptide at residues 15, 38, 54, 75, and 85 (Schmid et al., 1977). These carbohydrate groups account for approximately 45%, by weight, of the protein and approximately 12% is sialic acid. The sialyl residues are linked to the carbohydrate by a 2-ketosidic bond and are located terminally. (Popenoe and Drew, 1957; Popenoe, 1959).

Preliminary data on the structure and composition of the carbohydrate core and the degree of branching of the carbohydrate units were contradictory (Schmid, 1975; Jeanloz, 1972).

The early reports demonstrated the heterogeneity of the carbohydrate units observed following proteolytic digestion of the polypeptide (Izumi et al., 1961). Desialized orosomuroid had to be used since native orosomuroid is very resistant to digestion by proteolytic enzymes (Yamashina, 1966). The complex glycopeptide mixture resulting from proteolytic digestion prevented assignment of the glycopeptides to specific glycosylation sites.

It was not until Schmid and his collaborators (1977) identified five glycopeptides from five specific regions of the polypeptide chain that the structure of a single carbohydrate unit from a protein with multiple glycosylation sites was investigated.

The complete structures of 16 homogeneous glycopeptides isolated from

four of the five carbohydrate groups of this protein were determined using 360-MHZ ^1H NMR spectroscopy and permethylation analysis (Fournet et al., 1978). This study revealed that carbohydrate groups with different structures were present at each glycosylation site of pooled orosomucoid. The structures of these heteroglycans were grouped into five classes; three of which are new structures. These classes can be seen in Figure 3. Classes A and B are not new and possess known biantennary and triantennary chains, respectively. The biantennary structure, common to other glycoproteins, was found in only one of the 16 glycopeptides. The triantennary structure found is common to only a few glycoproteins.

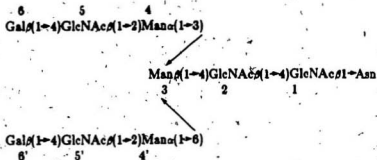
Class C is tetraantennary in its structure. Classes BF (triantennary + Fucose) and CF (tetraantennary + Fucose) compare with B and C except they contain a fucose. The occurrence and position of this fucose is unique and these structures have not been previously described in glycoproteins (Fournet et al., 1978).

The heterogeneity observed in the carbohydrate component of pooled orosomucoid by earlier workers can now be explained by different carbohydrate structures occurring at each glycosylation site. Carbohydrate groups linked to the second glycosylation site possess structures of classes A, B, C and BF. Carbohydrate groups from glycosylation sites three, four and five possess structures of classes B, C and CF. Fournet et al. (1978) speculates that the heterogeneity may be due to different carbohydrate groups at the same glycosylation site of individual protein molecules and may reflect differences in the biosynthesis and/or degradation of the

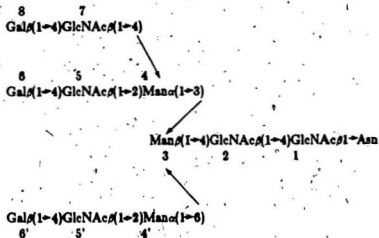
Figure 3: Orosomucoid oligosaccharide structure.

The structures of the five classes of orosomucoid oligosaccharides are given in the following three pages. Classes A and B are common to other glycoproteins whereas classes C, BF, and CF have not been reported in other glycoproteins. Adapted from Fournet et al. (1978).

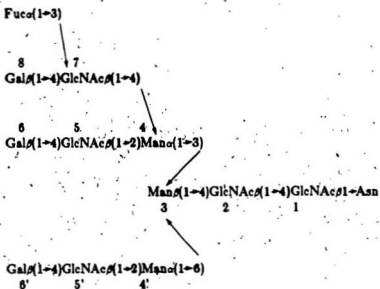
Class A structure:



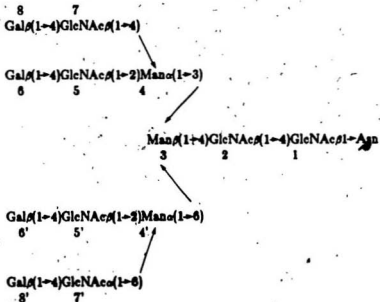
Class B structure:



Class BF structure:



Class C structure:



Class CF structure:

Fuco(1-3)

8 7
Gal(1-4)GlcNAc(1-4)

6 5 4
Gal(1-4)GlcNAc(1-2)Mano(1-3)

3 2 1
Man(1-4)GlcNAc(1-4)GlcNAc(1-Asn)

6' 5' 4'
Gal(1-4)GlcNAc(1-2)Mano(1-6)

8' 7'
Gal(1-4)GlcNAc(1-6)

carbohydrate unit.

Sialic acid deficient orosomucoid

Schmid et al. (1964b) and Caputo and Marcante (1964) have reported sialic acid-deficient orosomucoid is produced in certain pathological states. The sialic acid content of orosomucoid in certain chronic diseases is significantly lower. Individuals subjected to temporary stress produce the expected increased concentration of orosomucoid with sialic acid, but individuals who produce increased amounts of orosomucoid for a long time will eventually produce orosomucoid deficient in sialic acid (Schmid et al., 1964b).

Hormone effects on carbohydrates

Orosomucoid forms dissociable complexes with progesterone (Ganguly et al., 1967) and other steroid hormones (Kerkay and Westphal, 1968). The properties of the progesterone-orosomucoid complex are similar to those of the progesterone and corticosteroid complexes with the corticosteroid-binding globulin (Ganguly et al., 1967). This serum globulin, like orosomucoid, is a glycoprotein with a high carbohydrate content.

The influence of changes in serum concentration of female hormones on the heterogeneity of the carbohydrate units of orosomucoid was studied (Wells et al., 1981). The carbohydrate composition and possibly its metabolism or function may be affected by female hormones. During pregnancy and after oestrogen therapy a change in the pattern of carbohydrate part of orosomucoid was observed on crossed immuno-

affinoelectrophoresis. However, the post-partum pattern is slow to return to normal, indicating a non-direct hormonal effect (Wells et al., 1981).

Polymorphism and variants

Definition of terms

Orosomucoid is heterogeneous in both its carbohydrate and polypeptide components. *Polymorphism* is used exclusively to describe the electrophoretically detectable variations in the carbohydrate portion of this protein. These polymorphic forms result from the different linkages between sialic acid and the glycopeptides (Schmid et al., 1964a). *Variants* of orosomucoid describe the electrophoretically detectable variations arising from amino acid substitutions in the polypeptide chain.

Polymorphism

Starch gel electrophoresis at pH 2.8 of pooled native orosomucoid reveals seven bands for which the term polymorphism was applied (Schmid and Binette, 1961). There was evidence that the polymorphic forms were genetically determined when four different polymorphic patterns of either five, six, seven or eight bands were observed from the sera of normal individuals in family and twin studies. Desialized orosomucoid is always homogeneous under these electrophoretic conditions. Schmid et al. (1964a) proposed that these polymorphic forms were the result of differences in the sialic acids linked to C-2, C-3, C-4 or C-6 of the galactose residues. Most of the chemical and physical properties of the polymorphic forms of orosomucoid were similar (Schmid et al., 1962). The gross carbohydrate

and amino acid composition appeared the same but the isoelectric points were different.

Varlants

Starch gel electrophoresis at pH 5 of pooled orosomucoid from which the sialic acid was enzymatically hydrolyzed revealed two main bands (Tokita and Schmid, 1963). In individuals, one or two bands can be observed, depending on the individual from whom the sample was taken (Schmid et al., 1962). A single fast migrating (F) or slow migrating (S) major band could be seen as well as the combination of both (FS). The individual patterns do not change in response to stress or irradiation (Tokita et al., 1966; Yoshizaka et al., 1969), conditions known to increase the serum concentration of orosomucoid.

Family studies showed the mode of inheritance to be autosomal with codominant expression (Johnson et al., 1969). The differences in electrophoretic mobilities are due to differences in the polypeptide chain and further investigation revealed an amino acid substitution of an arginine for a glutamine at residue 20 (Nimberg et al., 1971).

A membrane form of orosomucoid

Gahmberg and Andersson (1978) investigated the possibility that leukocytes could be involved in the synthesis and release of orosomucoid and reported a membrane form of orosomucoid synthesized by lymphocytes. The involvement of orosomucoid in intracellular communication and recognition by leukocytes was supported by the

findings of Chiu et al. (1977) that the addition of orosomucoid to lymphocyte cultures alters the mixed lymphocyte cultures response.

The membrane form of orosomucoid is located on normal human lymphocytes, granulocytes and monocytes. It has a molecular weight of 52,000 and contains an additional fragment, probably hydrophobic. The increased serum concentration of orosomucoid in various conditions involving cell proliferation may be explained. It had been difficult to explain the elevated concentrations by stimulation of liver synthesis alone (Schmid, 1975). The sequence homology of orosomucoid and the immunoglobulins (Emura et al., 1971; Ikenaka et al., 1972; Schmid et al., 1973) may be understood since they both have their origin in similar cell types (Gahmberg and Andersson, 1978).

Biological role of orosomucoid

Many investigators have demonstrated that the serum concentration of orosomucoid, as well as other glycoproteins, is elevated in a number of physiological and pathological states, including cancer, pneumonia, and rheumatoid arthritis (Winzler, 1955). The increased serum level is also associated with induced inflammation (Jamieson et al., 1972), pregnancy (Adams and Wachter, 1968), and major surgery (Tokita et al., 1966). This increase in serum concentration of acute phase proteins is not clearly understood.

While a direct role for orosomucoid has not been found, this protein demonstrates biological activity in a number of conditions. It has been shown to prolong blood clotting time, possibly by inhibiting the conversion

of prothrombin to thrombin (Nilsson and Yamashina, 1958). Orosomucoid binds various steroids, particularly progesterone (Ganguly et al., 1967). Staprans et al. (1980) showed that orosomucoid is involved in the sequestration of a lipoprotein lipase cofactor. It has been demonstrated that this protein is an inhibitor of ADP- and epinephrine-induced platelet aggregation (Snyder and Coodley, 1976) and orosomucoid becomes a more potent inhibitor as the serum concentration increases (Snyder and Coodley, 1976). Orosomucoid acts as a depressant of phagocytosis (Oss and Bronson, 1974). The known increased serum levels of this protein in trauma patients plus the phagocytosis inhibiting properties may be a factor contributing to the decreased resistance to bacterial infections in trauma patients (Oss and Bronson, 1974). The spacing arrangement of the collagen fibers reformed from solubilized collagen upon dialysis is influenced by the presence of orosomucoid but other substances also have this effect (Gross et al., 1956).

More recently it has been suggested that orosomucoid may regulate the immune response (Bennett and Schmid, 1980) and a number of findings support this concept. Sequence homology of orosomucoid with the immunoglobulins (Emura et al., 1971; Ikenaka et al., 1972; Schmid et al., 1973) has been demonstrated and orosomucoid has been detected on the surface of lymphocytes (Gahmberg and Andersson, 1978).

The proliferation of lymphocytes stimulated with the mitogen phytohemagglutinin or with allogeneic cells can be inhibited with orosomucoid (Oss et al., 1974) and orosomucoid interferes with

phagocytosis of bacteria by macrophages (Chiu et al., 1977).

Bennett and Schmid (1980) have demonstrated the importance of the carbohydrate unit in the regulatory role of orosomucoid. It is the carbohydrate unit that is involved in specific protein to protein and protein to cell interactions. Conformational changes in the protein would not explain these interactions: the carbohydrate unit is the significant modulating factor.

Summary of methods of isolation and purification

Orosomucoid can be isolated from other plasma proteins by solubility, electrophoretic or ion-exchange chromatography procedures. These methods rely on the following two principles for their success: at values above its isoelectric point, pH 3.53 (Schmid et al., 1962) orosomucoid exhibits a high negative electrostatic charge; and that this protein is highly soluble, the most soluble of the plasma proteins,

Undenatured protein is preferred for non-chemical studies. The original method of isolation, to deproteinate serum with heat (Rimington, 1940) was discarded when it was realized that degradation of orosomucoid could occur. Low pH is also avoided because of the lability of the sialic acid ketosidic linkage.

Source

Orosomucoid is isolated from human blood. The development of methods of plasma fractionation, particularly the low temperature-low salt-ethanol procedure of Cohn et al. (1950) permitted isolation and

identification of proteins in the plasma fractions. Orosomucoid is one of the proteins in the supernatant of Cohn Fraction V (Ikenaka et al., 1965; Iwasaki and Schmid, 1967). Because of the availability of the supernatant of Cohn Fraction V, this is often the source. Urine of nephrotic patients has been investigated as another source (Popenoe, 1955); it has been shown that orosomucoid is one of the plasma proteins most easily passed into the urine and 0.2 to 0.4 g/l of orosomucoid can be recovered in nephrotic urine. The relatively low molecular weight of this protein may relate to this property.

Precipitation procedures

The early methods of isolating orosomucoid from human plasma involved precipitating the bulk of serum proteins with acidic reagents which left orosomucoid in solution. The strongly acidic reagents that have been used, perchloric acid (Winzler et al., 1948) or trichloroacetic acid (Popenoe, 1955), are now known to cleave the o-glycosidic linkage of sialic acid (Schmid, 1975). Precipitation with acidic reagents is still used in combination with other techniques when the orosomucoid recovered is used for chemical studies. The major disadvantage is that the acidic reagent has to be removed by dialysis.

Precipitation with ammonium sulfate

Essentially all of the plasma proteins are removed in step-wise precipitations with ammonium sulfate. Human plasma is diluted with sodium acetate and precipitated with ammonium sulfate at pH 4.9 and then at pH 3.7. Orosomucoid remains in solution and is then precipitated

by the addition of ammonium sulfate to saturation (Weimer et al., 1950).

Precipitation with ethanol at low temperatures

Cohn et al. (1946; 1950) demonstrated that by varying the ethanol concentration and temperature, fractionation of plasma proteins could be achieved in five main fractions. When albumin is precipitated in Fraction V, approximately 1% of the total plasma proteins remain in the supernatant. These are the most soluble plasma proteins and include orosomuroid (Ikenaka et al., 1965; Iwasaki and Schmid, 1967). The supernatant of Cohn Fraction V, although readily available, is very dilute. Schmid (1955) resolved this problem by precipitating the proteins from this supernatant by the addition of zinc salts, creating Fraction VI. The precipitated proteins are dissolved by adding neutralized EDTA. A disadvantage of this procedure is the precipitating steps require low temperatures, 0 to -5° C to prevent denaturation of the plasma proteins.

Orosomuroid was recovered from Fraction VI by decreasing the pH to 5.8 and recovering the supernatant which was treated at pH 9.5 with barium acetate. Orosomuroid was isolated from the supernatant by increasing the ethanol concentration, and purified through a second precipitation (Schmid, 1950; Schmid, 1953).

Ion-exchange chromatography

Both anion and cation exchange chromatography have been employed to isolate orosomuroid from whole or partially fractionated plasma.

One method of isolating orosomuroid from Fraction VI is to precipitate the proteins other than orosomuroid as zinc salts (Schmid, 1955), and

orosomuroid is then purified by anion-exchange chromatography on a DEAE-cellulose column (Schmid et al., 1982). An alternative method for recovering orosomuroid from Fraction VI is to use cation exchange chromatography: orosomuroid is not retained by an Amberlite IRC-50 column at pH 5.2 (Schmid et al., 1958) and can be separated from the other proteins in Fraction VI which are retained by the column.

Bezkorovainy and Winzler (1961) isolated orosomuroid from whole plasma or Fraction VI: chromatography of Fraction VI was done by anion-exchange CM-cellulose and a combination of chromatographic procedures with DEAE- and CM-cellulose was used with whole plasma.

Hao and Wickerhauser (1973) used batch-wise adsorption with DEAE-cellulose followed with chromatography on CM-cellulose to isolate orosomuroid from Fraction V. The batch-wise adsorption occurs under conditions close to the isoelectric point of albumin, the main contaminant, thereby requiring only a small bed volume of CM-cellulose. In many of the chromatographic techniques a high ratio of matrix to protein was required.

More recently, a two-step chromatographic method was employed to purify orosomuroid. DEAE-trisacryl and CM-trisacryl were used to isolate orosomuroid from plasma without any desialation or denaturation, as no acidic precipitation or low pH buffers are involved (Succari et al., 1985). Although only 50% of orosomuroid was recovered using this technique it is quite useful for studies on the variation in the sialic acid content.

Pseudo-affinity chromatography

A modified method of pseudo-ligand chromatography on Cibacron Blue F3-GA (Travis et al., 1976) retains albumin from plasma allowing albumin-free preparations.

To isolate orosomucoid from serum, the albumin-depleted eluate of the Cibacron Blue F3-GA was applied to another pseudo-affinity chromatography column, Procion Red HE3B. The fractions containing orosomucoid were removed from the column with a linear salt gradient. Any remaining contaminants in the orosomucoid fraction were removed by preparative isoelectric focusing (Laurent et al., 1984).

MATERIALS AND METHODS

I. DEVELOPMENT OF A METHOD

Some of the commonly employed methods for isolating orosomucoid were examined for a rapid, economical method that could be adapted to a small volume of starting material, 0.5 ml of human serum. Many of the techniques for isolating orosomucoid have been designed for large-scale purification of a pooled source of orosomucoid. A rapid method was desired since this protein was to be isolated separately from approximately 200 individual serum samples during the course of this investigation.

The criterion of purity was electrophoresis, using 7.5% polyacrylamide gels. Electrophoretically-detectable contaminants in the orosomucoid preparation would make it difficult to identify the specific products of CNBr cleavage of orosomucoid.

The starting material for many of the isolation methods is Cohn Fraction V supernatant (Cohn et al., 1948; Cohn et al., 1950) or Fraction VI (Schmid, 1955) not serum. These fractions were obtained from plasma fractionation and require several fractionation steps and sub-zero temperatures throughout the fractionation process.

When serum is the starting material, the first step in the isolation procedure is usually acidic precipitation to remove most of the plasma proteins, followed by one or more chromatography steps.

More recently two methods have been employed to minimize alteration of orosomuroid due to the strongly acidic conditions used to precipitate the bulk of the plasma proteins. Laurent et al. (1982) described a three-step purification of orosomuroid, with an 88% yield, using pseudo-ligand affinity chromatography on Cibacron Blue F3-GA, and then on Procion Red HE3B followed with preparative column isoelectric focusing. A two-step procedure, with a 50% yield, was described using DEAE- and CM-trisacryl chromatography (Succari et al., 1985). These methods would be preferred if undenatured orosomuroid was desired. Since for this project the purified orosomuroid would be used for chemical studies, mild denaturation of this protein did not have to be avoided.

The initial approach in this project to isolate and purify human serum orosomuroid was to remove the bulk of the serum proteins by precipitation. Orosomuroid would then be purified using a chromatography or electrophoresis step. A simple two-step method, avoiding multiple chromatography steps, would be ideal as a rapid and economical procedure readily adapted to processing approximately 200 individual samples.

As a first step, three precipitating agents were investigated: ammonium sulfate, ethanol and trichloroacetic acid. The most convenient and efficient of these agents would be the choice for the precipitation step.

Ion-exchange chromatography, both anionic and cationic, and polyacrylamide gel electrophoresis, using DATD in place of BIS, were also examined as possible techniques for the purification of orosomuroid.

Source

Aliquots of serum required for the development of a method of isolation and purification of orosomucoid were taken from a pooled source, 50 ml, from 20 individuals.

Ammonium sulfate precipitation

Ammonium sulfate precipitation was first described by Weimer and his collaborators as a method to isolate orosomucoid (Weimer et al., 1950; Weimer and Winzler, 1955). This procedure involves the precipitation of the less soluble serum proteins with ammonium sulfate under acidic conditions. Orosomucoid can then be precipitated by saturating the filtrate with ammonium sulfate. This precipitation step requires a minimum of 72 hours (Weimer et al., 1950; Weimer and Winzler, 1955), and the preparation has been reported to be nonhomogeneous (Travis et al., 1976).

Materials

0.1M sodium acetate

1N hydrochloric acid

1.4 g ammonium sulfate

Method

All operations were carried out at 4° C. An aliquot of the pooled serum (1.0 ml), was diluted with an equal volume of 0.1M sodium acetate and 1.4 g of ammonium sulfate was added with stirring. The precipitated proteins were removed after 16 hours by filtering through a double thickness of

Whatman No. 1 filter paper.

Most of the serum proteins were removed in the following two steps. The pH of the filtrate was first adjusted to pH 4.9 with 1N hydrochloric acid, and after 16 hours the precipitated proteins were removed by filtering through a double thickness of Whatman No. 1 filter paper. The filtrate was then adjusted to pH 3.7 with 1N hydrochloric acid. After a further 16 hours the precipitated proteins were removed by filtration.

The filtrate was dialysed against running tap water (5 to 10° C) for 14 to 16 hours to remove the low-molecular weight components and freeze-dried.

Ethanol precipitation

Orosomuroid is soluble in 50% ethanol (Schmid, 1955) but is precipitated by 70% ethanol at pH 3.5 (Schmid, 1953). Orosomuroid remains soluble in the supernatant of the Cohn Fraction V (Schmid, 1955) and material is frequently used as a source of this protein.

Materials

ethanol

0.02M Sodium acetate buffer, pH 5.0

sodium acetate 0.1 g

distilled water to 100.0 ml

adjust pH with glacial acetic acid

Methods

All operations were carried out at -20° C. An aliquot of pooled human serum, 1.0 ml, was diluted with 2.5 ml of 0.02M sodium acetate buffer, pH 5.0. 3.5 ml of ethanol was added slowly, with stirring. The supernatant was recovered after 16 hours by centrifuging at 2,200 g for 10 minutes.

The solution was dialysed against running tap water (5 to 10° C) for 14 to 16 hours and freeze-dried.

Trichloroacetic acid precipitation

Orosomucoid is soluble in 0.3M trichloroacetic acid (Schmid, 1975), although there is an erroneous report (Rimington, 1940) that orosomucoid is precipitated under these conditions.

Materials

0.6M trichloroacetic acid

Method

All operations were carried out at 4° C. An equal volume of 0.6M trichloroacetic acid was added dropwise, with continuous stirring to 1.0 ml of the pooled human serum. The 0.3M trichloroacetic acid soluble fraction was recovered after 1 hour by centrifuging 2,200 g for 10 minutes. The recovered supernatant was dialysed for 14-16 hours against running tap water (5 to 10° C) to remove the trichloroacetic acid and freeze-dried.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed on the freeze-dried products of ammonium sulfate, ethanol, and trichloroacetic acid precipitation.

Materials

Continuous Buffer: Ashton and Braden (1961), pH 8.0

Gel buffer, X5 stock

Tris (hydroxymethyl)methylamine [TRIS]	27.9 g
boric acid	5.9 g
citric acid	7.2 g
lithium hydroxide	0.6 g
distilled water to	1.0 l

Tank buffer X1

boric acid	11.8 g
lithium hydroxide	1.2 g
distilled water to	1.0 l

Sample buffer

gel buffer (X5)	1.0 ml
sucrose	10.0 g
bromphenol blue	5.0 mg
distilled water to	50.0 ml

7.5% Acrylamide gel with 5% N,N'-methylene-bis-acrylamide (BIS)

acrylamide	4.99 g
BIS	0.26 g
N,N,N',N'-tetramethyl- ethylenediamide (TEMED)	50.0 μ
gel buffer (X5)	14.0 ml
0.3M ammonium sulfate	0.7 ml
distilled water to	70.0 ml

Staining solution, X10 stock

amido black	0.1 g
glacial acetic acid	50.0 ml
distilled water to	100.0 ml

Dilute 1:9 with distilled water for use

Method**Preparation of gels**

Gels were prepared 2 to 3 hours in advance. To prepare a gel, 14.0 ml of the X5 Ashton and Braden (1961) gel buffer stock solution was added 50 μ TEMED, 4.655 g acrylamide, and 0.245 g BIS. The volume was brought to 69.3 ml with distilled water and the solution was mixed. Immediately before pouring the gel, 0.7 ml of 0.3M ammonium sulfate was added with mixing.

A Hoefer Scientific Instruments Vertical Slab Gel Unit (SE 600 series)

was used. The gels (3mm thick) were formed between two vertical glass plates, 16 cm long x 18 cm wide. The actual dimensions of the gel were 13 cm long x 14 cm wide. Ten sample wells were formed by the insertion of a comb.

Samples

Each sample was dissolved in a minimum of sample buffer, approximately 30 μ l and loaded on the gel.

Electrophoresis

The tank buffer was cooled by the continuous flow of tap water (5 to 10° C) through the cooling coil. Gels were run at 15 watts constant power until the tracking dye reached the bottom of the gel; approximately 2 to 2.5 hours were required.

Staining

Gels were stained by immersion in 100 ml of 0.01% Amido Black in 5% acidic acid (a 1:9 dilution of the X10 Stock) for 6 hours and destained in 5% acidic acid.

Results of 7.5% polyacrylamide gel electrophoresis

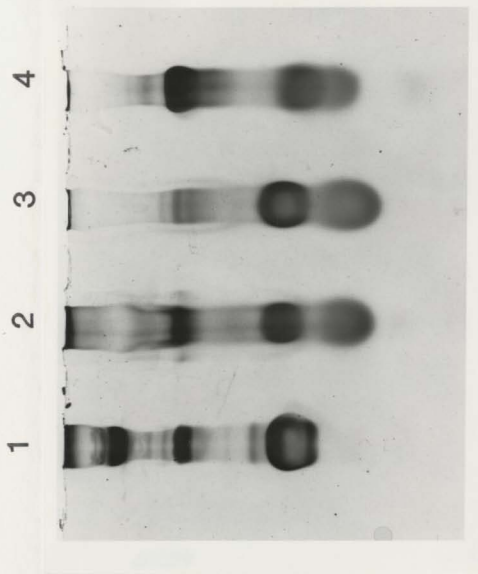
Polyacrylamide gel electrophoresis of serum precipitated with ammonium sulfate, ethanol or trichloroacetic acid did not reveal homogeneous preparations although a clear enrichment of orosomucoid was observed in all three preparations. Albumin was the major contaminant in each preparation, (Figure 4).

The 0.3M trichloroacetic acid non-precipitated sample was comparable

Figure 4: Electrophoretic patterns of serum precipitated by different techniques.

The electrophoretic patterns obtained in a 7.5% polyacrylamide gel (pH 8.0) are respectively;

1. human serum,
2. 0.3M trichloroacetic acid soluble fraction,
3. ammonium sulfate soluble fraction,
4. 50% ethanol soluble fraction.



to the other two procedures. As the trichloroacetic precipitation method involved a single precipitating step requiring only one hour, this was the precipitating agent chosen.

Purification step

Polyacrylamide gel electrophoresis, anion-exchange chromatography (DEAE-Sephadex) and cation-exchange chromatography (Amberlite IRC-50) were investigated as possible techniques for purifying orosomucoid on the scale required for genetic analysis.

N,N'-diallyl-tartardiamide (DATD)-substituted polyacrylamide gels

Gels cross-linked with DATD instead of BIS are soluble in 2% periodic acid (Anker, 1970). This provides a rapid, easy technique for the separation and isolation of proteins. Anker (1970) recommends using DATD mole for mole in place of BIS. Spath and Koblet (1970) modified the recipe for DATD cross-linked gels to overcome the problems of diffuse protein bands and swelling of the gels when DATD is used mole for mole instead of BIS: 10.27% acrylamide gels cross-linked with 27% DATD were used. No change in the retardation coefficients and free mobilities of proteins were observed at this concentration of cross-linker (Spath and Koblet, 1970).

Periodic acid solubilizes gels cross-linked with DATD by oxidatively cleaving the C-C bond of the cis-diol groups of DATD (Spath and Koblet, 1970). The gel matrix is completely solubilized in 20 to 30 minutes at

room temperature.

Materials

The conditions of electrophoresis were those outlined previously except 10.27% acrylamide gels with 27% DATD were used.

10.27% acrylamide gels, 27% DATD

acrylamide	5.25 g
DATD	1.94 g
TEMED	50.0 μ
gel buffer (X5 Stock)	14.0 ml
0.3M ammonium persulfate	0.7 ml
distilled water to	70.0 ml

100 mM periodic acid

Method

Solubilizing the gel

Preliminary staining of the gel was required to identify the position of the orosomucoid band. The protein band was cut from the gel, approximately 1.5 cm x 1.5 cm x 0.3 mm, and placed in 15 ml of 100 mM periodic acid for 30 minutes at room temperature. The solution was dialysed against running water, (5 to 10 ° C), for 14 to 16 hours and freeze-dried.

Results

DATD-substituted gels provide a rapid method for isolating orosomucoid from the other proteins present in the 0.3 M trichloroacetic acid soluble fraction. Although the technique provided electrophoretically homogeneous orosomucoid, extraction of the protein from the DATD gels was difficult. The solubilized gel that had been dialyzed and freeze-dried would not dissolve in distilled water or electrophoresis sample buffer.

Orosomucoid was recovered by soaking the freeze-dried gel in 5 ml of distilled water for 5 hours, centrifugation at 3400 g for 10 minutes and lyophilization of the supernatant.

Insufficient orosomucoid was recovered (2 ng) from 0.5 ml of trichloroacetic acid-precipitated serum to be detected by 7.5% polyacrylamide gel electrophoresis. The purity of orosomucoid obtained by this method was confirmed for a larger volume (5 ml) of starting material (not shown).

Anion-exchange chromatography (DEAE-Sephadex A50)

Materials

0.5M Sodium acetate buffer, pH 4.5; Stock buffer

- sodium acetate

distilled water to

adjust pH with glacial acetic acid

0.05M sodium acetate

dilute stock buffer 1:0 with distilled water

0.25M sodium acetate

dilute stock buffer 1:2 with distilled water

Method

Preparation of resin

Sephadex must be swollen prior to use. Approximately 0.5 g of DEAE-Sephadex dry powder was stirred into an excess of 0.05M sodium acetate buffer, pH 4.5 and placed in a boiling water bath for 2 hours. The swollen resin was washed with approximately 200 ml the 0.05M sodium acetate buffer, pH 4.5 on a Buchner funnel.

Samples

The freeze-dried 0.3M trichloroacetic acid soluble fraction from 1.0 ml of pooled human serum was dissolved in 0.5 ml of 0.05M sodium acetate buffer.

Chromatography

All operations were performed at 4° C. A 15.0 cm long, 1.0 cm diameter, column was filled with the swollen DEAE-Sephadex and equilibrated with 0.05M sodium acetate buffer, pH 4.5. The automatic fraction collector was then set up to give a flow rate of 12 ml per hour and collection of the eluate in 2 ml fractions.

The sample was applied to the column and washed with 10 ml of 0.05M sodium acetate buffer, pH 5.0, and the eluate was discarded.

Step-wise elution was achieved using 0.25M and 0.5M sodium acetate

buffer, pH 4.5. 38 ml of 0.25M sodium acetate buffer, pH 4.5 was added to the column and the eluate was collected in 2.0 ml fractions. Absorbance at 280 nm was used to identify the protein peak in the eluate and these fractions, number 10 to 14, were pooled (Figure 5).

46.0 ml of 0.5M sodium acetate buffer, pH 5.0 was added to the column and the eluate was collected in 2.0 ml fractions. Again the protein peak was identified using absorbance at 280 nm and the protein peak, fractions 26 to 29, were pooled (Figure 5). An aliquot, 0.5 ml, of the pooled fractions was reserved for "seromuroid" determination. The two pooled eluates were dialysed against running tap water, (5 to 10 ° C), for 14 to 16 hours and freeze-dried. Polyacrylamide gel electrophoresis was used to identify the components in each eluate.

Results

"Seromuroid" determination of the 0.25M and 0.5M sodium acetate buffer eluates from the DEAE-Sephadex column revealed the presence of glycoproteins in the 0.5M and not the 0.25M sodium acetate buffer eluates.

Polyacrylamide gel electrophoresis of both eluates are shown in Figure 6. Orosomuroid is eluted from the column with 0.5M sodium acetate buffer, pH 4.5.

This preparation is not homogeneous and albumin is one of the contaminants.

Figure 5: Absorbance (280nm) of DEAE-Sephadex column eluate.

Ion-exchange chromatography (DEAE-Sephadex A50) was performed on the 0.3M trichloroacetic acid soluble fraction of human serum. Fractions 1 through 19 and fractions 20 through 29 were eluted, respectively with 0.25M and 0.5M sodium acetate buffer, pH 4.5. Each fraction is 2 ml.

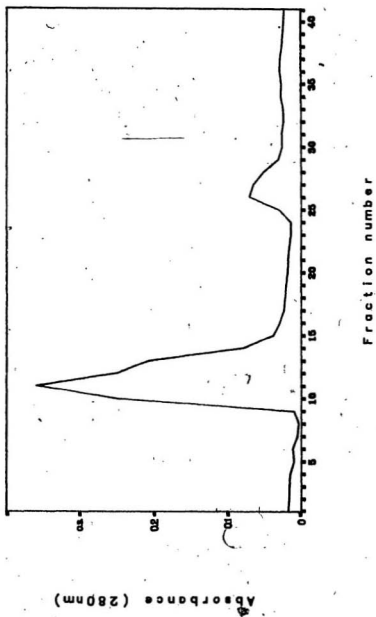


Figure 6/ Electrophoretic analysis of DEAE-sephadex A50 column eluate.

7.5% polyacrylamide gel electrophoresis (pH 8.0) of the ion-exchange column (DEAE-Sephadex A50) column eluate (Figure 5); 1. human serum, 2. fractions 10 through 14, 3. fractions 26 through 29.



Cation chromatography (Amberlite IRC-50)

Schmid et al. (1958) demonstrated that low molecular weight proteins with acidic isoelectric points could be absorbed and selectively displaced on carboxylated ion-exchange resins. These resins were usually employed to separate proteins with neutral or alkaline isoelectric points. Orosomuroid was isolated from Cohn Fraction VI using Amberlite XE-64 (Schmid et al., 1958). Charlwood et al. (1976) employed Amberlite IRC-50 (Amberlite XE-64 is a more finely divided form of Amberlite IRC-50) to separate orosomuroid from the soluble fraction of serum precipitated with a solution of 0.15M trichloroacetic acid and 3.2M ammonium sulfate. Critical selection of the buffer pH enabled direct elution of orosomuroid while the contaminants remained absorbed to the resin (Schmid et al., 1958; Charlwood et al., 1976).

Materials

0.05M sodium citrate buffer, pH 5.1 (Schmid et al., 1958)

sodium citrate	14.7 g
distilled water to	1.0 l
pH adjusted with solid citric acid	

1M sodium acetate buffer, pH 6.0

sodium acetate	7.79 g
distilled water to	100. ml
pH adjusted with acetic acid	

Methods

Samples

The freeze-dried 0.3M trichloroacetic acid soluble fraction from 1.0 ml of pooled serum was dissolved in 0.5 ml of the 0.05M sodium citrate buffer, pH 5.1.

Chromatography

A 10.0 cm long column, 1.0 cm diameter, of Amberlite IRC-50 was equilibrated, at 4° C, with 0.05M sodium citrate buffer, pH 5.1. The flow rate of the column was 0.2 ml per minute and 4.0 µl fractions were collected.

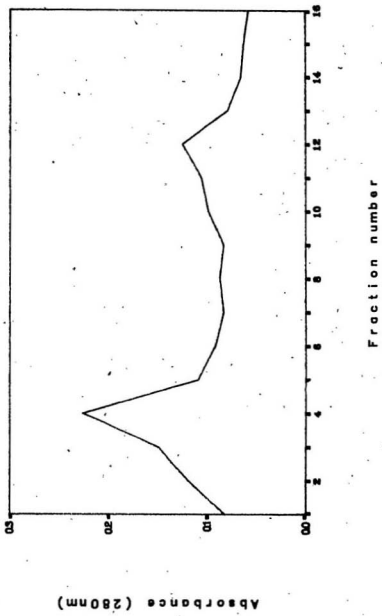
The sample was applied to the column and orosomuroid was directly eluted with 120 ml of 0.05M sodium citrate buffer, pH 5.1.

Absorbance at 280 nm was used to identify the protein peak in the eluate fractions. Two protein peaks were identified (Figure 7).

The fractions in each peak (fraction no. 1 to 7 and 8 to 13) were pooled and from each an aliquot, 0.5 ml, was reserved for glycoprotein determination. The two pooled fractions were dialysed against running tap water (5 to 10° C) for 14 to 16 hours and freeze-dried.

The bound fraction was eluted as a single fraction with 20 ml of 1.0M sodium acetate buffer, pH 6.0. This fraction was dialysed against running tap water (5 to 10° C), for 14 to 16 hours and freeze-dried. The purity of the recovered protein was determined by polyacrylamide gel electrophoresis.

Figure 7: Absorbance (280nm) of Amberlite IRC-50 column eluate. Cation-exchange chromatography (Amberlite IRC-50) was performed on the 0.3M trichloroacetic acid soluble serum fraction. The fractions ($V = 4$ ml) were eluted with 0.05M sodium citrate buffer, pH 5.1.



Results

The unbound fractions were combined when "seromuroid" determination revealed the presence of glycoproteins in both of the unbound fractions from the Amberlite IRC-50 column and not in the bound fraction.

Polyacrylamide gel electrophoresis of the unbound fraction is shown in Figure 8.

The orosomuroid preparation is not homogeneous and albumin is present in the preparation.

"Seromuroid" determination

The method of "seromuroid" determination used by Winzler (1955) was essentially that of Weimer and Moshin (1953).

This method of determining serum glycoproteins estimates the protein in a solution that cannot be precipitated by 1.8M perchloric acid and is precipitated by acidified phosphotungstic acid. This procedure is not quantitative as approximately 30% of the "seromuroid" components are coprecipitated with the serum proteins in the precipitation step. To control coprecipitation, careful attention must be given to the reproducibility of technique. The dilution of the serum at the precipitation step, the mixing of the serum with the perchloric acid and the time and temperature of contact between the supernatant and the precipitated proteins all influence coprecipitation losses.

Although the "seromuroid" fraction is nonhomogeneous, orosomuroid is the major component. The presence of orosomuroid can be inferred from

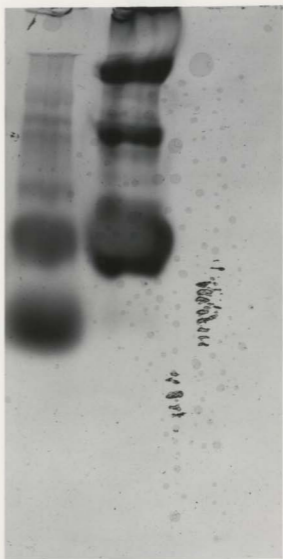
Figure 8: Electrophoretic analysis of Amberlite IRC-50
column eluate.

7.5% polyacrylamide gel electrophoresis (pH 8.0) of the
cation-exchange column, (Amberlite IRC-50); 1. fractions
1 through 13 (Figure 7), 2. human serum.

1 2

Albumin →

Orosomuroid →



the presence of the "seromuroid" fraction.

Materials

0.15M saline

1.8 M perchloric acid

0.02M phosphotungstic acid in 2N hydrochloric acid

0.1 N sodium hydroxide

biuret reagent of Mehl (Mehl, 1945)

ethelene glycol	50.0 ml
60% sodium hydroxide	20.0 ml
4% cupric sulfate	25.0 ml
distilled water to	200.0 ml

The mixture was heated until the precipitation was complete and filtered through Whatman No. 1 filter paper. Sufficient sodium hydroxide was added to make a final concentration of 10%. This stock was diluted 10 ml to 25 ml with distilled water for use.

Method

A 0.5 ml aliquot of the column eluate, or serum, was added to 4.5 ml of 0.15M saline and mixed. To this solution 2.5 ml of 1.8M perchloric acid was added dropwise, with stirring. The mixture was filtered through Whatman No. 50 filter paper after exactly 10 minutes. 5.0 ml of the filtrate was taken and 1.0 ml of the acidified 0.02M phosphotungstic acid was added with mixing. The precipitated "seromuroid" was recovered after 10 minutes by centrifuging for 10 minutes at 550 g. The precipitated

"seromuroid" was dissolved in 5.0 ml of 0.1N sodium hydroxide. 5.0 ml of the 0.1N sodium hydroxide was used as a blank and 1.0 ml of biuret reagent was added to both. The absorbance at 540 nm was determined.

Comment on column chromatographic methods

Neither of the chromatographic methods was successful in providing an electrophoretically homogeneous preparation by step elution. Difficulties arise in separating plasma proteins from albumin when the starting material is whole serum or plasma because the relative concentration of albumin is so great. Albumin is present in concentrations of 38 to 51 g/l and 40 to 52 g/l in sera from adult women and men, respectively (Winberg, 1979), whereas orosomuroid is present in concentrations of 0.50 to 1.00 g/l and 0.55 to 1.15 g/l in adult sera (Winberg, 1979). Albumin makes up 50 to 60% of the plasma proteins by weight. Therefore minor proteins with molecular weights and electrophoretic mobilities similar to those of albumin, are difficult to separate from albumin without significant contamination.

As albumin was the major contaminant, an alternative approach to isolating orosomuroid would be to remove albumin specifically in a first step, using Reactive Blue-2-Sepharose CL-6B, and then to purify orosomuroid by precipitating the remaining contaminants with trichloroacetic acid. This procedure is described in detail in the next section.

II. DEVELOPMENT OF A PRACTICAL TWO-STEP ISOLATION METHOD

The removal of essentially all albumin from the plasma proteins using Sepharose-Blue Dextran was first demonstrated by Travis and Pannell (1973). A modified method of pseudo-ligand chromatography, using Cibacron Blue F-3-GA, increased the efficiency of albumin binding and eliminated the problem of leaching of dye from the column (Travis et al., 1976). 98% of the plasma albumin and trace amounts of lipoproteins bind to the column. The strong interaction between human serum albumin and the dye Cibacron blue appears to be due to the binding of the dye to the bilirubin-binding site of albumin (Leatherbarrow and Dean, 1980).

Reactive Blue 2-Sepharose CL-6B (Pharmacia) is a commercial Cibacron blue F3G-A covalently attached to Sepharose CL-6B.

Reactive Blue 2-Sepharose CL-6B Chromatography

Materials

0.1M sodium phosphate, pH 7.0, containing 0.02% thimerosol

sodium phosphate, dibasic 8.52 g

sodium phosphate, monobasic 4.14 g

thimerosol (ethylmercurithiosalicylate) 0.18 g

distilled water to 900.0 ml

1.5M sodium thiocyanate in 0.1M sodium phosphate, pH 7.0.

sodium thiocyanate 12.01 g

0.1M sodium phosphate buffer, pH 7.0 to 100.0 ml

Method

A 15.0 cm x 1.5 cm column of Reactive Blue 2-Sepharose CL-6B (Pharmacia) was equilibrated with 0.1M sodium phosphate buffer, pH 7.0. The column was run at room temperature with a flow rate of 0.2 ml per minute. A fraction collector was used to collect 25 2.0 ml fractions.

Each serum sample was centrifuged at 1,200 g for 10 minutes before a 0.5 ml aliquot was drawn. The 0.5 ml aliquot of human serum, 1.0 ml of 0.1M sodium phosphate buffer pH 7.0, 1.0 ml of 1.5M sodium thiocyanate and 48.0 ml of 0.1M sodium phosphate buffer, pH 7.0 were applied sequentially to the column, care being taken to minimize mixing of the sample and eluent solutions. The unbound fraction containing orosomucoid was eluted with the 0.1M sodium phosphate buffer, pH 7.0 and the bound fraction, mainly albumin, was eluted with 1.5M sodium thiocyanate. Applying the 1.0 ml of 1.5M sodium thiocyanate before the 48 ml of 0.1M sodium phosphate bufer, pH 7.0 enabled the elution of the protein and the regeneration of the column in a single cycle. This facilitated the sequential application of serum specimens in a semi-automatic process.

The eluates containing orosomucoid ($V = 8$ ml) were pooled and stored

at -20° C, for up to several weeks until required for trichloroacetic acid precipitation.

Orosomucoid is a constituent in the break-through fraction of the column eluate (Gianazza and Arnaud, 1982), which was identified by the sharp peak in absorbance at 280 nm (Figure 9).

"Seromucoid" determination was carried out on fractions 7 through 13 (Figure 9) and the presence of "seromucoid" corresponds to the observed peak in absorbance at 280 nm.

Preparation of trichloroacetic acid soluble fraction

Materials

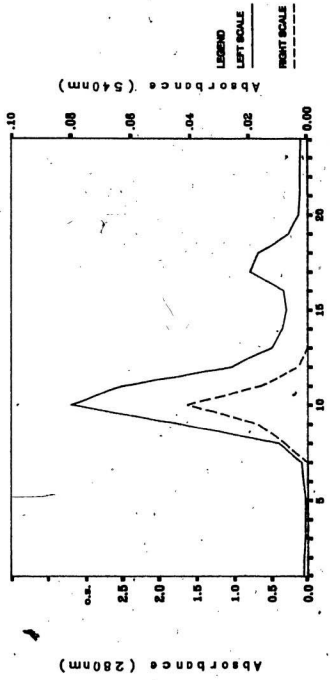
0.6M trichloroacetic acid

Method

The trichloroacetic acid precipitation of the albumin-depleted fractions was carried out at 4° C. To the 6.0 ml of pooled eluate was added an equal volume of 0.6M trichloroacetic acid drop-wise with continuous stirring. The precipitated proteins were removed after one hour by centrifugation, (2,200 g for 10 minutes). The recovered supernatant was dialysed 14 to 16 hours against running water (5 to 10° C) to remove trichloroacetic acid and filtered through Whatman No. 42 filter paper. The filtrate was freeze-dried.

Figure 9: Absorbance (280nm) of the Reactive Blue
2-Sepharose CL-6B column eluate and
absorbance (540nm) of the "seromuroid"
component.

Pseudo-ligand chromatography was performed on human
serum. 2 ml fractions were eluted with 0.1M sodium
phosphate buffer, pH 7.0. The "seromuroid" component
(measured at 540nm) of fractions 7 through 13 was
determined.



Quality and Yield

On electrophoresis in 7.5% polyacrylamide at pH 8.0 the preparation of orosomucoid thus prepared is pure. Orosomucoid migrates as a single band in the prealbumin region (Figure 10).

The yields of orosomucoid were 0.30 to 0.35 mg. A yield of at least 0.6 mg/ml can be obtained with this method.

Reduction and S-carboxymethylation

Orosomucoid has two disulfide bonds; residue 5 is linked to residue 147 and residue 72 is linked to residue 164 (Schmid et al., 1974). The procedure used to reduce and S-carboxymethylate the disulfide bonds of orosomucoid was essentially that of Crestfield et al. (1963) except that dithiothreitol, (Cleland's reagent) was used instead of 2-mercaptoethanol as the reducing agent. Dithiothreitol has a low oxidation potential and is a better reducing agent than 2-mercaptoethanol (Light, 1974). Also, it is less prone to spontaneous autoxidation when exposed to air.

Reduction of the disulfide bonds of cystine liberates thiol groups that are capable of being reoxidized in air. Reagents containing active halogen groups, such as iodoacetic acid or iodoacetamide, alkylate these groups and prevent reoxidation.

Exclusion of light from the solution during the alkylation step prevents the formation of elemental iodine, which may react with tyrosine, tryptophan or histidine residues. Excess iodoacetic acid is also avoided to minimise the iodine side reactions. The prevention of the iodine side reactions is most critical for subsequent amino acid sequencing.

Figure 10: Electrophoretic analysis of orosomuroid preparation.
7.5% polyacrylamide gel electrophoresis (pH 8.0).
Lanes 1, 3., and 5. human serum, lanes 2., and 4.
orosomuroid preparation.

1 2 3 4 5



Albumin →

Orosomucoid →

Materials

50 mM ethylenediaminetetra-acetic acid (EDTA),

disodium salt solution

1.5M TRIS-HCl, pH 8.6

3.61 g urea

23.2 mg dithiothreitol

31.19 mg iodoacetic acid, sodium salt

Method

3.61 g of urea was dissolved, by warming, in 0.3 ml 50mM sodium EDTA solution and 3.0 ml 1.5M TRIS-HCl, pH 8.6. This solution was added to the freeze-dried protein and the volume was brought to 7.5 ml with distilled water. Preincubation in this 8M urea solution at 37° C for 16 hours was required to completely denature orosomucoid (Kitamura and Yamashina, 1972). To reduce the disulfide bonds 23.2 g of dithiothreitol was added to the denatured protein solution at room temperature. After one hour S-carboxymethylation was achieved by the addition of 31.19 mg of sodium iodoacetate. The solution was kept dark for 20 minutes and then dialysed against running tap water (5 to 10° C) for 14 to 16 hours to remove the reagents used to reduce and alkylate orosomucoid, and then freeze-dried.

**Cyanogen bromide treatment of
reduced-carboxymethylated
orosomucoid**

Gross and Witkop (1962) have demonstrated the value of cyanogen bromide as a non-enzymatic reagent for the cleavage of methionyl-peptide bonds. Cyanogen bromide is specific for the methylmercapto-group and it does not react with other amino acids, except cystine, with which there is a slow reaction. The reaction proceeds at room temperature in an aqueous solution under acidic conditions. Excess cyanogen bromide and by-products of the reaction are volatile and can be removed by freeze-drying.

About 25% of the sialic acid is cleaved in this reaction (Schmid, 1975).

Materials

15M formic acid

0.5 mg cyanogen bromide

Method

The freeze-dried, reduced, carboxymethylated orosomucoid was dissolved in 1 ml of 15M formic acid and 0.5 mg of cyanogen bromide was added. Ikenaka et al. (1972) used a ratio of equal parts by weight of orosomucoid and cyanogen bromide. Orosomucoid is present in concentrations of 0.50 to 1.00 g/l and 0.55 to 1.15 g/l in adult female and male sera, respectively (Winberg, 1979). If one assumes a serum concentration of 100 mg% for orosomucoid, then 0.5 mg of cyanogen bromide provides a slight excess of a 1:1 ratio by weight for an initial

serum specimen of 0.5 ml.

The reaction proceeded at room temperature for 16 hours, with occasional stirring. Eight volumes of distilled water was added to dilute the solution and it was freeze-dried to remove the excess cyanogen bromide.

Molecular weight of cyanogen bromide fragments

A methionine residue at position 111 is universally present in human orosomucoid but approximately one-quarter of the orosomucoid molecules in a pooled sample from a large number of donors have a second methionine residue at position 158 (Emura et al., 1971). In no case was an amino acid other than methionine or valine found at this position. Cyanogen bromide treatment of single-methionine orosomucoid will generate two polypeptide fragments: CNBr-I and CNBr-II. Cyanogen bromide treatment of orosomucoid containing two methionine residues will generate three fragments: CNBr-I, CNBr-III and CNBr-IV.

The molecular weight of the peptide portion of orosomucoid calculated from the amino acid composition reported by Schmid et al. (1973) ranges from 21,095 to 21,780 daltons. The actual molecular weight of an individual polypeptide chain is dependent on its amino acid composition because 21 of the protein's 181 residues are sites of possible substitution (Emura et al., 1971; Schmid et al., 1973).

The molecular weights of the polypeptide fragments generated by cyanogen bromide treatment of orosomucoid are: CNBr-I, (residues 1-111) 12,779 to 13,145 daltons; CNBr-II (residues 112-181) 8,316 to 8,644 daltons;

CNBr-III (residues 112-156) 5,151 to 5,480 daltons; and CNBr-IV (157-181) 3,167 daltons. No amino acid substitutions are known for the CNBr-IV fragment and it has been termed the constant region (Emura et al., 1971).

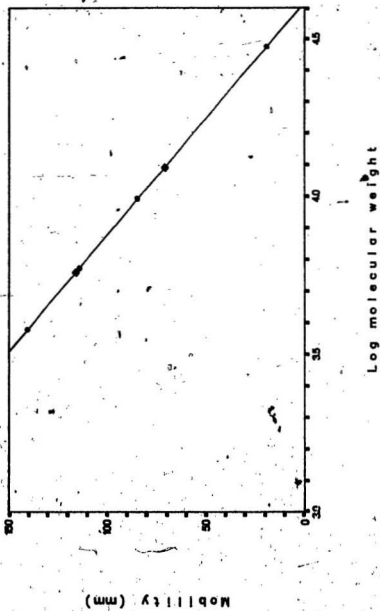
All five carbohydrate groups are attached to the CNBr-I fragment. If one assumes a molecular-weight of 40,000 for glycosylated orosomucoid than the molecular weight of this fragment plus the carbohydrate groups would be approximately 31,356 to 31,684 daltons. The experimentally-determined molecular weight of a glycosylated CNBr-I would be slightly less than theoretical because of partial carbohydrate degradation during the cleavage step.

The peptide fragments generated by cyanogen bromide cleavage of pooled human orosomucoid were resolved using SDS-polyacrylamide electrophoresis, the conditions for which are outlined in the following section. Although the cleavage products were not run simultaneously with the CNBr fragments from the lab of Schmid and his collaborators (Schmid et al., 1973), we can be certain that the peptides are as identified because the orosomucoid preparation was previously shown to be electrophoretically pure (Figure 10). The molecular weights of these fragments were estimated from their mobilities in SDS-polyacrylamide gels (Figure 11).

The experimentally determined molecular weights of CNBr-I, CNBr-II, CNBr-III and CNBr-IV were 28,208, 9,567, 5,927 and 3,839 daltons, respectively.

Figure 11: Molecular weight estimation of CNBr fragments.

Squares represent the markers; bovine insulin, (MW = 5,734) and horse heart cytochrome c, (MW = 12,384). Circles represent the fragments generated from cyanogen bromide cleavage of pooled human orosomucoid.



Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Molecular weights of peptide fragments can accurately be determined by sodium dodecyl sulfate polyacrylamide electrophoresis. The electrophoretic mobility of peptides in polyacrylamide gels, in the presence of the anionic detergent sodium dodecyl sulfate (SDS) is inversely proportional to the logarithm of their molecular weights (Shapiro et al., 1967). This relationship has been used for the estimation of the molecular weights of peptides.

The difficulties of separation and resolution when estimating the size of polypeptides with molecular weights less than 14,000 have been overcome in an anonymous publication (Bethesda Research Laboratories, Inc., 1981). Low molecular weight proteins (3,000 - 43,000 daltons) were resolved using a modified procedure of Shapiro et al. (1967).

Gels prepared with 8M urea have a decreased gel porosity and as a result an increase in the separation and resolution of peptides with molecular weights less than 10,000 daltons (Swank, 1971).

Materials

Running buffer and gel buffer

0.1M sodium phosphate, pH 7.2, 3.5 mM SDS

sodium phosphate, dibasic	10.51 g
sodium phosphate, monobasic	3.59 g
sodium dodecyl sulfate	1.00 g
distilled water to	1.0 l

Sample buffer

sodium phosphate, dibasic	0.105 g
sodium phosphate, monobasic	35.90 mg
sodium dodecyl sulfate	1.00 g
urea	42.04 g
bromphenol blue	10.00 mg
distilled water to	100.00 ml

Resolving Gel, 15% Acrylamide (BIS:monomer ratio 0.8:30)

acrylamide	10.23 g
BIS	0.27 g
urea	25.20 g
TEMED	50.00 μ
0.3M ammonium persulfate	0.70 ml
gel buffer to	70.00 ml

Stain: 0.1% Coomassie blue, 25% 2-propanol, 10% acetic acid,**0.1% cupric acetate**

Coomassie brilliant blue R 250	0.1 g
2-propanol	25.0 ml
glacial acetic acid	10.0 ml
cupric acetate	0.1 g
distilled water to	100.0 ml

Markers:

horse heart cytochrome c (MW = 12,384)

bovine insulin (MW = 5,734)

Methods**Preparation of 15% acrylamide gel**

Gels were prepared three to four hours in advance and kept at room temperature. No upper separating gel was used. Each gel was prepared by dissolving, with warming, 25.2 g of urea in approximately 25 ml of 0.1M sodium phosphate, pH 7.2, 3.5 mM SDS buffer. The following reagents were then added, 10.23 g acrylamide, 0.27 g BIS and 50 μ TEMED. The volume was brought to 69.3 ml with 0.1M sodium phosphate, pH 7.2, 3.5 mM SDS buffer and the solution was mixed. Immediately before pouring the gel between two vertical glass plates (16 cm long x 18 cm wide x 3 mm thick), 0.7 ml of 0.3M ammonium persulfate was added. Ammonium persulfate solutions were prepared weekly and kept at 4° C. A comb was inserted between the glass plates to make 10 wells. The dimensions, length and width, of the actual running gel was 13 cm x 14 cm.

Samples

Each individual sample of the cyanogen bromide treated orosomucoid was dissolved in 40 μ l of the sample buffer and loaded on the gel. 2-mercaptoethanol was omitted from the sample buffer and the samples were not boiled.

Electrophoresis

A Hoefer Scientific Instruments Vertical Slab Gel Unit (SE 600 series) was used. Gels were run at room temperature at constant power after an initial setting of 65 volts (5 v/cm) until the tracking dye reached the bottom of the gel in approximately 16 hours. The running buffer was 0.1M sodium phosphate, pH 7.2, 3.5 mM SDS.

SDS is less soluble at lower temperatures, therefore buffers containing SDS were kept at room temperature without external cooling during the electrophoresis run.

Staining

SDS must be removed from the gels before the proteins can be stained. Soaking gels in 25% 2-propanol for 10 to 15 hours removes the SDS. The Coomassie blue and acetic acid can be included in this fixative step. The addition of cupric acetate to the staining solution was recommended for improved staining of small peptides (Bethesda Research Laboratories, Inc., 1981).

Gels were soaked in 100 ml of the staining solution for 15 hours. Destaining was achieved through numerous changes of 10% acetic acid. All staining and destaining operations were performed on a mechanical shaking device set at minimum speed.

Results

The electrophoretic patterns of cyanogen bromide cleaved orosomucoid isolated from four individuals are given in Figure 12.

Population and familial samples

To determine the $156^{\text{Met,Val}}$ allele frequency 49 serum samples, randomly chosen from hospital patients, were processed using the previously described two-step purification method for orosomucoid.

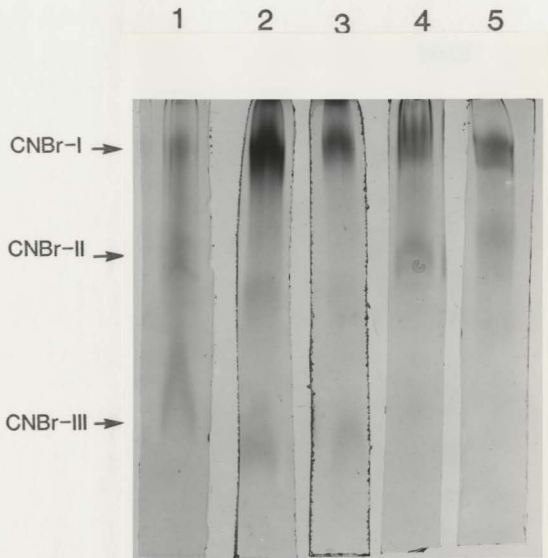
Serum samples from 25 nuclear families, collected by this laboratory for genetic studies, were processed using the previously described two-step purification method for orosomucoid to investigate the inheritance of the $156^{\text{Met,Val}}$ alleles. HLA and other genetic markers determined no parental exclusions in these families.

Figure 12: Electrophoretic patterns of orosomucoid CNBr fragments in SDS-polyacrylamide gels.

The electrophoretic patterns obtained in 15%

SDS-polyacrylamide gels are respectively;

1. CNBr-I, CNBr-II, CNBr-III (156^{Val, Met})
2. CNBr-I, CNBr-II, CNBr-III (156^{Val, Met})
3. CNBr-I, CNBr-III (156^{Met, Met})
4. CNBr-I, CNBr-II (156^{Val, Val})
5. CNBr-I, CNBr-II (156^{Val, Val})



RESULTS

Isolation of orosomuroid

A practical two-step isolation protocol for orosomuroid has been described in the Materials and Methods section. Although the techniques employed are not new, they have been combined successfully to isolate pure orosomuroid, as determined by 7.5% polyacrylamide electrophoresis, from 0.5 ml serum.

Allele frequency

Genotypes for the 156^{Met,Val} variants of orosomuroid were determined by separating the peptide products of CNBr treated orosomuroid in SDS-polyacrylamide electrophoresis. From the observed genotypes the allele frequencies were calculated using the Hardy-Weinberg equation for 49 samples, randomly chosen from hospital patients. These allele frequencies are given in Table 2.

Mendelian inheritance

A study of 25 families, consisting of both parents and at least one child, were used to demonstrate a Mendelian mode of inheritance of the 156^{Met,Val} variants. The actual and predicted number of progeny from the 25 mating pairs are reported in Table 3.

With the exception of one offspring from a Val,Val x Val,Val mating, all progeny genotypes are consistent with a Mendelian mode of inheritance for

Table 2: $156^{\text{Met,Val}}$ Frequency in 49 random samples

Genotype	Number	
	Observed	Expected
Val.Val	19	(19.45)
Val.Met	24	(22.84)
Met.Met	6	(6.71)
Total	49	

Estimation of allele frequencies:

$$156^{\text{Val}}: \frac{38+24}{98} = 0.63 \pm 0.1$$

$$156^{\text{Met}}: \frac{12+24}{98} = 0.37 \pm 0.1$$

$$(95\% \text{ Confidence limits: } \pm 2\sqrt{\frac{pq}{N}} = \pm 0.1)$$

Table 3: Inheritance of the 156 Val, Met alleles
in 25 mating pairs.

Mating pair	n	Offspring genotypes		
		Val, Val O (E)	Met, Val O (E)	Met, Met O (E)
Val, Val x Val, Val	5	13 (14)	0 (0)	1 (0)
Val, Val x Met, Val	13	27 (24.5)	22 (24.5)	0 (0)
Val, Val x Met, Met	1	0 (0)	3 (3)	0 (0)
Met, Val x Met, Met	1	0 (0)	2 (1.5)	1 (1.5)
Met, Val x Met, Val	5	7 (5.5)	11 (11)	4 (5.5)
Total mating pairs	25	total offspring		91

(O = observed; E = expected)

the 156^{Met,Val} variants.

DISCUSSION

Investigation of genetic variation in proteins has been at the forefront of genetic research since the development of electrophoretic methods of analysis in the 1950s. Protein polymorphisms have been of value from several perspectives: they permit direct, experimental analysis of the factors maintaining genetic variability in populations; they have been used as linkage markers in mapping the genome of many species, including humans; they have been implicated as inherited risk factors in disease susceptibility; and they give the protein chemist and enzymologist access to families of functionally equivalent proteins with amino-acid substitutions at various sites, giving insights on conformation and permitting development of concepts such as variable or highly conserved regions and sites within the primary structure.

Despite the importance of electrophoresis in biochemical genetics, the method has one major drawback: it can only detect genetic variation where there is a significant difference in molecular weight, or where there is a significant difference in net charge at the pH chosen for electrophoretic analysis. Genetic variation in molecular weight is an unlikely event; although examples are known, such as the human haptoglobin alleles, Hp^1 and Hp^2 , where Hp^2 has arisen from fusion of two Hp^1 genes (Giblett, 1969). Most genetic polymorphism involves a single amino acid

substitution, and the consequent change in molecular weight is less than that detectable by gel electrophoresis. Also, inspection of the genetic code indicates that approximately 60% of mutations will involve replacement of one uncharged amino acid side chain with another uncharged side chain; such mutations are unlikely to be detected by conventional gel electrophoresis. This problem has been resolved to some extent by the development of isoelectric-focusing. With this technique, minor changes in conformation arising from replacement of one amino acid with another of dissimilar size may induce subtle net electrical charge differences that can be detected as a shift in the isoelectric pH of the protein.

An alternative approach to the detection of electrically-neutral substitutions, is to cleave the protein with site-specific reagents and count the number of fragments generated. Cyanogen bromide, for example, cleaves specifically under standard conditions at the carboxyl position of methionine. Therefore, a mutation resulting in the replacement of an amino acid by methionine, or of methionine by another amino acid, can be detected by simply counting the number of fragments generated by cyanogen bromide treatment of the protein.

The use of cyanogen bromide cleavage as a specific tool to detect electrophoretically-cryptic polymorphism involving methionine has not been tested until now. This investigation can be subdivided logically and operationally into components: the choice of a suitable test protein; development of a simple technique to isolate and purify this protein from large numbers of individuals, as required for genetic investigations;

cyanogen bromide cleavage and SDS-electrophoresis of the selected protein; and confirmation by family and population studies that a genuine polymorphism has been detected.

The model protein should be one for which there is good evidence that a polymorphism involving methionine exists. Also, the protein should be readily available from a large number of subjects: in practice, only a blood protein meets these criteria. Orosomucoid was chosen as the model protein because it is present in human blood and the methionine content is known to be less than 1.3 moles of methionine per mole of protein (Schmid et al., 1968). Orosomucoid is a remarkably polymorphic protein: sequence analysis of pooled material indicates that 21 of the 181 residues are sites of substitution; yet only one substitution Arg/Gln at position 20, generates an electrophoretically-detectable polymorphism with allele frequencies of 0.54 and 0.46 for arginine and glutamine, respectively (Johnson et al., 1969; Nimberg et al., 1971). Even this polymorphism cannot be detected unless the protein is desialated before electrophoresis. This is a remarkable example of a highly polymorphic protein for which none of the variation is detectable by gel electrophoresis of the native protein.

Sequence analysis (Schmid et al., 1968) indicates that methionine is present at position 111 universally but a variant exists with either methionine or valine at position 156: presumably the polymorphism is due to a GUG-AUG base substitution. The relative proportions of methionine and valine at position 156 in pooled material suggests that the allele frequencies are 0.75 and 0.25 for 156^{Val} and 156^{Met}, respectively (Emura et

al., 1971). Cyanogen bromide cleavage of a 156^{Val} protein should yield two fragments, CNBr-I and CNBr-II, while 156^{Met} should yield three fragments, CNBr-I, CNBr-III and CNBr-IV. Can these fragments be detected electrophoretically in cyanogen-bromide treated orosomucoid derived from single individuals?

It is essential that the starting material should be as pure as possible, otherwise cyanogen bromide will engender a series of contaminating fragments. Most of the work of this investigation was directed to the development of a protocol that would permit isolation of pure orosomucoid from small volumes of blood.

Many of the isolation methods reported isolate orosomucoid from a large pooled source and very often the starting material is Cohn Fraction V or VI, a by-product of plasma fractionation. Of the reported isolation methods using serum as a starting source none seemed practical and time-efficient for isolating orosomucoid from 0.5 ml of serum from approximately 200 samples.

The high solubility and the high net negative electrostatic charge of this protein above pH 4 would suggest that isolation methods based on these two physicochemical properties would successfully isolate orosomucoid from the other plasma proteins. In practice there are difficulties in separating minor plasma proteins from albumin because the relative concentration of albumin is so great.

Depleting serum of albumin, using Reactive Blue-2-Sepharose CL-6B, provided a preparation free of the major contaminant and allowed for

quick isolation of orosomucoid by trichloroacetic acid precipitation of the remaining contaminants. Although acid precipitation of serum proteins has been criticized, mild denaturation of orosomucoid did not have to be avoided as the preparation would be used for chemical studies. The protocol developed permitted the isolation of sufficient electrophoretically-pure orosomucoid from as little as 0.5 ml of serum to enable subsequent cyanogen bromide cleavage and electrophoretic analysis of the resulting fragments.

The presence of a methionine residue at position 111 was confirmed for all samples typed (100 samples). In no individual was less than two CNBr fragments resolved in SDS-polyacrylamide electrophoresis and the relative mobilities of the fragments were appropriate for the molecular weights calculated for these fragments. Patterns of two, three, or four bands could be observed depending on the individual sera sample, confirming the methionine substitution at position 156.

CNBr-IV was difficult to stain and in some samples could not be detected. Genotypes could still be determined as CNBr-III was readily detectable and CNBr-IV could not be present without CNBr-III.

The final test of this approach was to determine if the fragments detected were indicative of a genuine polymorphism. The criteria selected were that the inheritance of CNBr fragments should strictly follow a Mendelian pattern in families where HLA-typing and segregation of other genetic markers confirm true paternity and that the frequencies of variants in a random sample of the population should be in concordance with the

postulates of the Hardy-Weinberg Equilibrium: these criteria were met. With only one exception, an analysis of 141 individuals in 25 nuclear families demonstrated a clear-cut Mendelian segregation. The exception was probably the result of a mislabelled specimen being supplied. In addition, the phenotype frequencies observed from 49 randomly-selected specimens were in good agreement with the expectations of the Hardy-Weinberg equation. Estimated allele frequencies are 0.37 and 0.63 for 156^{Met} and 156^{Val} , respectively.

In conclusion, this investigation clearly demonstrates that genetic variation involving methionine can be detected by specific cleavage of proteins with cyanogen bromide followed by SDS-electrophoresis. Using orosomucoid as a model, a method has been developed to purify this protein from as little as 0.5 ml of serum, to cleave the purified material with cyanogen bromide and analyse the fragments by electrophoresis, and to confirm these results by genetic analysis of familial and population material.

REFERENCES

- Adams, J.B. and Wachter, A. (1968). Specific changes in the glycoprotein components of seromucoid in pregnancy. *Clin. Chim. Acta*, **21**, 155-157.
- Anker, H.S. (1970). A solubilizable acrylamide gel for electrophoresis. *FEBS. Lett.*, **7**, 293.
- Ashton, G.C. and Braden, A.W.H. (1961). Serum β -globulin polymorphism in mice. *Aust. J. Biol. Sci.*, **14**, 248-253.
- Athineos, M., Thornton, M. and Winzler, R.J. (1962). Comparative antigenicity of native and "desialized" orosomucoid in rabbits. *Proc. Soc. Exptl. Biol. Med.*, **111**, 353-356.
- Barker, S.A. and Whitehead, P.H. (1963). Some immunochemical studies on orosomucoid. *Clin. Chim. Acta*, **8**, 848-854.
- Bennett, M. and Schmid, K. (1980). Immunosuppression by human plasma α_1 -acid glycoprotein: importance of the carbohydrate moiety. *Proc. Natl. Acad. Sci. USA.*, **77**, 6109-6113.
- Bethesda Research Laboratories, Inc. (1981). *A procedure for gel electrophoresis of low molecular weight proteins*. Bethesda Research Laboratories, Inc. Spring 1981 Biologue, pg. 6.
- Bezkorovainy, A. (1965). Some hydrodynamic properties of native and modified serum orosomucoids. *Biochim. Biophys. Acta*, **101**, 336-342.

- Bezkorovainy, A. and Winzler, R.J. (1961). Isolation and characterization of an acidic α_2 -acid glycoprotein. *Biochim. Biophys. Acta*, **49**, 559-565.
- Caputo, A. and Marcante, M.L. (1964). Studies on the structure of α_1 -acid glycoprotein isolated from Yoshida ascites tumor. I. Enzymatic and acid degradation of the carbohydrate unit. *Arch. Biochem. Biophys.*, **105**, 193-196.
- Charlwood, P.A., Hatton, M.W.C. and Regoecki, E. (1976). On the physicochemical and chemical properties of α_1 -acid glycoprotein from mammalian and avian in plasmas. *Biochem. Biophys. Acta*, **459**, 81-92.
- Chiu, K.M., Mortensen, R.F., Osmand, A.P. and Gewurz, H. (1977). Interactions of α_1 -acid glycoprotein with the immune system. I. Purification and effects upon lymphocyte responsiveness. *Immunology*, **32**, 997-1005.
- Cohn, E.J., Strong, L.E., Huges, W.L. Jr., Mulford, D.J., Asworthy, J.N., Melin, M. and Taylor, H.L. (1948). Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J. Am. Chem. Soc.*, **68**, 459-475.
- Cohn, E.J., Gurd, F.R.N., Surgenor, D.M., Barnes, B.A., Brown, R.R., Derouaux, G., Gillespie, J.M., Kabat, F.W., Lever, W.F., Lui, C.H., Mittelman, D., Mouton, R.F., Schmid, K. and Uroma, E. (1950). A system for the separation of the components of human blood:

- quantitative procedures for the separation of the protein components of human plasma. *J. Am. Chem. Soc.*, **72**, 465-474.
- Doolittle, R.F. (1972). Terminal-purrolidonecarboxylic acid: cleavage with enzymes. *Methods Enzymol.*, **25**, 231-249.
- Emura, J., Ikenaka, T., Collins, J.H. and Schmid, K. (1971). The constant and variable regions of the carboxyl-terminal CNBr fragment of α_1 -acid glycoprotein. *J. Biol. Chem.*, **246**, 7821-7823.
- Fournet, B., Montreuil, J., Strecker, G., Dorland, L., Haverkamp, J., Vilegenthart, F.G., Binette, J.P. and Schmid, K. (1978). Determination of the primary structures of 16 asialo-carbohydrate units derived from human plasma α_1 -acid glycoprotein by 360-MHz ^1H NMR spectroscopy and permethylation analysis. *Biochemistry*, **17**, 5208-5214.
- Gahmberg, C.G. and Andersson, L.C. (1978). Leucocyte surface origin of human α_1 -acid glycoprotein (orosomucoid). *J. Exp. Med.*, **148**, 507-521.
- Ganguly, M., Carnighan, R.,H. and Westphal, U. (1967). Steroid protein interaction. XIV. interactions between human α_1 -acid glycoprotein and progesterone. *Biochemistry*, **6**, 2803-2814.
- Gianazza, E. and Arnaud, P. (1982). A general method for fractionation of plasma proteins. Dye-ligand affinity chromatography on immobilized Cibacron Blue F3-GA. *Biochem. J.*, **201**, 129-136.
- Giblett, E.R. (1969). *Genetic Markers in Human Blood*, pg. 79-81.

- Goa, J. (1961). The components of orosomucoid. *Acta Chem. Scand.*, **15**, 1975-1980.
- Gross, E. and Witkop, B. (1962). Nonenzymatic cleavage of peptide bonds: the methionine residues in bovine pancreatic ribonuclease. *J. Biol. Chem.*, **237**, 1858-1859.
- Gross, J., Highberger, J.H. and Schmitt, E.O. (1956). Some factors involved in the fibrogenesis of collagen in vitro. *Proc. Soc. Exptl. Biol. Med.*, **80**, 462-465.
- Hao, Y. and Wickerhauser, M. (1973). Development of large-scale fractionation methods. IV. A simple method for the large-scale preparation of α_1 -acid glycoprotein. *Biochim. Biophys. Acta*, **322**, 99-108.
- Ikenaka, T., Bammerlin, H., Kaufmann, H. and Schmid, K. (1966). The amino-terminal peptide of α_1 -acid glycoprotein. *J. Biol. Chem.*, **241**, 5560-5563.
- Ikenaka, T., Ishiguro, M., Emura, J., Kaufmann, H., Isemura, S., Bauer, W. and Schmid, K. (1972). Isolation and partial characterization of the cyanogen bromide fragments of α_1 -acid glycoprotein and the elucidation of the amino acid sequence of the carboxyl-terminal cyanogen bromide fragment. *Biochemistry*, **11**, 3817-3829.
- Ikenaka, T., Gitlin, D. and Schmid, K. (1965). Preparation and characterization of the low molecular weight human plasma 3S γ_1 -globulins. *J. Biol. Chem.*, **240**, 2868-2876.
- Iwasaki, T. and Schmid, K. (1967). Purification and characterization of

- the 2S₇-globulin of normal human plasma. *J. Biol. Chem.*, **242**, 2356-2361.
- Izumi, K., Makino, M. and Yamashina, I. (1961). Isolation and analysis of a carbohydrate-amino acid complex from an enzymic digest of α_1 -acid glycoprotein of human plasma. *Biochim. Biophys. Acta*, **50**, 196-197.
- Jager, B.V. (1960). Estimation of serum protein fractions by immunochemical techniques. *Federation Proc.*, **12**, 723-728.
- Jamieson, J.C. and Ashton, F.E. (1973). Studies on acute phase proteins of rat serum. IV. Pathway of secretion of albumin and α_1 -acid glycoprotein from liver. *Can. J. Biochem.*, **51**, 1281-1291.
- Jamieson, J.C., Ashton, F.E., Friesen, A.D. and Chou, B. (1972). Studies on acute phase proteins of rat serum. II. Determination of the contents of α_1 -acid glycoprotein, α_2 -macroglobulin, and albumin in serum from rats suffering from induced inflammation. *Can. J. Biochem.*, **50**, 871-880.
- Jeanloz, R.W. (1972). α_1 -acid glycoprotein. In A. Gottschalk (Ed.), *Glycoproteins*, pg. 565-611. Amsterdam, London, New York: Elsevier Publishing Company.
- Johnson, A.M., Schmid, K. and Alper, C.A. (1969). Inheritance of human α_1 -acid glycoprotein (orosomucoid) variants. *J. Clin. Invest.*, **48**, 2293-2299.
- Kalous, V. (1965). Some hydrodynamic properties of native and modified serum orosomucoids. *Biochim. Biophys. Acta*, **101**, 336-342.
- Kawahara, K., Ikenaka, T., Nimberg, R.B. and Schmid, K. (1973). The

effect of the sialyl residues on the thermodynamic and hydrodynamic properties of α_1 -acid glycoprotein. *Biochim. Biophys. Acta*, **295**, 505-513.

Kawasaki, T., Koyama, J. and Yamashina, I. (1966). Isolation and characterization of α_1 -acid glycoprotein from rat serum. *J. Biochem. (Japan)*, **60**, 554-560.

Kerkay, J. and Westphal, U. (1968). Steroid-protein interactions. XIX. Complex formation between α_1 -acid glycoprotein and steroid hormones. *Biochim. Biophys. Acta*, **170**, 324-333.

Kitamura, K. and Yamashina, I. (1972). Studies of amino acid sequences of α_1 -acid glycoprotein of human plasma: sequences of glycopeptides obtained from the NH_2 -terminal cyanogen bromide peptide. *J. Biochem.*, **71**, 63-76.

Krotoski, W.A. and Weimer, H.E. (1966). Effects of dilute acid on the chemical and immunochemical properties of human orosomucoid. *Clin. Chem. Acta*, **13**, 329-340.

Laurent, P., Miribel, L., Bienvenu, J., Vallve, C. and Arnard, P. (1984). A three-step purification of human α_1 -acid glycoprotein. *FEBS. Lett.*, **168**, 79-83.

Leatherbarrow, R.J. and Dean, P.D.G. (1980). Studies on the mechanism of binding of serum albumins to immobilized cibacron blue F3GA. *Biochem. J.*, **189**, 27-34.

Li, Y.T. and Li, S.C. (1970). Isolation and characterization of α_1 -acid glycoprotein from chimpanzee plasma. *J. Biol. Chem.*, **245**, 825-832.

- 87
- Light, A. (1974). *Protein: Structure and Function*, pg. 79-81. Englewood Cliffs, N.J.: Prentice-Hall.
- Marshall, W.E. and Porath, J. (1968). The structure of glycoproteins. *J. Biol. Chem.*, *240*, 209-217.
- Mehl, J.W. (1945). The biuret reaction of proteins in the presence of ethylene glycol. *J. Biol. Chem.*, *157*, 173-180.
- Mehl, J.W., Humphrey, J. and Winzler, R.J. (1949). Mucoproteins of human plasma. IV. Electrophoretic demonstration of mucoproteins in serum at pH 4.5. *Proc. Soc. Exptl. Biol. Med.*, *72*, 110-114.
- Morell, A.G. Gregoriadis, G., Scheinberg, I.H., Hickman, J., and Aswell, G. (1971). The role of sialic acid in determining the survival of glycoproteins in the circulation. *J. Biol. Chem.*, *246*, 1461-1467.
- Nilsson, I.M. and Yamashina, I. (1958). Effects of human α_1 -acid glycoprotein on blood coagulation. *Nature (Lond.)*, *181*, 711-712.
- Nimberg, R., Motoyama, T. and Schmid, K. (1971). The amino acid substitutions found in the genetic variants of α_1 -acid glycoprotein. *J. Biol. Chem.*, *246*, 5817-5821.
- Oss, C.J. van and Bronson, P.M. (1974). Isolation and purification of serum alpha₁-acid glycoprotein. *Prep. Biochem.*, *4*, 115-126.
- Oss, C.J. van, Gillman, C.F., Bronson, P.M. and Border, J.R. (1974). Phagocytosis-inhibiting properties of alpha₁-acid glycoprotein. *Immun. Commun.*, *4*, 321-328.
- Popnoe, E.A. (1955). Characterization of a glycoprotein in the urine of patients with proteinuria. *J. Biol. Chem.*, *217*, 61-66.

- Popenoe, E.A. (1959). The linkage of neuraminic acid in orosomucoid. *Biochim. Biophys. Acta*, *32*, 584-585.
- Popenoe, E.A. and Drew, R.M. (1957). The action of an enzyme of *Clostridium perfringens* on orosomucoid. *J. Biol. Chem.*, *228*, 673-683.
- Rimington, C. (1940). Seromucoid and the bound carbohydrate of the serum proteins. *Biochem. J.*, *34*, 931-940.
- Sarione, E.J. (1963). Synthesis of α_1 -acid glycoprotein by the isolated perfused rat liver. *Arch. Biochem. Biophys.*, *100*, 516-519.
- Schmid, K. (1950). Preparation and properties of an acidic glycoprotein prepared from human plasma. *J. Am. Chem. Soc.*, *72*, 2816.
- Schmid, K. (1953). Preparation and properties of serum and plasma proteins. XXIX. Separation from human plasma of polysaccharides, peptides, and proteins of low molecular weight, crystallization of an acid glycoprotein. *J. Am. Chem. Soc.*, *75*, 60-68.
- Schmid, K. (1954). The N-terminal residue in acid-glycoprotein. *Biochim. Biophys. Acta*, *14*, 437.
- Schmid, K. (1955). Isolation of a group of α_2 -glycoproteins from human plasma. *J. Am. Chem. Soc.*, *77*, 742-745.
- Schmid, K. (1975). α_1 -acid glycoprotein. In F.W. Putman (Ed.), *The Plasma Proteins*, 2nd. ed., pg.185-228. New York, San Francisco, London: Academic Press.
- Schmid, K. and Binette, J.P. (1961). Polymorphism of α_1 -acid glycoprotein. *Nature (Lond.)*, *190*, 630-631.
- Schmid, K., MacNair, M.B. and Burgi, A.F. (1958). The chromatographic

separation and purification of acidic proteins on carboxylated ion exchange resins. *J. Biol. Chem.*, 290, 856-864.

Schmid, K., Benzze, W.L., Nussbaumer, T. and Wehrmuller, J.O. (1959). Studies on the structure of α_1 -acid glycoprotein. *J. Biol. Chem.*, 234, 529-533.

Schmid, K., Binette, J.P., Kamiyama, S., Pfister, V. and Takahashi, S. (1962). Studies on the structure of α_1 -acid glycoprotein. III. Polymorphism of α_1 -acid glycoprotein and the partial resolution and characterization of its variants. *Biochemistry*, 1, 959-966.

Schmid, K., Polis, A., Nanziker, K., Fricke, R. and Yayoshi, M. (1967). Partial characterization of the sialic-acid free forms of α_1 -acid glycoprotein from human plasma. *Biochem. J.*, 104, 361-368.

Schmid, K., Okuyama, T. and Kaufmann, H. (1968). Isolation and chemical composition of the human plasma α_1 -acid glycoprotein variants. *Biochim. Biophys. Acta*, 154, 565-572.

Schmid, K., Kaufmann, H., Isemuro, S., Bauer, F., Emura, J., Motoyama, T., Ishiguro, M. and Nanno, S. (1973). Structure of α_1 -acid glycoprotein. The complete amino acid sequence, multiple amino acid substitutions, and homology with immunoglobulins. *Biochemistry*, 12, 2711-2724.

Schmid, K., Burgi, W., Collins, J.H. and Nanno, S. (1974). The disulfide bonds of α_1 -acid glycoprotein. *Biochemistry*, 13, 2694-2697.

Schmid, K., Chen, L.H., Occhino, J.C., Foster, J.A. and Sperandio, K. (1976). Topography of human plasma α_1 -acid glycoprotein.

Biochemistry, 15, 2245-2254.

Schmid, K., Nimberg, R.B., Kimura, A., Yamaguchi, H. and Binette, J.P. (1977).

The carbohydrate units of human plasma α_1 -acid glycoprotein. *Biochim. Biophys. Acta*, 492, 291-302.

Schmid, K., Binette, J.P., Tokita, K., Moroz, L. and Yoshizaki, H. (1964a). The polymorphic forms of α_1 -acid glycoprotein of normal caucasian individuals. *J. Clin. Invest.*, 43, 2347-2352.

Schmid, K., Burke, J.F., Debray-Sachs, M. and Tokita, K. (1964b). Sialic acid-deficient α_1 -acid glycoprotein produced in certain pathological states. *Nature (Lond.)*, 204, 75-76.

Schultze, H.E. and Heremans, J.F. (1966). *Molecular Biology of Human Proteins I*, pp. 250-254. New York: Elsevier Publishing Co.

Shapiro, A.L., Vinuela, E. and Maizel, J.V. Jr. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. Biophys. Res. Comm.*, 28, 815-820.

Smith, E.L., Brown, D.M., Weimer, H.E. and Winzler, R.J. (1950). Sedimentation, diffusion, and molecular weight of a mucoprotein from human plasma. *J. Biol. Chem.*, 185, 569-575.

Snyder, S. and Coodley, E.L. (1976). Inhibition of platelet aggregation by α_1 -acid glycoprotein. *Arch. Intern. Med.*, 136, 778-781.

Spath, P.J. and Koblet, H. (1979). Properties of SDS-polyacrylamide gels highly cross-linked with N,N' -diallyltartardiamide and the rapid isolation of macromolecules from the gel matrix. *Anal. Biochem.*, 89,

275-285.

Staprans, I., Anderson, C.D., Lurz, F.W. and Feltz, J.M. (1980).

Separation of a lipase cofactor from the α_1 -acid glycoprotein fraction from the urine of nephrotic patients. *Biochim. Biophys. Acta*, 617, 514-523.

Stilberg, S., Goodman, M., Kefalides, N.A. and Winzler, R.J. (1955).

Immunochemical determination of orosomucoid. *Proc. Soc. Exptl. Biol. Med.*, 90, 641-647.

Succari, M., Foguetti, M.-J. and Percheron, F. (1985). Two-step

purification of human α_1 -acid glycoprotein. *J. Chromatography*, 341, 457-461.

Swank, R.T. and Munkres, K.D. (1971). Molecular weight analysis of

oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. *Anal. Biochem.*, 39, 462-477.

Toh, H., Hayashida, H., Kikuno, R., Yasunaga, T. and Miyata, T. (1985).

Sequence similarity between EGF receptor and α_1 -acid glycoprotein. *Nature (Lond.)*, 314, 199.

Tokita, K. and Schmid, K. (1963). Variants of α_1 -acid glycoprotein.

Nature (Lond.), 200, 266.

Tokita, K., Burke, J., Yoshizaka, H., Fisher, S. and Schmid, K. (1966).

The consistency of the α_1 -acid glycoprotein variants of normal adults under conditions of severe stress. *J. Clin. Invest.*, 45, 1624-1630.

Travis, J. and Pannell, R. (1973). Selective-removal of albumin from

plasma by affinity chromatography. *Clin. Chim. Acta*, 49, 49-52.

Travis, J., Bowen, J., Tewksbury, D., Johnson, D. and Pannell, R. (1976).

Isolation of albumin from whole human plasma and fractionation of albumin-depleted plasma. *Biochem. J.*, 157, 301-306.

Weimer, H.E. and Moshin, J.R. (1953). Serum glycoprotein concentrations in experimental tuberculosis of guinea-pigs. *Am. Rev. Tuberculosis*, 68, 594-602.

Weimer, H.E. and Winzler, R.J. (1955). Comparative study of orosomucoid preparations from sera of six species of mammals. *Proc. Soc. Exp. Biol. Med.*, 90, 458-460.

Weimer, H.E., Mehl, J.W. and Winzler, R.J. (1950). Studies on the mucoproteins of human plasma. V. Isolation and characterization of a homogeneous mucoprotein. *J. Biol. Chem.*, 185, 561-567.

Weimer, H.E. and Ricé, E.H. (1957). Effects of perchloric acid upon electrophoretic and chemical composition of human orosomucoid. *Federation Proc.*, 16, no.1880, 438.

Wells, C., Cooper, E.H. and Glass, M.R. (1981). The detection of hormone-associated variations in α_1 -acid glycoprotein using concanavalin a crossed immuno-affinoelectrophoresis. In Bøg-Hansen, T.C. (Ed.), *Lectins- Biology, Biochemistry, Clinical Biochemistry*, 1, pg.339-346. Berlin, New York: Walter de Gruyter & Co.

Winberg, H. (1979). *Plasma Proteins*, pg. 340. Chichester, New York, Brisbane, Toronto: John Wiley and Sons.

Winzler, R.J. (1955). Determination of serum glycoproteins. *Methods of Biochem. Analysis*, 2, 279-311.

Winzler, R.J. (1960). Glycoproteins, pg. 309-347. In F.W. Putnam (Ed.),
The Plasma Proteins. New York, London: Academic Press.

Winzler, R.J. (1965). Metabolism of glycoproteins. *Clin. Chem.*, - 11,
339-347.

Winzler, R.J., Devor, A.W., Mehl, J.M. and Smyth, I.M. (1948). Studies
on the mucoproteins of human plasma. I. Determination and
isolation. *J. Clin. Invest.*, 27, 609-616.

Yamashina, I. (1966). On the carbohydrate components of the α_1 -acid
glycoprotein of human plasma. *Acta Chem. Scand.*, 10, 1666-1668.

Yoshizaka, H., Hunziker, K. and Schmid, K. (1969). The constancy of the
types of α_1 -acid glycoprotein variants in patients with hysterectomy
and irradiation. *Clin. Chim. Acta*, 23, 147-151.

APPENDIX

The 156^{Met,Val} genotypes for the mating pairs and offspring are given in the following pages. The sample numbers are all FS numbers unless otherwise indicated.

<u>sample number</u>	<u>genotype</u>	<u>sample number</u>	<u>genotype</u>
1812	Val,Val	2314	Val,Val
1817	Val,Val	2624	Val,Val
1820	Val,Val	2625	Met,Val
1824	Met,Met	2632	Met,Val
1825	Val,Val	2633	Val,Val
1826	Val,Val	2635	Val,Val
1827	Val,Val	2647	Met,Val
1828	Val,Val	2649	Val,Val
1829	Val,Val	2665	Met,Val
1830	Val,Val	2666	Met,Val
1839	Val,Val	2709	Val,Val
1845	Val,Val	2710	Met,Val
1846	Val,Val	2711	Val,Val
1847	Val,Val	2712	Met,Val
1849	Val,Val	1713	Met,Val
2258	Val,Val	2715	Met,Val
2259	Met,Val	2716	Val,Val
2260	Met,Val	1717	Met,Val
2261	Met,Val	1718	Met,Val
2262	Met,Val	2969	Met,Val
2277	Val,Val	2970	Met,Val

<u>sample number</u>	<u>genotype</u>	<u>sample number</u>	<u>genotype</u>
2311	Val,Val	2972	Val,Val
2312	Val,Val	2973	Met,Met
2313	Val,Val	2974	Met,Val
2975	Met,Val	4424	Met,Val
3267	Met,Val	4460	Val,Val
3272	Val,Val	4463	Met,Val
3273	Val,Val	4539	Val,Val
3274	Met,Val	4540	Met,Val
3381	Val,Val	4541	Met,Val
3570	Val,Val	4542	Met,Val
3571	Val,Val	4543	Met,Met
3572	Met,Val	4545	Met,Val
3573	Val,Val	4562	Met,Met
3574	Val,Val	4594	Met,Val
3575	Met,Val	4595	Met,Val
3577	Met,Val	4596	Met,Met
3579	Met,Val	4597	Met,Val
3581	Met,Val	4598	Met,Val
3583	Met,Val	4599	Val,Val
4399	Met,Val	4600	Val,Val
4400	Met,Val	4654	Met,Met

<u>sample number</u>	<u>genotype</u>	<u>sample number</u>	<u>genotype</u>
4402	Val,Val	4655	Met,Val
4403	Val,Val	4661	Met,Met
4404	Met,Val	4662	Met,Val
4405	Val,Val	4663	Met,Val
4411	Met,Val	4664	Met,Val
4415	Met,Val	4665	Met,Val
4666	Met,Val	5684	Met,Val
4667	Val,Val	5685	Val,Val
4668	Met,Met	5686	Val,Val
4815	Val,Val	6417	Val,Val
4816	Met,Val	6418	Met,Val
4817	Val,Val	6419	Val,Val
4833	Val,Val	6468	Met,Val
4835	Met,Val	6482	Val,Val
4836	Val,Val	6493	Val,Val
4840	Met,Val	6494	Met,Val
4842	Val,Val	6495	Val,Val
4843	Val,Val	7162	Met,Val
5531	Val,Val	7173	Val,Val
5532	Val,Val	7174	Val,Val
5533	Val,Val		

<u>sample number</u>	<u>genotype</u>	<u>sample number</u>	<u>genotype</u>
5534	Met,Val	BB.1213	Val,Val
5535	Met,Val	BB 1231	Val,Val
5617	Met,Val	BC 1209	Val,Val
5622	Val,Val	BC 1210	Val,Val
5661	Met,Val	BC 1211	Val,Val
5662	Met,Val	BC 1212	Val,Val
5681	Val,Val		
5682	Val,Val		
5683	Val,Val		

