Studies on the Intracellular Mechanism of Biosynthesis of Rat α_1-Acid Glycoprotein

by

A.D. Friesen, B.Sc.(H), M.Sc.

A Thesis submitted to the Faculty of Graduate Studies in partial fulfilment of the requirement for the Degree of Doctor of Philosophy

Department of Chemistry

Winnipeg Manitoba

June, 1982

STUDIES ON THE INTRACELLULAR MECHANISM OF BIOSYNTHESIS OF RAT α_1-ACID GLYCOPROTEIN

BY

ALBERT D. FRIESEN

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

The mechanism of glycosylation of rat α_1-acid glycoprotein (α_1-AGP) was studied by following the kinetics of incorporation of radiolabelled mannose into α_1-AGP isolated from the rough membrane, smooth membrane and Golgi fractions of rat liver. The study showed that the total protein fraction and, more specifically, α_1-AGP associated with rough membrane fractions had the highest specific radioactivities at early times of incorporation. One explanation for the kinetic data is that α_1-AGP exists in a high mannose form at early times of assembly within the channels of the rough endoplasmic reticulum. This idea was confirmed by the isolation of a high mannose-containing intracellular form of α_1-AGP from rough membrane fractions of liver. This form (referred to as form 1) contained 53 residues of hexose (mainly mannose) compared with 35 residues of hexose (composed of about equal amounts of mannose and galactose) which are present in serum α_1-AGP. Further studies involving electrophoresis on SDS polyacrylamide gels indicated the presence of a second intracellular form of -AGP (referred to as form 2). This form had an amino acid composition similar to that found in form 1, but had much lower mannose content. Both forms of intracellular α_1-AGP were detected in rough, smooth and Golgi fractions from liver. The results from carbohydrate analysis showed that α_1-AGP is initially synthesized as high mannose-containing species and is rapidly processed by removal of mannose to form a low mannose-containing species before addition of

terminal sugar residues occur to form the secreted serum protein. Amino acid analyses indicated that the intracellular forms of α_1-AGP isolated from rough membrane, smooth membrane and Golgi fractions all contained similar amino acid compositions and that the composition of intracellular α_1-AGP contained an additional complement of amino acids which were not present in serum α_1-AGP. One explanation for the additional segment of amino acids is that the intracellular forms of α_1-AGP contained an amino acid extension of the pre- or pro-type. The existence of a pro-type extension was supported by the observation that the additional amino acid segment could be removed by a limited tryptic treatment which had been shown to convert proalbumin to serum-type albumin.

Abbreviations

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
UDP	Uridine 5'-diphosphate
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
CM-cellulose	Carboxymethyl cellulose
ConA-Sepharose	Concanavalin A-Sepharose
α_1-AGP	α_1-acid glycoprotein
RER	Rough endoplasmic reticulum
SER	Smooth endoplasmic reticulum
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
V	Volt
EDTA	Ethylenediamine tetraacetate
Asp	Aspartic acid
Asn	Asparagine
Asx	Asparagine or aspartic acid
Thr	Threonine
Ser	Serine
Glu	Glutamic acid
Gln	Glutamine
Glx	Glutamine or glutamic acid
Pro	Proline
Cys	Cysteine
Gly	Glycine
Ala	Alanine
Val	Valine
Met	Methionine
Ile	Isoleucine
Leu	Leucine
Tyr	Tyrosine
Phe	Phenylalanine
His	Histidine
Lys	Lysine
Arg	Arginine
Glc	Glucose
Man	Mannose
Gal	Galactose
GlcNAc	N-acetyl glucosamine
NeuAc	N-acetyl neuraminic acid

TABLE OF CONTENTS

INTRODUCTION

Plasma proteins

The term "protein" was introduced by Mulder in the late 1830's after his chemical analysis of blood fibrin and other substances. Since that time plasma has been one of the most popular sources of proteins studied by biochemists and clinicians because plasma proteins are readily available with a wide variety of biological functions. The classification of plasma proteins developed in parallel with the development of fractionation techniques. The introduction of fractional precipitation with ammonium sulfate at the beginning of the 20th century resulted in the division of plasma proteins into two broad categories: "globulin" and "albumin". The globulin fraction precipitated in 48% saturated ammonium sulfate, while the albumin fraction remained in solution. These two very general terms were used extensively for protein classification in the first half of the 20th century, but they have since been replaced by more descriptive nomenclature. However, it is interesting to note that in some cases clinical diagnosis is still based on albumin: globulin ratios.

The development of the moving-boundary electrophoretic technique by Tiselius in the late 1930's (1) resulted in the separation of plasma proteins into five major fractions. Although the apparatus developed by Tiselius is no longer widely used for electrophoretic characterization of plasma proteins, the classification into albumin,

α_1-, α_2-, β and γ-globulins on the basis of electrophoretic mobilities is still a standard procedure in the field of plasma protein chemistry. Characterization of plasma proteins has, however, become much more sophisticated as a result of the very rapid development of numerous protein separation and characterization techniques. For example, T. Svedberg (2) developed the analytical ultracentrifuge which separated proteins on the basis of size and shape; Sober and Peterson (3) made ion exchange chromatography of plasma proteins possible by the introduction of DEAE- and CM-cellulose; Porath's (4) development of cross linked dextran beads, trade name "Sephadex", led to chromatographic separation of plasma proteins on the basis of size; Grabar and Williams (5) introduced the powerful method of immunoelectrophoresis; and Vesterberg and Svensson (6) introduced the isoelectric focussing technique of separating proteins based on their isoelectric points.

Despite the enormous volume of publications on isolation and characterization of plasma proteins and studies on their metabolism and biosynthesis, very few of the more than one hundred proteins in plasma have as yet had recognized functions assigned to them. With the exception of albumin and the retinal-binding protein, most well characterized plasma proteins are glycoproteins which contain one or more covalently bound oligosaccharide chains. Although serum glycoproteins have been the subject of research for many years (7,8), interest in these molecules has increased substantially in the last decade mainly because they represent a group of molecules which appear to

have well defined biological functions. Schwick et al.(9) have recently listed and described fifty-two well characterized plasma glycoproteins, information obtained primarily from the literature of the 1970's. The functions assigned to these glycoproteins are as diverse as the proteins themselves and include transport of metal ions (e.g. transferrin and ceruloplasmin), blood coagulation (fibrinogen and the blood clotting factors) and defence against invasion of the host by foreign cells or molecules [(immunoglobulins and complement factors (9,10)]. Another group of plasma glycoproteins for which a biological role has not been clearly elucidated, but which have been implicated with a broad group of pathological conditions, are the plasma glycoproteins that are elevated during the acute inflammatory process (11). These proteins are now referred to as acute phase reactants. This group includes proteins such as haptoglobin, fibrinogen and α_1-AGP. The latter acute phase protein is the one studied in this thesis and since the acute phase response is used as a means of elevating liver and serum levels of α_1-AGP prior to isolation, an understanding of the main features of the inflammatory process is of importance to the present studies.

The acute inflammatory response

In mammals, inflammation may be caused by chemical inflammatory agents, neoplastic diseases, bacterial infections or endotoxin injections, rheumatoid arthritis, major surgery, and mechanical injury

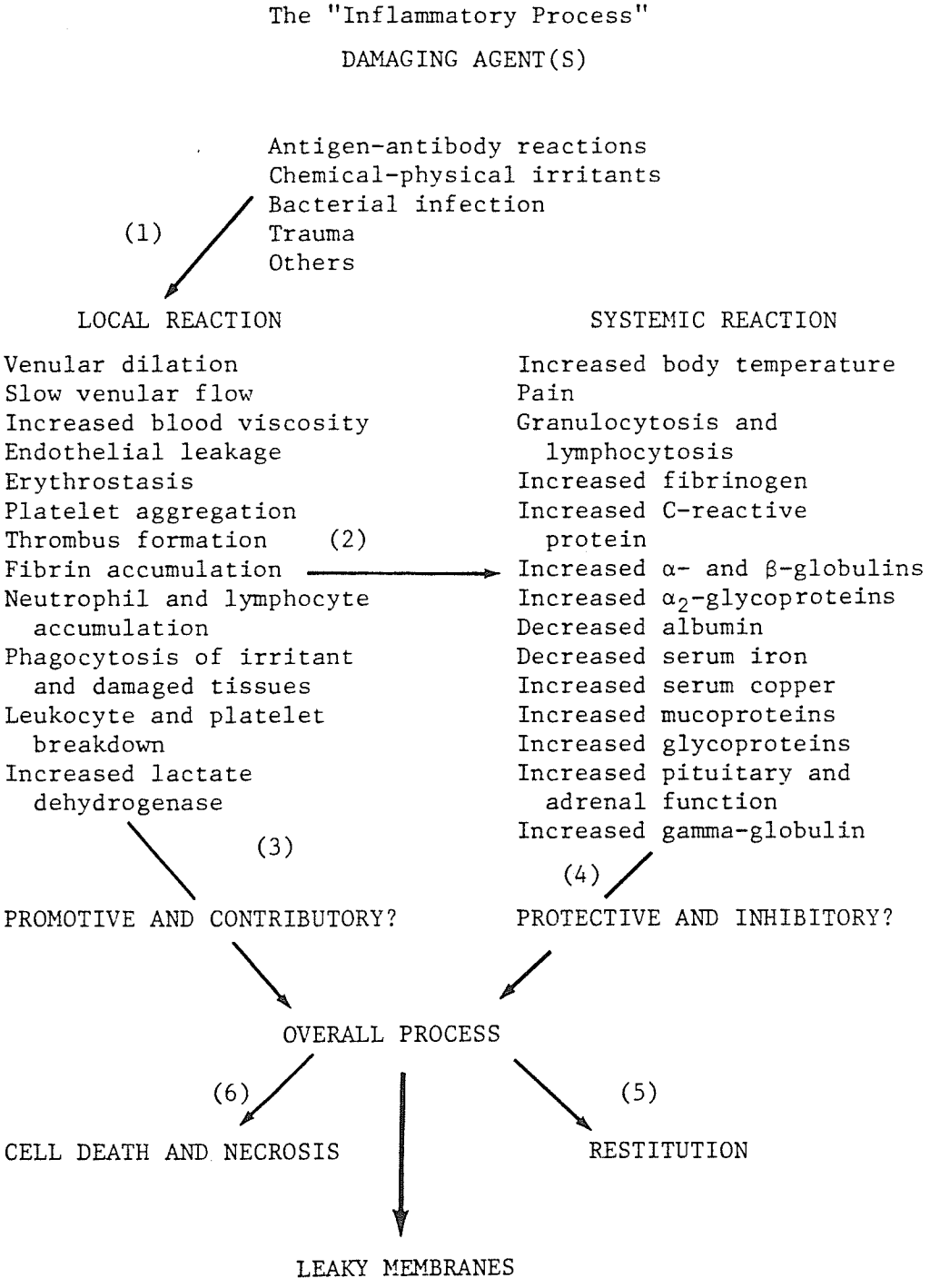
(11-13). Although the exact biochemical mechanisms of the acute phase response remain unknown, a great deal of information has accumulated about the events which occur. These events are appropriately summarized by the scheme proposed by Glenn et al.(14) and shown in Fig. 1. In brief, Glenn et al.(14) separated the acute inflammatory response into two reactions, the local reaction in the immediate area of tissue damage, and the systemic reaction induced by the local reaction.

The first events in the local reaction to inflammation are believed to be venular dilation and alterations in blood flow (15). This is followed by platelet aggregation, the formation of thrombi, and then the migration of neutrophil and lymphocyte cells into the area of tissue damage (16). This process is thought to be accompanied by a release of cytoplasmic and lysosomal enzymes into the extracellular compartment of the damaged tissue (17). The released lysosomal enzymes appear to activate cutaneous collagenase which degrades collagen in the structural components of tissue into soluble breakdown products which then drain away from the area of tissue damage (18). Weissman (19) has referred to the release of lysosomal enzymes as the "final common pathway" in inflammation representing a common "trigger" mechanism linking a variety of injuries with the systemic response.

The systemic response consists, primarily, of increased synthesis of the acute phase reactants. A considerable body of information has

Fig. 1 Schematic representation of the inflammatory process. The numbers in parenthesis describe the order in which the events are believed to occur. Taken from (14).

The "Inflammatory Process"



been accumulated on the changes in plasma levels of acute phase reactants found following inflammation. Table 1 lists the changes induced by trauma for some of the more important acute phase reactants. At least two common features have been ascribed to these acute phase reactants; almost all are glycoproteins with a significant amount of carbohydrate and all are synthesized in liver parenchymal cells (20-22). The local reaction to injury is believed to result in the formation of hormonal-like factors which are transported by blood (23) to the liver where they stimulate increased synthesis of the acute phase reactants (11,24). Eddington et al. (25) have suggested that polymorphonuclear leukocytes may be the source of factors which exert an indirect effect on the stimulation of the liver by formation (or release) of such chemical mediators as pyrogens (26), histamines (27), lymphokines (26) or other unidentified products of proteolysis. Leukocyte factors, collectively referred to as lymphokines or cytokines,have been known to be involved in the general response to tissue injury for some time (28). Recent studies have been directed at the isolation of some of these leukocyte factors in order to delineate their mechanisms of action during the acute phase response. One such factor, termed leukocyte endogenous mediator, is now known to be released by stimulated polymorphonuclear leukocytes. Crude preparations of leukocyte endogenous mediator have been shown to induce many of the changes observed following inflammation and it is believed that leukocyte endogenous mediator

Table 1

Proteins of human plasma showing altered concentrations after trauma[a]

Protein		% of preoperative values
Increased:	Fibrinogen	>200
	Haptoglobin	206
	orosomucoid[b]	>200
	C-reactive protein	>200
	α_1-anti-trypsin	>200
	slow α-globulin	173
	complement	122
	ceruloplasmin	124
Decreased:	Albumin	80
	α-lipoprotein	--
	β-lipoprotein	77
	Transferrin	78
	thyroxine-binding globulin	69

[a] The information in the table was taken from (13). The data were obtained from protein concentrations in plasma of patients who had undergone minor surgery 8 h or more before the second blood sample was collected.

[b] Orosomucoid is α_1-acid glycoprotein which is the major component of the seromucoid fraction of serum or plasma. The seromucoid fraction consists of those proteins soluble in 0.6 M perchloric acid, but precipitated by 5% phosphotungstic acid.

may contain components that are mediators of the acute phase response (29). The mechanism by which hormones and hormonal-like factors increase hepatic synthesis of acute phase reactants is not clear. A number of groups have shown that turpentine induced inflammation dramatically increases mRNA synthesis (30-34). Taylor's group (34) recently reported that hepatic levels of the mRNA for α_1-AGP increased up to 90-fold at about 36 h after induction of inflammation. In addition, it has been shown that actinomycin D, an inhibitor of transcription, effectively blocks the elevated synthesis of fibrinogen normally found following inflammation (35). These studies strongly suggested that there is increased formation of mRNA transcripts for acute phase reactants during inflammation. Since serum cortisol levels are known to increase within a few hours following injury and since it has been shown that cortisol can enhance transcription of DNA to produce more functional mRNA,attention has been directed at the possible involvement of hormones like cortisol in the acute phase response (36, 37). Unfortunately, attempts to duplicate the acute phase response with cortisol alone have usually resulted in a general increase in liver protein synthesis. For example, John and Miller (23) successfully elevated synthesis of fibrinogen, haptoglobin and an α_1-globulin with cortisol in an <u>in vitro</u> perfusion system, but albumin, which is a negative acute phase protein, was also increased. In addition, inclusion of insulin and growth hormone with cortisol enhanced the stimulation of protein synthesis observed

by cortisol alone, but no stimulation was observed by these hormones without cortisol. Thus, although cortisol may be involved in the acute phase response, the current ideas suggest that other factors, such as those derived from leukocytes, are needed for full expression of the acute phase response to inflammation.

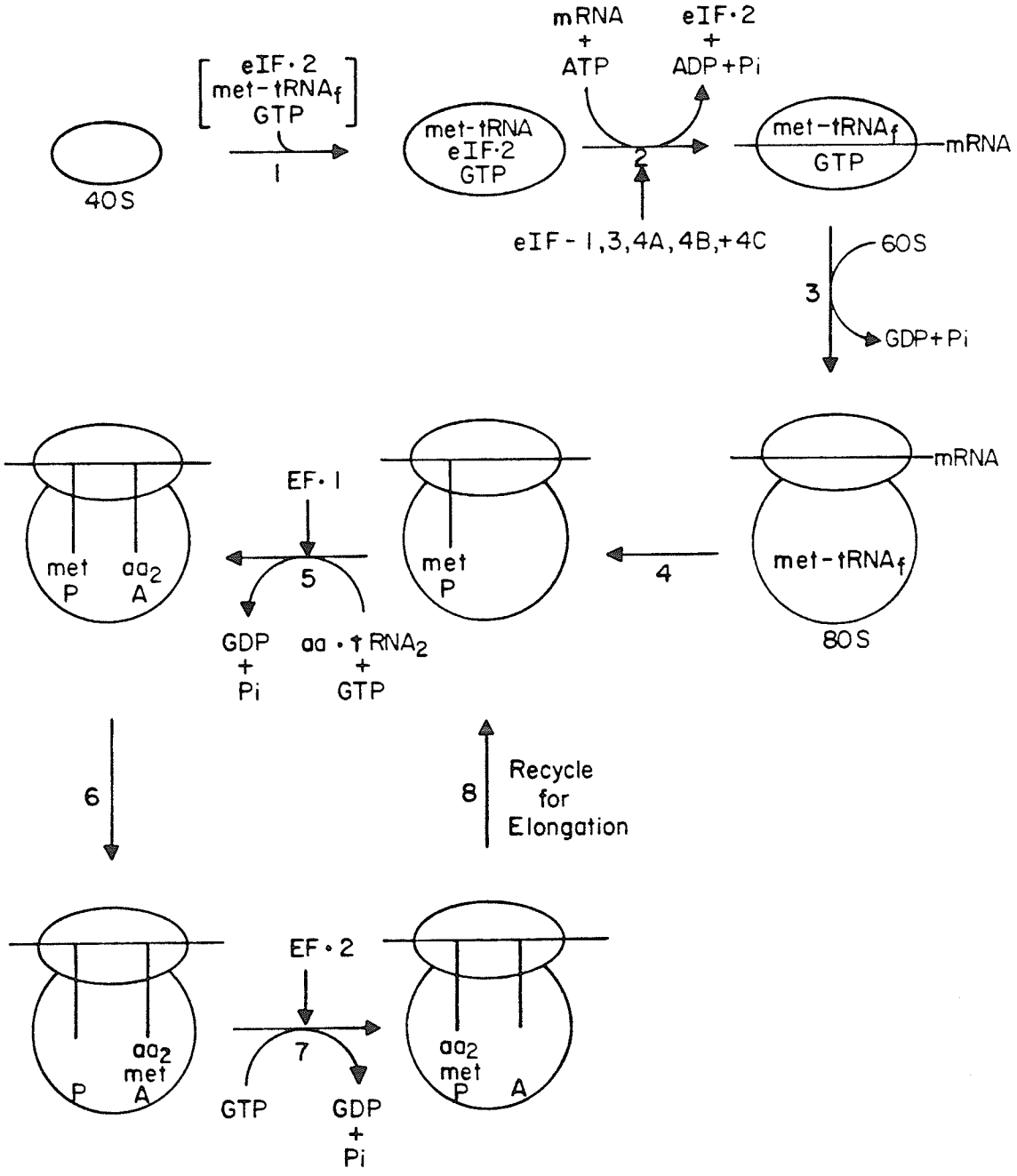
Protein synthesis-polypeptide

The basic principles and many of the details of protein biosynthesis are now well understood (38-41). In eukaryotic cells, the genetic information encoded in the cellular DNA is mainly confined to the nucleus while protein synthesis takes place mainly in the cytoplasm. The genetic information is transferred from DNA by transcription to precursor RNA molecules which are subsequently modified to produce translatable mRNA, tRNA or ribosomal RNA (rRNA). It is believed that conversion of precursor RNA forms occurs in the nucleus and the modified forms pass into the cytoplasm where they are operative for the synthesis of proteins. The current ideas on elongation of polypeptide chains in eukaryotic systems, such as liver, are outlined in Fig. 2. It now appears that, in general, secretory proteins such as the acute phase glycoproteins, and some intracellular proteins, such as lysosomal hydrolases, are manufactured on polyribosomes bound to the endoplasmic reticulum membranes, whereas most intracellular proteins are manufactured on free polyribosomes in the cytoplasm of the cell (42,43). Albumin was the first secretory protein to be studied in detail in liver. It was found

Fig. 2 Initiation and elongation reactions in eukaryotic protein synthesis (38,39).

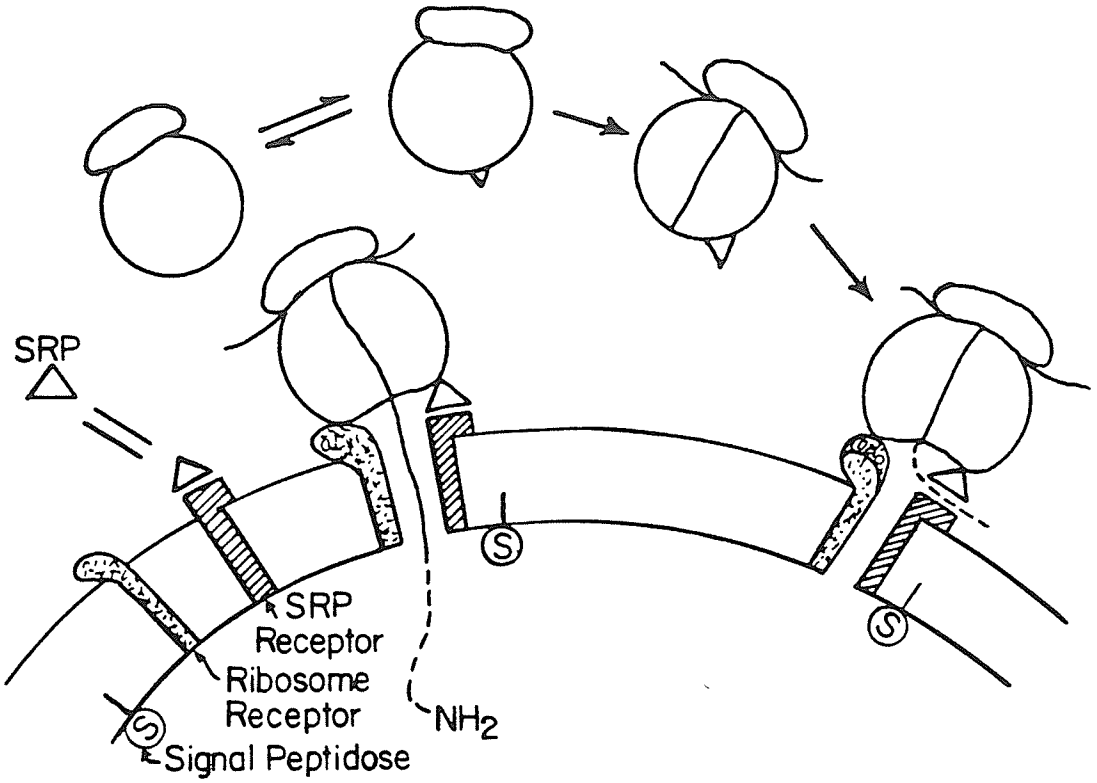
Initiation involves the formation of a complex involving formylmethionine tRNA (met-$tRNA_f$) mRNA, a 40S ribosomal subunit and initiation factors (eIF factors) with energy for the reactions supplied by GTP and ATP (reactions 1 and 2). An 80S complex is then formed by interaction of a 60S subunit with the 40S initiation complex (reaction 3).

Elongation begins with the met-$tRNA_f$ occupying the peptidyl site (P) in the 60S subunit in such a way that the tRNA anticodon pairs with the AUG codon on mRNA (reaction 4). Elongation proceeds with the incoming aminoacyl tRNA occupying the aminoacyl site (A) on the 60S ribosome subunit followed by the transfer reaction (reaction 5 and 6) in which the methionine forms a peptide bond with the incoming aminoacyl tRNA. The next phase of elongation is the translocation of the peptide into the peptide binding site (reaction 7). Elongation continues by repeated cycling of reactions 5-8. Polypeptide chain termination eventually occurs when the mRNA termination codons UAA, UGA or UAG arrive in position to be read by the incoming aminoacyl tRNA.

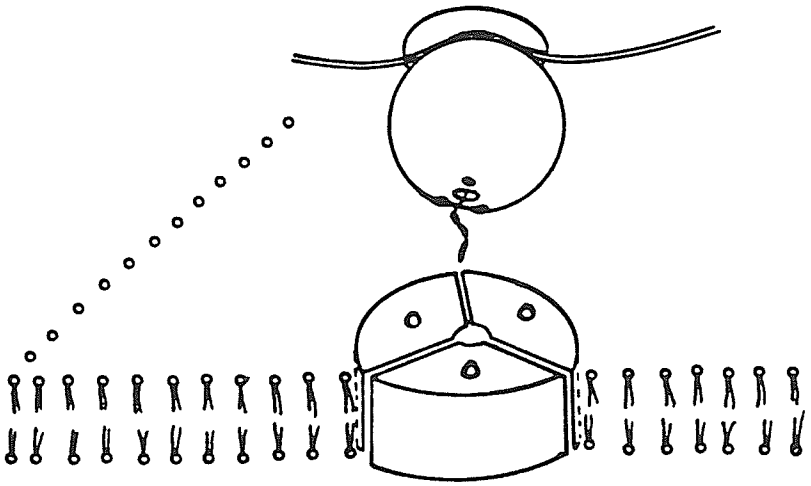

40S
eIF·2
met-tRNA$_f$
GTP
1
met-tRNA
eIF·2
GTP
mRNA
+
ATP
eIF·2
+
ADP+Pi
2
met-tRNA$_f$
GTP
mRNA
eIF-1,3,4A,4B,+4C
3
60S
GDP+Pi
mRNA
met-tRNA$_f$
80S
4
met
P
EF·1
5
GDP
+
Pi
aa·tRNA$_2$
+
GTP
met
P
aa$_2$
A
6
8
Recycle
for
Elongation
aa$_2$
met
P
A
EF·2
7
GTP
GDP
+
Pi
aa$_2$
met
P
A

that the polypeptide was synthesized mainly on bound polyribosomes and was transferred into the intracisternal space of the RER and subsequently secreted from the cell by transfer from the channels of the RER to the channels of the SER and finally to the Golgi complex before secretion from the cell (44-47). Rat α_1-AGP has been shown to be secreted from liver in a similar way (48). The mechanism of polypeptide chain translocation through the membrane of the RER has been studied by several groups of workers (49-55) and has resulted in the development of the signal hypothesis which provides a mechanistic basis for transfer of nascent polypeptide chains through membranes. Fig. 3 outlines the current ideas on the signal hypothesis as presented by Blobel and Dobberstein (49,53-55). Briefly, proteins that are destined for secretion from the cell are synthesized initially on free polyribosomes. The mRNA for a secretable protein contains a unique sequence which codes for an N-terminal signal peptide consisting of 15-30 amino acids. The emergence of this signal peptide triggers the attachment of the ribosome to the membrane. This process of attachment requires a second protein termed the "signal recognition protein" (SRP) in order to cause a specific interaction between the polyribosome and the endoplasmic reticulum membrane (Fig. 3). Once the complex is formed a channel is created in the membrane to allow passage of the nascent polypeptide either into the lumen, or into the membrane of the endoplasmic reticulum. The signal is thought to be removed by a signal pepti-

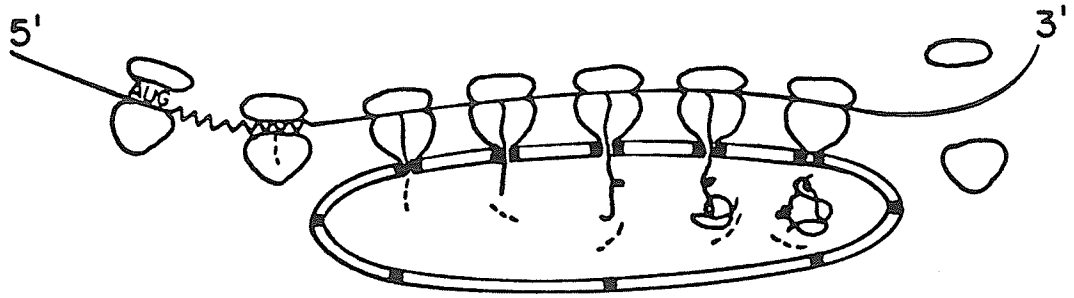
Fig. 3 The signal hypothesis as proposed by Blobel and Dobberstein (49,54,55). According to the scheme polypeptide synthesis is initiated on free ribosomes. The mRNA for a protein that is synthesized on membranes contains a nucleotide sequence which codes for a unique peptide sequence referred to as the signal sequence (illustrated by ------). Attachment to membrane is mediated by a signal recognition protein (SRP) which binds to the ribosome and to an SRP receptor on the membrane; the SRP also has an affinity for the signal peptide and if it binds this peptide prior to the SRP receptor, transfer across the membrane is blocked (these steps are illustrated in diagram A). Successful association of ribosome and SRP with membrane results in aggregation of membrane proteins causing a tunnel to be formed through which the nascent polypeptide can pass (diagram B). The signal is rapidly removed by signal peptidases as the nascent polypeptide is elongated on the ribosome (diagram C).


SRP
SRP
Receptor
Ribosome
Receptor
Signal Peptidose
NH2
S
S
S

A



B


5'
AUG
3'

C

dase at an early stage in polypeptide chain elongation. The existence of N-terminal signal segments has been established for all eukaryotic secretory proteins so far examined (52). The signal segment is usually referred to as the "pre" peptide.

Although the amino acid composition of "pre" peptides is variable, all have been found to be rich in hydrophobic amino acids which presumably aids in transporting the protein across the highly hydrophobic membrane. Examples of some "pre" peptides are given in Table 3. Although most signal peptides have been observed on the N-terminus of secretory proteins, at least one example of an internal signal sequence has been reported (60). Ovalbumin was shown to contain an internal signal with striking homology to N-terminal cleaved signals of other oviduct secretory proteins. It was also shown that although the peptidase activity for cleavage of a signal sequence was found in the RER, ovalbumin could be isolated from a microsomal fraction with the internal uncleaved signal sequence.

Another form of protein precursor with a peptide extension on the N-terminus has been described for albumin (61). Similar N-terminus extensions have been described for other proteins, such as parathyroid hormone which are synthesized by tissues other than liver (44,59,61). These intracellular precursor forms possess N-terminal oligopeptide extensions which are situated immediately to the carboxyl side of the signal peptide (examples shown in Table 2). Cleavage of the signal peptide reveals another N-terminal peptide

Table 2

Examples of secretory protiens with intracellular amino acid extensions

Lysozyme (oviduct)(56)	Met-Arg-Ser-Leu-Leu-Ile-Leu-Val-Leu-Cys-Phe-Leu-Pro-Leu-Ala-Ala-Leu-Gly-↑-Lys-Val
Growth hormone (bovine)(57)	Met-Met-X-X-X-Pro-X-X-X-Leu-Leu-Leu-X-Phe-X-Leu-Leu-X-Leu-Pro-X-X-X-X-X-↑-X-Pro
Albumin (rat)(58)	Met-Lys-Trp-Val-Thr-Phe-Leu-Leu-Leu-Leu-Phe-Ile-Ser-Gly-Ser-Ala-Phe-Ser-↑-Arg-Gly-Val-Phe-Arg-Arg-↑↑-Glu
Parathyroid hormone (bovine)(59)	Met-Met-Ser-Ala-Lys-Asp-Met-Val-Lys-Val-Met-Ile-Val-Met-Leu-Ala-Ile-Cys-Phe-Leu-Ala-Arg-Ser-Asp-Gly-↑-Lys-Ser-Val-Lys-Lys-Arg-↑↑-Ala

↑ indicates the position where the presegment is attached to the remaining polypeptide

↑↑ indicates the position where the prosegment is attached to the remaining polypeptide

extension which is subsequently released to produce the mature form of the protein. These peptide extensions, referred to as "pro" segments differ from the presegments in that they do not contain the highly hydrophobic region, but rather are rich in basic amino acids (44,59). In both albumin and parathyroid hormone the prosegment is believed to be removed at later stages of the biosynthetic secretory pathway, but before secretion from the cells. Other examples, however, exist where the prosegment appears to remain intact following secretion. These include collagen (62), zymogens, like trypsin, and chymotrypsin (63), and the blood clotting proteins (64). For proteins in which the prosegment is removed before secretion, conversion to the mature form is believed to occur in the Golgi complex (44). Edwards et al.(65) tested various subcellular fractions from rat liver for the ability to convert exogenous radioactive proalbumin into native serum albumin. They found that RER contained very little converted proalbumin, SER contained about equal proportions of pro- and native serum albumin, Golgi contained about twice as much native serum albumin as precursor and only native serum albumin was detected in the cytoplasmic fraction. Conversion from the pro- to the secreted form appears to occur in the Golgi complex or in a Golgi-derived vesicle just prior to secretion (66). It is not yet known whether the amino acids of the prosegment extensions are removed sequentially during conversion or whether several oligopeptides are released en bloc. Quinn et al.(61) showed that for

albumin isolated from the rat liver microsome fraction the prosegment could be removed *in vitro* by trypsin or other "serine" proteases. The function of the prosegment remains unknown, although it has been suggested (67,68) that the positively charged amino acid prosegment could form tight salt linkages with negatively charged phosphatidyl-serine in the membrane anchoring the polypeptide to the membrane. Both Judah *et al.*(69) and Schreiber *et al.* (68) have suggested that cleavage of the prosegment from proalbumin may serve to regulate the flow of secreted albumin into the blood stream. The prosegment released during the conversion of proalbumin to albumin may mediate a feed back control on the rate of conversion. The third function postulated for a prosegment is to facilitate the formation of the correct tertiary structure (70).

Several functions for general compartmentalization of protein have been presented in Shore and Tata's (43) review on protein synthesis. Concentration of protein within the lumen of the endoplasmic reticulum presumably serves to properly channel this protein through the secretory apparatus. Certain proteins are also required inside membrane vesicles like the endoplasmic reticulum, Golgi and mitochondria. The signal hypothesis of Blobel and Dobberstein (49) describes a mechanism for the polypeptide insertion into these vesicles. Localization of proteins in close proximity to membrane enzymes can serve to appropriately orient nascent polypeptide for modification by proteolytic cleavage, disulphide bridge

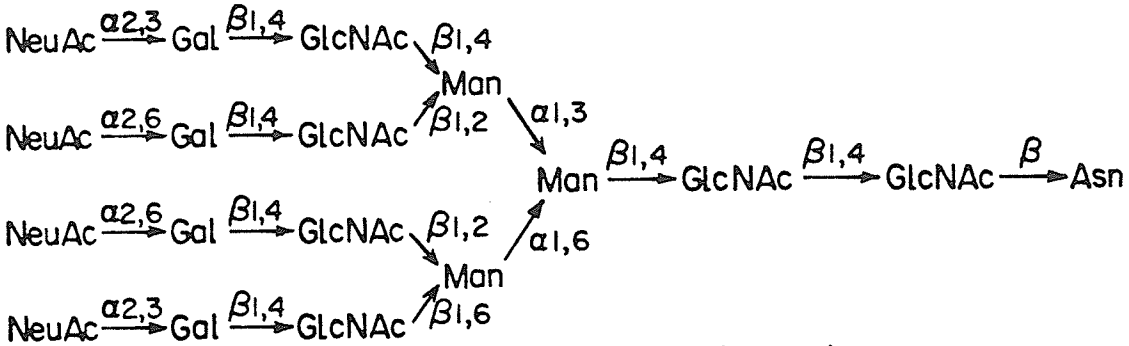
formation, or further chemical modification such as phosphorylation, hydroxylation, iodination, lipidation and glycosylation. Clearly, although all of these chemical modifications are of great significance it would be impossible to discuss them in this review. The one exception is glycosylation, an understanding of which is important for the work described in this thesis.

Glycoprotein synthesis-glycosylation

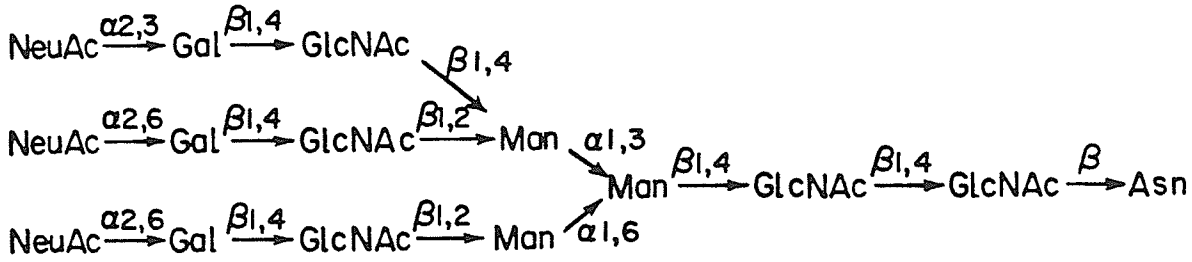
The weight of evidence suggests that the carbohydrate chains of serum glycoproteins are covalently linked to polypeptide via an N-glycosylamine bond involving the amide group of asparagine and the hydroxyl on C-1 of a GlcNAc residue. This type of linkage between polypeptide and carbohydrate was first characterized by Marshall and Neuberger (71) in the early 1960's and has since been found widely distributed in nature (72).

The asparagine-linked oligosaccharides of serum glycoproteins fall into two general categories (72): the simple or high mannose-type and the complex-type. Examples of the different types of structures are shown in Fig. 4. It can be seen that the simple type of chain contains only two sugars: mannose and GlcNAc, whereas the complex type contains, in addition, a characteristic terminal triplet of sugars with the sequence GlcNAc-Gal-NeuAc. In addition, complex chains can also contain the deoxy sugar fucose. Both types of N-glycosidically linked oligosaccharides appear to have the common core structure shown in Fig. 5. This suggests a common

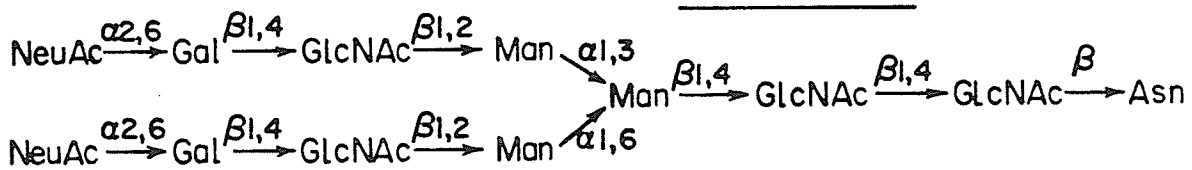
Fig. 4 Structures of N-glycosidically linked oligosaccharide structures. The high mannose structure shown is found in bovine thyroglobulin, but a variety of this type of structure with varying amounts of mannose and varying degrees of branching has been characterized. The complex type of oligosaccharide chains are of the bi-, tri-, and tetra-antennary type. The bi- and tri-antennary structures have been reported to be present in rat serum α_1-AGP and the tetra-antennary was found in human α_1-AGP. Taken from (72).



tetraantennary

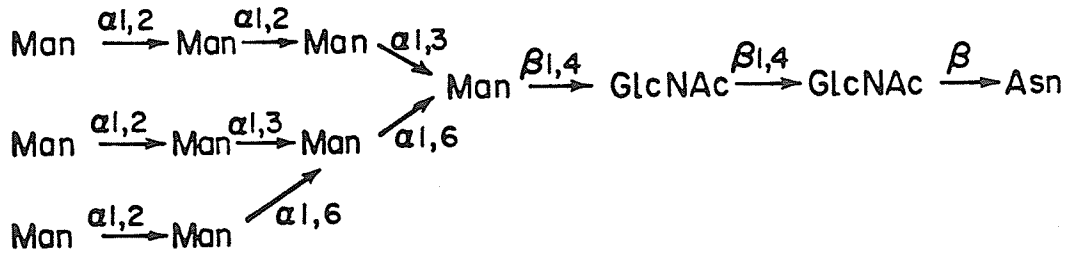


triantennary



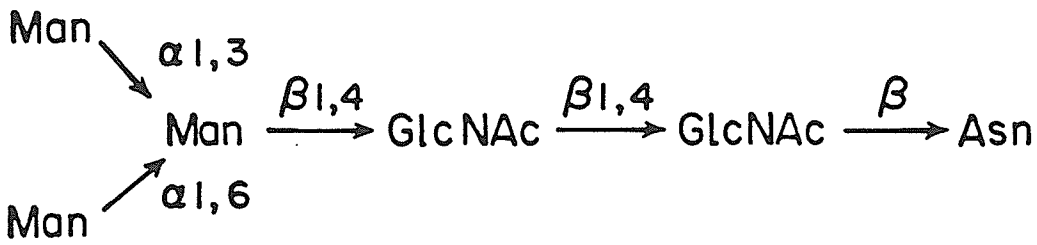
biantennary

Complex



High mannose

Fig. 5 Oligosaccharide core structure of asparagine linked glycoprotein sugar chains. Taken from (72).



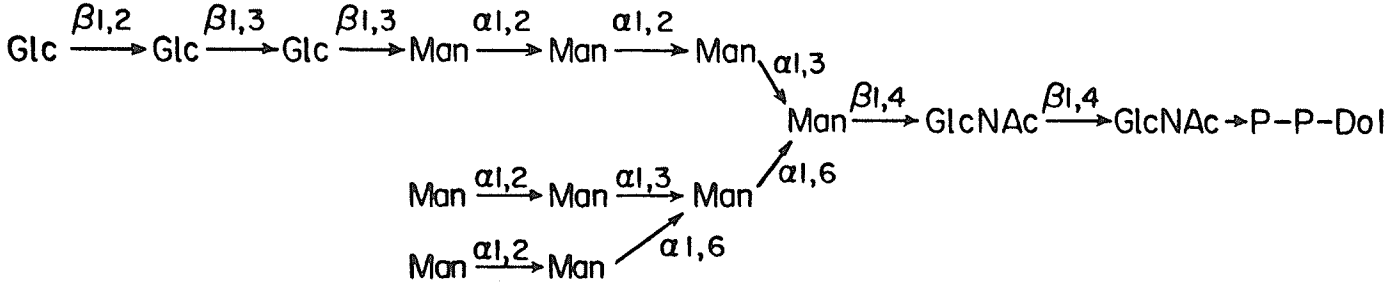
Core Structure

biosynthetic pathway for at least the internal region of the saccharide chain. Before the 1970's the most widely accepted theory for the biosynthesis of glycoproteins assumed that the oligosaccharide chain was assembled by the stepwise addition of sugars from nucleotide sugar derivatives to the growing oligosaccharide chain. The sugar additions were thought to be specified by glycosyltransferase-catalyzed reactions as the polypeptide chain was synthesized and secreted within the lumen of the endoplasmic reticulum and Golgi apparatus. This concept is still accepted as the mechanism for the addition of terminal triplet sugars of complex oligosaccharide chains, but not for the synthesis of the core regions of these chains. Studies since 1970 [for a review see Parodi et al.(74)] have demonstrated that the core oligosaccharide is preassembled on a lipid carrier. After assembly on the lipid carrier is complete, the oligosaccharide is transferred en bloc to the protein acceptor. A mechanism involving preassembly of activated lipid derivatives of various saccharides in the synthesis of a variety of complex glycans has been known to exist in bacteria for some time (75). In 1970 Behrens and Leloir (76) provided the first firm evidence that a similar mechanism was involved in eukaryotic systems. The work of Leloir's group (74) and Lennarz's group (77) has shown that the hydrophobic moiety of the intermediate complex is a compound from a family of polyisoprenol alcohols with about 17 to 23

isoprene units, known as the dolichols. Hemming's (78) group demonstrated that other lipids could also function as acceptors in liver, but dolichol phosphate was the most efficient. More recently, retinyl phosphate has also been shown to act as an acceptor of mannose in liver, but it is not yet clear what role these complexes play in the synthesis of liver glycoproteins (77,79). The assembly of the oligosaccharide chains of glycoproteins involving phosphorylated polyisoprenol sugar intermediates can be considered in three steps: (1) assembly of the intermediate phosphorylated dolichol carbohydrate complex; (2) transfer of the oligosaccharide from dolichol-oligosaccharide to polypeptide; (3) removal of some sugar residues by glycosidases in a process referred to as oligosaccharide processing, followed by synthesis of terminal triplet structures.

1. Assembly Parodi et al.(80) in 1972 reported that a glucose-containing lipid-linked oligosaccharide containing approximately 20 monosaccharide units could be synthesized by liver and the oligosaccharide transferred to protein in cell-free preparations from rat liver. Subsequently, evidence from a variety of studies on liver and other tissues has led to the identification of a dolichol pyrophosphate oligosaccharide complex which represents the end product of a series of reactions before transfer of the oligosaccharide to protein (reviewed in 75 and 81). The structure of the dolichol pyrophosphate oligosaccharide is given in Fig. 6 and the series

Fig. 6 Structure of the glucose-containing dolichol-oligosaccharide complex based on that proposed by Kornfeld's group (72) and Robbins' group (73).

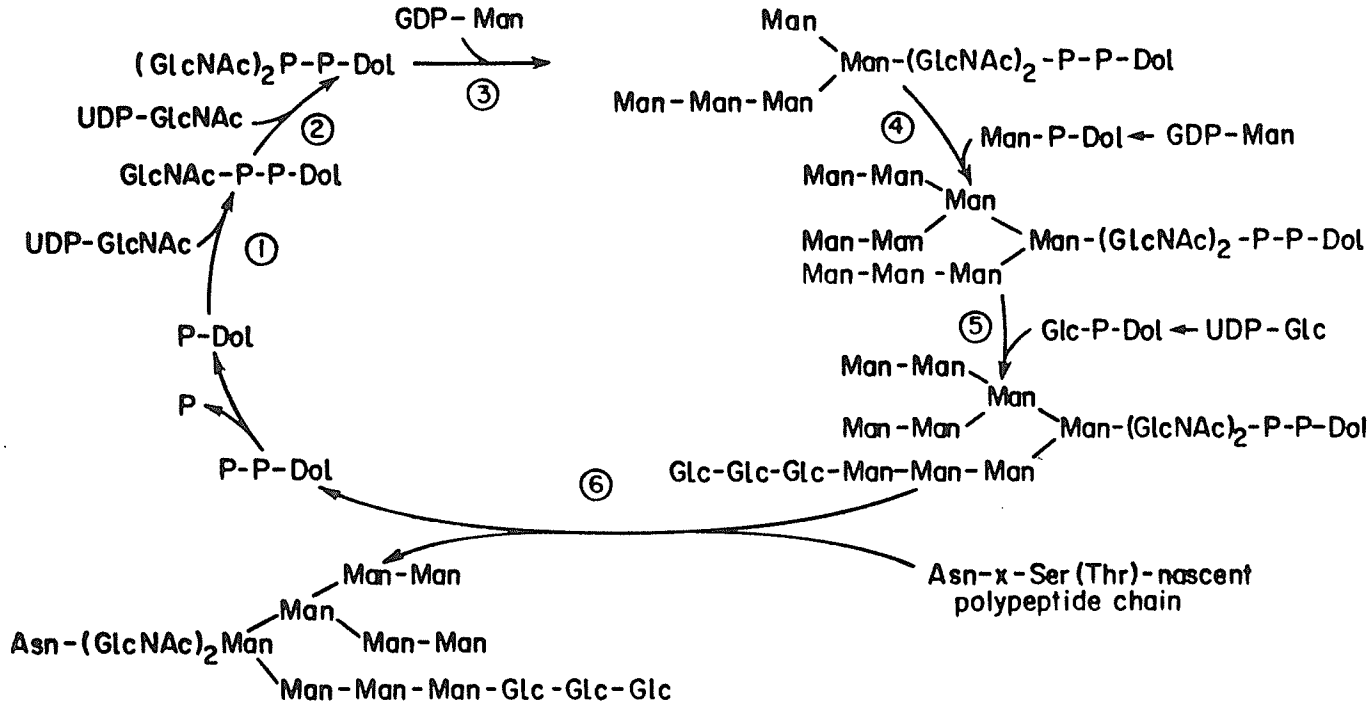

Glc
β1,2
Glc
β1,3
Glc
β1,3
Man
α1,2
Man
α1,2
Man
α1,3
Man
β1,4
GlcNAc
β1,4
GlcNAc→P-P-Dol
Man
α1,2
Man
α1,3
Man
α1,6
Man
α1,2
Man
α1,6

of reactions leading to its formation, now called the dolichol cycle, is shown in Fig. 7. The cycle can be divided into six main reactions (74) and although evidence in support of the cycle has come from sources other than liver there is evidence that all of the reactions indicated in Fig. 7 are operative in liver (74,75,82). The first two reactions involve the formation of $GlcNAc_2$-P-P-Dol with UDP-GlcNAc being the source of GlcNAc; the next two steps involve the addition of nine mannose residues with the first five residues coming from GDP-Man and the other residues coming from Man-P-Dol. The final steps in the assembly of the dolichol pyrophosphate oligosaccharide involve addition of three glucose residues, the donor being Glc-P-Dol.

2. Transfer N-Glycosylation of secretory proteins involves the transfer of the high mannose oligosaccharide from the dolichol pyrophosphate oligosaccharide complex to suitable asparagine residues in a nascent polypeptide chain. A pre-requisite for the attachment of the carbohydrate to the polypeptide is the occurrence of the acceptor asparagine in a tripeptide sequence of the type, -Asn-X-Thr (or Ser)-, referred to as the "asparagine sequon" (83). The amino acid -X- can be any of the 20 amino acids except aspartic acid (83) and proline (84) which prevent glycosylation. Pless and Lennarz (85) have suggested that an additional requirement for glycosylation of asparagine is that the polypeptide structure in the region of the sequon be unfolded.

The polypeptide moiety of serum glycoproteins is translated

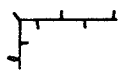
Fig. 7 The dolichol phosphate cycle showing the assembly reactions for the synthesis of a dolichol pyrophosphate oligosaccharide from which the oligosaccharide is transferred in N-glycosylation of proteins (74).


GDP-Man
(GlcNAc)2P-P-Dol
③
UDP-GlcNAc
②
GlcNAc-P-P-Dol
UDP-GlcNAc
①
P-Dol
P
P-P-Dol
⑥
Man
Man-(GlcNAc)2-P-P-Dol
Man-Man-Man
④
Man-P-Dol ← GDP-Man
Man-Man
Man
Man-Man
Man-(GlcNAc)2-P-P-Dol
Man-Man-Man
⑤
Glc-P-Dol ← UDP-Glc
Man-Man
Man
Man-Man
Man-(GlcNAc)2-P-P-Dol
Glc-Glc-Glc-Man-Man-Man
Asn-x-Ser(Thr)-nascent polypeptide chain
Man-Man
Man
Asn-(GlcNAc)2Man
Man-Man
Man-Man-Man-Glc-Glc-Glc

on membrane-bound polyribosomes and is transported across the ER membrane. The nascent polypeptide being transported across the membrane would be unfolded and therefore the appropriate acceptor asparagine sequon should be readily accessible for transfer of oligosaccharide from dolichol-pyrophosphate oligosaccharide as it emerges on the luminal side of the membrane (Fig. 8). This mechanism suggests that glycosylation of polypeptide occurs solely during elongation of the polypeptide on ribosomes. There is strong evidence that glycosylation does occur within the lumen of the ER membrane (86,87) and that at least in some cells, the transfer of oligosaccharide from dolichol pyrophosphate oligosaccharide occurs during the elongation of the polypeptide on membrane-bound polyribosomes (87-90). However, in vivo studies in liver following incorporation of glucosamine and mannose into the carbohydrate chains of nascent glycoproteins showed that transfer of oligosaccharide to polypeptide was mainly a post ribosomal event (91). The intracellular site of transfer of oligosaccharide thus remains unclear and may, in fact, involve at least two different mechanisms. The particular mechanism used may depend on the cell type, the accessibility of the sequon and the location of the oligosaccharide transferase and dolichol pyrophosphate oligosaccharide complex in the membrane.

As mentioned above the site of assembly of the lipid-oligosaccharide complex also remains unclear. Snider et al.(92) reported that in liver the enzymes involved in the dolichol

Fig. 8 Model for the concerted translation and glycosylation of secretory glycoproteins. The model shows the proposed sequence of processing of peptide and peptide-bound N-linked oligosaccharide chains from high mannose-type to complex-type. The abbreviations are:


dolichol pyrophosphate

▲ glucose

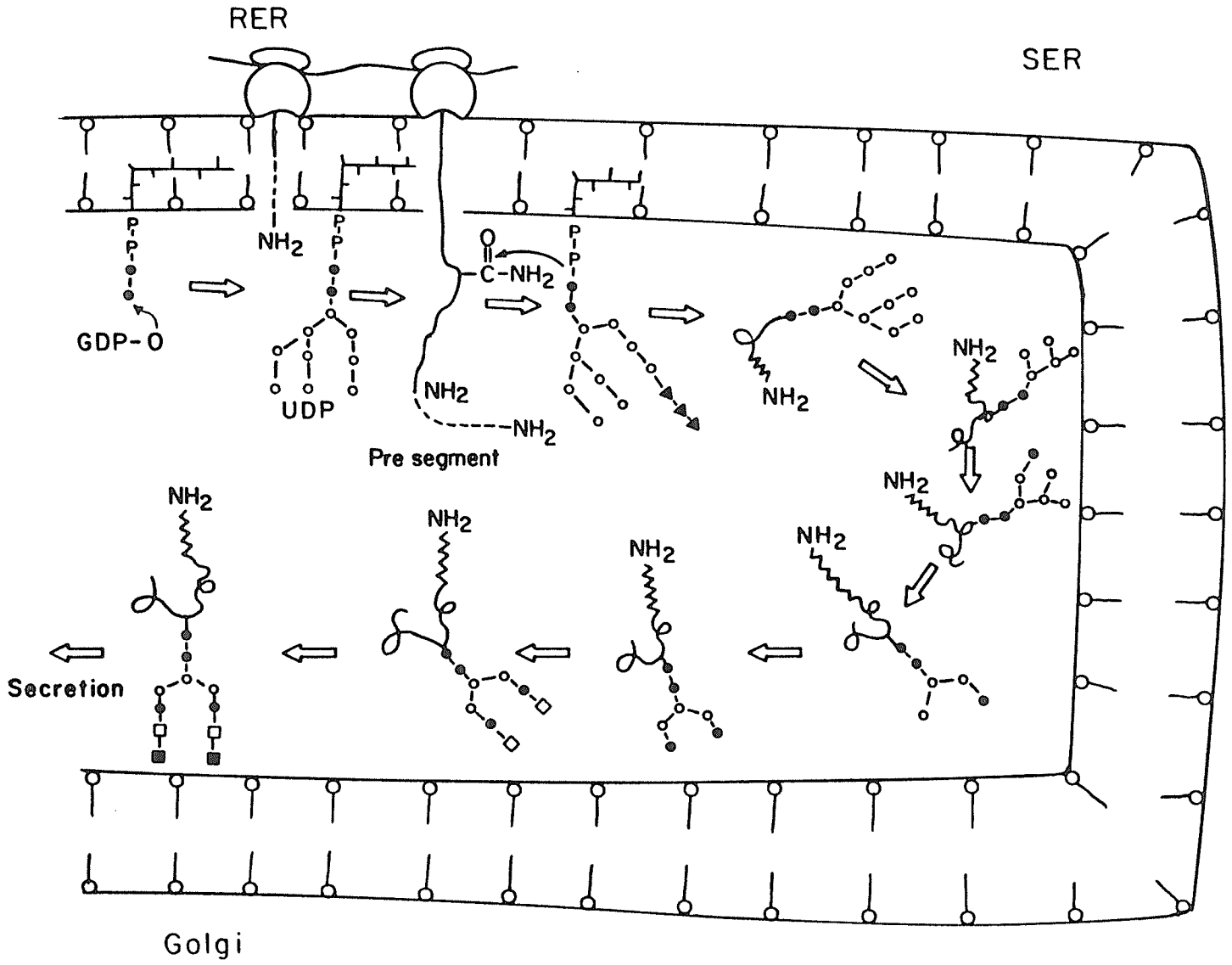
● N-acetyl glucosamine

○ mannose

□ galactose

■ N-acetyl neuraminic acid

---- presegment


RER
SER
GDP-O
UDP
NH2
C-NH2
Pre segment
Secretion
Golgi

phosphate cycle seemed to be present on the cytoplasmic side of the ER membrane suggesting that the lipid-oligosaccharide complexes were manufactured at that site. This is, however, in conflict with Hanover and Lennarz's (93) report which indicated that at least one lipid complex ($GlcNAc_2$-P-P-Dol) was present on the luminal side of the membrane. Since transfer of the oligosaccharide occurs within the lumen of the ER membrane, assembly of the lipid-oligosaccharide complex outside the membrane would require the transfer of the complex across the membrane in a process which has not yet been reported.

Clearly, more work is needed to answer several unresolved questions about the precise mechanisms involved in glycosylation of secretory proteins. The presence of glucose on the lipid-oligosaccharide complex is somewhat surprising since this sugar is not present in mature carbohydrate structures in secretory glycoproteins (for structure see Fig. 4). Obviously, the glucose must be required for some process and then removed. Turco et al. (94) showed that glucose containing oligosaccharides were transferred from lipid donors to endogenous acceptors at a 4- to 9-fold higher initial rate than oligosaccharide without glucose. This suggestion that glucose is required for transfer of oligosaccharide from dolichol pyrophosphate to polypeptide, was supported in studies by Spiro et al. (95). It appears that glucose residues on the terminal position of the lipid-oligosaccharide structure may serve as a

recognition factor for the enzymes involved in the transfer of oligosaccharide to polypeptide.

3. Processing The presence of glucose on the lipid-linked oligosaccharide involved in the synthesis of asparagine-linked glycoproteins was a surprise since this sugar residue is not present on the complex carbohydrate chains of these glycoproteins (structures shown in Fig. 4 and 6). Examination of these structures also indicates that the complex carbohydrate chain only contains three mannose residues rather than the nine found in the lipid-linked carbohydrate. Thus,it appears that glucose and some mannose must be removed from the oligosaccharide after transfer from dolichol pyrophosphate oligosaccharide to the nascent polypeptide chain. This process of removal of glucose and mannose, now referred to as oligosaccharide processing (depicted in Fig. 8), begins with trimming of glucose immediately after the transfer of the oligosaccharide to the protein (81). From studies in liver cell-free systems, it appears that at least two separate enzymes participate in the reaction (96-99). One enzyme (glucosidase I) removes the terminal glucose, while the second enzyme (glucosidase II) converts $Glc_2Man_9GlcNAc_2$ to $Man_9GlcNAc_2$. Both enzyme activities were found in the RER and SER of liver cells (96,97) although the glucosidase I activity was highest in the RER (99).

The second stage of processing is the removal of mannose. The fact that six non-equivalent mannose residues (see Fig. 6) must be

cleaved from the $Man_9GlcNAc_2$- containing precursor during biosynthesis of complex oligosaccharide chains suggests that more than one α-mannosidase is involved in processing. It appears that three different mannosidases are involved. The cleavage of α-1,2 linked mannose residues can be accounted for by two enzymes; mannosidase IA reported by Tabas and Kornfeld (100), and mannosidate IB reported by Tulsiani et al.(101). These two enzymes appear to be involved in the conversion of $Man_9GlcNAc_2$- to $Man_5GlcNAc_2$-. Tulsiani et al.(101) demonstrated the existence of a third enzyme, mannosidase II, which catalyses the removal of the α-1,3 and α-1,6 mannose residues in the species $Man_5GlcNAc_2$- to produce $Man_3GlcNAc_2$-, which is the core structure of the complex-type oligosaccharide. Tabas and Kornfeld (100) found that the enzyme activity which was capable of removing the α-1,2 linked mannose residues from deglucosylated oligosaccharide attached to polypeptides were located mainly in the Golgi complex of rat liver. An α-1,2-mannosidase similar to that described by Tabas and Kornfeld was isolated by Forsee and Schutzbach (102). This enzyme appeared to be modulated by the phospholipid environment. This suggests that the removal of α-1,2 mannose could be a controlling step in determining whether an N-linked oligosaccharide retained a high mannose structure or was converted to a complex-type. The primary structure near the glycosylation site influences the secondary and tertiary folding and this may also play a role in control of the α-mannosidase

activity and determine the nature of the mature carbohydrate structure. Mannosidase II (101) has also been shown to be located in the Golgi complex so it appears that removal of mannose to form the basic core structure occurs late in the secretory process.

The third stage in oligosaccharide processing is the addition of the terminal triplet of sugars (GlcNAc, Gal and NeuAc) (See Fig. 8). This process, referred to as elongation, has been shown to involve the reactions of nucleotide sugars, transferase enzymes and a suitable core oligosaccharide structure (103). The first step is the addition of GlcNAc to the $Man_5GlcNAc_2$- structure, so this step obviously begins before the removal of all the mannose residues. The addition of the first GlcNAc is catalyzed by GlcNAc transferase I (104,105) and is donated from UDP-GlcNAc to α-linked mannose to form a GlcNAc-Man α-linkage. The addition of this first GlcNAc by transferase I is apparently essential for further processing of the α-1,3 and α-1,6 linked mannose from the $Man_5GlcNAc_2$-structure (104,105) by mannosidase II. GlcNAc-transferase II the second enzyme involved in the addition of GlcNAc appears to have a specific requirement for the branched structure, $GlcNAc_1Man_3GlcNAc_2$- and therefore cannot act until GlcNAc-transferase I has added the first GlcNAc residue. The lack of GlcNAc-transferase I activity prevents subsequent addition of GlcNAc and also results in the retention of the five mannose

containing oligosaccharide structures. The mannosidase II activity cannot release the α-1,6 and α-1,3 mannose residues until the first GlcNAc is β-1,2 linked to an α-1,3 linked mannose (104).

Following the addition of the first GlcNAc and subsequent release of α-1,3 and α-1,6 linked mannose the mature complex oligosaccharide is synthesized by sequential addition of GlcNAc, galactose, NeuAc and fucose from appropriate nucleotide sugars. Wilson et al.(106) showed that a fucosyl transferase activity from the rat liver Golgi complex was capable of catalyzing the transfer of fucose from GDP-β-L-fucose to $GlcNAc_1Man_3GlcNAc_2$-Asn and that the attachment was α-linked to the innermost GlcNAc residue. They also showed that although fucosyl transferase can act on products of either GlcNAc-transferase I or II it cannot act until at least one GlcNAc has been added to the oligosaccharide core structure.

Galactosyl transferase catalyzes the addition of galactose from UDP-α-D-Gal to the terminal GlcNAc residues to become either the ultimate terminal nonreducing sugar or the penultimate sugar to NeuAc. The order in which the fucose and galactose are added is not known, but they are both catalyzed by Golgi-associated enzymes and consequently catalyze late reactions in the processing of the oligosaccharide chain (103,107,108).

In N-glycosidically linked oligosaccharides, NeuAc is usually found at the nonreducing terminus in an α-linkage to a penultimate

galactosyl residue (109). Sialyl transferases, which catalyze the transfer of NeuAc from CMP-NeuAc to an acceptor with a β-linked terminal galactose residue, have been identified in numerous mammalian tissues as membrane-bound enzymes associated predominantly with the Golgi complex (104). Both α-2,3- and α-2,6-NeuAc-Gal linkages are found in glycoproteins suggesting that at least two different transferase enzymes are involved in the addition of NeuAc to complex oligosaccharides. The sialyl transferase activity capable of catalyzing the synthesis of both NeuAc -α-2,3- lactose and NeuAc -α-2,6- lactose has been observed in mammalian liver (110). Over all, the data suggest the possible existence of at least four sialyl transferases. In general, the reactions described above for the synthesis of terminal regions of complex chains appear to support the "one linkage-one glycosyltransferase" hypothesis proposed by Schachter (111,112) which suggests that a separate transferase is required for every known sugar-sugar linkage.

Many complex oligosaccharides contain more than two external branches, but as yet, little is known about the enzymes that catalyze the reactions which result in triantennary and tetraantennary structures. In some cases, increased branching of complex oligosaccharide appears in transformed cells (113) and appears to be due to an anomaly in the elongation process, while in other cases, tetraantennary structures appear to be the normal structure (114).

The structure of the protein being processed may be a factor in the transferase activity during elongation.

Other modifications of the elongation process have recently been observed. For example, several lysosomal enzymes have been demonstrated to contain mannose-6-phosphate groups attached to the α-1,2 mannose residues of the high mannose oligosaccharide (115). The presence of the phosphate group prevents the removal of mannose residues from the high mannose oligosaccharide and subsequent conversion to a complex-type. Thus, most lysosomal enzymes contain high mannose oligosaccharide chains. The mannose-6-phosphate structure is believed to function as a signal directing subcellular transfer of newly synthesized lysosomal enzyme from the Golgi to the lysosome. In general, the Golgi complex appears to function as a packaging and sorting compartment from which the completed protein is either transferred to another cellular compartment or secreted from the cell to the extracellular space (116).

Introduction to the work presented in this thesis

Previous studies in this laboratory have led to the isolation and characterization of α_1-AGP from rat serum (117). In subsequent studies (22,48) α_1-AGP was shown to be a major acute phase reactant which was elevated about 6-fold in serum and about 4-fold in liver following turpentine induced inflammation.

In the study presented in this thesis, inflammation was used to elevate the liver content of α_1-AGP so that the larger quantities of

intracellular forms of α_1-AGP would be available for study. According to the information presented above on the biosynthesis of glycoproteins,the earliest intracellular forms of α_1-AGP should be found in the RER where it might be expected that high mannose forms of α_1-AGP would exist in which the oligosaccharide chains were unprocessed. In addition, partially processed forms might be expected to exist in SER and Golgi. Therefore, the work presented in this thesis is directed towards the isolation and characterization of intracellular forms of α_1-AGP containing largely unprocessed or partially processed oligosaccharide chains. In addition, preliminary information is presented to suggest that intracellular forms of α_1-AGP contain an additional amino acid extension of the pro- or pre- type.

EXPERIMENTAL

Materials

Chemicals Whenever possible, chemicals were of analytical reagent grade obtained from Fisher Scientific Ltd., Toronto; or J.T. Baker Chemical Co., Phillipsburg, New Jersey. Other chemicals and reagents were obtained as follows: Lubrol-W flakes were a gift from Imperial Chemical Industries Ltd., Blackley, Manchester, England; cesium chloride, Sigma Chemical Co., St. Louis, Missouri; pyroglutamate amino peptidase from calf liver, Boehringer Mannheim GmbH, West Germany; phenylmethanesulfonyl fluoride (PMSF), Eastman Kodak Co., Rochester, New York; acetic anhydride, Aldrich Chemical Co., Milwaukee, Wisconsin; N-methyl-bis (trifluoroacetamide) (MBTFA), Pierce Chemical Co., Rockford, Illinois; sodium borohydride, Fisher Scientific Ltd., Toronto; aqueous counting scintillant (ACS), Amersham Corp., Oakville, Ontario. Radioactive chemicals: D-[1-^{14}C]-glucosamine (55 mCi/mmole; 1 Ci = 37 GBq); and D-[1-^{14}C]-mannose (50 mCi/mmole); were purchased from Amersham Corp., Oakville, Ontario.

Chromatographic and electrophoretic media Sephadex G-100, and ConA-Sepharose, Pharmacia (Canada) Ltd., Montreal; Ultrogel AcA44, Fisher Scientific Ltd., Toronto, Ontario; CM-cellulose (CM-52), Dowex 50 X8-400 and Dowex 2 X8-400,Sigma Chemical Co., St. Louis, Missouri; acrylamide; N,N' methylene-bis-acrylamide (Bis); N,N,N',N'-tetramethylethylene-diamine (TEMED), ammonium persulfate, sodium

dodecyl sulfate (SDS), Coomassie Brilliant Blue R-250, and dithiothreitol, Bio-Rad Laboratories, Richmond, California; 2-aminodiphenyl hydrogen oxalate; 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride), Pierce Chemical Co., Rockford, Illinois; agarose (Seakem), Marine Colloids Inc., Rockland, Maine.

Proteins, amino acids and sugars D(+) glucose, D(+) galactose, meso-inositol, D(+) mannose, D(+) glucosamine HCl, N-acetyl-D-glucosamine, N-acetylneuraminic acid (type IV), α-methyl mannoside, D-gluconic acid lactone, D-galactonic acid-γ-lactone, L-mannonic acid-γ-lactone, sucrose, bovine serum albumin (crystalline), Human γ-globulin, trypsin (type XI) and trypsin inhibitor (type 1-S), Sigma Chemical Co., St. Louis, Missouri; calibration kit for molecular weight determination (ribonuclease, chymotrypsinogen, ovalbumin, aldolase, blue dextran 2000), Pharmacia Fine Chemicals, Piscataway, New Jersey; human serum albumin, Winnipeg Rh Institute, Winnipeg, Canada; phenylthiohydantoin-amino acid (PTH) quantitative standard kit, Dansyl amino acid kit (individual dansyl amino acid derivatives), amino acid standard H kit (quantitative, 2.5μ moles/ml of 17 amino acids) and norleucine, Pierce Chemical Co., Rockford, Illinois.

Physical measurements

Radioactivity was determined with a Packard Tricarb model 3003 or an LKB Rac-Beta II model 2115 liquid scintillation counter. Aqueous solutions of protein (up to 0.4 ml and 3 mg protein) were counted following the addition of 10 ml of ACS scintillation cocktail.

With the Packard, mixtures of ^{3}H and ^{14}C were counted with the red channel at pulse height settings of 10-1000 divisions (60% gain) and the green channel at pulse height settings of 200-1000 divisions (8% gain). When counting ^{14}C alone the pulse height settings were 10-1000 divisions (8% gain). With the LKB counter ^{14}C was counted with the channel 1 gate setting of 60-165. For double label counting of ^{3}H and ^{14}C, the gates were set at: channel 1, 16-100; channel 2, 100-165. The LKB had an automatic external standard which automatically corrected for quenching to calculate disintegrations per minute (DPM).

Extinctions at 280 nm were measured with a Bausch and Lomb Spectronic 700; a Gilford 2400-2; or a Zeiss Model PMQII spectrophotometer. The Spectronic 700 or the Zeiss Model PMQII were also used for measuring extinctions in the visible range of the spectrum. Measurements of pH were made with an Orion model 701A pH meter, an Orion model 801 pH meter or a Radiometer model 28 pH meter.

Ultrafiltration

Concentration by ultrafiltration of protein solutions in the preparative procedures was carried out with Amicon stirred cell ultrafiltration equipment. Diafiltration of Lubrol-W extracts was based on the method of Sober et al.(118); dialysis tubing was attached to drying tubes and placed in an evacuated chamber with the dialysis tubing immersed in the dialyzing medium. Routine dialysis against buffer or distilled water was with Spectraphor dialysis

membrane at 4°.

Preparation of Dowex ion-exchangers

Dowex 2 and Dowex 50 were washed successively with the following organic solvents: acetone-water (1:1, v/v), acetone, petroleum ether (40°-60°), acetone and acetone-water (1:1, v/v) (119). Dowex 2 was first converted to the hydroxyl form by washing with 6 N NaOH and then with water. It was converted to the formate form by washing successively with 2 N HCl, water and 2 M sodium formate until the effluent gave a negative reaction for chloride (120). Excess formate was removed by washing with water. Dowex 50 was converted to the hydrogen form by washing successively with 2 N NaOH, water, 2 N HCl and water; the procedure was repeated.

Electrophoretic methods

Electrophoresis with polyacrylamide was performed in three ways; with buffer at pH 8.9 in tube gels, with SDS buffer, pH 6.4, in tube gels and with SDS buffer in a vertical slab gel system.

For examination at pH 8.9, electrophoresis was performed by a modification of the method of Ornstein (121) and Davis (122). Disc gels with 7.5% monomer in 0.188 M Tris-glycine buffer, pH 8.9, were used with a Bio-Rad model 155 electrophoresis cell at 4°. Samples containing approximately 10 to 40 µg of protein were mixed with 0.04 M Tris-glycine buffer, pH 8.9, 30% sucrose and 0.5% bromophenol blue as tracking dye in a total volume of 30 µl to 60 µl. Samples were introduced into the gels at 60 V and electro-

phoresis performed at 200 V until the tracking dye migrated 90% of the length of the gel. The electrode compartments of the tank contained 0.188 M Tris-glycine buffer, pH 8.9.

Polyacrylamide electrophoresis in presence of SDS was performed by a modification of the method of Weber and Osborn (123). Gels were prepared with 7.5% monomer in 0.205 M Tris-acetate buffer, pH 6.4; 0.1% SDS was used in both tubes and the vertical slab system. Proteins were denatured prior to application by the method of Fairbanks et al. (124). This involved incubation at 100° for 5 min in electrophoresis buffer containing 0.04 M dithiothreitol, 1% SDS and 0.001 M EDTA. After cooling, bromophenol blue (0.5%) and sucrose (10%) were added to the samples which were introduced into the gel by electrophoresis at 60 V. Electrophoresis was performed in gels containing 0.205 M Tris-acetate buffer, pH 6.4,0.1% SDS at 160 V until the tracking dye migrated 90% of the length of the gel.

Both basic and SDS gels were stained by the method of Fairbanks et al. (124) using a Bio-Rad model 172A diffusion destainer for disc gels and a Bio-Rad model 222 for slab gels. Gels were stained overnight in a solution containing 0.03% Coomassie Brilliant Blue R-250, 10% acetic acid and 25% isopropanol. Gels were destained by diffusion in a solution containing 0.0025% Coomassie Brilliant Blue R-250, 10% acetic acid and 60% isopropanol for 4 to 6 h followed by a final diffusion in a solution of 10% acetic acid and recirculation through an activated charcoal filter until the back-

ground cleared.

Measurement of radioactivity in gel slices was performed by incubation of the slices in a tightly capped scintillation vial with 0.5 ml of 30% hydrogen peroxide at 70° until the slices had dissolved (about 1 h). Following cooling, 10 ml of ACS scintillation cocktail was added to the vial and radioactivity determined by scintillation counting as previously described.

Immunological methods

Antiserum to rat α_1-AGP was prepared in albino rabbits as described by Simkin et al. (125). An emulsion of 0.75 mg α_1-AGP, 0.25 ml sterile 0.15 M sodium chloride and 0.4 ml Freund's complete adjuvant was injected intramuscularly, one-half of the dose being injected into each thigh of the rabbit. After 6 days, a second preparation containing 1.25 mg α_1-AGP with Freund's complete adjuvant was injected as before. After a further 22 days, a series of intravenous injections was given of a suspension of a coprecipitate of α_1-AGP with aluminum hydroxide. This was prepared by adding 0.4 ml 10% (w/v) $Al_2(SO_4)_3$, $K_2SO_4.24\ H_2O$ to a mixture of 3.0 mg α_1-AGP in 0.2 ml water to which 0.08 ml M $NaHCO_3$ were added, stored at 2° for 16 h after which the precipitate was resuspended in 0.4 ml 0.15 M sodium phosphate buffer, pH 7.4. Samples of 0.05 ml, 0.10 ml and 0.25 ml of this suspension were injected intravenously on alternate days. Blood was collected after five days.

Double diffusion analysis was based on the method of

Ouchterlony (126). The media contained 1.0% agarose in 0.0075 M sodium phosphate pH 7.2, 0.15 M sodium chloride, 0.01% sodium azide. Gels were 2 mm thick and 4 mm diameter wells were cut 8 mm apart. Plates were developed at room temperature in a humidity chamber and photographs were taken using an illumination device as described by Hunter (127).

The quantitative precipitin technique described by Jamieson et al. (48) was applied to serum samples and Lubrol-W extracts of rough, smooth and Golgi fractions of rat liver microsome material. For the preparation of the precipitation curves, mixtures were prepared (total volume 0.45 ml) containing up to 40 µg α_1-AGP, 0.15 M NaCl, 1 mM sodium azide and 4.7% Dextran T70 followed by the addition of 0.15 ml of antiserum to α_1-AGP. Mixtures were incubated at 37° for 45 min and then allowed to stand for 48 h at 2°. The precipitates which formed were collected by centrifugation at 2000 rpm (750 g_{av}) for 10 min at 2° and washed with 0.3 ml 0.15 M NaCl containing 4% Dextran T70 and then with 3 x 0.3 ml 0.15 M NaCl. Precipitates were dissolved in 1 ml 0.1 N NaOH and suitable volumes were removed for determination of protein and radioactivity. When the technique was applied to rat serum 0.05 - 0.35 ml volumes of 80- to 120-fold dilutions of serum were reacted with antisera as described above.

For the isolation of α_1-AGP from Lubrol-W extracts of liver microsome material, Lubrol-W extracts were first concentrated 6-fold by ultrafiltration with concurrent dialysis against 0.15 M

NaCl, 1% Lubrol-W and 10 mM D-mannose and the precipitation procedure was scaled up 10-fold. Prior to precipitation with antiserum to α_1-AGP, precipitation with a heterologous immune system was performed in order to remove any nonspecific precipitating material. This involved the preparation of a mixture of 3 ml concentrated Lubrol-W extracts (equivalent to microsome material from 7.5 g liver), 500 µg human albumin, 0.15 M NaCl, 1 mM sodium azide, 4.7% Dextran T70 (4.5 ml total volume), and 1.2 ml antiserum to human serum. Solutions were incubated at 37° for 45 min and then allowed to stand for 24 h at 2°. Precipitates were removed by centrifugation at 2000 rpm for 10 min at 2° and 360 µg human fraction VI[1] dissolved in 0.1 ml, 0.15 M NaCl were added to the supernatants and solutions incubated as described above. The complete precipitation procedure was repeated. After removal of the final precipitate, supernatants were reincubated at 37° for a further 45 min and allowed to stand at 2° for 24 h in order to check that there was no further precipitation of material. Quantitative precipitation with antiserum to α_1-AGP was performed in triplicate employing up to 0.45 ml supernatant and 0.15 ml antiserum to α_1-AGP. Precipitates were washed with 0.3 ml 0.15 M NaCl containing 4% Dextran T70 and then with 3 x 0.3 ml 0.15 M NaCl. Washed precipitates were dissolved in 1 ml 0.1 N NaOH and suitable volumes removed for deter-

[1] human fraction VI is an α-globulin-containing fraction, a major component of which is human α_1-AGP; human α_1-AGP together with antiserum to human serum effectively clears the extracts of non-specific precipitating material which would otherwise precipitate with antiserum to rat α_1-AGP.

mination of protein and radioactivity.

Subcellular fractionation

A Beckman model L5-50 refrigerated ultracentrifuge was used for the subcellular fractionation of rat liver. A No. Ti50 angle head was used for the preparation of rough and smooth membrane fractions of microsome material and a No. SW27-1 swinging bucket rotor was used for the preparation of the Golgi-enriched fraction.

Homogenates were prepared and fractionated by a modification of the method described by Dallner (128). All experimental procedures were performed in ice. Livers were perfused with ice-cold saline, excised and cut into small pieces. For the preparation of rough and smooth membrane fractions of microsome material liver was homogenized with 3 vol. of 0.25 M sucrose in a Potter-Elvehjem type homogenizer with a motor-driven polytetrafluoroethylene pestle; the pestle rotated at 2000 rpm and about 10 up and down strokes of the pestle over approximately 30 sec were carried out. Rough and smooth membrane fractions were prepared according to the scheme outlined in Fig. 9.

For the preparation of Golgi-enriched fractions, liver was homogenized as described above except that 4 ml of 0.5 M sucrose in medium A (0.1 M Tris-HCl, pH 7.6; 0.01 M $MgCl_2$, 1% Dextran T70) were added per g of liver and only 5 up and down strokes of the pestle were used. Aliquots of 8 ml of homogenate were layered on discon-

Fig. 9 Subcellular fractionation scheme for the preparation of rough and smooth membrane fractions from rat liver microsome material based on the procedure described by Dallner (128). For kinetic studies on incorporation of labelled sugars the volumes used were those indicated in the scheme; the procedure was scaled up about 10-fold when intracellular α_1-AGP was isolated from liver.

Liver homogenate

(5 g liver + 15 ml 0.25 M sucrose)

centrifuged at 12,200 rpm (10,000 g_{av}) for 20 min

sediment (discarded)

supernatant I

CsCl added to 15 mM and 3.5 ml volumes layered on 2.25 ml 1.3 M sucrose, 15 mM CsCl and centrifuged at 40,000 rpm (106,000 g_{av}) for 150 min

sediment

rough membrane fraction

supernatant II; material at and above interface was aspirated and diluted with equal vol 0.25 M sucrose; 0.01 vol 1 M $MgCl_2$ was added; and centrifuged at 40,000 rpm (106,000 g_{av}) for 150 min

sediment

smooth membrane fraction

supernatant III

cell sap fraction

Fig. 10 Subcellular fractionation scheme for the preparation of the Golgi-enriched fractionation of rat liver microsome material based on the procedure of Schachter et al.(129). This scheme was used for kinetic studies on incorporation of sugars and for preparation of intracellular α_1-AGP from liver.

Liver homogenate

(5 g liver + 20 ml 0.5 M sucrose in Medium A)

8 ml homogenate layered on following discontinuous sucrose gradient in medium A

8 ml 0.7 M sucrose/Medium A

8 ml 1.3 M sucrose/Medium A

10 ml 1.7 M sucrose/Medium A

centrifuged at 24,000 rpm ($70,000\ g_{av}$) for 45 min

remainder (discarded)

Material at interface between 0.7 M and 1.3 M sucrose solutions aspirated and diluted with 3 vol 0.25 M sucrose; centrifuged at 40,000 rpm ($106,000\ g_{av}$ with Ti50 head) for 150 min

sediment

supernatant (discarded)

Golgi-enriched fraction

tinuous sucrose density gradients and fractionation performed according to the scheme outlined in Fig. 10.

Extraction of subcellular fractions

Extraction of the rough and smooth membrane fractions and Golgi-enriched fractions of liver microsome material with Lubrol-W was performed as described by Simkin et al.(130). Prior to extraction, the inside of the walls of the centrifuge tubes and surface of the pellets were washed 3 times with 1 ml vol. of 0.25 M sucrose. For the preparation of detergent extracts, 1 ml Lubrol-W was added per 7-10 mg protein in the pellets. The pellet was dispersed in extractant by homogenization as described above and the insoluble material was removed by centrifugation at 40,000 rpm (106,000 g_{av}). Lubrol-W extracts were normally concentrated 6-fold by ultrafiltration with concurrent dialysis against 1% Lubrol-W.

For preparation of ultrasonic extracts subcellular fractions of rat liver were suspended in 10-15 ml of 0.15 M NaCl and homogenized by 5 up and down strokes of a Potter-Elvehjem pestle at 2,000 rpm. The membranes were disrupted by exposure to ultrasonic vibrations for 1 min at 100 W with a 12 mm diameter titanium probe using a Sonic Dismembrator supplied by Artek Corporation. The ultrasonic extracts were isolated from the membrane debris by centrifugation at 40,000 rpm (106,000 g_{av}) for 150 min. The supernatant, containing mainly the intravesicular proteins, was dialyzed against 0.15 M NaCl, 0.1% sodium azide at 4° and concentrated

10-fold with an Amicon stirred cell ultrafiltration apparatus using a UM10 membrane.

Isolation of protein for measurement of radioactivity

For the measurement of radioactivity an equal volume of ice - cold 10% trichloroacetic acid was added to serum or liver samples containing about 5 mg/ml protein and 20 mM D-mannose. Precipitates were collected by centrifugation at 2,000 rpm (750 g_{av}) for 5 min. The precipitates were washed by resuspension followed by centrifugation with the following solvents: once with 5% trichloroacetic acid at 50°; once with acetone-0.1 M NaCl (4:1 v/v); twice with ethanol-ether-chloroform (2:2:1 v/v/v) for 15 min; and finally twice with ether alone (131). In all of the above washes the concentration of protein was maintained at above 5 mg/ml. All protein samples were dried in air, dissolved in 0.1 N NaOH, and samples removed for determination of protein by the Lowry technique and for measurement of radioactivity. For determination of trichloroacetic acid soluble radioactivity the supernatant collected following the initial precipitation with trichloroacetic acid was pooled with the supernatant from the first washing with trichloroacetic acid; a suitable volume of the mixture was removed for determination of radioactivity.

Incorporation studies in vivo

Male Long Evans hooded rats of 300 g body weight were purchased locally or from Canadian Breeding Farms, St. Constance, Quebec.

Rats were maintained on a diet of Purina Laboratory Chow and tap water and were starved 16 h prior to sacrifice. Inflammation was induced by subcutaneous injection of 0.5 ml turpentine per 100 g body weight into the dorsolumbar region; controls received injections of sterile 0.15 M NaCl (132).

When experiments involved incorporation of radioactive mannose, rats were lightly anaesthetized with ether and were injected with a solution containing 15 μCi D[^{14}C]-mannose in 0.2 ml sterile 0.15 M NaCl *via* the femoral vein. Rats were sacrificed at 5-60 min after injection (see Results). Blood was collected by severing the jugular veins, allowed to clot for 2 h at room temperature and serum prepared by centrifuging at 2,000 rpm for 10 min. Serum was stored at -20° until required. Livers were perfused with 0.15 M NaCl *via* the portal vein, a small sample was retained for preparation of total liver trichloroacetic acid soluble and insoluble fractions (see below) and the remainder was subjected to subcellular fractionation to prepare rough and smooth membrane fractions and Golgi fractions. Trichloroacetic acid soluble and insoluble fractions were prepared from total liver, serum and samples of rough and smooth membrane fractions and Golgi fractions from liver. Lubrol-W extracts were also prepared from rough and smooth membrane fractions and Golgi fractions; α_1-AGP isolated by application of the quantitative precipitin tehcnique utilizing antiserum to α_1-AGP.

Isolation of α_1-AGP from rat serum

Rat serum α_1-AGP was prepared by a method based on procedures described by Simkin et al.(133) and Jamieson et al.(117). Rats suffering from inflammation for 48 h were used since the serum levels of α_1-AGP were highest at this time after inflammation (22). Volumes of 50-100 ml serum were diluted with 9 volumes of 0.15 M NaCl stirred vigorously with a magnetic stirrer, and 1.8 M perchloric acid added, dropwise, to a final concentration of 0.6 M. The solution was allowed to stand at room temperature for 10 min and then centrifuged in a Sorvall RC-3 centrifuge at 5,000 rpm (7,000 g_{av}) for 20 min. The supernatant, referred to as the perchloric acid-soluble fraction, was decanted, cooled in ice, and the pH adjusted to pH 6.5 with 2 M NaOH. The solution was dialyzed against three changes of distilled water over three days, concentrated by ultrafiltration using a UM10 Amicon membrane, and freeze dried. The α_1-AGP in the perchloric acid-soluble fraction was purified by chromatography on CM-cellulose. CM-cellulose was suspended in 0.05 M sodium acetate buffer, pH 4.9, packed in a 2.5 cm x 45 cm column and equilibrated with the same buffer. The freeze dried perchloric acid-soluble fraction was dissolved in 10 ml of equilibrating buffer, applied to the CM-cellulose column and eluted with the equilibrating buffer at a flow rate of 20 ml/h. Protein was detected in the eluate by monitoring the absorbance at 280 nm with an LKB Uvicord II. Samples of 5 ml were collected and frac-

tions excluded from the CM-cellulose were pooled, concentrated by ultrafiltration and freeze dried. This fraction is referred to as the CMC-I fraction. α_1-AGP which was the major component in CMC-I was isolated in pure form by gel filtration on a 2.5 cm x 120 cm column of Ultrogel AcA44 equilibrated with 0.05 M NH_4HCO_3, pH 8.0; typically, 2 ml containing about 40 mg CMC-I in 0.05 M NH_4HCO_3, pH 8.0 were applied to the column and eluted at 10 ml/h. The column effluent was monitored as above and 4 ml fractions were collected. A narrow cut from the major peak was collected, dialyzed and freeze-dried, yielding α_1-AGP of over 95% purity as judged by electrophoresis on basic and SDS PAGE.

Isolation of α_1-AGP from subcellular fractions of rat liver

Intracellular forms of α_1-AGP were isolated from rough and smooth membrane fractions and Golgi-enriched fractions of liver excised from 24 h experimental rats. This time after inflammation was used since earlier studies had shown that α_1-AGP in liver was at a maximum at about 24 h after induction of inflammation (48).

For the isolation of intracellular forms of α_1-AGP, livers were perfused with ice-cold 0.15 M NaCl to minimize contamination with serum α_1-AGP. Batches of 60-70 g liver were transferred to ice-cold 0.25 M sucrose and subcellular fractions prepared as described in Figs. 9 and 10 with the following modifications. A large capacity Ti60 rotor was used in place of the Ti50 head because of the larger quantities of liver homogenate prepared. Ultrasonic extraction of

subcellular fractions was used instead of Lubrol-W since the latter treatment dissolved membrane proteins in addition to those present in the intracisternal space of subcellular fractions; in addition, detergent would be difficult to eliminate by chromatographic procedures. D-gluconic acid lactone, D-galactonic acid-γ-lactone and L-mannonic acid-γ-lactone (2 mM each) were added to the ultrasonic extracts to inhibit glycosidase activities which might result in processing of oligosaccharide chains of glycoproteins during the isolation procedure.

In initial experiments, the ultrasonic extract was applied to a 3 cm x 130 cm column of Sephadex G-100 equilibrated with 0.025 M phosphate buffer, pH 7.5. The column was eluted at a flow rate of 50 ml/h at 4° and 5 ml fractions were collected. In later experiments it was found that superior separations were accomplished with a 2.5 cm x 120 cm column of Ultrogel AcA44 eluted with a 0.2 M NH_4HCO_3 buffer, pH 8.0 at a flow rate of 35 ml/h. Column effluents were routinely monitored for protein at 280 nm and 5 ml fractions collected as before. In some experiments rats were given injections of 20 μCi D-[^{14}C]-mannose as described previously to label glycoproteins; in these experiments 0.2 ml aliquots were removed from each fraction for counting. Each fraction was also examined for immunological activity using antisera to rat α_1-AGP and rat albumin by the Ouchterlony double diffusion technique. Fractions containing material reacting with antiserum to α_1-AGP were pooled, concen-

trated by ultrafiltration and rechromatographed on Sephadex G-100 or Ultrogel AcA44 as appropriate. The fractions containing material reacting immunologically with antiserum to α_1-AGP were pooled, concentrated by ultrafiltration with an Amicon system, dialyzed against 0.1 M sodium acetate buffer, pH 6.0, containing 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EDTA, and 1.0 M NaCl and applied to a 1 cm x 5 cm column of ConA-Sepharose previously equilibrated with the same buffer (referred to as ConA-Buffer I), 3 ml fractions were collected and protein in the effluent was monitored as before. Elution was continued until the 280 nm signal returned to baseline; the column was then eluted with ConA-Buffer II (ConA-Buffer I containing 0.5 M α-methyl D-mannoside); elution was performed at 30°. ConA-Buffer II eluted a second protein peak; both peaks I and II were examined immunologically by double diffusion analysis using antiserum to α_1-AGP and albumin. Fraction ConA-II was also examined by electrophoresis on basic PAGE at pH 8.9 and on SDS PAGE at pH 6.4.

Fraction ConA-II which contained material reacting immunologically with antiserum to α_1-AGP was further fractionated on CM-cellulose as described by Jamieson et al.(117); ConA-II was dialyzed against 0.05 M sodium acetate, pH 4.9, and applied to a 1 cm x 5 cm column of CM-cellulose previously equilibrated with the same buffer. The column effluent was monitored for protein as before and 2 ml fractions were collected. The column was eluted with

sodium acetate, pH 4.9, until the 280 nm signal returned to baseline; under these conditions any residual serum α_1-AGP was excluded from the column. This fraction is referred to as fraction CMC-I. The column was then eluted with 0.4 M sodium acetate, pH 6.0; protein eluting under these conditions is referred to as fraction CMC-II. This procedure produced CMC-I and CMC-II fractions from rough and smooth membrane fractions and from the Golgi fractions; all were examined immunologically with antiserum to α_1-AGP and electrophoretically by basic PAGE at pH 8.9 and SDS PAGE at pH 6.4.

Limited proteolysis with trypsin

Judah et al.(61,134) showed that albumin isolated from rat liver existed in a precursor form, and that the precursor form (containing an additional N-terminus polypeptide segment) could be converted into serum-type albumin by limited tryptic hydrolysis. This procedure was applied to intracellular forms of α_1-AGP isolated from the rough membrane fraction of rat liver. Samples of 1 mg of α_1-AGP isolated from the rough fraction were incubated at 20° for 1 h or 2 h with 5-10 µg trypsin in 1 ml 50 mM Tris-HCl, pH 7.8, containing 50 mM $CaCl_2$. At the end of the incubation an amount of soya bean trypsin inhibitor was added corresponding to twice the weight of trypsin used. The mixture was immediately fractionated on ConA-Sepharose as described above. The trypsin, trypsin inhibitor, and the complex were eluted with ConA-Buffer I and the α_1-AGP was eluted with ConA-Buffer II as described above.

Chemical Analyses

Protein Protein was determined by the method of Lowry et al.(135) with modified reagents and volumes as described by Miller (136). Low levels of protein were determined by the microassay procedure of Bradford (137). In both procedures crystalline bovine serum albumin was used as standard.

Amino acids Amino acid analysis was performed with an NC-2P Technicon amino acid analyzer. Proteins were hydrolyzed at 110° for 24 h with 6 N HCl in vacuo. Hydrolyzates were dried in a vacuum desiccator over NaOH pellets and redissolved in distilled water for analysis. The amino acid composition of rat serum α_1-AGP was determined by performing analysis on 12, 24, 48 and 72 h hydrolyzates; results were extrapolated to zero time. Using these data, it was determined that for all other amino acid compositions in which amino acid analyses were performed on 24 h hydrolyzates, contents of threonine and serine were corrected for losses of 5% and 10%, respectively. Tyrosine and tryptophan were determined spectrophotometrically by the method of Edelhoch (138). The optical density of 1 ml protein solution in 6 M guanidine-HCl at or below pH 9 was measured at 280 nm, 288 nm, 295 nm and 300 nm. An aliquot of 0.10 ml of 5 N NaOH was added to the protein solution and optical densities measured again; this time at 30 sec intervals for 2 min at 295 nm and 300 nm. The values were extrapolated to zero time and the tyrosine and tryptophan concentration calculated with the Edel-

hoch equations:

for tyrosine ΔE_M(pH 9→12) = 2480 at 295 nm

= 2270 at 300 nm

for tryptophan OD_{280} = 5690 [tryptophan] + 1280 [tyrosine]

OD_{288} = 4815 [tryptophan] + 385 [tyrosine]

where ΔE_M is the difference in extinction coefficient of 1 M solution at pH 9 and pH 12

and OD is the optical density at 280 nm and 288 nm

Amino acid and carbohydrate analyses of protein eluted from SDS PAGE gels were performed by the method described by Sreekrishna et al.(139) with the following modifications. Proteins were labelled prior to electrophoresis with dansyl groups by the method of Talbot et al.(140) to enable rapid visualization of protein. The procedure used for dansyl labelling involved adding a 10% solution of dansyl chloride in acetone to protein in 0.1 M Tris-acetate buffer, pH 8.2, containing 5% SDS protein solution and vortexing. The mixture was placed in a boiling water bath for 5 min after which the solution was mixed with an equal volume of SDS reducing buffer (see SDS PAGE method) and boiled for an additional 5 min. Sucrose and bromophenol blue were added and electrophoresis carried out on a slab gel as described above. Following electrophoresis, the dansyl labelled proteins were visualized using a Blakray UVL 21 long wavelength UV lamp. The gel portion containing the dansyl labelled protein was excised with a razor blade, washed with distilled water and the protein eluted by

mincing the gel in 3 ml of 0.05 M NH_4HCO_3 containing 0.05% SDS followed by incubation at room temperature for 6 h and then at 4° for 20 h. The gel suspension was centrifuged and the supernatant aspirated from the gel. Elution with 0.05 M NH_4HCO_3 was repeated with 3 ml at 4° overnight; the supernatant was combined with the first supernatant and dialyzed at 4° against 0.005% SDS. The freeze dried sample was hydrolyzed with 6 N HCl *in vacuo* at 110° for 24 h and amino acid analysis carried out on the Technicon NC-2P amino acid analyzer. Carbohydrate analysis was attempted with the gas liquid chromatographic technique employing trifluoroacetylated derivatives referred to below.

Carbohydrate For qualitative analysis, sugars were identified using paper chromatography procedures previously described by Jamieson *et al.*(117). Quantitative analysis was performed in two ways: in the first procedure sugars were separated into neutral, basic and acidic fractions by chromatography on Dowex resins and analyzed using spectrophotometric methods. In the later studies quantitative analysis was performed directly on hydrolyzates of glycoproteins by gas liquid chromatography following formation of alditol acetate derivatives by the method described by Torello *et al.*(141).

For quantitative analysis of sialic acid by the column fractionation method a solution containing 1-2 mg glycoprotein in 2 ml 0.05 N H_2SO_4 was heated in a glass stoppered tube at 90° for 60 min (133). The hydrolysate was applied to a 1 cm x 7 cm column of Dowex 2 (formate form). The tube and column were washed with a

total of 7 ml distilled water and the sialic acid eluted with 15 ml of 0.3 N formic acid; the eluate was collected in a pear shaped flask. Formic acid was removed by rotary evaporation *in vacuo* at 35° to one-third the original volume; diluted to the original volume and evaporated back to one-third the original volume. The process was repeated. Finally, the eluate was evaporated to dryness and analyzed for sialic acid by the periodate-thiobarbituric acid method of Aminoff (142). The standard curve was scaled down to a maximum of 25 µg N-acetylneuraminic acid.

For qualitative and quantitative analysis of hexose and hexosamine, glycoproteins were hydrolyzed with Dowex 50 and HCl by a method based on that described by Simkin *et al.*(133). Dowex 50 (H+ form) was washed with five volumes 0.46 N HCl and then suspended in an equal volume of 0.46 N HCl. Samples of 1-2 mg glycoprotein dissolved in 0.25 ml distilled water were mixed with 0.6 ml Dowex 50, 0.46 N HCl resin suspension, and placed in a 1 cm x 14 cm bomb tube. Tubes were sealed and heated in an oven at 100° for 30-36 h; tubes were positioned at a 45° angle and rotated several times during hydrolysis. The hexose and hexosamine fractions were separated by filtration through a Dowex 50 (H+ form) column followed by a Dowex 2 (formate form) column. The columns were arranged such that the filtrate from a small funnel fitted with a glass wool plug could pass through a 0.5 cm x 7 cm column of Dowex 50 (H+ form) and the effluent from the latter could pass

directly through a 0.5 cm x 7 cm Dowex 2 (formate form) column. The hydrolysate was transferred to the small funnel and the bomb tube washed with 5 x 0.5 ml distilled water; the washings were transferred to the funnel. The filtrates were allowed to pass successively through the Dowex 50 and Dowex 2 columns. The effluent from the Dowex 2 column was collected and evaporated to dryness *in vacuo* at 35°. The residue is referred to as the neutral sugar fraction. When the flow of liquid through the Dowex 50 column stopped it was clipped off and 1 ml distilled water applied to the top of the column. Resin in the top funnel was washed successively with 0.25 ml 2 N HCl, 2 x 0.25 ml water and 0.25 ml 2 N HCl; the filtrates were allowed to pass into the 1 ml on the top of the Dowex 50 column. When all washings had passed through the funnel, the clip on the Dowex 50 column was removed and the liquid allowed to pass through the column; the eluate was discarded. The Dowex 50 column was then eluted with 3 ml of 2 N HCl (143); the eluate was collected and evaporated to dryness *in vacuo* at 35°. The residue is referred to as the hexosamine fraction. Controls included the hydrolysis of 0.25 ml of 1-2 mg bovine serum albumin with 50-100 µg of glucosamine HCl or hexose (an equimolar mixture of galactose and mannose). For quantitative analysis the neutral sugar fraction was assayed for hexose by the orcinol method of Winzler (144) with an equimolar mixture of galactose and mannose as standard; the method was scaled down to a total volume of 4.75 ml. The hexosamine fraction was

assayed by the method of Rondle and Morgan (145) with D-glucosamine HCl as standard; the method was scaled down to a total volume of 5.0 ml. For qualitative analysis the neutral sugar fraction and hexosamine fraction were examined by paper chromatography. The neutral sugar fraction was chromatographed on Whatman 3MM using butan-1-ol-ethanol-water (10:1:2, v/v) as solvent and on Whatman No. 1 using butan-1-ol-pyridine-0.1 N HCl (5:3:2, v/v) as solvent (146) with downward development at room temperature. Hexose was detected by spraying with the 2-amino biphenyl hydrogen oxalate reagent described by Gordon et al.(147). Semiquantitative information on the distribution of hexose was obtained by elution of the stained spots with methanol and by measuring the optical density at 400 nm as described by Li (148). Qualitative analysis of hexosamine was performed by paper chromatography on Whatman No. 1 using butan-1-ol-acetic acid-water (4:1:5, v/v) as the solvent with downward development at room temperature. Hexosamines were detected by spraying with Ehrlich's reagent as described by Partridge (149). Since only small quantities of precursor forms of α_1-AGP were available for carbohydrate analysis, an attempt was made to develop a sensitive gas liquid chromatographic analytical procedure employing trifluoroacetylated derivatives of sugars coupled with the use of an electron capture detector(150). Unfortunately, complete separation of glucose, mannose and galactose could not be achieved with this procedure. However, following the development of the improved isolation procedure using Ultrogel AcA44

carbohydrate analyses of intracellular forms of α_1-AGP isolated from subcellular fractions of rat liver were successfully carried out by the alditol acetate procedure of Torello et al. (141). The gas chromatographic spectrum of glucose, galactose, mannose, meso-inositol and GlcNAc contained single peaks for each sugar with complete separation. For routine analysis, 2 to 5 nmoles of glycoprotein were placed in a 1 ml Reacti-vial with 4 N HCl, degassed with nitrogen, capped tightly with Teflon-lined screw caps and placed in a heating block at 100° for 3 h. The vials were removed from the heating block, cooled and dried over NaOH in vacuo. An aliquot of 100 mM of meso-inositol as internal standard was added to each vial followed by 0.5 ml freshly prepared $NaBH_4$ in 1 M NH_4OH (2 mg/ml); each vial was capped, vortexed and incubated at room temperature for 1 h. Excess reducing reagent was destroyed by dropwise addition of glacial acetic acid until effervescence ceased; the solution was taken to near dryness with nitrogen at 60-80°. The viscous boric acid remaining was removed by adding 1 ml methanol-benzene mixture (5:1, v/v); capped tightly, vortexed, heated to 90° for 5 min, and evaporated to near dryness with nitrogen at 60-80°. This procedure was repeated five times with pure methanol and finally samples were taken completely to dryness; 0.75 ml acetic anhydride was added, capped tightly, vortexed, heated to 100° for 1.5 h in a heating block, and taken to dryness with nitrogen at 38°. Samples of 0.5 ml of chloroform were added and the salts removed by partitioning against 0.5 ml distilled

water; the partitioning with water was repeated five times. The desalted samples were taken to dryness at 38° with nitrogen and stored under P_2O_5 at room temperature overnight. The alditol acetate derivatives were dissolved in 100 µl of chloroform; vortexed and transferred to 100 µl Reacti-vials attached to the auto loader on the gas chromatograph. Analysis was performed with a Hewlett-Packard gas chromatograph Model 5700A equipped with a flame-ionization detector and a 6 ft x 2 mm glass column of 3% SP 2330 on 100-120 mesh Supelcoport (Supelco, Bellefonte, Pennsylvania, U.S.A.). Samples of 3 µl were injected and chromatographed with a temperature program of 230° for 8 min followed by a 4°/min gradient to 250° and held at 250° for 16 min. The nitrogen carrier gas was set at a flow rate of 20 ml/min; injection port at 250° and detector at 200°. The peaks were integrated with a Hewlett-Packard 3373B integrator. Standards consisted of 100 nM each of D-glucose, D-galactose, D-mannose, D-GlcNAc and meso-inositol. Relative response factors determined from the standard analysis were used in the calculation of sugars hydrolyzed from the glycoprotein.

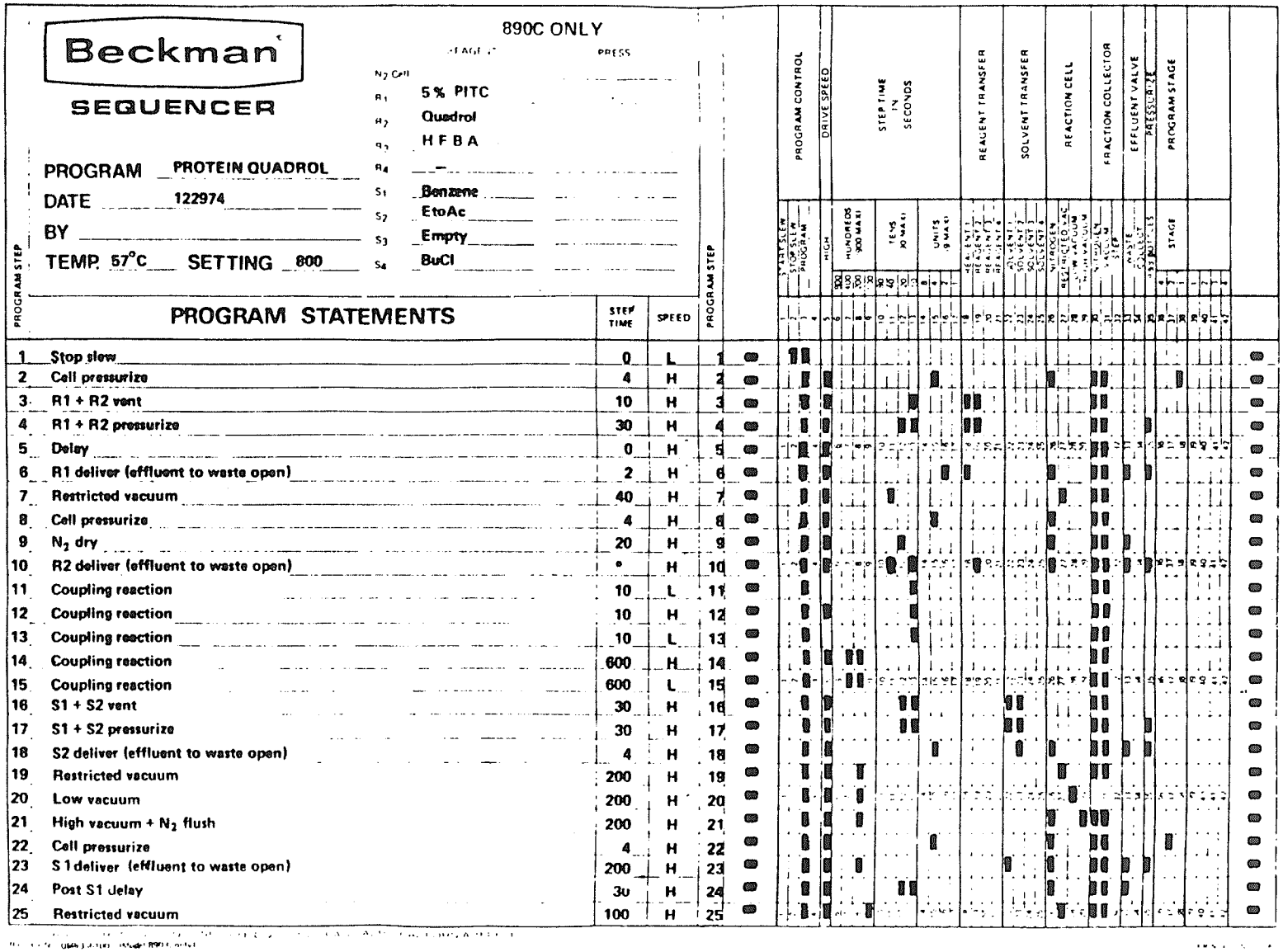
Primary structural analysis of rat α_1-AGP

Cleavage of N-terminal pyroglutamate N-terminal pyroglutamate was removed by treatment with L-pyroglutamate amino peptidase as described by Podell et al.(151). Protein (1 mg/ml) was dialyzed at 4° against a solution of 0.1 M sodium phosphate buffer, pH 8.0, containing 5 mM dithiothreitol, 10 mM EDTA, and 5% (v/v) glycerin.

The protein solution was transferred to a scintillation vial and pyroglutamate amino peptidase from calf liver was added; a fresh solution of 1 mg/ml was prepared and sufficient solution transferred to add 0.5 mg enzyme per 10 mg blocked protein. The tube was flushed with nitrogen, capped and incubated for 9 h at 4° with occasional mixing. After 9 h an additional 0.5 mg enzyme per 10 mg blocked protein was added; the vial flushed with nitrogen, capped and mixed. This solution was incubated at room temperature for 20 h with occasional mixing. The reaction was stopped by dialysis against 0.05 N acetic acid and lyophilized. The unblocked protein was analyzed by automatic amino acid sequencing with the Beckman 890C sequencer as described below. In initial experiments the pyroglutamate amino peptidase was removed from the treated protein by gel permeation chromatography. Later a control experiment, which consisted of a sequence analysis of about five times the weight of pyroglutamate amino peptidase (2 mg) present with the treated protein, was performed and in subsequent experiments peptidase enzyme was not removed for sequence analysis.

N-terminal sequence Automated N-terminal amino acid sequence analysis was performed by the Edman degradation technique described by Niall (152,153) using a Beckman Model 890C Sequencer with a 1 M Quadrol program 122974 supplied by Beckman with modifications shown in Fig. 11. All chemicals used in the Sequencer were sequence grade supplied by Beckman Instruments Inc., Palo Alto, California or Pierce Chemical Co., Rockford, Illinois. Between 30 nM and 250 nM

Fig. 11 Sequencer Program. Modified Beckman 122974 program used for automated N-terminal Edman degradation sequencing of protein with a Beckman 890C Sequencer



Beckman

SEQUENCER

890C ONLY

PROGRAM PROTEIN QUADROL

DATE 122974

BY

TEMP. 57°C SETTING 800

N_2 Cell

R_1 5% PITC

R_2 Quadrol

R_3 HFBA

R_4 —

S_1 Benzene

S_2 EtoAc

S_3 Empty

S_4 BuCl

PROGRAM STEP	PROGRAM STATEMENTS	STEP TIME	SPEED	PROGRAM STEP
1	Stop slew	0	L	1
2	Cell pressurize	4	H	2
3	R1 + R2 vent	10	H	3
4	R1 + R2 pressurize	30	H	4
5	Delay	0	H	5
6	R1 deliver (effluent to waste open)	2	H	6
7	Restricted vacuum	40	H	7
8	Cell pressurize	4	H	8
9	N_2 dry	20	H	9
10	R2 deliver (effluent to waste open)	*	H	10
11	Coupling reaction	10	L	11
12	Coupling reaction	10	H	12
13	Coupling reaction	10	L	13
14	Coupling reaction	600	H	14
15	Coupling reaction	600	L	15
16	S1 + S2 vent	30	H	16
17	S1 + S2 pressurize	30	H	17
18	S2 deliver (effluent to waste open)	4	H	18
19	Restricted vacuum	200	H	19
20	Low vacuum	200	H	20
21	High vacuum + N_2 flush	200	H	21
22	Cell pressurize	4	H	22
23	S1 deliver (effluent to waste open)	200	H	23
24	Post S1 delay	30	H	24
25	Restricted vacuum	100	H	25

PROGRAM CONTROL

DRIVE SPEED

STEP TIME IN SECONDS

REAGENT TRANSFER

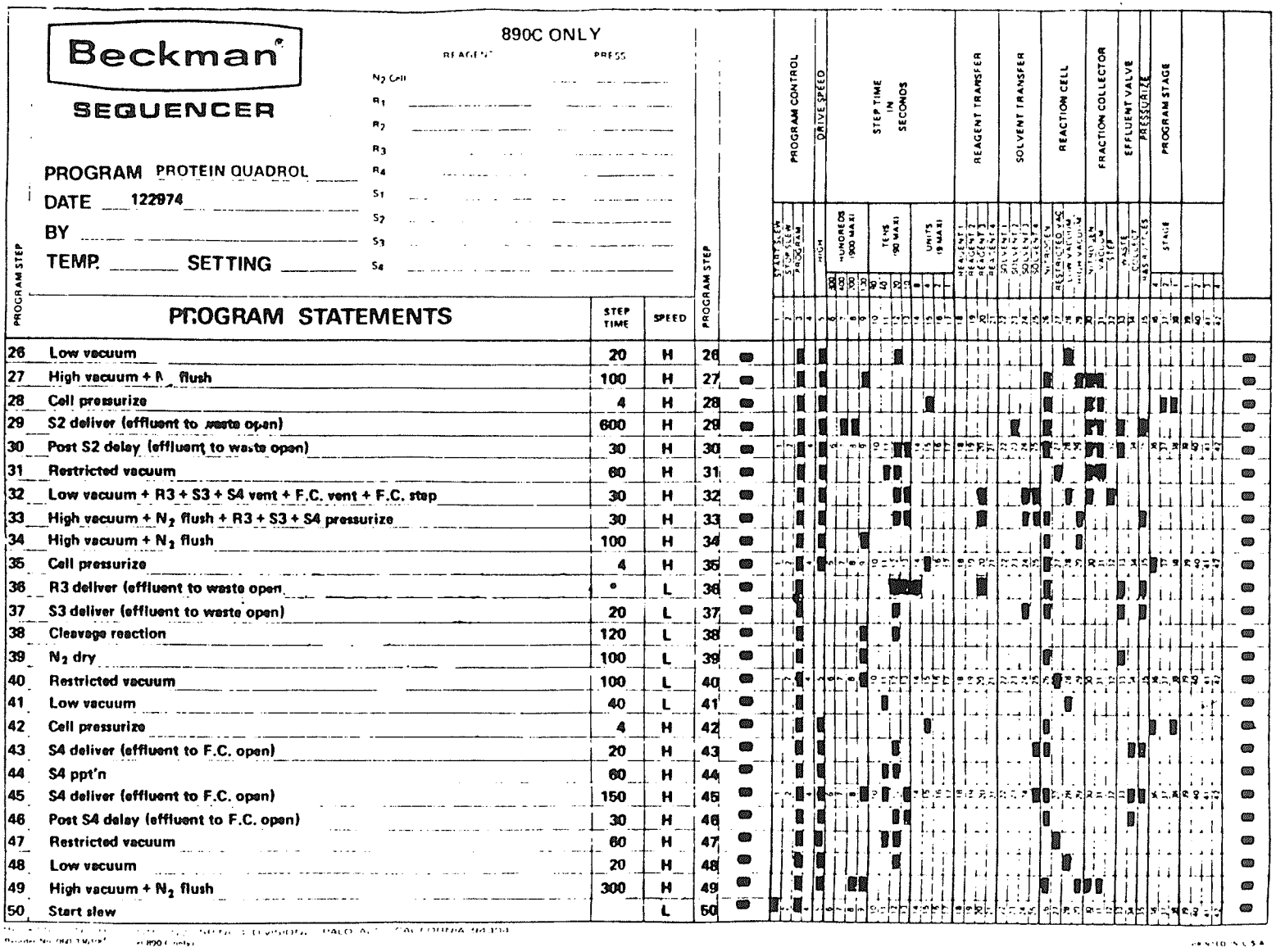
SOLVENT TRANSFER

REACTION CELL

FRACTION COLLECTOR

EFFLUENT VALVE

PROGRAM STAGE


Beckman
SEQUENCER
890C ONLY
REAGENT
PRESS
N_2 Cell
R1
R2
R3
R4
S1
S2
S3
S4
PROGRAM PROTEIN QUADROL
DATE 122974
BY
TEMP. SETTING
PROGRAM CONTROL
DRIVE SPEED
STEP TIME IN SECONDS
REAGENT TRANSFER
SOLVENT TRANSFER
REACTION CELL
FRACTION COLLECTOR
EFFLUENT VALVE
PROGRAM STAGE
PROGRAM STEP
PROGRAM STATEMENTS
STEP TIME
SPEED
26 Low vacuum 20 H
27 High vacuum + R_ flush 100 H
28 Cell pressurize 4 H
29 S2 deliver (effluent to waste open) 600 H
30 Post S2 delay (effluent to waste open) 30 H
31 Restricted vacuum 60 H
32 Low vacuum + R3 + S3 + S4 vent + F.C. vent + F.C. step 30 H
33 High vacuum + N_2 flush + R3 + S3 + S4 pressurize 30 H
34 High vacuum + N_2 flush 100 H
35 Cell pressurize 4 H
36 R3 deliver (effluent to waste open 0 L
37 S3 deliver (effluent to waste open) 20 L
38 Cleavage reaction 120 L
39 N_2 dry 100 L
40 Restricted vacuum 100 L
41 Low vacuum 40 L
42 Cell pressurize 4 H
43 S4 deliver (effluent to F.C. open) 20 H
44 S4 ppt'n 60 H
45 S4 deliver (effluent to F.C. open) 150 H
46 Post S4 delay (effluent to F.C. open) 30 H
47 Restricted vacuum 60 H
48 Low vacuum 20 H
49 High vacuum + N_2 flush 300 H
50 Start stew L

of protein were transferred to the reaction cup in one of two ways: either freeze-dried protein was transferred to the cup followed by the addition of heptafluorobutyric acid (HFBA) to dissolve the sample, or a solution of protein in aqueous acetic acid or in distilled water was added to the cup. In both cases a film of the protein was created on the wall of the cup using a vacuum drying program. Sequencing was started by dissolving the dried protein film in 1 N Quadrol buffer [N,N,N'N' tetrakis (2-hydroxypropyl)-ethylenediamine] followed by a coupling reaction with phenylisothiocyanate (PITC) at 50°. Excess PITC was washed out of the cup with benzene which also precipitates the protein to prevent protein washout. This was followed by an ethyl acetate wash to remove Quadrol and the breakdown products of PITC. Anhydrous heptafluorobutyric acid was added to cleave off the N-terminal amino acid as the anilinothiazolinone derivative of that amino acid; and finally butyl chloride was used to extract the cleaved product from the cup to the fraction collector. The butyl chloride contained 0.1% dithiothreitol (DTT) to protect the serine and threonine derivatives from excess decomposition. The butyl chloride extracts were removed from the fraction collector and divided into two parts. One part, which consisted of two-thirds of the extract, was converted to free amino acids, the remainder was converted to phenylthiohydantin (PTH) amino acids. For conversion to free amino acids the butyl chloride extract was transferred to hydrolysis tubes followed by removal of the butyl

chloride *in vacuo*. The dry thiazolinone derivatives were hydrolyzed with 6 N HCl containing 0.1% stannous chloride *in vacuo* at 150° for 4 h. The HCl was removed *in vacuo* over solid NaOH and the free amino acids analyzed with a Technicon NC-2P amino acid analyzer.

For conversion to PTH amino acid, the butyl chloride extract was taken to dryness with nitrogen at 50-60°: 0.2 ml of 1 N HCl was added; the tube flushed with nitrogen and stoppered. Conversion was carried out at 80° for 10 min and the PTH derivatives extracted twice from the aqueous acid with aliquots of 0.7 ml of ethyl acetate. The ethyl acetate PTH extracts were taken to dryness with nitrogen and the residues analyzed for PTH amino acids by gas chromatography (154). Assignments of amino acids were made on the basis of the amino acid analysis and these results checked with the gas chromatographic analysis of the PTH derivatives.

The phenylthiohydantin derivative of norleucine (50 nmol) was added to each tube in the Sequencer fraction collector as an internal standard. The content of norleucine in the amino acid analysis data was used to standardize the recovery of the amino acids obtained at each cycle of the Edman degradation. Repetitive yields from cycle to cycle were obtained by calculating the relative yields of a specific amino acid at two different cycles.

Preparation of CNBr peptides from rat α_1-AGP For cleavage with cyanogen bromide (CNBr), rat α_1-AGP (100 mg) was dissolved in 10 ml of 70% formic acid. Approximately 1000-fold molar

excess (200 mg) of CNBr was added and the mixture stirred in the dark under nitrogen at room temperature for 20 h (155). The reaction was stopped by the addition of 100 ml distilled water and the solution lyophilized. For reduction of the disulfide bonds, CNBr-treated α_1-AGP was dissolved in 10 ml of 0.1 M Tris-HCl buffer, pH 8.6, containing 6 M guanidine hydrochloride and 10 mg EDTA followed by the addition of 200 µl β-mercaptoethanol. The solution was stirred at room temperature for 7 h and the α_1-AGP was alkylated by the addition of 540 mg iodoacetic acid which had been dissolved in 1 ml 1 N NaOH. The reaction proceeded for 30 min during which time the pH was maintained at 8.0 by the addition of 1 N NaOH.

The reduced and alkylated CNBr peptides of rat α_1-AGP were chromatographed on columns of ConA-Sepharose and Sephadex G-100. The mixture of peptides was loaded onto a 1 cm x 5 cm column of ConA-Sepharose. Stepwise elution of the fraction was performed with ConA-Buffer I and ConA-Buffer II (as described above) at a flow rate of 20 ml/h and 3 ml fractions were collected. Fractions were analyzed for protein by measuring the absorbance at 280 nm; protein fractions eluted with ConA-Buffer I were pooled and referred to as CNBr ConA-I; protein fractions eluted with ConA-Buffer II were pooled and referred to as CNBr ConA-II. Each fraction was freeze dried and chromatographed on a 2 cm x 150 cm column of Sephadex G-100. Elution was carried out with 20% acetic acid at a flow rate of 15 ml/h and 5 ml fractions were collected. Fractions were analyzed for protein

by measuring the absorbance at 280 nm. Fractions containing protein were pooled, freeze dried, and analyzed with SDS PAGE on 12% gels.

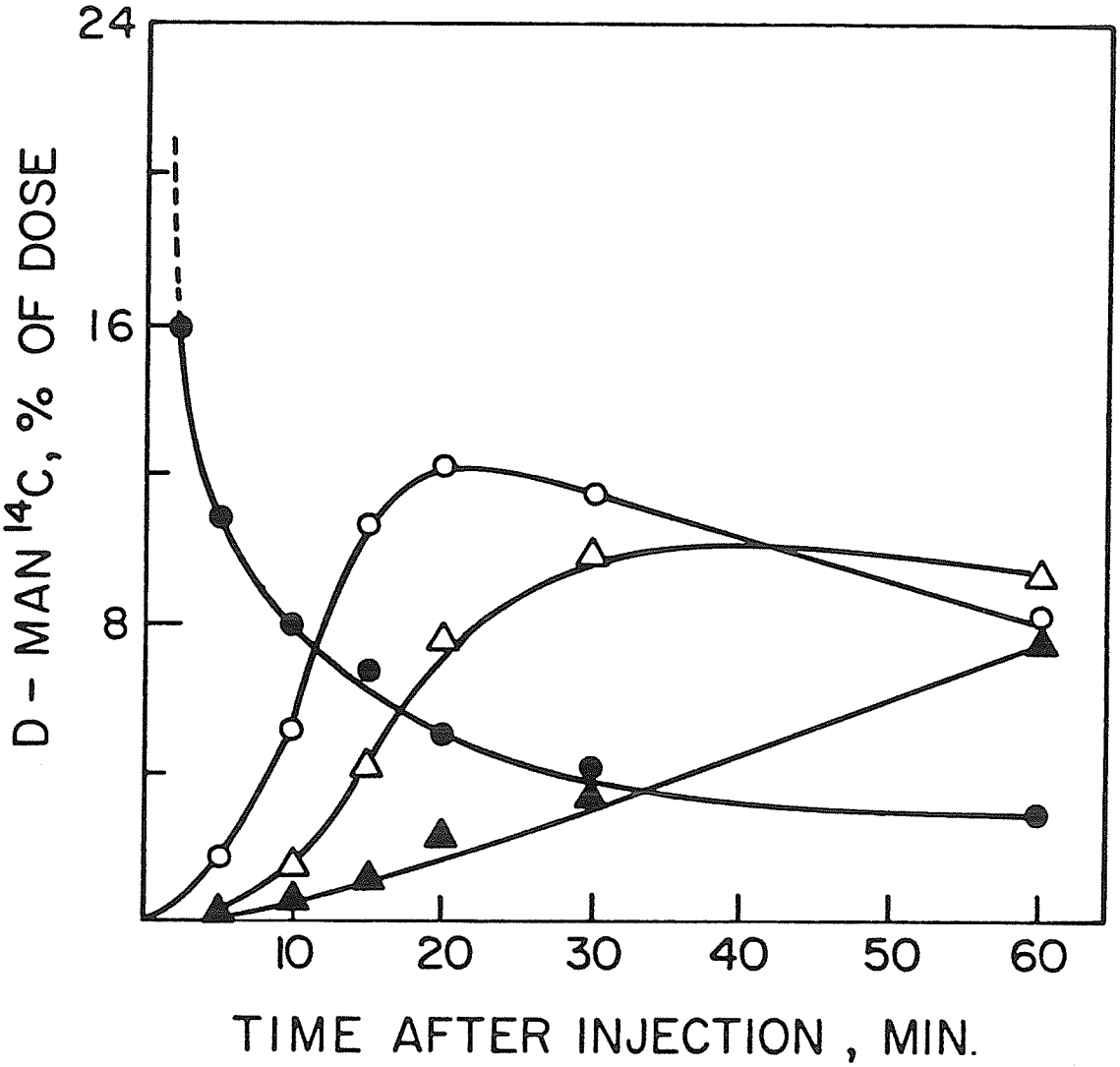
RESULTS

Uptake of D-[^{14}C]-mannose from serum

According to the current understanding of the synthesis of oligosaccharides of secretory glycoproteins (Fig. 8) a high mannose containing form of α_1-AGP should be present in the rough membrane fraction of rat liver. Incorporation of radioactive mannose should result in a high content of label in this fraction since each carbohydrate chain should contain nine mannose residues. Therefore, as a preliminary to an attempt to isolate a high mannose form of α_1-AGP, a kinetic study on the incorporation of D-[^{14}C]-mannose into liver proteins was performed. Rats suffering from inflammation were used in addition to control rats because of the higher glycoprotein biosynthetic capacity associated with liver from inflamed animals (22).

The first experiments followed the uptake of radioactive mannose from serum into liver and the incorporation of radioactivity into total serum protein. Fig. 12 shows that most of the radioactive mannose was cleared from the serum within 5 min after injection. Uptake into the liver occurred first in the non-protein bound fraction reaching a maximum at 20 min after injection, and then into the protein-bound fraction reaching a maximum at 30 min after injection. The D-[^{14}C]-mannose appeared in serum protein at 15 min to 20 min after injection increasing to about 8% of the initial dose at 60 min after injection.

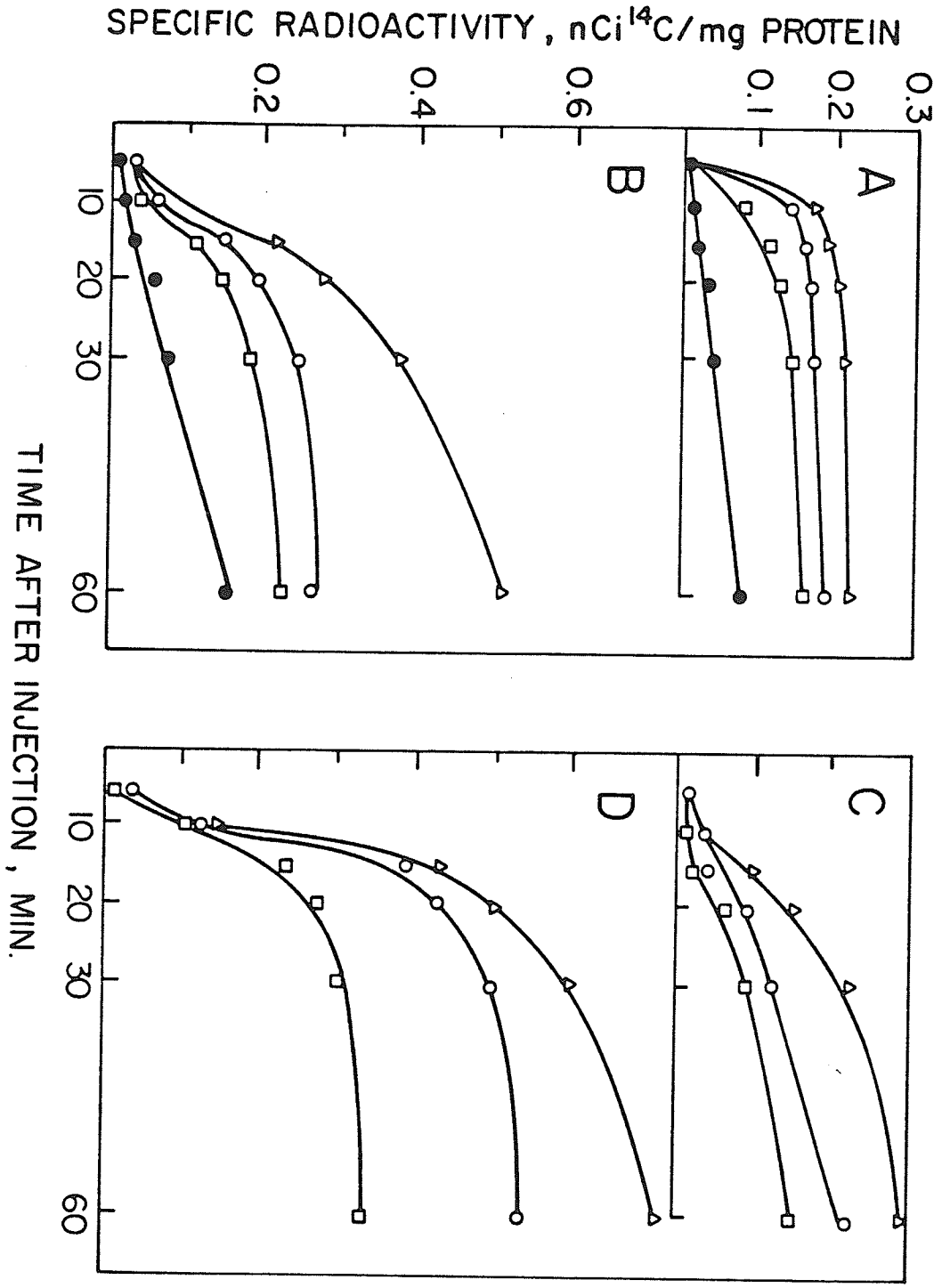
Fig. 12 Total radioactivity in trichloroacetic acid soluble and protein-bound fractions from rat liver and serum following intravenous injection of 15 μCi D-[^{14}C] mannose; —●—, trichloroacetic acid soluble fraction of serum; —○—, trichloroacetic acid soluble fraction of liver; —▲—, protein-bound fraction of serum; —△—, protein-bound fraction of liver. To construct the graph it was assumed that serum represented 3.5% and liver 3.8% of body weight (see 22). Each point represents the mean from three to four experiments; reproducibility was within ± 12%


24
16
8
D - MAN ^{14}C, % OF DOSE
10
20
30
40
50
60
TIME AFTER INJECTION , MIN.

The second set of experiments involved the incorporation of D-[^{14}C]-mannose into liver subcellular fractions. Fig. 13 shows the results of incorporation into serum protein and total protein of subcellular liver fractions and Lubrol extracts of the same fractions from experiments with control and 24 h experimental rats.

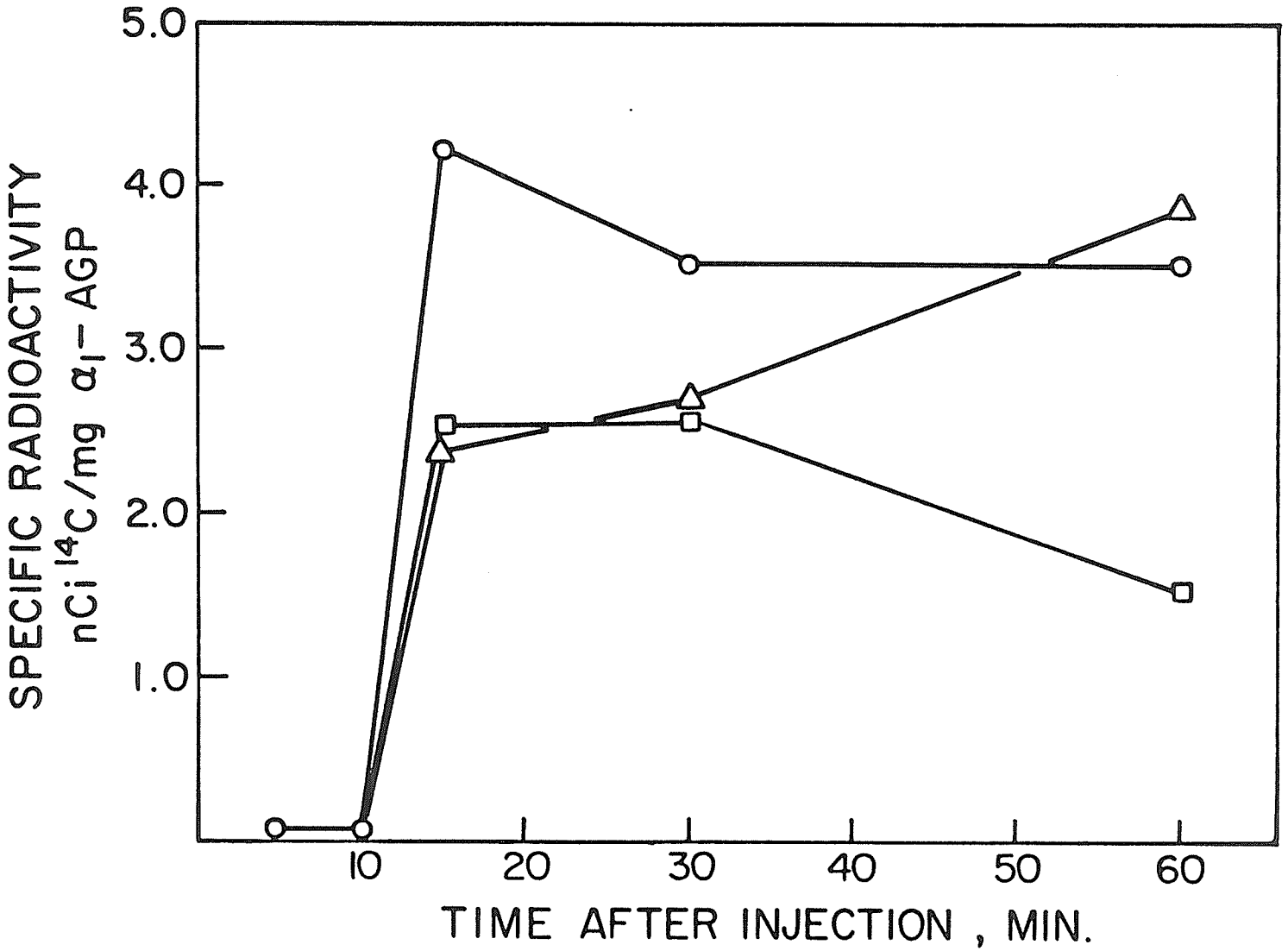
It was found that incorporation into serum and liver fractions from experimental rats was always about twice that found in corresponding fractions from control rats (Fig. 13, compare panels A with B and C with D). The highest specific radioactivities were found associated with Lubrol extracts of Golgi fractions (Fig. 13, panels C and D) although Lubrol extracts of rough fractions were also highly labelled, particularly at short times of incorporation. Lubrol extracts of smooth fractions had specific radioactivities which were significantly lower than those found for Lubrol extracts of rough and Golgi fractions. The pattern of labelling of total protein from liver subcellular fractions (Fig. 13, panels A and B) was generally similar to that found for the Lubrol extracts (Fig. 13, panels C and D) except that the specific radioactivities in the total protein fractions were lower than those found for the corresponding Lubrol-soluble fractions. A variance to this generalization was observed in the total protein fractions from control livers at early times of labelling.

Fig. 13 Incorporation of D-[^{14}C]-mannose into serum protein from subcellular fractions of liver, and Lubrol extracts of subcellular fractions of liver from control and 24 h experimental rats following intravenous injection of 15 μCi D-[^{14}C]-mannose. Panels A and B, total protein from control (panel A) and experimental (panel B) rats; —△—, total Golgi protein; —○—, total rough fraction protein; —□—, total smooth fraction protein; —●—, total serum protein. Panels C and D, Lubrol-soluble protein from control (panel C) and experimental (panel D) rats; —△—, Lubrol-soluble protein from Golgi fraction; —○—, Lubrol-soluble protein from rough fraction; —□—, Lubrol-soluble protein from smooth fraction. Each point represents the mean from three to four experiments; reproducibility was within ±12%.


SPECIFIC RADIOACTIVITY, nCi ^{14}C/mg PROTEIN
0.2
0.4
0.6
0.1
0.2
0.3
A
B
C
D
10
20
30
60
TIME AFTER INJECTION, MIN.

The third part of this study involved the incorporation of D-[^{14}C]-mannose into α_1-AGP, a specific glycoprotein which has been extensively characterized in this laboratory (22,48,117). Rat α_1-AGP was isolated from serum and Lubrol extracts of subcellular fractions of liver by immune precipitation as described in Experimental. Fig. 14 shows the kinetics of incorporation of D-[^{14}C]-mannose into α_1-AGP isolated immunologically from Lubrol extracts of rough, smooth and Golgi fractions of liver from 24 h experimental animals. A lag of 10 min was observed before label appeared in α_1-AGP isolated from extracts of the subcellular fractions of liver. Following the lag there was a rapid increase in specific radioactivities of α_1-AGP with the highest level found in extracts of rough fractions. The maximum specific radioactivity was found in rough fractions at 15 min; this declined at 30-60 min after injection. The specific radioactivities of α_1-AGP isolated from extracts of smooth fractions also reached a maximum at 15 min after injection followed by a decline at 30-60 min after injection in a similar pattern to that observed in the rough fraction. The specific radioactivities of α_1-AGP isolated from the extracts of Golgi fractions increased rapidly at 15 min in parallel with the increase observed in the smooth fraction but continued to increase at 30-60 min after injection (Fig. 14). One explanation for the high specific radioactivities of α_1-AGP in extracts of the rough membrane

Fig. 14 Specific radioactivities of α_1-AGP isolated from Lubrol extracts of liver subcellular fractions after intravenous injection of 15 μCi D-[^{14}C]-mannose into 24 h experimental rats: —○—, rough fractions; —□—, smooth fractions; —△—, Golgi fractions. Each point represents the mean of three experiments; reproducibility was within ±12%.


SPECIFIC RADIOACTIVITY
nCi ^{14}C/mg α_1– AGP
5.0
4.0
3.0
2.0
1.0
10
20
30
40
50
60
TIME AFTER INJECTION , MIN.

fraction at short times of labelling is that the protein isolated from the rough fraction contained a higher proportion of mannose. This explanation would be consistent with the current understanding of the mechanism of glycosylation of secretory glycoproteins discussed above.

Distribution of radioactivity in sugars following labelling of protein with D-[^{14}C]-mannose

Labelling studies described above will only be valid if the label remains associated with mannose residues. The results of a study to examine the distribution of label in sugars following injection of D-[^{14}C]-mannose are shown in Table 3. Total protein isolated from Lubrol extracts and α_1-AGP precipitated immunologically with antiserum to α_1-AGP from these extracts were hydrolyzed and the distribution of radioactivity in various sugars was determined. At 20 min and 30 min after incorporation, the ^{14}C label was mainly found in mannose. At 60 min after incorporation more label became associated with the fucose and galactose fractions isolated from the total protein isolated from Lubrol extracts of smooth and Golgi fractions. Some conversion to galactose was also observed in α_1-AGP isolated from extracts of smooth and Golgi fractions, but at all times up to 60 min after injection at least 70% of the label remained associated with mannose in both total protein and α_1-AGP. This indicated that the specific radioactivity measured in the

Table 3

Distribution of radioactivity in sugars after incorporation of D-[^{14}C]-mannose into total protein and α_1-AGP isolated from Lubrol extracts of liver subcellular fractions

Fraction	Incorporation time min	Radioactivity recovered, %									
		Total protein from Lubrol extracts					α_1-AGP from Lubrol extracts				
		Origin	Man	Gal	Fuc	Total	Origin	Man	Gal	Fuc	Total
rough	20	5.0	64.5	2.0	3.0	74.5	8.0	60.5	4.0	5.0	77.5
smooth	20	4.0	63.0	3.0	2.0	72.0	7.0	59.0	4.0	7.5	77.5
Golgi	20	5.0	61.0	4.0	5.0	75.0	4.0	62.0	6.0	7.0	79.0
rough	30	8.0	56.5	4.0	5.0	73.5	9.0	67.0	4.0	4.0	84.0
smooth	30	9.0	59.0	6.0	8.0	82.0	10.0	64.0	3.0	7.5	85.5
Golgi	30	5.0	63.0	7.0	7.5	82.5	11.0	62.0	9.0	6.0	88.0
rough	60	5.0	66.0	4.0	5.0	80.0	9.0	66.0	5.0	3.0	83.0
smooth	60	4.0	58.0	7.0	12.0	81.0	6.0	59.0	9.5	4.0	78.5
Golgi	60	5.0	57.0	9.0	14.0	85.0	8.5	59.0	10.0	4.0	81.5

Radioactivity in Glc, glucosamine and galactosamine was also determined, but was found to be neglibible and is not reported. Radioactivity recovered is expressed as a percentage of the radioactivity applied to the origins: for total protein of Lubrol extracts 5.5-6.0 x 10^3 cpm were applied; for α_1-AGP immune complexes 4-5 x 10^3 cpm were applied. Results are typical values; when replicates were analyzed recoveries were within ±10% of those reported above.

incorporation experiments was mainly due to mannose and not other sugars in the glycoproteins.

Isolation of intracellular forms of α_1-AGP from subcellular fractions of rat liver

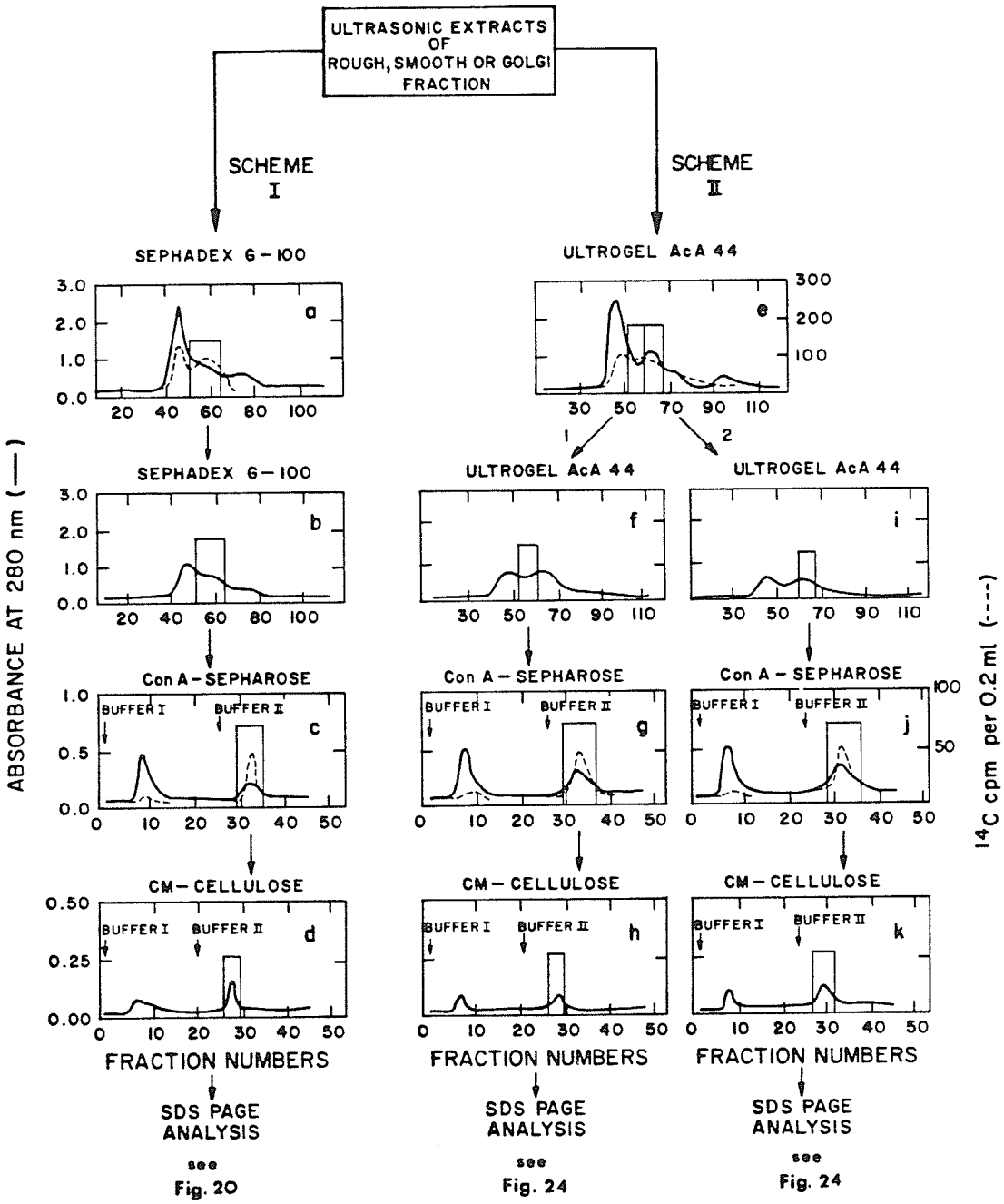
The kinetic studies suggested that a high mannose form of α_1-AGP might be present in the rough fraction of rat liver. An attempt was made to isolate this form to study how it might be involved in biosynthesis and secretion from liver. Livers from 24 h experimental rats were used for the preparation of intracellular forms of α_1-AGP because earlier studies (22) showed that turpentine induced inflammation resulted in a 4- to 5- fold increase in α_1-AGP content in liver. Rats were also injected with D-[^{14}C]-mannose 15 min to 20 min prior to sacrifice to label glycoproteins and the liver was perfused with ice-cold saline to minimize contamination by residual serum α_1-AGP present in blood in the liver. Subcellular fractionation of homogenized liver was carried out as described in Experimental and proteins associated with liver fractions were extracted by treatment with ultrasound for 60 sec. In the case of secretable proteins these would normally be found in the intracisternal space or loosely bound to the membranes. Ultrasonic extraction was used instead of Lubrol for two reasons: first, in order to eliminate the necessity of removing the detergent; secondly, because membranes disrupted by ultrasonic vibrations can be removed by centrifugation, whereas Lubrol ex-

traction dissolves the membrane proteins and includes them in solution with the intracisternal material. Other workers (134) who attempted to isolate intracellular forms of albumin from detergent extracts employed antiserum to albumin. The immune precipitate that formed was separated by centrifugation, dissociated in acid, and the intracellular precursor albumin separated from the immune globulin. This approach was tried with the rat α_1-AGP, but the immune complex did not dissociate at high salt, in glycine-HCl buffer, pH 2.5, in 3 M KSCN, or in 4 M urea. Affinity chromatography using the immunoglobulin fraction of the antiserum to rat α_1-AGP bound to Sepharose was also tried but was found to be unsuccessful. The approach that was used to isolate intracellular forms of α_1-AGP from ultrasonic extracts of rough, smooth and Golgi fractions involved initial gel permeation chromatography followed by affinity chromatography on ConA-Sepharose with the final step being ion exchange chromatography on CM-cellulose. Two fractionation procedures were used, both of which are presented in Fig. 15. In initial studies, intracellular forms of α_1-AGP were isolated from the ultrasonic extracts of each of the three subcellular fractions of liver by the procedure presented as scheme I in Fig. 15 which involved the use of Sephadex G-100 in the two gel permeation chromatographic steps. The elution of intracellular α_1-AGP was monitored by examination of fractions for radioactivity due to D-[^{14}C]-mannose and by double diffusion analysis with antiserum to α_1-AGP. Fractions eluting from the Sephadex G-100 column (Fig. 15a) which contained both α_1-AGP immunological activity and

Fig. 15 Flow diagram of the chromatographic procedures used in the preparation of intracellular forms of α_1-AGP from ultrasonic extracts of rough, smooth and Golgi fractions of rat liver.

The solid lines in each graph indicate the absorbance at 280 nm and the dashed lines the radioactivity in cpm per 0.2 ml. The blocked area indicates the fractions which contained material reacting immunologically with anti-α_1-AGP; these fractions were pooled. The elution conditions for each chromatography step are described in Experimental.

Two schemes were followed for the preparation of intracellular forms of α_1-AGP. In scheme I, the initial gel permeation chromatography step was performed with Sephadex G-100 (a). The α_1-AGP material collected was recycled on Sephadex G-100 (b) followed by purification on a column of ConA Sepharose (c) and finally chromatography on CM-cellulose (d). In scheme II, the gel permeation steps were performed on Ultrogel AcA44 (e), (f) and (i); use of Ultrogel AcA44 resulted in the separation of two fractions (f) and (i) which were separately purified on ConA-Sepharose (g) and (j) and CM-cellulose (h) and (k).



see Fig. 20

see Fig. 24

see Fig. 24

mannose label were pooled and recycled on the column of Sephadex G-100 (Fig. 15b). The fractions containing α_1-AGP immunologically reactive material from the second Sephadex column were pooled and then further fractionated on ConA-Sepharose to remove non-glycoprotein components such as albumin (Fig. 15c). Examination of fractions eluted from the ConA-Sepharose column with antiserum to α_1-AGP and albumin indicated that all the albumin was eluted with Buffer I and that the α_1-AGP was eluted with Buffer II. Material eluting with Buffer II, referred to as ConA-II, was pooled and applied to a column of CM-cellulose (Fig. 15d). The buffer conditions used in stepwise elution from CM-cellulose were those used for the purification of α_1-AGP from serum (117). With these conditions Buffer I eluted serum-type α_1-AGP and Buffer II eluted forms of α_1-AGP which were more basic in nature (see below).

In later studies, an improvement in the separation of intracellular forms of α_1-AGP was achieved by replacing the Sephadex G-100 step with Ultrogel AcA44 chromatography (Fig. 15e). The intracellular forms of α_1-AGP eluted from Ultrogel AcA44 appeared to separate into two fractions, one eluting slightly ahead of the other. The two fractions referred to as 1 and 2 were purified separately using the same approach described above; that is, two cycles of chromatography with Ultrogel AcA44 followed by ConA-Sepharose and CM-cellulose chromatography (Figs. 15f-h and 15i-k).

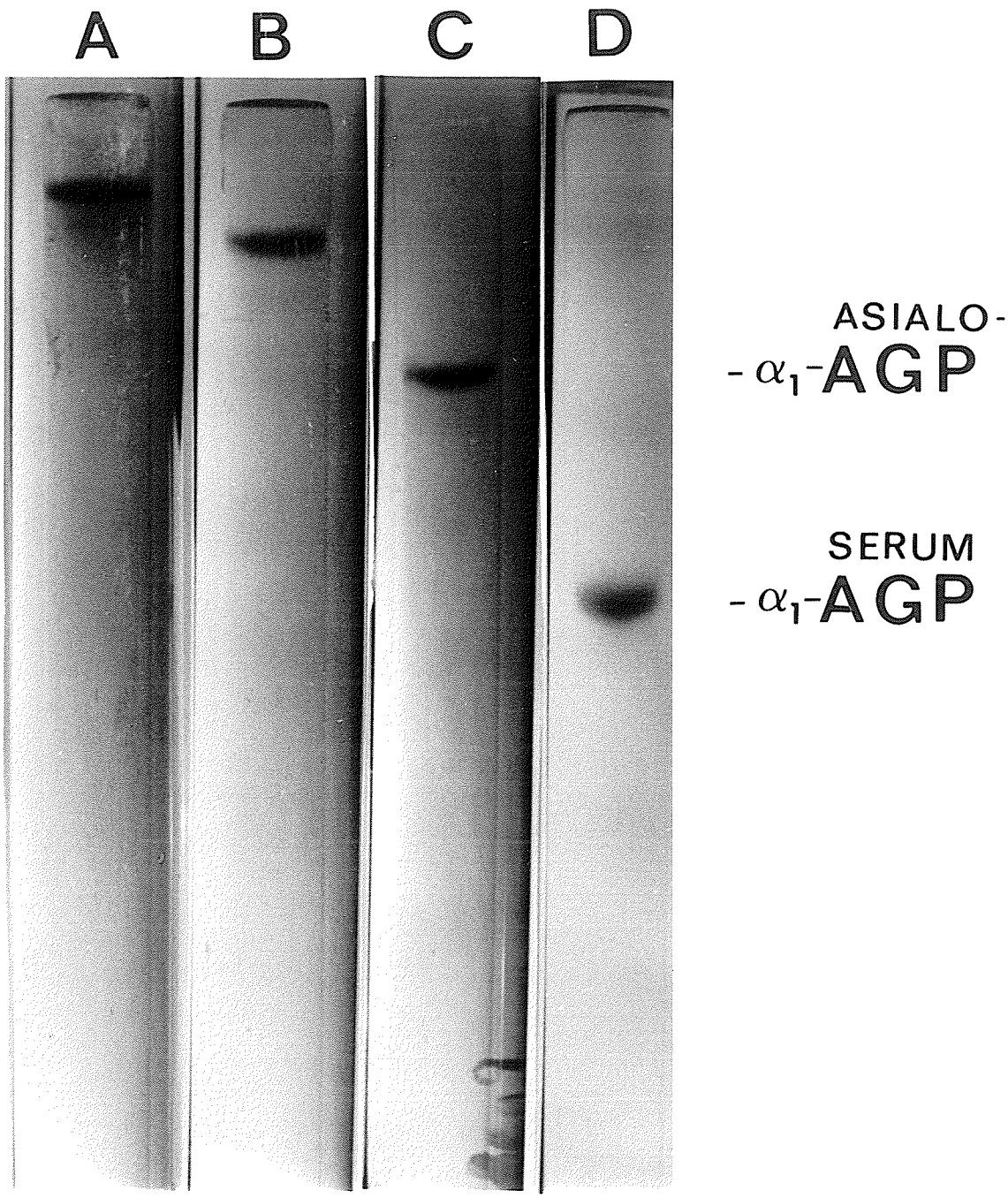
Characterization of intracellular forms of α_1-AGP isolated from the rough membrane fraction of rat liver by scheme I in Fig. 15

The development of the separation procedure outlined in Fig. 15 scheme I made possible the isolation of intracellular forms of α_1-AGP from rough fractions for analysis.

Electrophoretic analysis The intracellular form of α_1-AGP prepared from the ultrasonic extract of rough fractions was examined electrophoretically with the basic PAGE system at pH 8.9, and with gels containing SDS. The basic PAGE pattern of intracellular forms of α_1-AGP prepared from the rough fraction by scheme I (see Fig. 15) showed one major band with a few minor contaminants (Fig. 16 gel A). The major band had a much lower electrophoretic mobility than serum α_1-AGP (Fig. 16, gel D) and serum α_1-AGP from which sialic acid had been removed (Fig. 16, gel C). It can be seen that removal of the acidic NeuAc residues resulted in a significant reduction in electrophoretic mobility of α_1-AGP due to the more basic nature of the asialo-form. However, the mobility of the asialo-α_1-AGP was still higher than that of the intracellular form (Fig. 16, compare gel A and C).

This suggests that the intracellular form is likely to be more basic in nature than asialo-α_1-AGP although it is possible that the electrophoretic behavior of the intracellular form could be explained by the presence of a high molecular weight species which migrates more slowly through the gel than asialo-α_1-AGP.

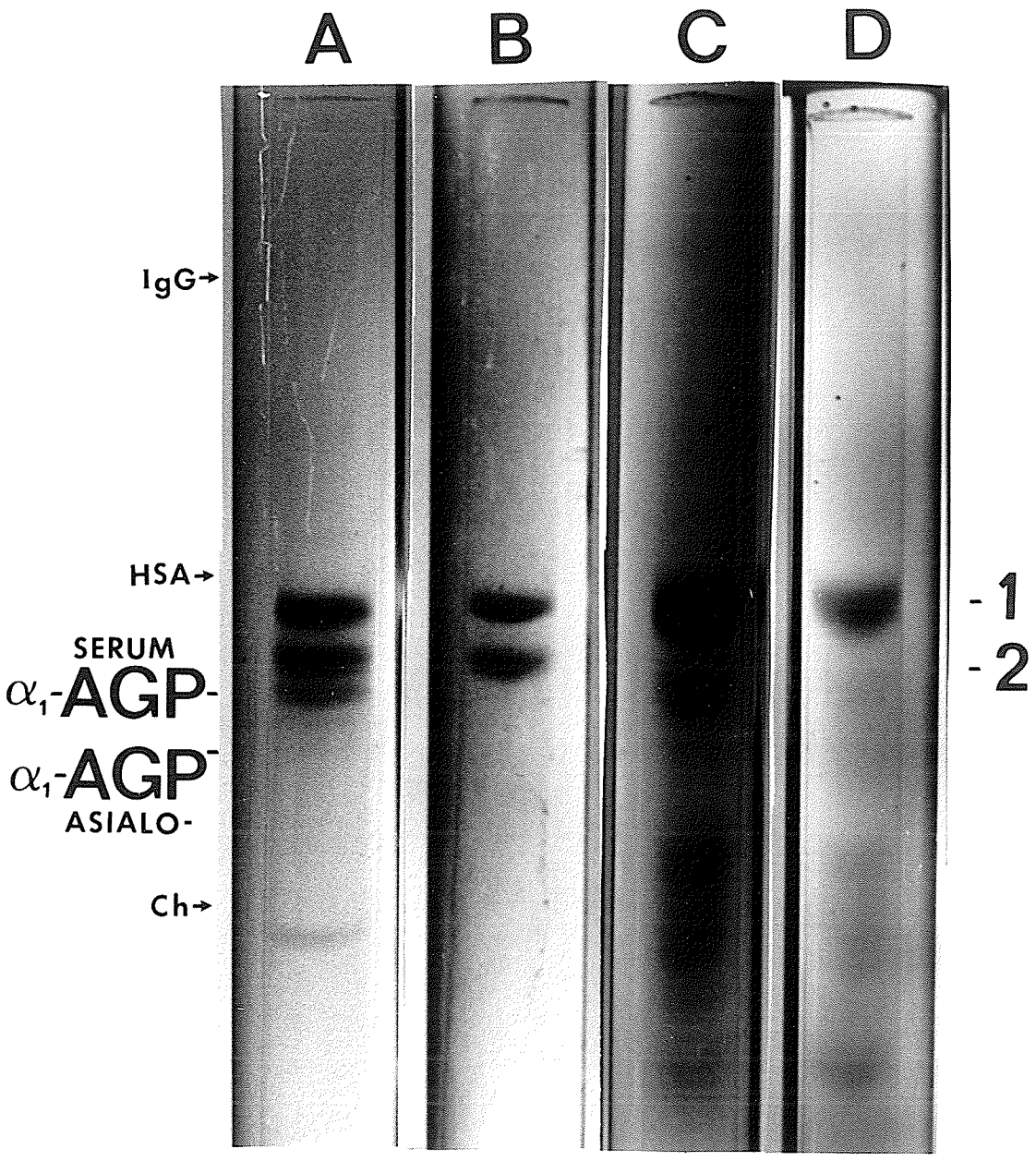
Fig. 16 Basic PAGE on 7.5% gels of: gel A, intracellular form of α_1-AGP isolated from rough membrane fraction of rat liver; gel B, intracellular form of α_1-AGP isolated from rough membrane fraction of rat liver and treated with trypsin for 2 h as described in Experimental; gel C, serum α_1-AGP treated to remove NeuAc (asialo-α_1-AGP); gel D, serum α_1-AGP.


A
B
C
D
ASIALO-
-α_1-AGP
SERUM
-α_1-AGP

Analysis of the intracellular forms of α_1-AGP isolated from rough membrane fractions according to scheme I by SDS PAGE is shown in Fig. 17, gel B. The gel pattern contained two major bands of about equal intensity and with lower electrophoretic mobilities than α_1-AGP and asialo-α_1-AGP. This is shown in Fig. 17, (gel A), where the analysis was performed on a sample which had not been chromatographed on CM-cellulose. As indicated earlier this step would normally remove native α_1-AGP which comes from residual serum in the liver. The presence of the two bands on SDS PAGE suggests the presence of two forms of intracellular α_1-AGP which have slightly different molecular weights, but which appear to have similar charge/mass ratios by virtue of the fact that only one band was observed on electrophoresis on basic PAGE (Fig. 16, gel A). Since the two bands observed on SDS PAGE appear to constitute different forms of intracellular α_1-AGP they are referred to below as intracellular α_1-AGP forms 1 and 2. Forms 1 and 2 correspond to the bands of lower and higher electrophoretic mobilities, respectively, when analyzed on SDS PAGE.

Limited trypsin treatment of intracellular forms of α_1-AGP isolated from rough membrane fractions of rat liver In studies on intracellular forms of serum albumin it was found that limited trypsin treatment of a pro-form released a hexapeptide which was located on the N-terminus (see Table 2) resulting in the formation of native serum albumin with an accompanying increase in isoelectric point (134). This approach was employed in the present studies to determine if the

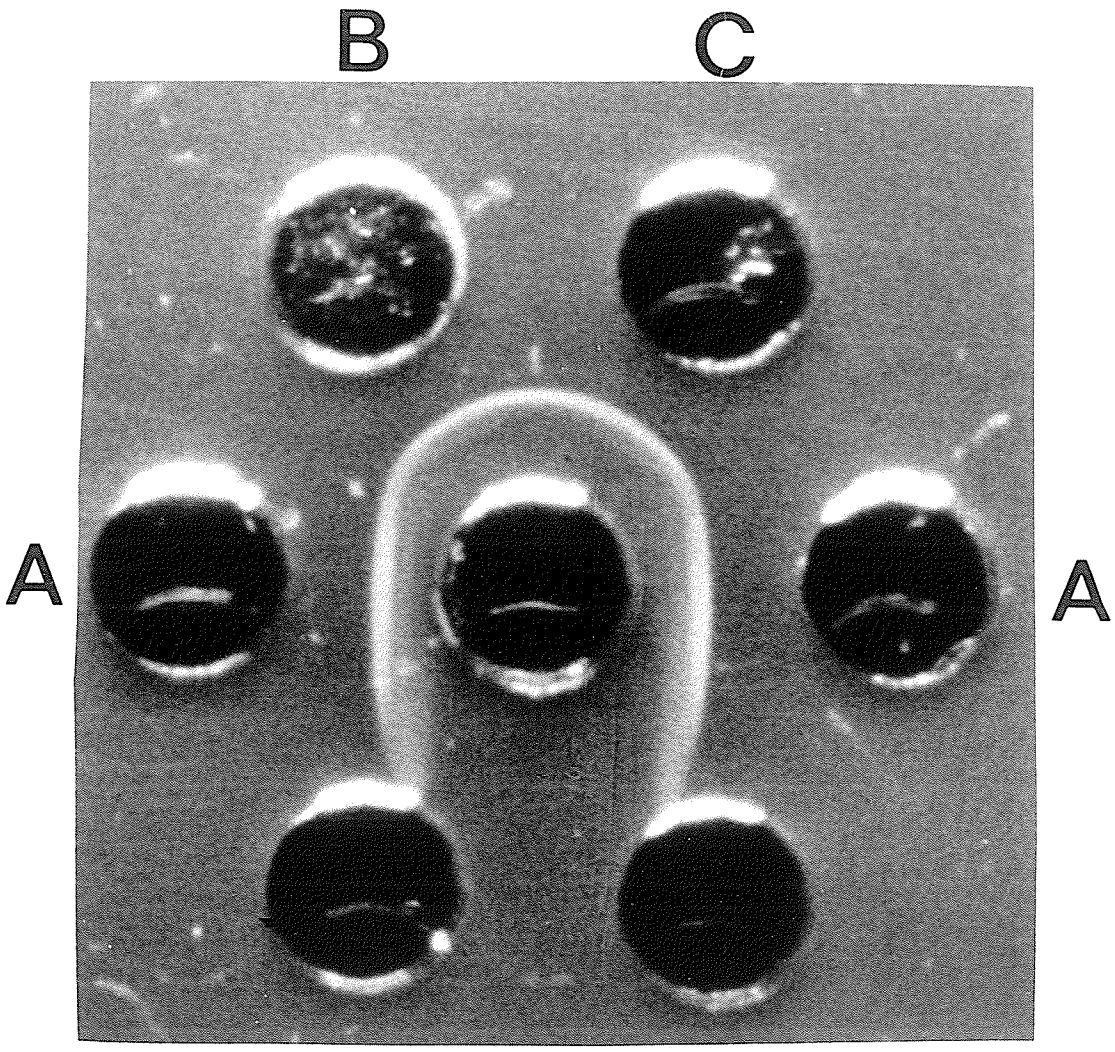
Fig. 17 SDS PAGE on 7.5% gels of: gel A, intracellular forms of α_1-AGP isolated from rough membrane fraction of rat liver, analysis following ConA-Sepharose chromatography step; gel B, intracellular forms of α_1-AGP isolated from rough membrane fraction of rat liver, analysis following CM-cellulose chromatography step; gel C, intracellular forms of α_1-AGP isolated from rough membrane fraction and treated with trypsin for 1 h; gel D, intracellular forms of α_1-AGP isolated from rough membrane fraction and treated with trypsin for 2 h. In these experiments the trypsin reaction was stopped by addition of reducing buffer containing 0.04 M dithiothreitol, 1% SDS and 0.001 M EDTA, the mixture was then incubated at 100° for 5 min followed immediately by electrophoresis on SDS PAGE. The arrows indicate the electrophoretic mobility of other proteins; IgG, human immune globulin (M.W. 150,000); HSA, human serum albumin (M.W. 66,000); Ch, chymotrypsinogen (M.W. 25,000).


A
B
C
D
IgG→
HSA→
SERUM
α1-AGP-
α1-AGP⁻
ASIALO-
Ch→
- 1
- 2

precursor α_1-AGP preparation was trypsin sensitive and whether the electrophoretic mobilities of the components of the preparation could be altered significantly in this way. Fig. 16 (gel B) shows that limited tryptic treatment of intracellular α_1-AGP from rough membrane fractions resulted in one band on basic PAGE with an electrophoretic mobility slightly greater than that of the untreated protein. The electrophoretic mobility of the trypsin treated form was still less than that of asialo-α_1-AGP. Fig. 17 (gels C and D) shows the results of tryptic treatment of intracellular α_1-AGP when examined on SDS PAGE. It was observed that intracellular α_1-AGP form 1 was less sensitive to trypsin treatment than form 2. Trypsin treatment of intracellular α_1-AGP for 1 h (Fig. 17, gel C) resulted in a reduction of form 2 relative to form 1 and, in addition, appeared to cause an increase in the electrophoretic mobility of form 2. Treatment of intracellular α_1-AGP with trypsin for 2 h resulted in the almost complete elimination of form 2, whereas form 1 appeared to be largely trypsin resistant under these conditions of treatment.

Immunological analysis of trypsin treated and untreated forms of intracellular α_1-AGP Double diffusion analysis of serum α_1-AGP, and trypsin treated and untreated forms of intracellular α_1-AGP from rough fractions is shown in Fig. 18. Tryptic treated and untreated forms of intracellular α_1-AGP both gave single lines on double diffusion analysis which showed reactions of immunological

Fig. 18 Double diffusion analysis with antiserum to α_1-AGP present in the centre well. The outer wells contained: A, α_1-AGP prepared from rat serum; B, intracellular forms of α_1-AGP isolated from the rough membrane fraction of rat liver; and C, intracellular forms of α_1-AGP isolated from the rough membrane fraction of rat liver and treated with typsin for 2 h as described in Experimental.


B
C
A
A

identity with serum α_1-AGP. This suggests that both fractions contained proteins that are capable of reacting immunologically like α_1-AGP and that the tryptic treated protein which showed a single band on SDS PAGE (Fig. 17, gel D) is an intracellular precursor form of α_1-AGP.

Amino acid and carbohydrate analyses of trypsin-treated and untreated forms of α_1-AGP Amino acid analyses of α_1-AGP and trypsin treated and untreated forms of intracellular α_1-AGP are given in Table 4. It can be seen that the amino acid composition of intracellular α_1-AGP isolated from rough fractions is similar to that of serum α_1-AGP except that it contains an extra complement of amino acid residues. In addition, it appears that trypsin treated intracellular α_1-AGP has lost most of these extra amino acid residues since it has an amino acid composition close to that found in serum α_1-AGP. Carbohydrate analyses of α_1-AGP and the intracellular forms are given in Table 5. It is clear that both tryptic treated and untreated forms of intracellular α_1-AGP contain substantially higher contents of hexose and lower contents of hexosamine. The intracellular forms were devoid of NeuAc; this is not surprising, since this sugar is believed to be added to glycoprotein after it passes from the rough fraction to the Golgi fraction. Qualitative analysis of the hexose fraction by paper chromatography showed that the hexose was composed mainly of mannose with only trace quantities of glucose; the hexosamine fraction was entirely composed of glucosamine.

Table 4

Amino acid composition of serum α_1-AGP and intracellular forms of α_1-AGP isolated from rough membrane fractions of rat liver

	Protein, residues/mole[a]		
	Untreated fraction(5)[b]	Trypsin treated fraction(3)[c]	Serum α_1-AGP(5)
Asx	23.0 ± 0.9	21.5 ± 1.2	22.1 ± 0.9
Thr	15.7 ± 1.5	16.5 ± 0.9	16.6 ± 1.7
Ser	16.6 ± 0.8	13.6 ± 1.8	13.2 ± 1.2
Glx	26.5 ± 0.9	26.4 ± 2.6	27.7 ± 1.5
Pro	13.9 ± 0.5	10.0 ± 0.9	9.5 ± 1.2
Gly	18.6 ± 0.9	12.9 ± 1.0	11.7 ± 0.5
Ala	16.8 ± 1.3	13.7 ± 1.3	13.2 ± 1.1
Val	15.0 ± 1.4	12.8 ± 1.0	12.6 ± 0.5
Met	3.0 ± 0.6	3.0 ± 0.8	3.0 ± 0.5
Ile	9.0 ± 0.9	9.5 ± 0.6	10.0 ± 0.6
Leu	20.4 ± 0.8	16.5 ± 1.3	16.5 ± 0.7
Tyr	6.1 ± 0.2	6.1 ± 0.6	5.6 ± 0.5
Phe	11.0	11.0	11.0 ± 0.7
His	5.8 ± 0.2	4.3 ± 0.2	4.2 ± 0.3
Lys	17.0 ± 1.3	16.3 ± 0.8	16.5 ± 1.4
Arg	8.8 ± 0.4	6.1 ± 0.7	6.0 ± 0.3
Trp[d]	ND[e]	ND	3.9

[a] The values were determined by normalizing phenylalanine to 11.0 residues per mole. The number of analyses are indicated in parenthesis and the standard deviation presented after each mean value.

[b] Untreated fraction refers to intracellular α_1-AGP isolated from rough membrane fraction.

[c] Treated fraction refers to intracellular α_1-AGP isolated from rough membrane fraction that was treated with trypsin for 2 h. The trypsin and trypsin inhibitor were removed following incubation by ConA-Sepharose chromatography as described in Experimental.

[d] Determined by the method of Edelhoch (138); results for tyrosine agreed with those from amino acid analysis.

[e] Not determined

Table 5

Carbohydrate composition of serum α_1-AGP and intracellular forms of α_1-AGP isolated from rough membrane fractions of rat liver

Residue	Protein source, residues/mole[a]		
	Untreated fraction(3)[b]	Trypsin treated fraction(3)[c]	Serum α_1-AGP(3)
Total hexose	$53.2 \pm {}_{2.9}$	$56.2 \pm {}_{2.6}$	$34.6 \pm {}_{2.5}$
GlcNAc	$9.8 \pm {}_{1.3}$	$10.5 \pm {}_{2.3}$	$23.0 \pm {}_{2.2}$
NeuAc	---	---	$15.4 \pm {}_{2.1}$

[a] The number of analyses are indicated in paranthesis and the standard deviation is presented after each mean value. The values were calculated using the molar concentration determined from the amino acid analysis of an aliquot of the sample analyzed for carbohydrate; carbohydrate analysis was by the spectrophotometric method.

[b] Untreated fraction refers to intracellular α_1-AGP isolated from rough membrane fractions.

[c] Treated fraction refers to intracellular α_1-AGP isolated from rough membrane fractions that was treated with trypsin for 2 h. The trypsin and trypsin inhibitor were removed by ConA-Sepharose chromatography as described in Experimental.

Characterization of intracellular forms of α_1-AGP isolated from rough, smooth and Golgi fractions of rat liver

The results described above showed the presence of a high mannose form of α_1-AGP in rough fractions of rat liver. A second intracellular form of α_1-AGP which was more sensitive to trypsin was also present in rough fractions and it might be expected that other forms with extensively processed oligosaccharide chains could be present in smooth and Golgi fractions of rat liver. In order to investigate this possibility an attempt was made to isolate for study the intracellular forms of α_1-AGP from the smooth and Golgi fractions. The approach used was the same as that described for the isolation of intracellular forms of α_1-AGP from rough fractions.

Electrophoretic and immunological analyses In initial experiments, ultrasonic extracts of rough, smooth and Golgi fractions were fractionated according to scheme I, Fig. 15. Electrophoretic examination on basic PAGE showed that the smooth and Golgi fractions also seemed to contain one major component which appeared to be identical to the form isolated from rough fractions (Fig. 19). Examination of the fractions by SDS PAGE also showed that the electrophoretic patterns of the intracellular α_1-AGP from the smooth and Golgi fractions were similar to those found with intracellular α_1-AGP isolated from rough fractions (Fig. 20). Each showed two major protein bands of about equal intensity with slightly slower

Fig. 19 Basic PAGE on 7.5% gels of intracellular forms of α_1-AGP prepared from subcellular fractions of rat liver; A, rough membrane fraction; B, smooth membrane fraction; C, Golgi fraction; D, asialo-α_1-AGP; E, serum α_1-AGP.

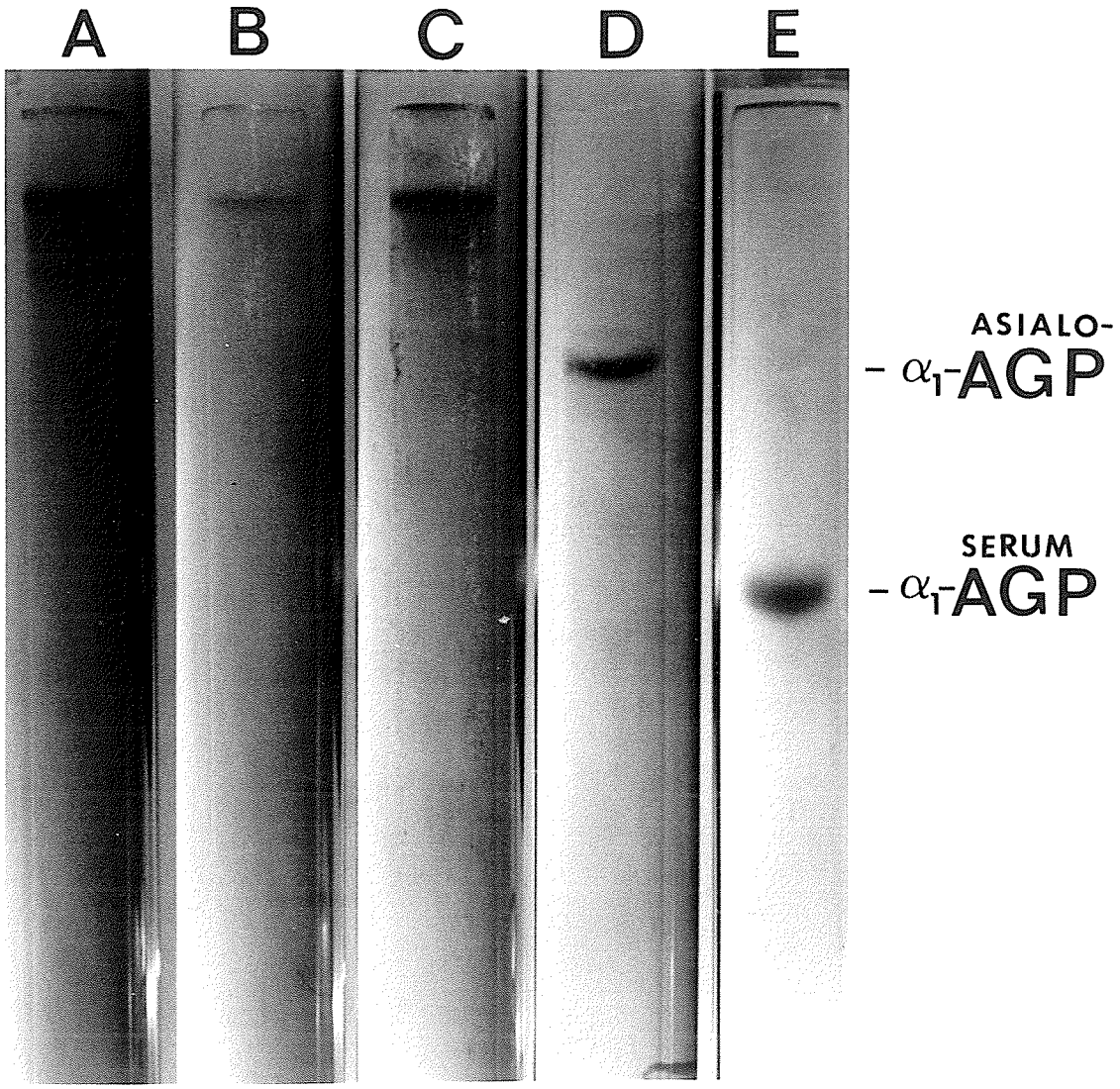
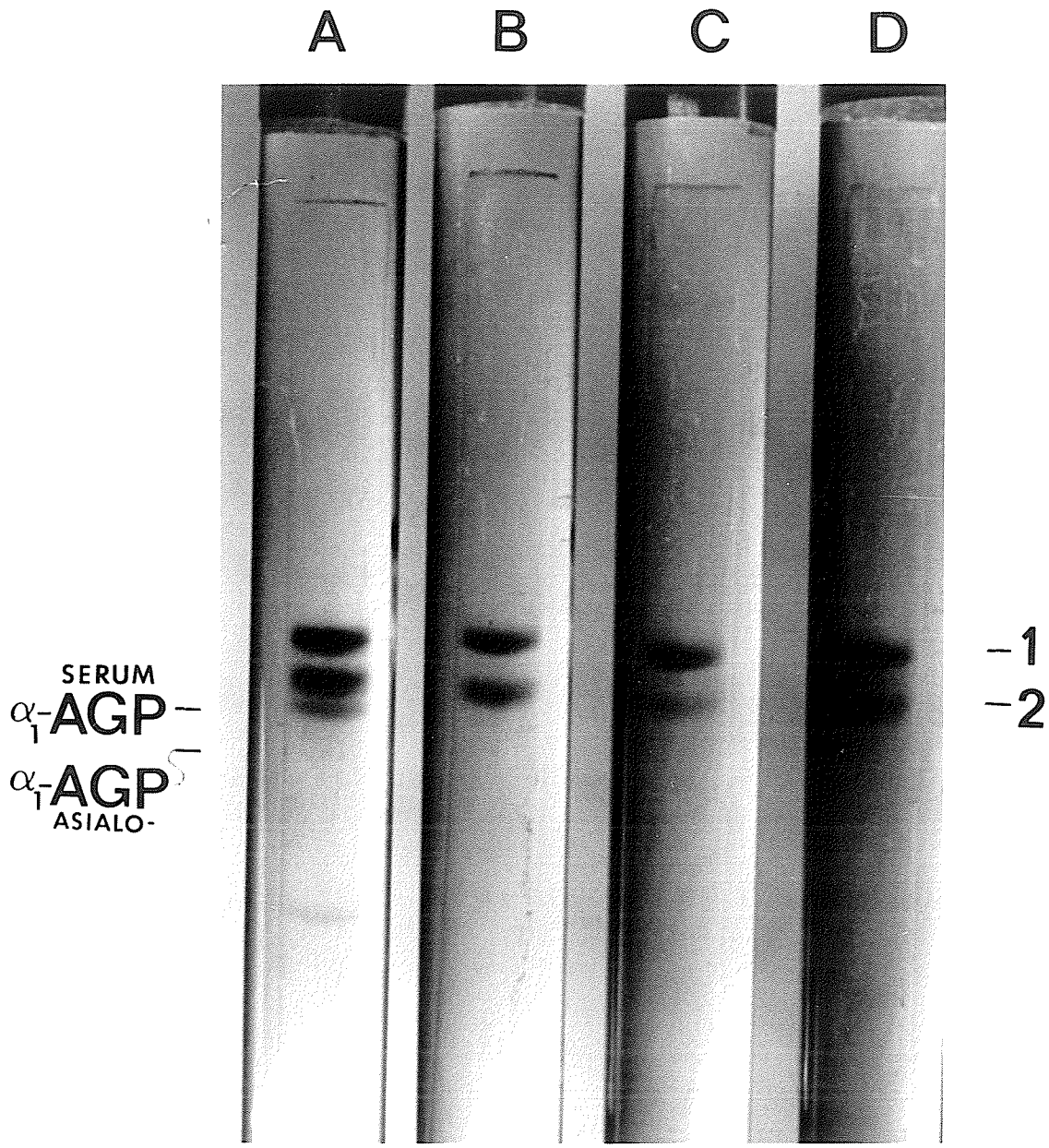

A
B
C
D
E
ASIALO-
- α_1-AGP
SERUM
- α_1-AGP

Fig. 20 SDS PAGE on 7.5% gels of α_1-AGP isolated from subcellular fractions of rat liver. A, α_1-AGP fraction prepared from the rough membrane fraction, examined following the ConA-Sepharose step, but prior to CM-cellulose chromatography; B, C and D, α_1-AGP fractions prepared from the rough membrane fraction, the smooth membrane fraction and the Golgi fraction, respectively, examined following CM-cellulose chromatography. The positions at which serum α_1-AGP and asialo-α_1-AGP migrate are indicated in the Fig.; 1 and 2 refer to intracellular α_1-AGP forms 1 and 2, respectively. It should be noted that serum α_1-AGP is effectively removed by CM-cellulose chromatography; compare gel A with gels B, C and D.


A
B
C
D
SERUM
α_1-AGP
α_1-AGP
ASIALO-
1
2

mobility than serum α_1-AGP and asialo-α_1-AGP. The relative intensities of the bands formed by the two intracellular proteins were determined with a scanning densitometer; a typical scan of a gel corresponding to that shown in gel A in Fig. 20 is shown in Fig. 21. The values for the relative intensities of the two major bands varied from preparation to preparation, but there was no indication from densitometer scans that there was any pattern of distribution of the two intracellular proteins between the rough, smooth and Golgi fractions. Thus, it would appear that using the procedures of isolation presented in scheme I of Fig. 15 each subcellular fraction contained the two proteins found on SDS PAGE as shown in Fig. 20, and that at least one and perhaps both proteins were intracellular forms of α_1-AGP.

A partial separation of the two intracellular forms appeared to occur when the fractionation of ultrasonic extracts of subcellular fractions was performed using Ultrogel AcA44 instead of Sephadex G-100 (scheme II, Fig. 15). Double diffusion analysis with antiserum to α_1-AGP showed that two fractions (59 and 61) in the middle of the fractionation seemed to contain forms of α_1-AGP which reacted with antiserum, but which did not react with total identity. The difference was indicated by the formation of a spur between the immunological precipitin lines formed by fractions 59 and 61 (Fig. 22). The fact that the two precipitin lines joined showed that the forms of α_1-AGP in each fraction had common antigenic determinants. The occurrence of the spur suggested the possibility

Fig. 21 Densitometer scan of SDS PAGE analysis of intracellular α_1-AGP isolated from the rough fraction; examined following the ConA-Sepharose chromatography step (ConA-II). A photograph of the preparation is also shown in Fig. 25; gel A. The numbers 1 and 2 identify the intracellular α_1-AGP forms 1 and 2; serum-type α_1-AGP is the smaller peak to the left of 1 and 2. The relative quantities of each protein are indicated in percentages.

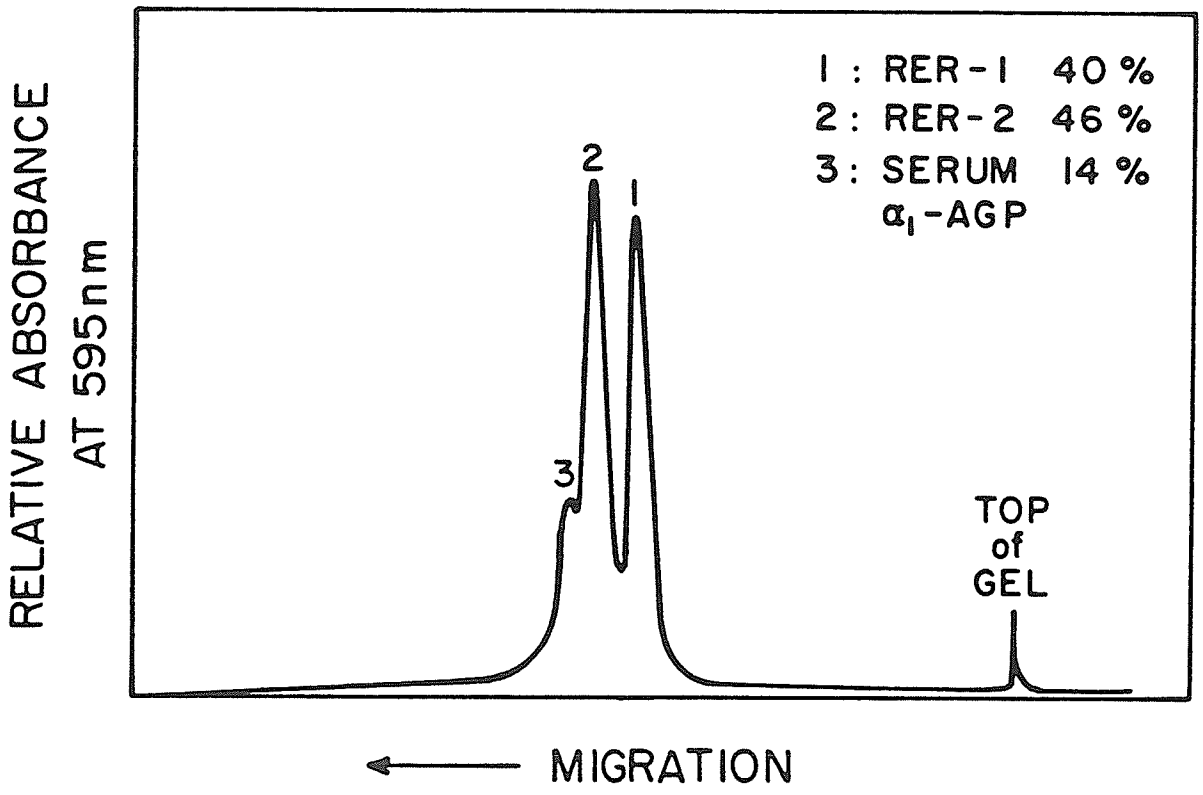
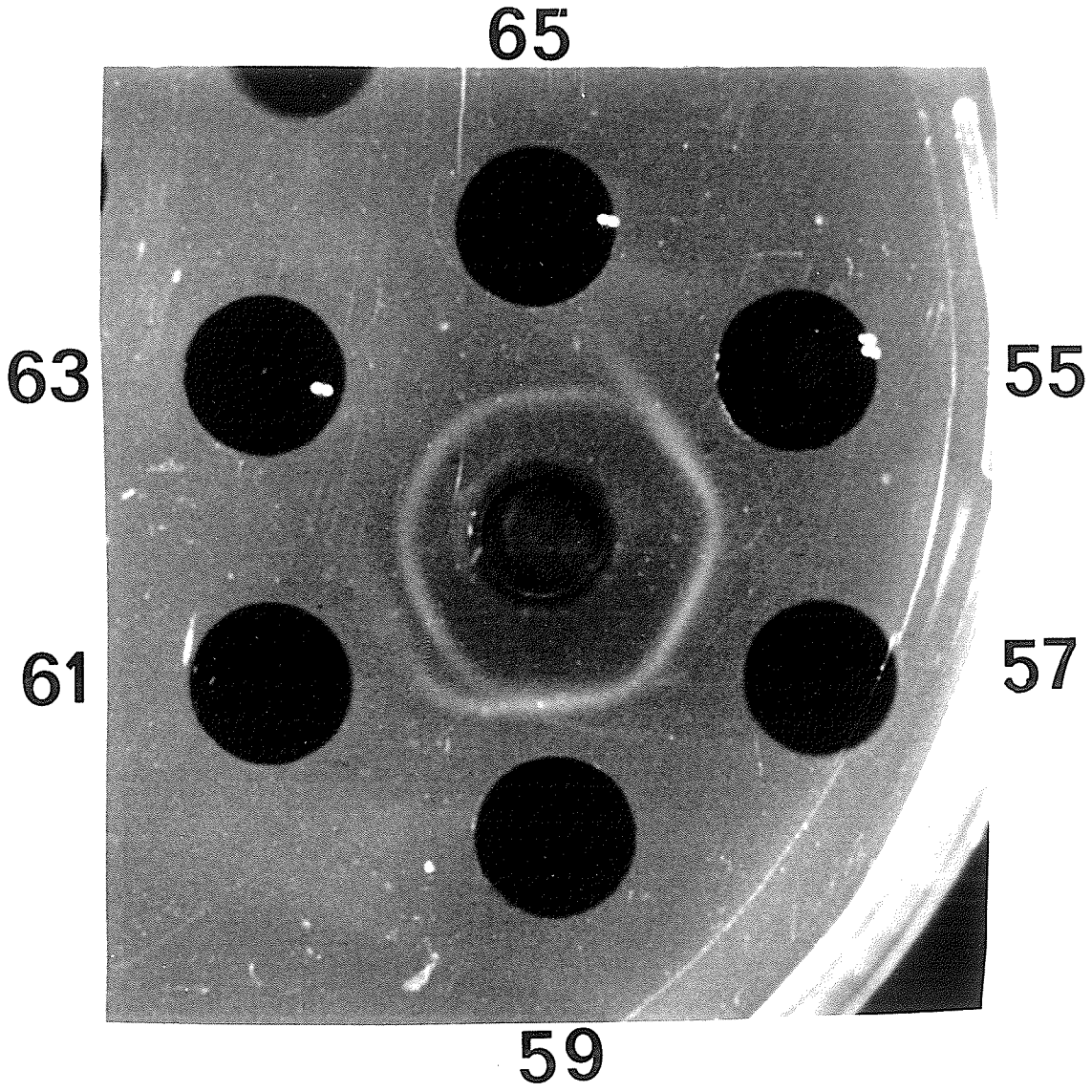

RELATIVE ABSORBANCE
AT 595nm
1 : RER-1 40 %
2 : RER-2 46 %
3 : SERUM 14 %
α1-AGP
2
1
3
TOP
of
GEL
MIGRATION

Fig. 22 Double diffusion analysis with antiserum to α_1-AGP present in the centre well. The other wells contained samples from appropriate tubes collected after Ultrogel AcA44 chromatography of the ultrasonic extract of rat liver rough membrane fractions (Fig. 15e). Diffusion was at room temperature for 24 h.


65
63
55
61
57
59

that fraction 59, and fractions eluting ahead of it, contained an antigenic determinant not present in fraction 61 and fractions eluted thereafter. The spur formation in double diffusion analysis was used to separate the two fractions of intracellular α_1-AGP eluted from Ultrogel AcA44 (Fig. 15e). The two fractions from each of the three subcellular fractions were purified as outlined in Fig. 15 scheme II and the six resulting preparations were analyzed by double diffusion analysis (Fig. 23). All the intracellular forms of α_1-AGP produced immune precipitin lines against antiserum to α_1-AGP and spur formation which appeared to occur between forms 1 and 2 was indicative of partial identity between the two forms. For reasons that are not clear great diffuculty was experienced in attempts to produce high quality double diffusion analysis of the fractions isolated following repeated chromatography on Ultrogel AcA44 (compare Fig. 22 with Fig. 23).

Analysis on SDS PAGE of the intracellular forms of α_1-AGP, prepared by scheme II using Ultrogel AcA44 is shown in Fig. 24. The use of Ultrogel AcA44, and the division of the fractions depending on the results of double diffusion analysis, resulted in at least partial separation of two intracellular forms of α_1-AGP as determined on SDS PAGE. Fig. 24 shows that preparations of form 1 were contaminated with form 2, but preparations of form 2 isolated from all three subcellular fractions were relatively free of form 1 (e.g. Fig. 24, gels D and F).

Fig. 23 Double diffusion analysis with antiserum to α_1-AGP present in the centre well. The outer wells contained intracellular forms of α_1-AGP prepared from subcellular fractions of rat liver: A, rough, form 1; B, rough, form 2; C, smooth, form 1; D, smooth, form 2; E, Golgi, form 1; F, Golgi, form 2. High quality double diffusion results could not be obtained with fractions that were subjected to repeated chromatography, particularly with smooth and Golgi fractions where limited quantities of material were available.

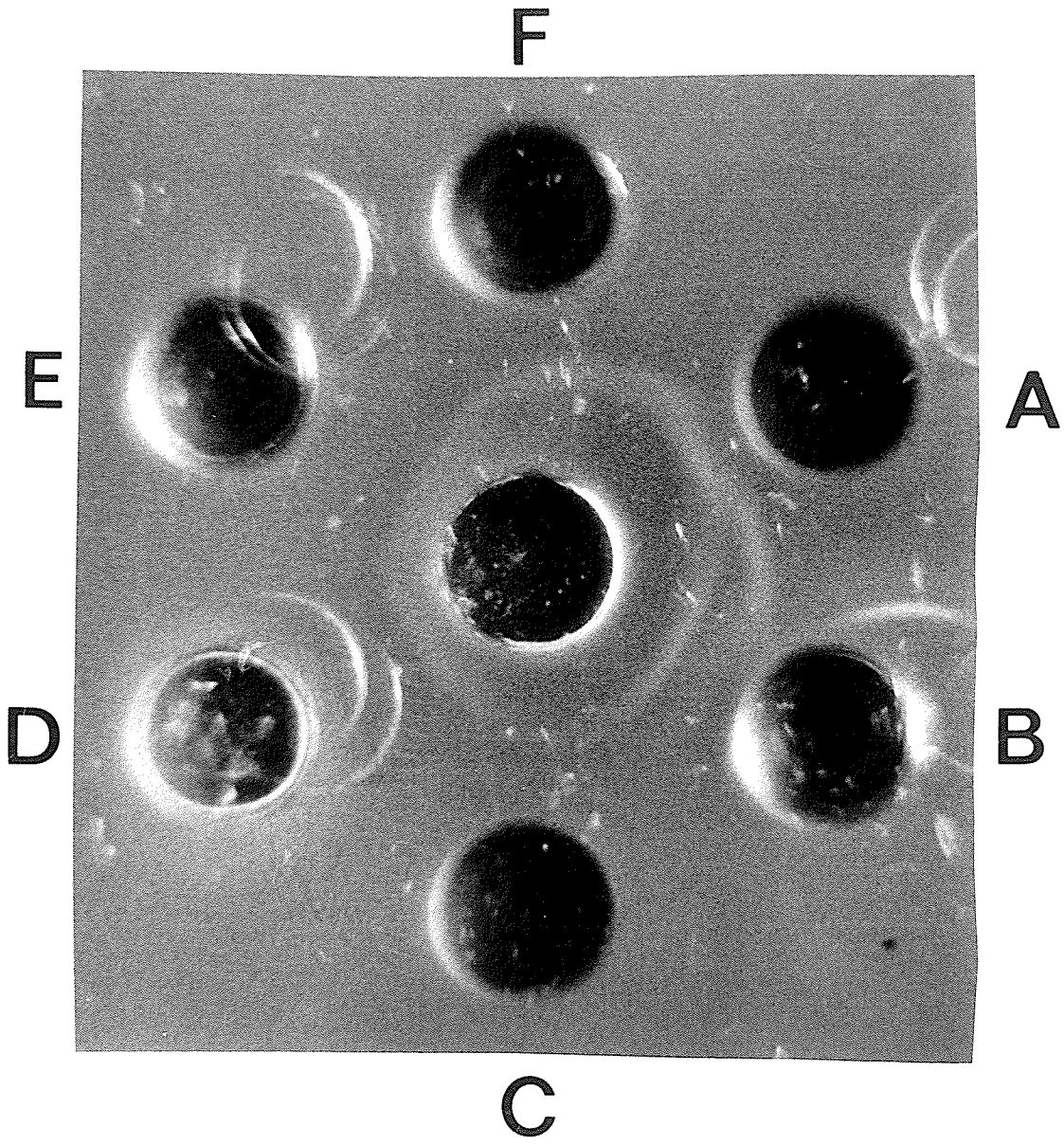
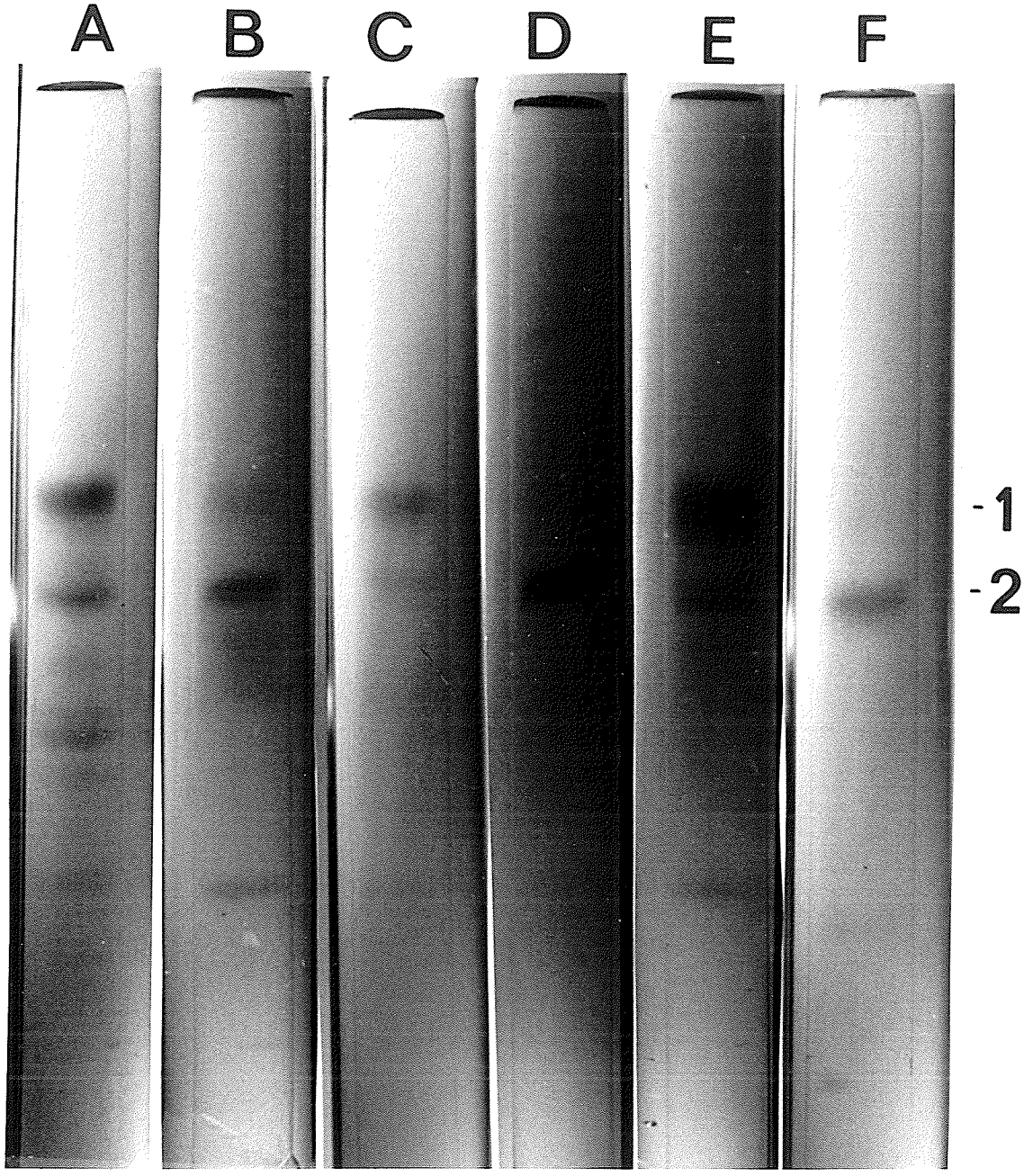

F
E
A
D
B
C

Fig 24 SDS PAGE on 7.5% gels of intracellular forms of α_1-AGP isolated from subcellular fractions of rat liver by scheme II: A, rough, form 1; B, rough, form 2; C, smooth, form 1; D, smooth, form 2; E, Golgi, form 1; F, Golgi, form 2.


A
B
C
D
E
F
1
2

Amino acid and carbohydrate analyses Amino acid analyses of intracellular α_1-AGP isolated from rough fractions by scheme I (Fig. 15) indicated the presence of extra amino acid residues not present in serum α_1-AGP (see Table 4). Table 6 shows that α_1-AGP isolated from smooth and Golgi fractions also contained the extra complement of amino acids found in α_1-AGP from rough fractions. Amino acid analyses were also performed on the two intracellular forms of α_1-AGP isolated from the rough, smooth and Golgi fraction by scheme II. Table 7 shows the results of these analyses compared with the amino acid composition of serum α_1-AGP. The amino acid compositions of forms 1 and 2 were found to be similar regardless of which subcellular fraction they were isolated from. The results of the amino acid analyses suggested that the differences between forms 1 and 2 which caused these two forms to have different electrophoretic mobilities on SDS PAGE did not reside in the polypeptide moiety.

Because of the small amounts of form 1 and 2 that were available it was found that it was impossible to perform accurate carbohydrate analysis using the method employed earlier (Table 5) which involved ion exchange chromatography on Dowex resins coupled with spectrophotometric methods. An attempt was made to develop a highly sensitive analytical procedure for sugars using trifluoroacetylated derivatives and analysis by gas liquid chromatography

Table 6

Amino acid composition of serum α_1-AGP and intracellular forms of α_1-AGP prepared by scheme I

Residue	Protein source, residues/mole[a]			
	Serum α_1-AGP (5)	rough fraction (5)	smooth fraction (3)	Golgi fraction (3)
Asp	22.1 ± 0.9	23.0 ± 0.9	30.8 ± 1.7	26.2 ± 2.0
Thr	16.6 ± 1.7	15.7 ± 1.5	18.8 ± 1.9	15.3 ± 2.2
Ser	13.2 ± 1.2	16.6 ± 0.8	24.6 ± 0.9	19.7 ± 2.1
Glu	27.7 ± 1.5	26.5 ± 0.9	33.1 ± 1.8	25.4 ± 1.1
Pro	9.5 ± 1.2	13.9 ± 0.5	16.6 ± 0.6	12.8 ± 1.2
Gly	11.7 ± 0.5	18.6 ± 0.9	26.4 ± 0.7	19.7 ± 1.0
Ala	13.2 ± 1.1	16.8 ± 1.3	22.1 ± 1.4	20.4 ± 1.7
Val	12.6 ± 0.5	15.0 ± 1.4	18.1 ± 1.5	14.9 ± 0.6
Met	3.0 ± 0.5	3.0 ± 0.6	4.3 ± 0.6	3.7 ± 0.7
Ile	10.0 ± 0.6	9.0 ± 0.9	9.4 ± 0.9	11.7 ± 1.8
Leu	16.5 ± 0.7	20.4 ± 0.8	22.9 ± 0.6	21.8 ± 2.5
Tyr	5.6 ± 0.5	6.1 ± 0.2	6.0 ± 0.4	6.0 ± 0.6
Phe	11.0 ± 0.7	11.0	11.0	11.0
His	4.2 ± 0.3	5.8 ± 0.2	7.8 ± 0.1	5.3 ± 1.0
Lys	16.5 ± 1.4	17.4 ± 1.3	21.6 ± 1.3	17.9 ± 2.1
Arg	6.0 ± 0.3	8.8 ± 0.4	9.6 ± 0.6	11.1 ± 0.6

[a] Values are means of the number of analyses indicated in parenthesis and standard deviations are included after each value. The values were normalized by assuming 11 residues of phenylalanine. The analyses of intracellular forms of α_1-AGP were performed on 24 h hydrolyses.

Table 7

Amino acid composition of serum α_1-AGP and intracellular forms of α_1-AGP prepared by scheme II

Residue	Protein source, residues/mole[a]						
	Serum(5)	rough fraction form 1(5)	rough fraction form 2(5)	smooth fraction form 1(2)	smooth fraction form 2(3)	Golgi fraction form 1(2)	Golgi fraction form 2(3)
Asp	22.1 ±0.9	27.8 ± 1.5	26.4 ± 1.5	27.0 ± 1.2	32.1 ± 1.4	29.5 ± 2.6	27.1 ± 2.0
Thr	16.6 ±1.7	17.6 ± 1.3	16.1 ± 1.9	15.9 ± 1.1	15.3 ± 1.6	14.1 ± 1.5	13.6 ± 0.7
Ser	13.7 ±1.2	19.4 ± 1.5	18.6 ± 1.8	18.2 ± 0.9	17.7 ± 4.4	24.6 ± 3.0	16.6 ± 0.5
Glu	27.7 ±1.5	29.6 ± 1.0	25.9 ± 1.4	25.6 ± 0.6	32.0 ± 1.6	34.5 ± 3.0	22.0 ± 0.6
Pro	9.5 ±1.2	15.7 ± 0.9	14.0 ± 0.8	12.5 ± 1.4	16.7 ± 1.0	14.4 ± 1.7	14.2 ± 0.9
Gly	11.7 ±0.5	21.4 ± 0.9	19.3 ± 1.0	18.6 ± 0.8	27.8 ± 1.4	24.6 ± 3.0	18.3 ± 0.4
Ala	17.2 ±1.1	21.8 ± 2.8	20.9 ± 1.1	17.2 ± 0.5	27.6 ± 1.3	23.6 ± 0.2	18.3 ± 2.0
Val	12.6 ±0.5	20.4 ± 2.6	15.8 ± 1.6	15.6 ± 1.9	16.7 ± 0.9	15.2 ± 0.2	12.3 ± 0.1
Met	3.0 ±0.5	3.7 ± 0.9	3.6 ± 0.5	3.5 ± 0.7	4.6 ± 0.8	2.3 ± 0.6	2.3 ± 0.4
Ile	10.0 ±0.6	10.7 ± 0.6	9.7 ± 1.0	8.0 ± 0.4	12.7 ± 0.6	7.5 ± 0.9	8.2 ± 0.2
Leu	16.5 ±0.7	23.0 ± 0.6	20.2 ± 1.0	18.1 ± 0.1	24.0 ± 1.8	20.2 ± 1.2	17.8 ± 1.7
Tyr	5.6 ±0.5	8.1 ± 0.5	6.4 ± 0.2	6.5 ± 0.6	7.4 ± 0.4	8.0 ± 0.6	6.4 ± 0.7
Phe	11.0 ±0.7	11.0	11.0	11.0	11.0	11.0	11.0
His	4.2 ±0.3	7.1 ± 0.2	5.5 ± 0.6	5.4 ± 0.8	6.7 ± 0.3	8.9 ± 0.6	5.6 ± 0.8
Lys	16.5 ±1.4	22.0 ± 1.9	19.8 ± 2.3	19.9 ± 3.0	23.6 ± 1.7	20.5 ± 0.2	19.4 ± 2.6
Arg	6.0 ±0.3	10.6 ± 0.7	9.0 ± 1.2	7.3 ± 2.0	10.1 ± 0.6	11.1 ± 1.1	9.3 ± 1.1

[a] Values are means of the number of analyses indicated in parenthesis and standard deviations are included after each value. The values were normalized by assuming 11 residues of phenylalanine. The analyses of intracellular forms of α_1-AGP were performed on 24 h hydrolyses.

using an electron capture detector as described by Pritchard and Niedermeier (150). However, this approach was abandoned after many unsuccessful attempts when it was learned by direct communications with Dr. Niedermeier that the authors were also experiencing difficulties in achieving satisfactory separation using the trifluoroacetylation method because the column packing used in the original work was no longer available. Satisfactory carbohydrate analysis was subsequently achieved by hydrolysis to release free sugars which were then converted to alditol acetates and separated by gas liquid chromatography. Typical chromatograms obtained using this approach to carbohydrate analysis are shown in Fig. 25. It can be seen that the sugars that might be present in intracellular forms of α_1-AGP (i.e. mannose, galactose, glucose and GlcNAc) are well separated. The method also proved to be quite sensitive; the chromatogram of a 3 µl injection of a 100 µl sample containing 100 nmoles of each sugar shown in Fig. 25A produced peaks of approximately half of full scale deflection and peaks which are readily distinguished from the baseline noise.

Table 8 shows the results of analyses for carbohydrate of intracellular forms of α_1-AGP isolated from rough, smooth and Golgi fractions using the alditol acetate procedure. Although there was some variability in sugar composition determined from preparation to preparation it was clear that form 1 from all three subcellular fractions was a high mannose form of α_1-AGP and form 2 from all

Fig. 25 Chromatograms of alditol acetate sugar derivatives on a Hewlett-Packard Model 5700A gas liquid chromatograph equipped with a flame-ionization detector and a 6 ft x 2 mm glass column containing 3% SP2330 on 100-120 mesh Supercoport.

A. 3 µl injection of a standard 1 mM mixture of: 1 - mannose, 2 - galactose, 3 - glucose, 4 - meso-inositol, 5 - N-acetylglucosamine.

B. 3 µl injection of a hydrolysate of 5 nmoles of rat serum α_1-AGP with meso-inositol as the internal standard.

C. 3 µl injection of a hydrolysate of 2 nmoles of α_1-AGP isolated from the rough membrane fraction with meso-inositol as the internal standard.

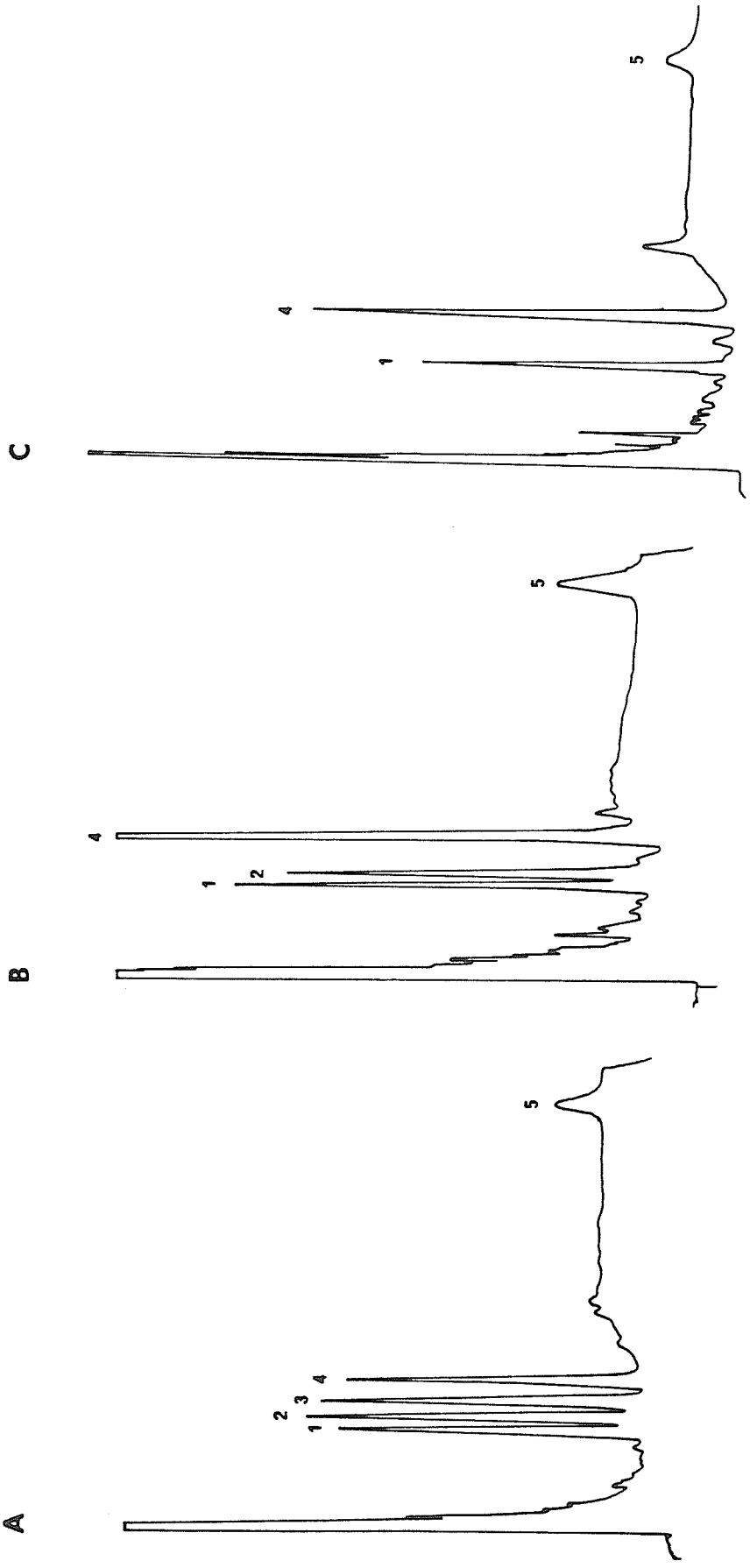

A
5
4
3
2
1
B
5
4
2
1
C
5
4
1

Table 8

Analyses of serum α_1-AGP and intracellular forms of α_1-AGP for mannose, galactose and hexosamine

Residue	Serum (3)	Protein source, residues/mole[a]					
		rough fraction		smooth fraction		Golgi fraction	
		form 1(4)	form 2(4)	form 1(3)	form 2(3)	form 1(3)	form 2(2)
Man	17 ± 3	38 ± 6	12 ± 3	32 ± 7	4 ± 2	24 ± 3	4 ± 1
Gal	17 ± 3	2 ± 1	3 ± 2	4 ± 2	3 ± 2	2 ± 1	2 ± 1
GlcNAc	28 ± 6	19 ± 8	6 ± 1	11 ± 4	14 ± 3	12 ± 7	12 ± 5

[a] Values are means of the number of analyses indicated in parenthesis and the standard deviation is indicated after each value. The values were calculated using the molar concentration of protein determined from amino acid analysis of an aliquot of the sample analyzed for carbohydrate.

three fractions contained substantially less mannose. Measurement of galactose was difficult because of the presence of only trace quantities of this sugar, but the results have been reported in Table 8 since the galactose peak was always greater than baseline. Analysis for hexosamine indicated that all intracellular forms contained substantially less hexosamine than serum α_1-AGP. The results from hexosamine and galactose analysis of the intracellular forms when compared with analyses of these sugars in serum α_1-AGP clearly show that all the intracellular forms contain incomplete carbohydrate chains. In the case of the high mannose form it is clear that this must represent a form of α_1-AGP with unprocessed chains since it contained about twice the amount of mannose associated with serum α_1-AGP (Table 8). One possible exception was the high mannose form isolated from the Golgi fraction which appeared to contain less mannose than the corresponding forms isolated from rough and smooth fractions.

Determination of N-terminal amino acid sequence of serum α_1-AGP

Since the work presented above on the amino acid analyses of intracellular forms of α_1-AGP indicated that they contained an additional complement of amino acids not present in serum α_1-AGP and that trypsin could remove these amino acids, the possibility of the existence of pre- or pro- forms of α_1-AGP was investigated. Since most pre- and pro- forms of proteins (see Introduction) contain the amino acid extension on the N-terminus, a study of the

presence of a pre- or pro- form of α_1-AGP would require the determination of the amino acid sequence of rat serum α_1-AGP, particularly the amino acid sequence of the N-terminus.

Studies by Schmid's group (156,157) on the amino acid sequence of human α_1-AGP showed that sequencing of the N-terminus of the serum protein was possible after removal of a blocking N-terminal cyclized pyroglutamate and that the entire sequence could be determined from N-terminal sequencing of CNBr peptides coupled with tryptic, chymotryptic and peptic hydrolysates of the CNBr peptides. This approach used by Schmid's group on human α_1-AGP was applied to rat α_1-AGP with the main objective being the determination of the N-terminal sequence of the serum protein for comparison with the N-terminal sequence of intracellular α_1-AGP. The N-terminal sequencing of the CNBr peptides of rat α_1-AGP might also allow some comparison to be made with the amino acid sequence of human α_1-AGP.

Initial N-terminal sequence analysis by automated Edman degradation indicated that the N-terminus of rat serum α_1-AGP was blocked to reaction with phenylisothiocyanate (PITC) presumably by the same N-terminal pyroglutamate residue found on the human protein (156). Schmid *et al.*(156) showed that this blocking pyroglutamate could be removed to free the new N-terminus to Edman degradation sequencing by treatment of the protein with pyrrolidonecarboxylyl peptidase from *Pseudomonas fluorescens*. Podell *et al.*(151) reported that a commercially available pyroglutamate amino peptidase isolated from

calf liver was also capable of removing N-terminal pyroglutamate from protein. Treatment of rat serum α_1-AGP with pyroglutamate amino peptidase as described in Experimental freed the N-terminus for reaction with PITC resulting in successful automated Edman degradation sequencing using the Beckman Model 890C Sequencer. The results of two separate sequencing runs on serum α_1-AGP treated with pyroglutamate amino peptidase are shown in Fig. 26. In the first analysis (Fig. 26A) the pyroglutamate amino peptidase was removed from the treated α_1-AGP before sequence analysis by gel permeation chromatography on a 2.5 x 120 cm column of Ultrogel AcA44. In the second analysis (Fig. 26B) the pyroglutamate amino peptidase was not removed before sequencing. From a control experiment in which 2 mg of pyroglutamate amino peptidase was sequenced (Fig. 27) it was shown that the presence of about 10% of this enzyme with the protein to be sequenced would not affect the assignment of the amino acid sequence.

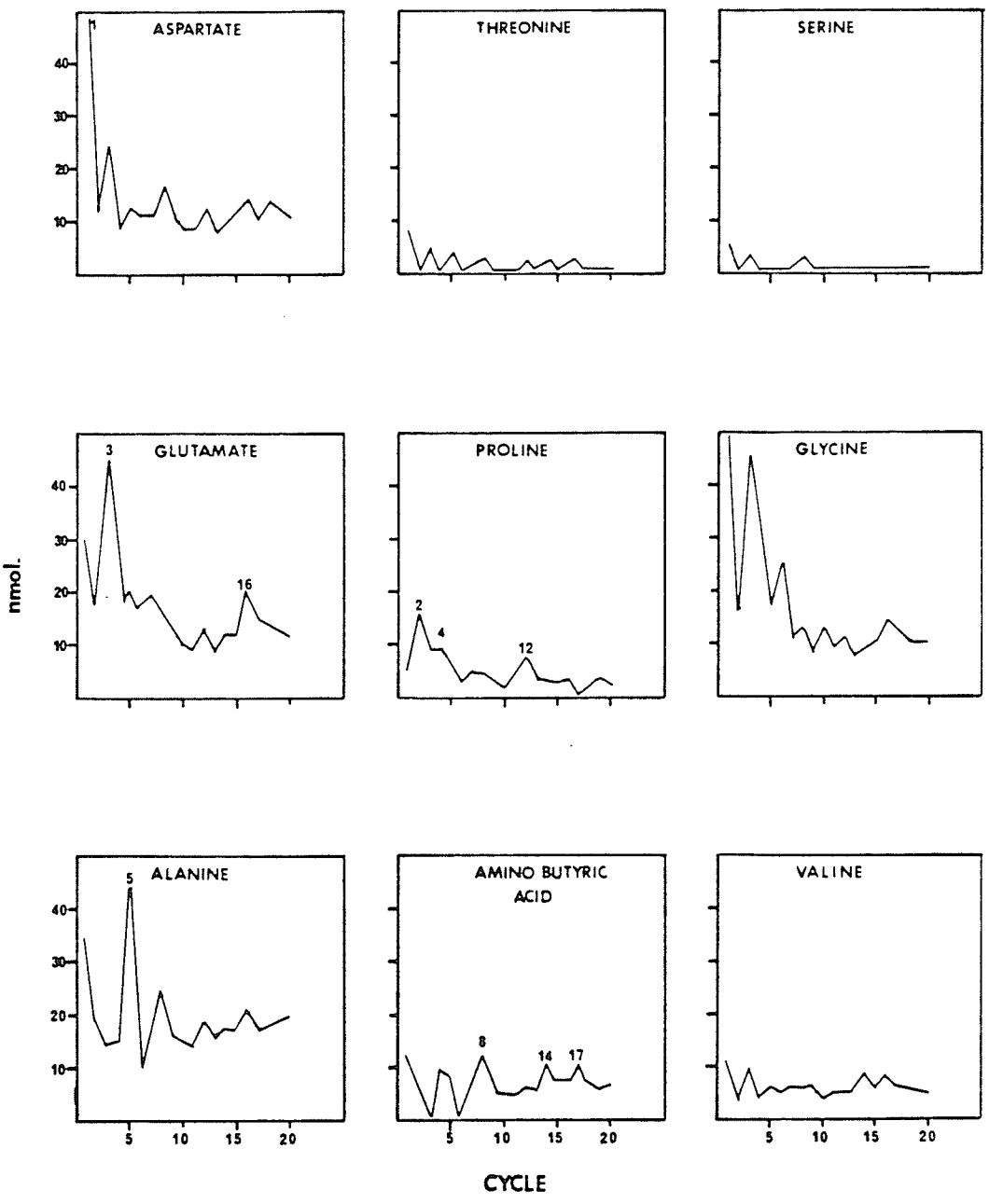
Identification of the amino acid cleaved at each cycle of the Edman degradation procedure was made from amino acid analysis following hydrolysis of the thiazolinone derivatives extracted. The conditions for hydrolysis have been shown to cause conversion of amino acid derivatives such that serine and carboxymethyl cysteine are converted to alanine; threonine is converted to aminobutyric acid and isoleucine is converted to a mixture of isoleucine and allo-isoleucine. In addition, tryptophan in completely destroyed and asparagine and glutamine are converted to aspartic acid and glutamic

Fig. 26 Yields of amino acids from automated Edman degradation sequencing of rat serum α_1-AGP. Amino acids were quantitated by amino acid analysis using the Technicon NC-2P Analyzer following acid hydrolysis of the thiazolinone derivatives produced by the Beckman 890C sequencer. Assignments of amino acids are indicated by the cycle number above the peak.

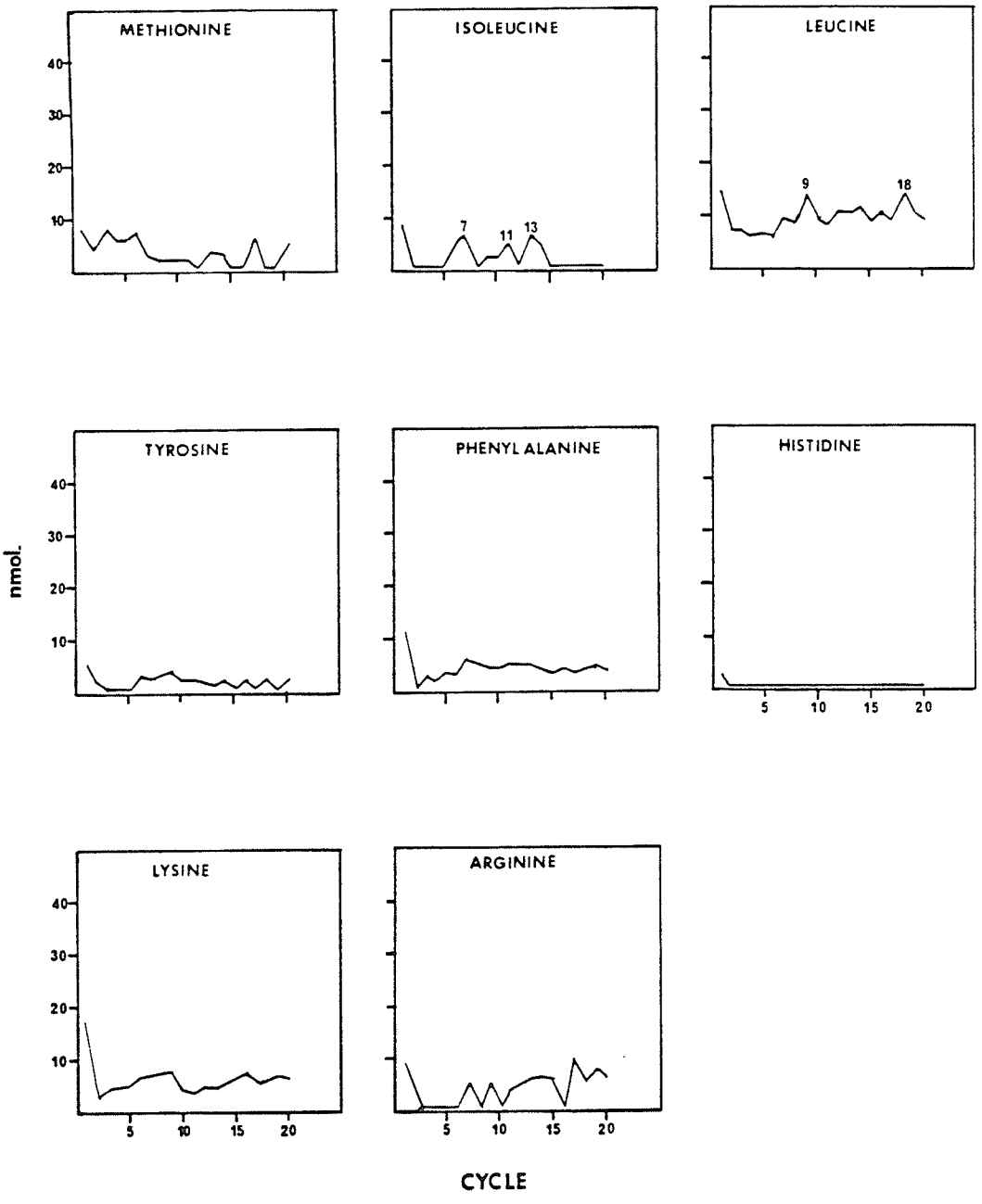
A. The amino acid sequence was determined with 200 nmole pyroglutamate amino peptidase-treated rat serum α_1-AGP following separation of the enzyme by gel permeation chromatography. The repetitive yields calculated for proline were: for cycle 2-4, 78.7%; for cycle 2-12, 93.2%; for cycle 4-12, 97.1%.

B. The amino acid sequence was determined with 250 nmole of pyroglutamate amino peptidase-treated rat serum α_1-AGP; pyroglutamate amino peptidase was not removed. The repetitive yields calculated for proline were: for cycle 2-4, 75.9%; for cycle 2-12, 91.5%; for cycle 4-12, 95.8%.

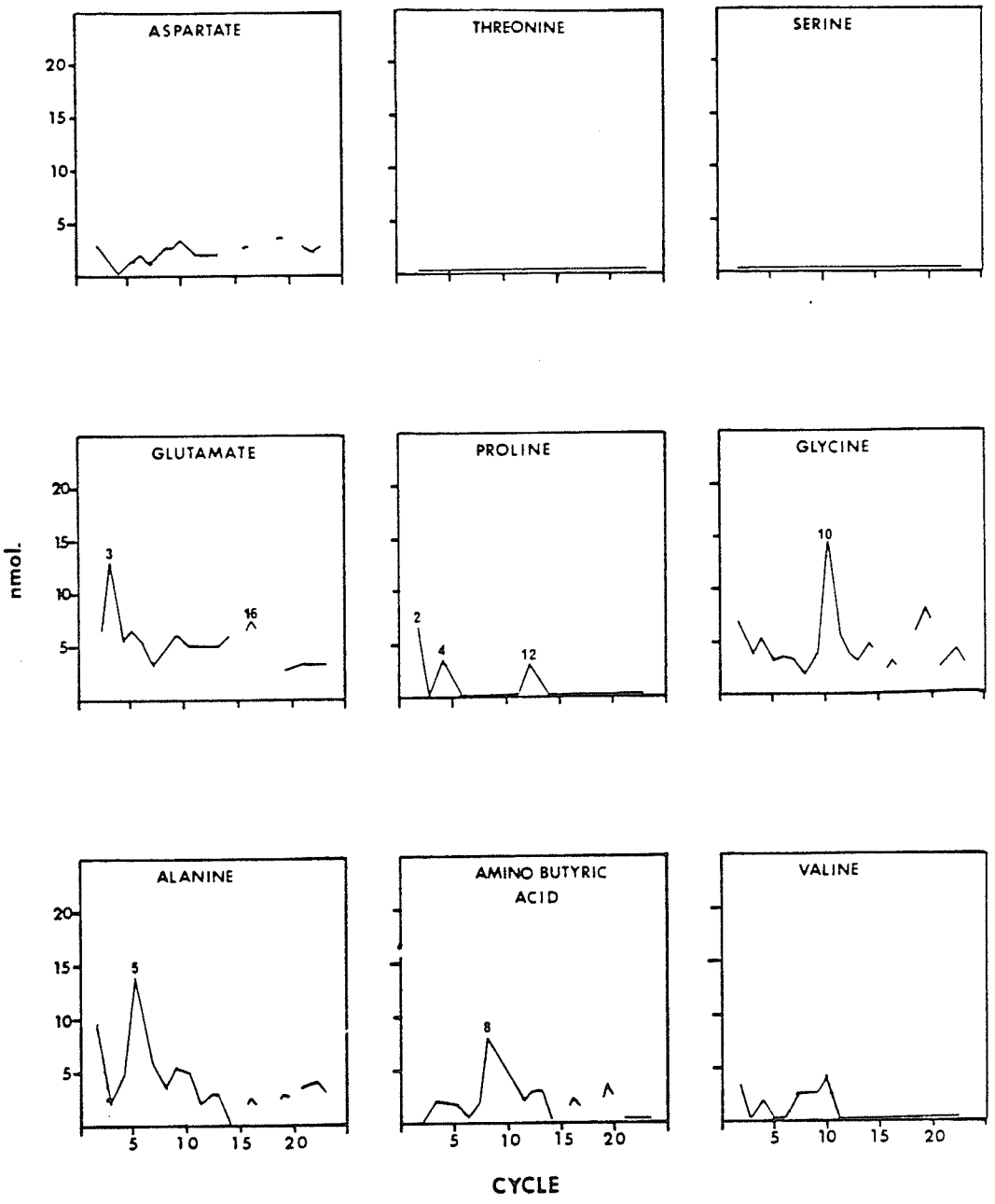
A


ASPARTATE
THREONINE
SERINE
GLUTAMATE
PROLINE
GLYCINE
ALANINE
AMINO BUTYRIC ACID
VALINE
nmol.
CYCLE
40
30
20
10
5
10
15
20

A


METHIONINE
ISOLEUCINE
LEUCINE
TYROSINE
PHENYL ALANINE
HISTIDINE
LYSINE
ARGININE
nmol.
CYCLE
7
11
13
9
18
10
20
30
40
5
10
15
20

B


ASPARTATE
THREONINE
SERINE
GLUTAMATE
PROLINE
GLYCINE
ALANINE
AMINO BUTYRIC ACID
VALINE
nmol.
CYCLE
3
16
2
4
12
10
5
8
5
10
15
20

B

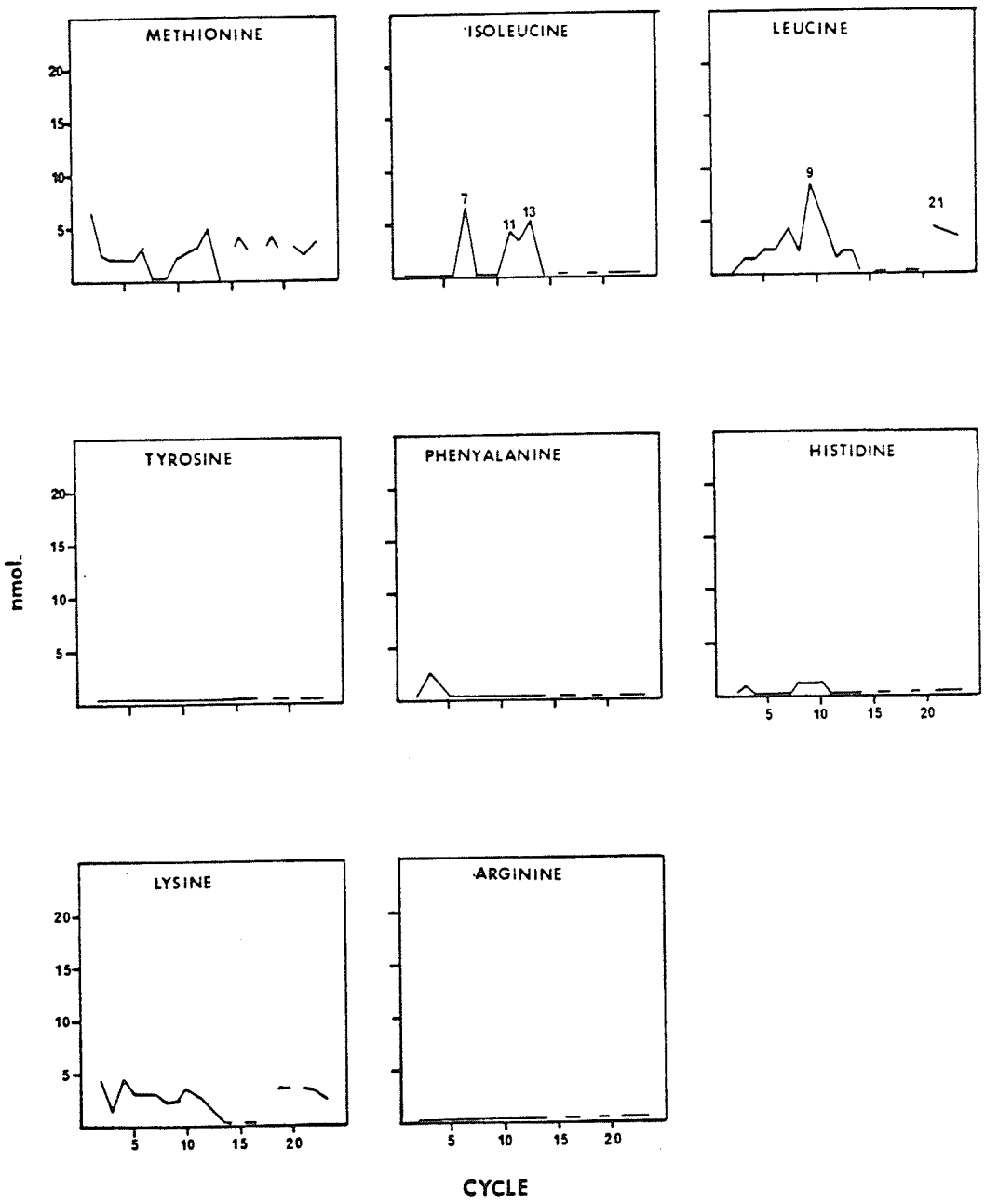
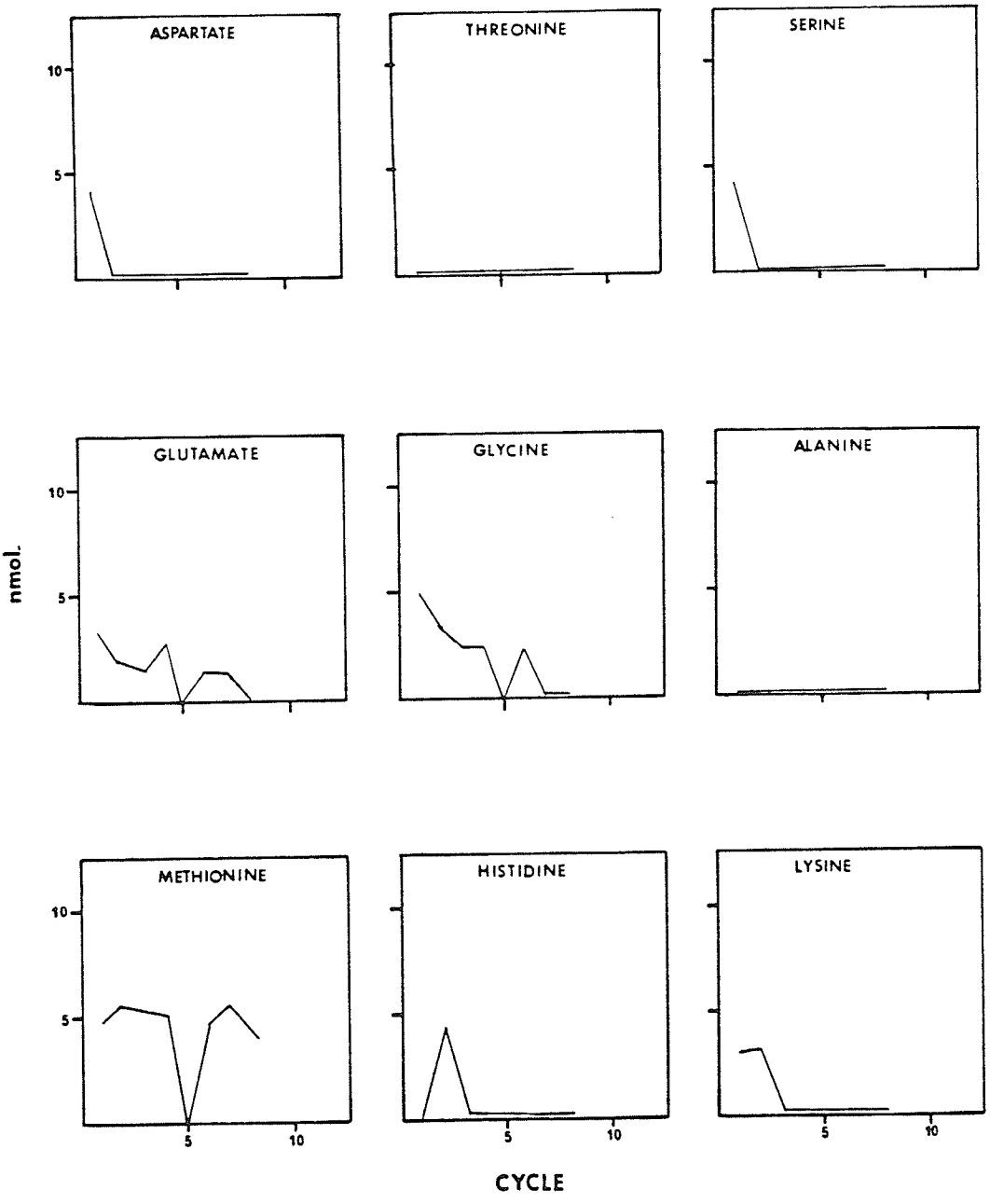

METHIONINE
ISOLEUCINE
LEUCINE
TYROSINE
PHENYALANINE
HISTIDINE
LYSINE
ARGININE
nmol.
CYCLE
7
11
13
9
21
5
10
15
20

Fig. 27 Yields of amino acids from automated Edman degradation of 2 mg of pyroglutamate amino peptidase with 5 mg polybrene present. Amino acids were quantitated by amino acid analysis on a Technicon NC-2P Analyzer following acid hydrolysis of the thiazolinone derivatives produced by a Beckman 890C sequencer.


ASPARTATE
THREONINE
SERINE
GLUTAMATE
GLYCINE
ALANINE
METHIONINE
HISTIDINE
LYSINE
10
5
nmol.
5
10
CYCLE

acid, respectively.

The relative recoveries of thiazolinone derivatives from cycle to cycle were determined by normalizing with the internal standard PTH-norleucine. The results of two analyses of rat serum α_1-AGP are presented in Fig. 26 with each graph showing the relative recoveries of a particular amino acid at the cycles analyzed. An indication of the performance of the sequence run was obtained by calculating repetitive yields for proline. It should be noted that these analyses were performed on protein which has had the N-terminal residue removed and consequently one must be added to the cycle number on the graph when referring to the position of that residue on the polypeptide. The interpretation of the sequence analysis presented in Figs. 26 A and B, into an amino acid sequence of the N-terminal 19 resideus of rat serum α_1-AGP is shown in Fig. 28. The residues at positions 7 and 16 could not be clearly identified and are consequently unassigned.

In order to confirm this sequence and in order to obtain additional amino acid sequence information about the remainder of the polypeptide, CNBr peptides were prepared from rat serum α_1-AGP. Preparation and sequencing of cyanogen bromide peptides of rat serum α_1-AGP α_1-AGP isolated from rat serum was treated with cyanogen bromide (CNBr) as described in the Experimental section. In the initial phase of this study the reduced and alkylated CNBr fragments were fractionated on a 2 cm x 150 cm column of Sephadex G-100 eluted with 20% acetic acid. The elution profile of

Fig. 28 The N-terminal amino acid sequence of:

A - α_1-AGP isolated from rat serum;

B - CNBr-4 prepared from rat serum

α_1-AGP

A

1	2	3	4	5	6	7	8	9	10
(pyroGlu)-	Asp -	Pro -	Glu -	Pro -	Ala -	X -	Ile -	Thr -	Leu

11	12	13	14	15	16	17	18	19
Gly -	Ile -	Pro -	Ile -	Thr -	X -	Glu -	Thr -	Leu

B

1	2	3	4	5	6	7	8	9
(pyroGlu)-	Asp -	Pro -	Glu -	Gly -	Ala -	Gly -	Ile -	Thr

protein and hexose are shown in Fig. 29. The first peak was assigned an identification number of 0 because SDS PAGE analysis on 12% gels indicated that it contained a composite of all the CNBr fragments which apparently aggregated and consequently eluted in the void volume of the column. Since analysis of the eluting fractions for hexose did not clearly indicate which CNBr peptide contained protein-bound hexose (20% acetic acid used as the elution buffer apparently eluted hexose from the Sephadex G-100 packing material) in subsequent experiments the CNBr peptide mixture was initially fractionated on a 1 cm x 5 cm column of ConA-Sepharose (Fig. 30). The first fraction which eluted unadsorbed from the column of ConA-Sepharose, was further fractionated on a 2 cm x 150 cm column of Sephadex G-100 (Fig. 31). The fraction of CNBr fragments adsorbed by ConA-Sepharose was eluted by the addition of 0.5 M α-methyl-D-mannoside and then fractionated on a 2 cm x 150 cm column of Sephadex G-100 (Fig. 32). Since the peak which was eluted in the largest elution volume (referred to as CNBr-4) was shown to be a relatively pure peptide by electrophoretic analysis on SDS PAGE using 12% gels (Fig. 33) it was the first peptide chosen for N-terminal amino acid sequence analysis. The initial sequence analysis by automated Edman degradation on the Beckman 890C sequencer resulted in a very low yield of thiazolinone derivatives. Subsequent treatment of CNBr-4 with the enzyme pyroglutamate amino peptidase, which removed the

Fig. 29 Gel filtration on a 2 cm x 150 cm column of Sephadex G-100 of reduced and alkylated CNBr peptides from rat serum α_1-AGP. The column was eluted with 20% acetic acid at a flow rate of 15 ml/h; 5 ml fractions collected. The fractions were collected and analyzed for protein by measuring the absorbance at 280 nm and for hexose by the Orcinol method as described in Experimental. The fractions were pooled and identified as indicated.

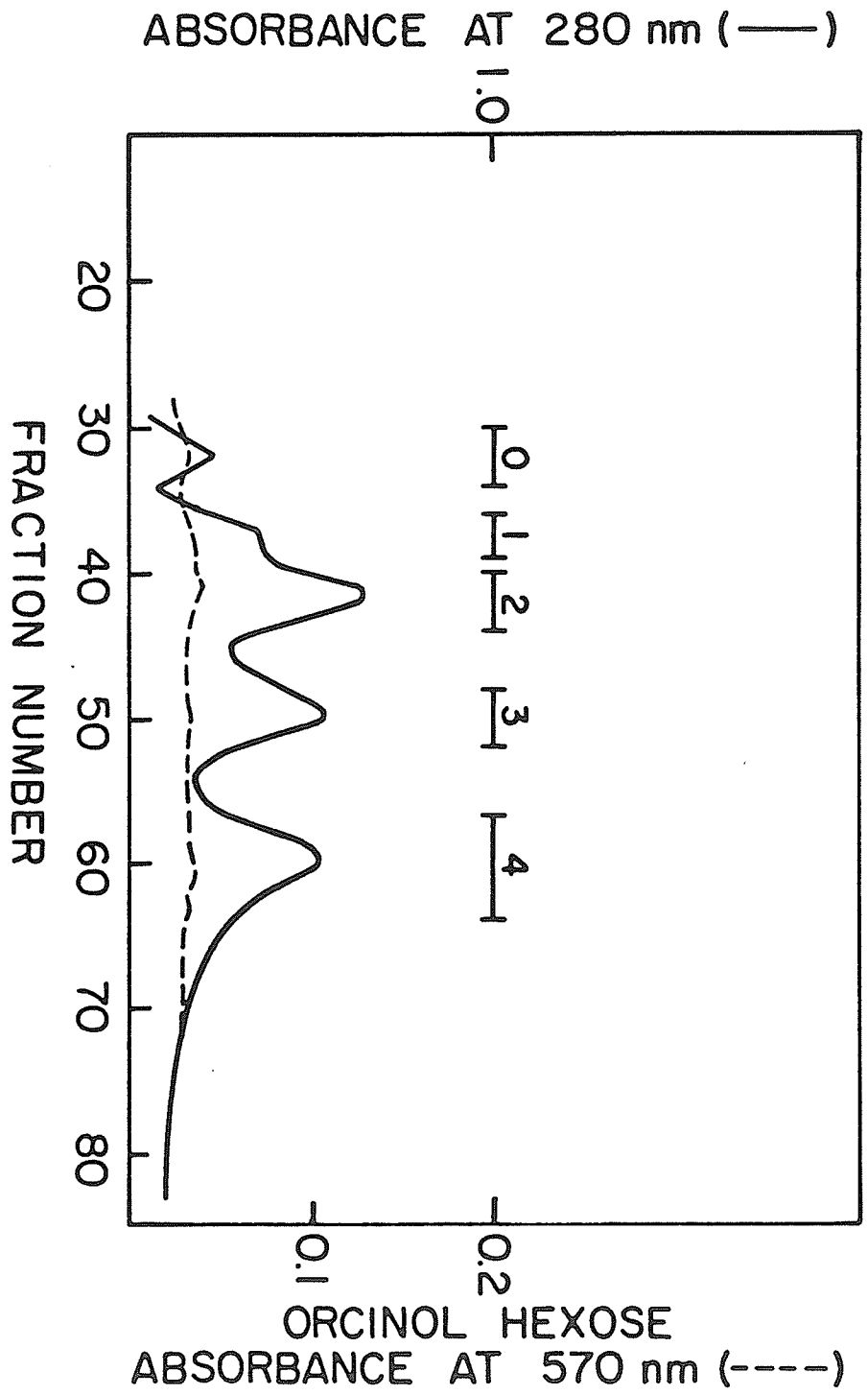

ABSORBANCE AT 280 nm (——)
1.0
0
1
2
3
4
20
30
40
50
60
70
80
FRACTION NUMBER
0.1
0.2
ORCINOL HEXOSE
ABSORBANCE AT 570 nm (----)

Fig. 30 Affinity chromatography of a mixture of rat serum α_1-AGP CNBr peptides on a 1 cm x 5 cm column of ConA-Sepharose. The column was eluted with 0.1 M sodium acetate buffer, pH 6.0, containing 0.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mM $CaCl_2$ and 0.5 M NaCl until the unadsorbed peptides were removed; the arrow indicates the position at which initial buffer containing 0.5M α-methyl mannoside (α-MeM) was applied. 3 ml fractions were collected and analyzed for protein by measuring the absorbance at 280 nm. Fractions I and II were pooled as indicated.

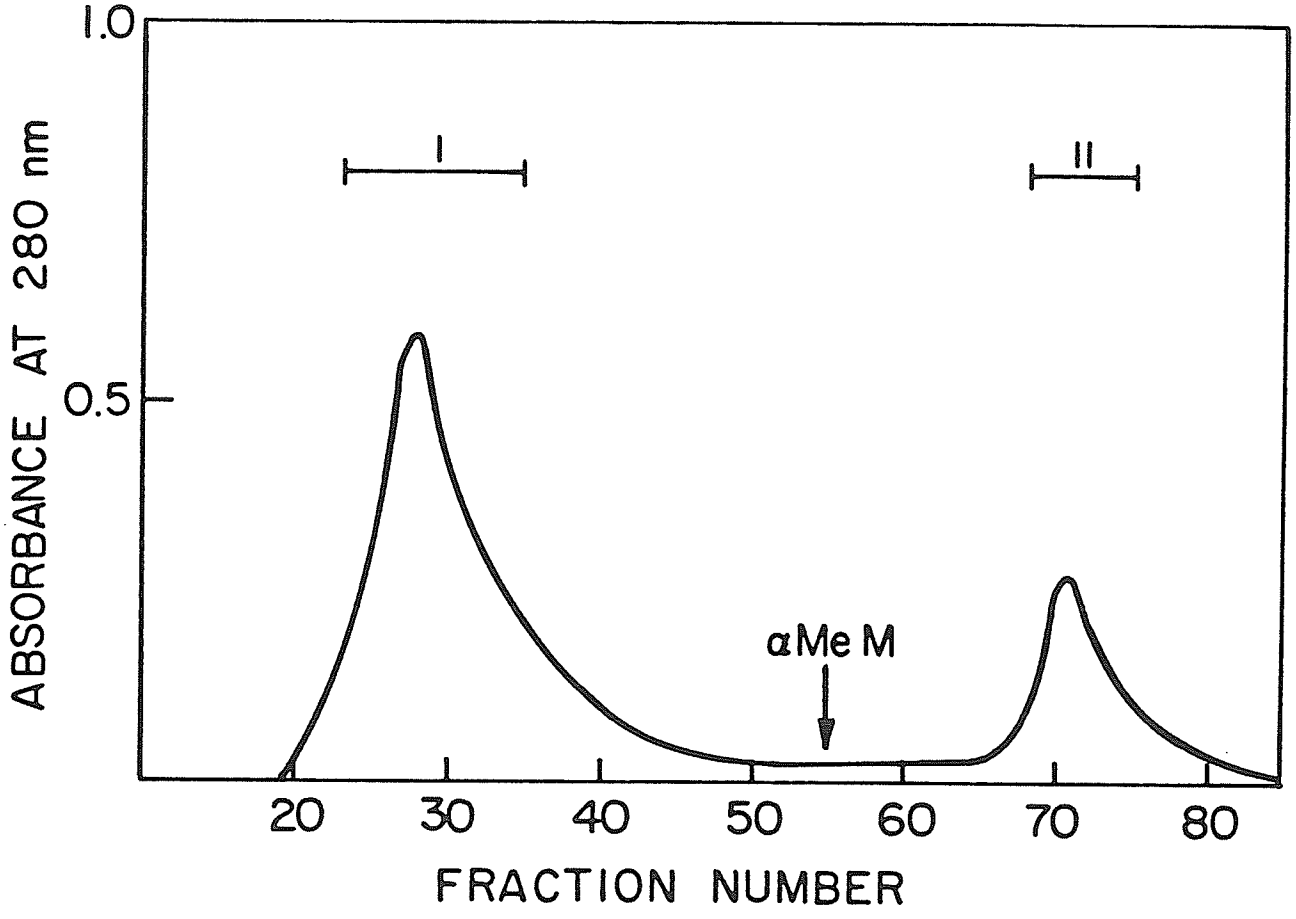

1.0
0.5
ABSORBANCE AT 280 nm
I
II
αMe M
20
30
40
50
60
70
80
FRACTION NUMBER

Fig. 31 Gel filtration of ConA-Sepharose I on a 2 cm x 150 cm column of Sephadex G-100. The column was eluted with 20% acetic acid at a flow rate of 15 ml/h; 5 ml fractions were collected and analyzed for protein by measuring the absorbance at 280 nm. Fractions were pooled and identified as shown.

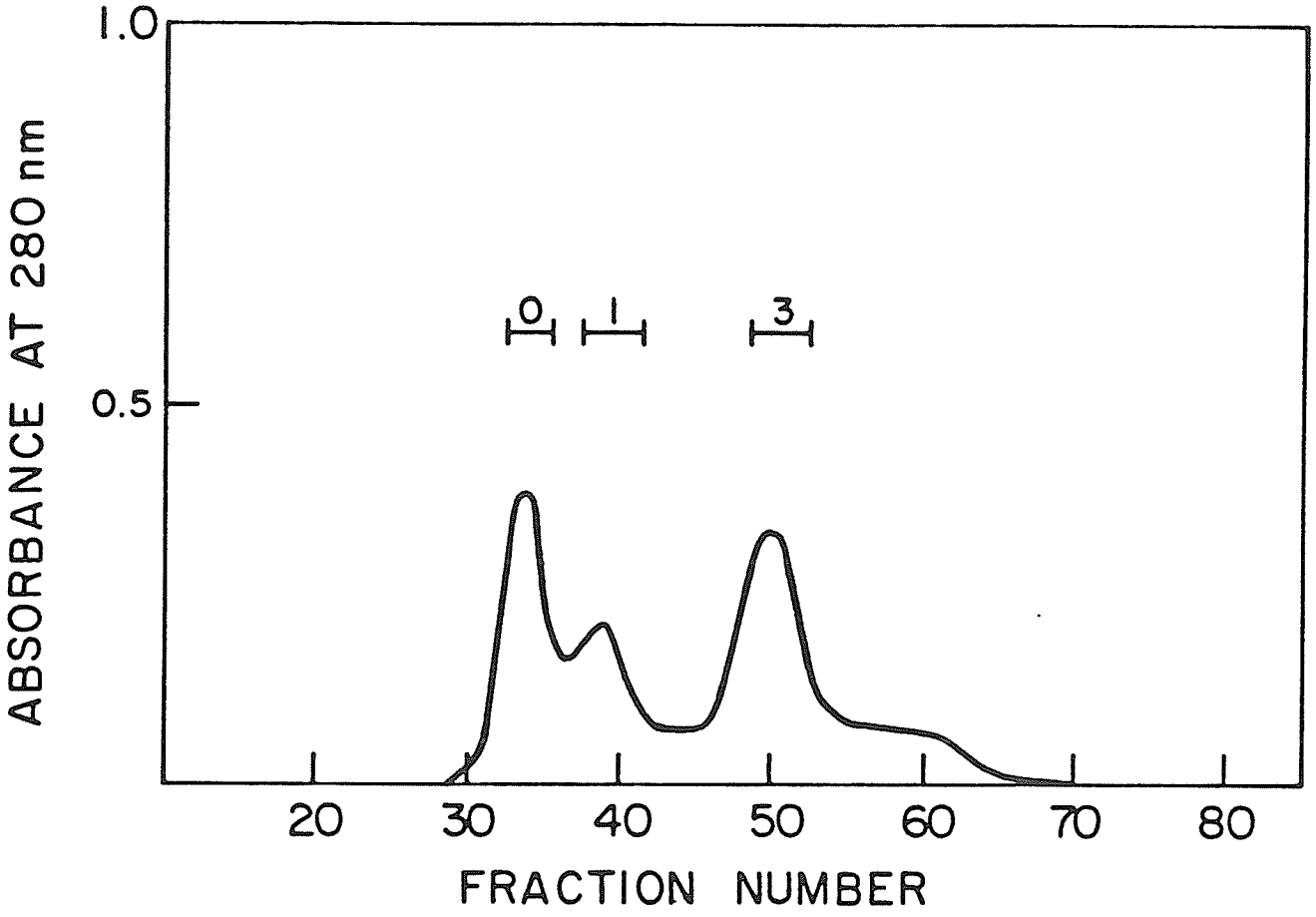

1.0
0.5
ABSORBANCE AT 280 nm
0
1
3
20
30
40
50
60
70
80
FRACTION NUMBER

Fig. 32 Gel filtration of ConA-Sepharose II on a 2 cm x 150 cm column of Sephadex G-100. The column was eluted with 20% acetic acid at a flow rate of 15 ml/h; 5 ml fractions were collected and analyzed for protein by measuring the absorbance at 280 nm. Fractions were pooled and identified as shown.

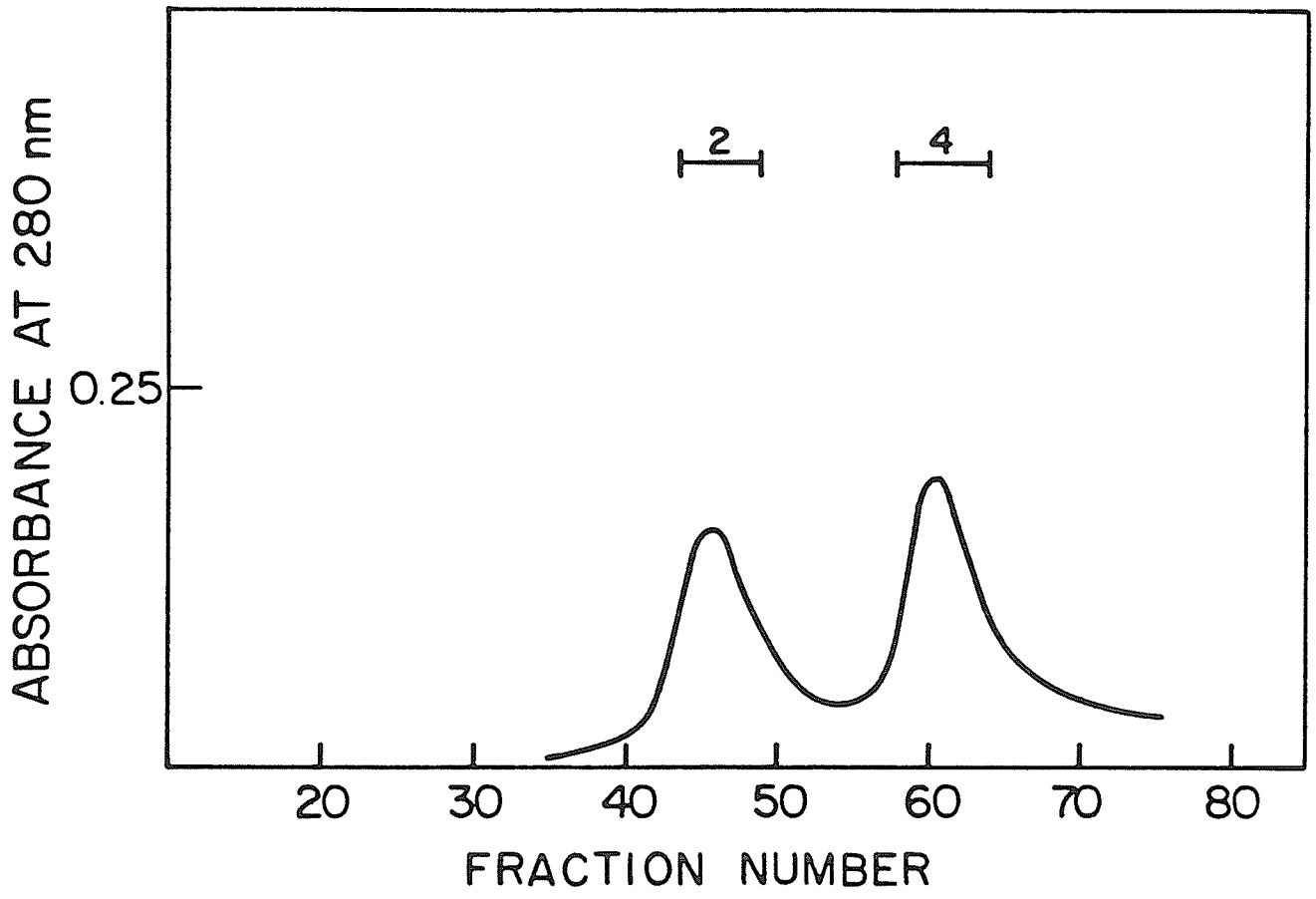
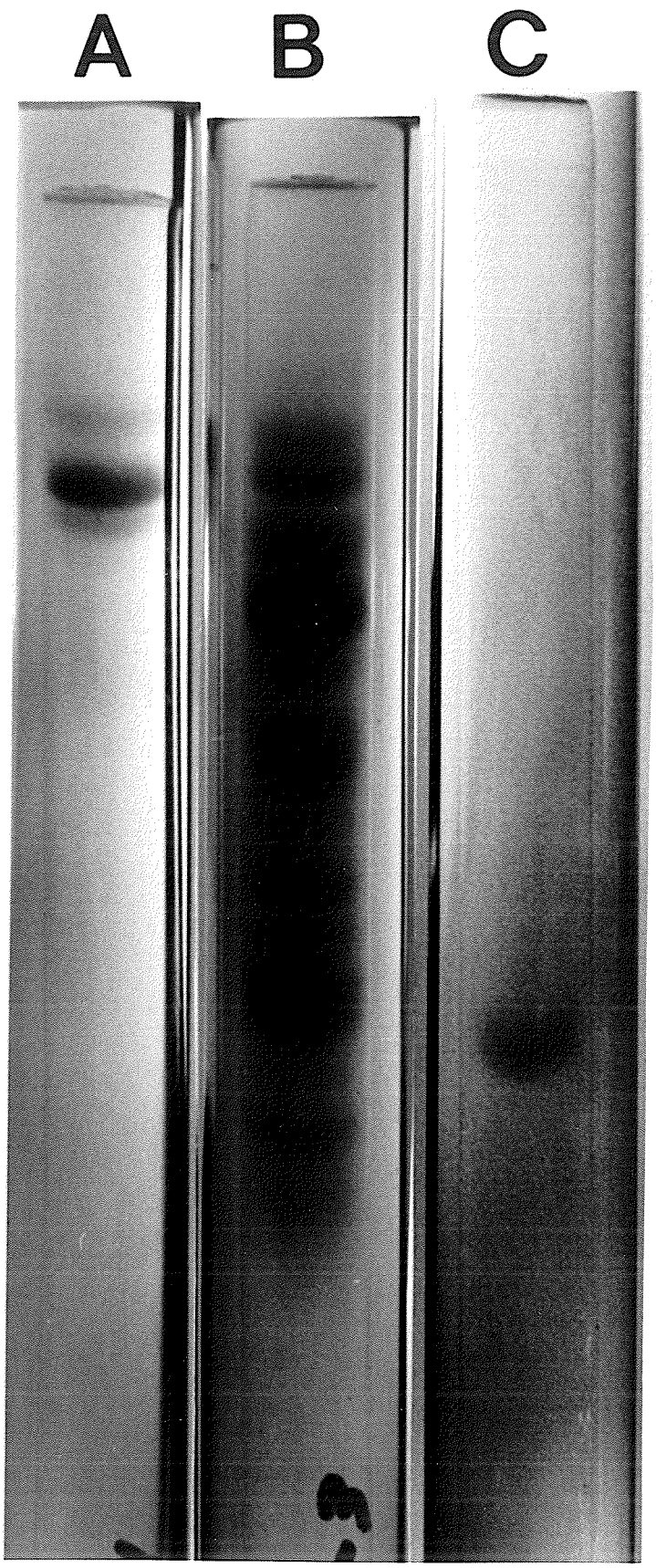

ABSORBANCE AT 280 nm
0.25
2
4
20
30
40
50
60
70
80
FRACTION NUMBER

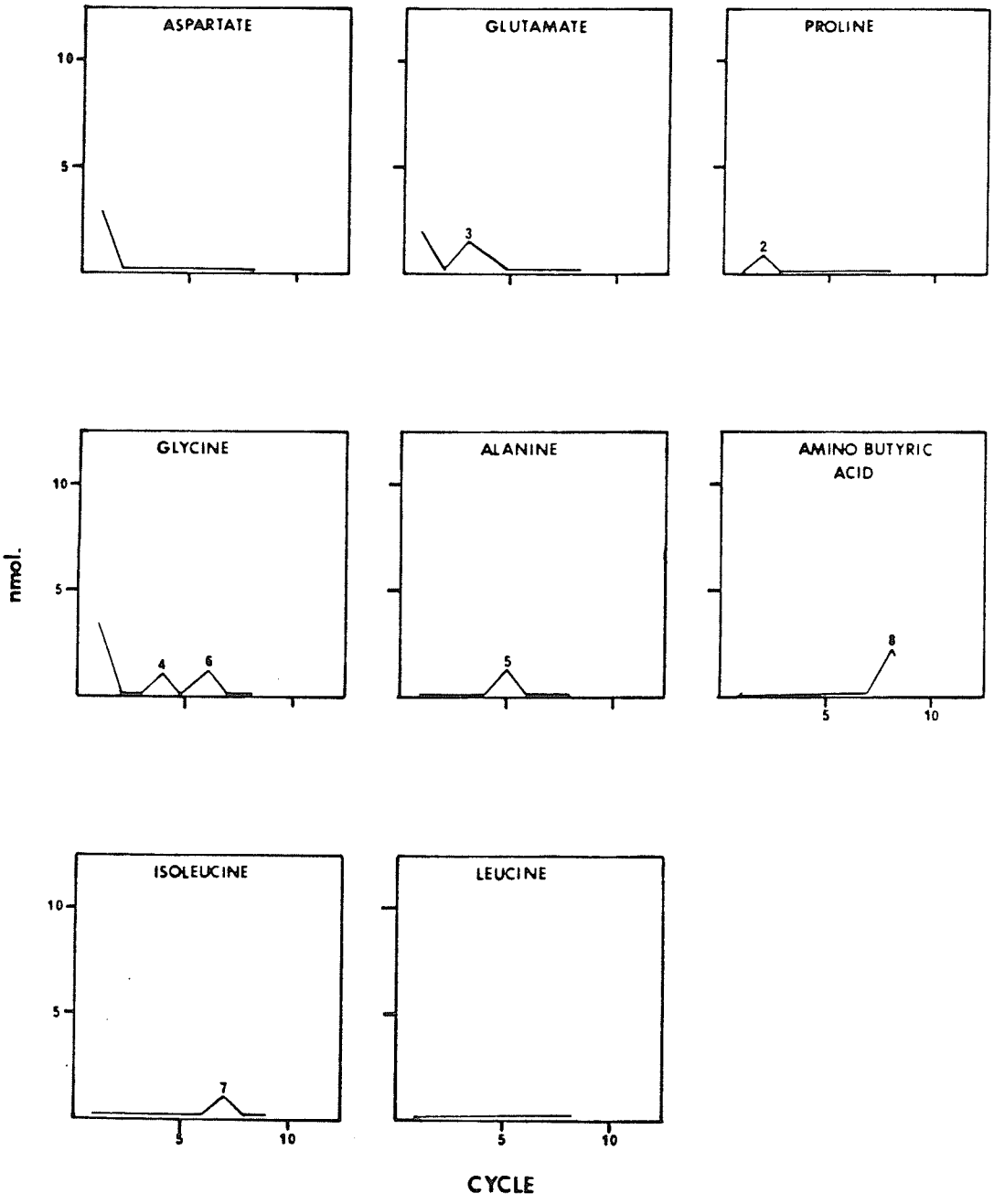
Fig. 33 SDS PAGE on 12% gels of: A, serum α_1-AGP;
B, mixture of serum α_1-AGP CNBr peptides;
C, CNBr-4 which was sequenced by automated
Edman degradation (Fig. 34).


A
B
C

blocking pyroglutamate from the N-terminus of serum α_1-AGP, resulted in the successful N-terminal sequence analysis shown in Fig. 34. The interpretation of the relative amino acid yields at each cycle resulted in the sequence assignment shown in Fig. 28. The N-terminal sequence of CNBr-4 was identical to the N-terminal sequence of serum α_1-AGP except for positions 5 and 7. The amino acid analysis of cycles 5 and 7 from the CNBr sequence run showed that glycine was present in the greatest amount, whereas sequence analysis of serum α_1-AGP showed that proline was present at position 5 and the amino acid at position 7 could not be clearly assigned. Since the assignment of amino acids for all other positions were identical and since the sequence analysis indicated that the N-terminus of both CNBr-4 and serum α_1-AGP contained a cyclized pyroglutamate residue, it is clear that CNBr-4 is the N-terminal peptide and its sequence provides confirmation of the N-terminal sequence of serum α_1-AGP.

During the course of the work of determining the amino acid sequence of the α_1-AGP isolated from rat serum in our laboratory, Ricca and Taylor (158) reported the complete amino acid sequence of rat α_1-AGP from a cloned double stranded cDNA of the mRNA (shown in Appendix I). Since the sequence of the first 19 residues of the serum α_1-AGP isolated in our laboratory was identical to the equivalent portion of the Ricca and Taylor sequence it appeared that their report made the complete sequence available

Fig. 34 Yields of amino acids from automated Edman degradation sequence analysis of 1 mg of CNBr-4 prepared from rat serum α_1-AGP and which was treated with pyroglutamate amino peptidase. Amino acids were quantitated by a Technicon NC-2P amino acid analyzer following acid hydrolysis of the thiazolinone derivatives produced by a Beckman 890C sequencer. Assignments are identified by the number above peaks.


ASPARTATE
GLUTAMATE
PROLINE
GLYCINE
ALANINE
AMINO BUTYRIC ACID
ISOLEUCINE
LEUCINE
nmol.
10
5
3
2
4
6
5
8
7
CYCLE

to us. Consequently it seemed that more fruitful information could be obtained in other studies with the carbohydrate and amino acid characterization of the intracellular forms of α_1-AGP.

Attempted N-terminal sequence analysis of intracellular α_1-AGP

As mentioned above, amino acid analyses of intracellular forms of α_1-AGP isolated from subcellular fractions of rat liver suggested that the intracellular forms might contain an extra amino acid segment of the pre- or pro- type. Automated N-terminal Edman degradation sequence analysis was performed on intracellular α_1-AGP isolated from the rough fraction of rat liver. The yields of amino acids in the initial sequence analyses were low suggesting the possibility that intracellular α_1-AGP was blocked to reaction with PITC. The obvious possibility was that a pyroglutamate residue occupied the N-terminus of the intracellular form of α_1-AGP as was found with the native serum protein. Both intracellular α_1-AGP form 1 and the mixture of forms 1 and 2 obtained from rough fractions were subjected to treatment with pyroglutamate amino peptidase in the hope of removing a blocking group. Sequence analysis using the approach described above for α_1-AGP failed after several attempts to provide any meaningful sequence information and so no report on these experiments can be provided.

DISCUSSION

Previous studies in this laboratory showed that rat α_1-AGP is initially assembled in association with RER and is then secreted from the liver cell by way of the SER and Golgi complex (22,48). This biosynthetic mechanism has been widely accepted as the general pathway for most, if not all, secretory proteins (45). Initiation and elongation of polypeptide biosynthesis occurs within the ribosomal complex and as the chain extends out of the large ribosomal subunit the ribosomes become associated with the RER. The nascent polypeptide is then vectorially transported across the RER membrane into the intracisternal space where further processing and secretion occurs. For secretory glycoproteins, glycosylation is believed to occur by the *en bloc* transfer of a high mannose oligosaccharide from lipid to the polypeptide chain within the intracisternal space of the RER at an early stage of the secretory process (see Introduction). The high mannose oligosaccharide is then believed to be processed to the complex-type oligosaccharide commonly found on secretory glycoproteins by removal of some mannose and synthesis of the terminal triplet of sugars with the sequence GlcNAc-Gal-NeuAc. This process has been suggested mainly from *in vitro* studies using membrane preparations from a variety of cells, or by using cells grown in culture; in both types of study mannose or GlcNAc were used as labelled precursor for glycoprotein biosynthesis (74,75,81 and 159). However, at the time the studies reported in this thesis were

initiated the existence of a high mannose containing form of a liver synthesized glycoprotein had not been demonstrated. It was the main objective of this work to show the existence of such an intermediate in the synthesis of a liver glycoprotein. Since past work in the laboratory had led to the isolation and characterization of rat serum α_1-AGP (117) this protein was chosen for study with the aim of isolating it in its high mannose form, and then extending the studies to the isolation of other forms with partially processed oligosaccharide chains.

Earlier studies (48), which involved the incorporation of labelled glucosamine into rat α_1-AGP, showed that there appeared to be two main sites for the addition of this sugar during the secretory process. The RER appeared to contain the site where addition of GlcNAc to the inner core regions of the oligosaccharide occurred, whereas the Golgi complex appeared to be the site of addition of GlcNAc to the terminal triplet regions of the oligosaccharide chains of α_1-AGP.

These studies were repeated in the project presented in this thesis using labelled mannose, since this sugar should be incorporated into α_1-AGP mainly in the RER if a high mannose form of the protein was initially formed at this intracellular site. The observed uptake of labelled mannose from rat serum into liver was similar to that reported earlier for labelled glucosamine. The label appeared first in the non-protein-bound fraction of liver,

followed by incorporation into liver protein and finally appearing in glycoprotein secreted into serum. The pattern of incorporation of labelled mannose specifically into α_1-AGP of the three subcellular fractions of rat liver was different than that reported earlier with labelled GlcNAc (48). The main difference was that at early times after injection, α_1-AGP isolated from the rough membrane fraction had the highest specific radioactivity, whereas following labelling with glucosamine, α_1-AGP isolated from all three fractions had the same specific radioactivity. The incorporation of D-[^{14}C]-mannose into both total and Lubrol-soluble protein also showed a higher mannose label in the rough fraction compared to the smooth fraction, but with these crude fractions labelling of Golgi protein was the highest. The difference in kinetics of labelling of the crude fractions and α_1-AGP probably reflects the nature of the glycoproteins in the crude fractions. These crude fractions would contain numerous proteins which could have different kinetic patterns of labelling with respect to mannose. At later times of labelling with mannose the Golgi fraction seemed to be the source of α_1-AGP with the highest specific radioactivity. However, experiments in which sugars were recovered from α_1-AGP or total protein fractions, indicated that at these longer times after injection of labelled mannose (60 min) there was significant conversion of label from mannose to galactose and fucose. Since galactose and fucose are known (103) to be

added to glycoproteins in the Golgi complex, conversion to these sugars is most likely responsible for a significant amount of the labelling of α_1-AGP and total protein in the Golgi at later times.

The most likely explanation for the higher specific radioactivity of α_1-AGP at early times after injection of labelled mannose is that a high mannose form of the glycoprotein is associated with the RER. This explanation was investigated further by the isolation and characterization of intracellular forms of α_1-AGP.

Isolation and characterization of intracellular forms of α_1-AGP from subcellular fractions of rat liver

A number of reports have described the isolation of pro-forms of secretable proteins[(160,161) and reviewed in(162)]. The best characterized pro-form isolated from liver is proalbumin, the isolation and characterization of which was described by Judah's group (61,69,134,160,163). In these studies, Judah et al.(160) isolated an intracellular form of albumin from the rat liver microsome fraction by immunoprecipitation with antiserum to serum albumin, followed by dissociation of the antibody-albumin complex in acid and fractionation on DEAE-cellulose. This approach was attempted initially for the isolation of α_1-AGP from rat liver subcellular fractions, but it proved to be unsuitable because the antibody-antigen complex was not readily dissociated.

However, intracellular forms of α_1-AGP were isolated from subcellular fractions of rat liver by a combination of gel permeation chromatography, affinity chromatography on ConA-Sepharose and ion exchange chromatography on CM-cellulose. Ultrasonic extracts were used as the starting material in these studies to avoid additional steps that would be needed to remove the detergent. ConA-Sepharose was used to remove non-glycoprotein, particularly albumin, which was found to be a major component of the α_1-AGP-containing fraction following Sephadex G-100 chromatography. CM-cellulose chromatography was added as the final step to the purification scheme to remove any serum-type α_1-AGP which was always found to be present, although care was taken to remove blood from liver by exhaustive perfusion. Using this approach, a form of α_1-AGP was isolated from rough fractions which gave one band on basic PAGE and which gave immunological reactions which corresponded with serum α_1-AGP. Analyses of this form of α_1-AGP showed that it contained a substantially higher hexose content, most of which was mannose, and also an additional complement of amino acid residues when compared to serum α_1-AGP. Analysis of this intracellular form on SDS PAGE, however, revealed the presence of two protein bands which suggested the presence of two species with slightly different molecular weights. Treatment with trypsin prior to SDS PAGE showed that the form of higher electrophoretic mobility, referred

to as form 2, could be eliminated, whereas the form with lower electrophoretic mobility, referred to as form 1, was much more trypsin resistant. This trypsin resistant fraction gave a reaction of immunological identity with serum α_1-AGP, had a high hexose content like the fraction prior to trypsin treatment, and also had an amino acid composition very similar to that of α_1-AGP. It appeared that although this protein could resist complete destruction by trypsin under the conditions used, the amino acid analyses showed that there was a loss of amino acids as a result of trypsin treatment. The amino acids lost during treatment did not appear to significantly affect electrophoretic mobility of intracellular form 1 on SDS PAGE. This was surprising since amino acid analysis suggested that the loss in molecular weight should have been in the region of 3,000. Analysis of the trypsin treated fraction on basic PAGE showed that tryptic treatment resulted in a substantial increase in electrophoretic mobility which would correlate with a loss of some basic amino acids. This observation was corroborated by amino acid analysis which showed that the trypsin treated fraction had indeed lost three arginine and one or two histidine residues with no apparent change in the composition with respect to acidic amino acids. However, the electrophoretic mobility of the fraction on basic PAGE was still much less than that of asialo-α_1-AGP which had the same amino acid composition as the tryptic treated material, but

was devoid of the acidic sialic acid which is responsible in large part for the high electrophoretic mobility of α_1-AGP on basic PAGE. It is not clear why trypsin-treated intracellular α_1-AGP and asialo-serum α_1-AGP had different electrophoretic mobilities on SDS PAGE, or why there was no apparent increase in electrophoretic mobility of form 1 on SDS PAGE following trypsin treatment, even though there was a molecular weight reduction due to loss of amino acids. However, it is known (164) that glycoproteins exhibit anomalous behavior on acrylamide gels due to the extensive hydration of carbohydrate chains. This results in these molecules having a more expanded structure in free solution than would be suggested by the molecular weight determined from amino acid and carbohydrate analysis. It is possible that the high mannose forms described above are showing anomalous behavior on acrylamide gels due to the presence of a higher carbohydrate content (in the case of a comparison with asialo-α_1-AGP on basic PAGE), or due to some different conformational form (after trypsin treatment and analysis by SDS PAGE).

Although it is not clear why the intracellular forms of α_1-AGP described above show anomalous behavior on acrylamide gels, it is without question that the fraction isolated from rough membrane fractions contains a high mannose form of α_1-AGP which on trypsin treatment gives a single high mannose-containing protein with an amino acid composition similar to that of serum α_1-AGP.

The carbohydrate analysis of the trypsin treated material showed it also contained 53 residues of hexose which were mostly mannose as well as 10 residues of GlcNAc per mole. If it is assumed that rat α_1-AGP contains five oligosaccharide chains, as reported by Yoshima et al.(165), then the most likely structure for each oligosaccharide chain of intracellular α_1-AGP would be $Man_{10}GlcNAc_2$. This structure is very similar to the high mannose precursor that would be transferred to the polypeptide in the RER from the oligosaccharide-lipid structure $Glc_3Man_9GlcNAc_2$-P-P-Dol that is believed to be the end product of the dolichol cycle (95,166,167), except that the structure apparently does not contain glucose. Trace amounts of glucose were detected in both qualitative analysis on paper chromatography and quantitative analysis on gas liquid chromatography of hydrolyzed samples of intracellular α_1-AGP, but quantitation was considered unreliable because the presence of glucose could be attributed to elution of this sugar from the Sephadex and cellulose packing used in the columns employed for the preparation of this material.

Investigation into the structural differences between the proteins that gave the two bands on SDS PAGE was possible by the improved separation achieved with Ultrogel AcA44 (Fig. 15, scheme II). This, together with the observation of a spur in the immunodiffusion analysis, led to the isolation and characterization of each form without the use of trypsin to prepare a single protein. Both proteins were found to react immunologically with antiserum

to α_1-AGP and had similar amino acid compositions, although as discussed above, they differed slightly when compared to the amino acid composition of serum α_1-AGP. Thus, both proteins, referred to as forms 1 and 2, appear to be intracellular forms of α_1-AGP.

Carbohydrate analysis of these intracellular forms showed that form 1 from rough fractions, as expected, was a high mannose form. However, it was not clear why the gas liquid chromatographic analysis suggested that this form contained slightly fewer mannose residues than indicated earlier with the spectrophotometric method. Carbohydrate analysis of form 2 from rough membrane fractions suggested that it was a low mannose form of α_1-AGP. Because of the small amount of material available for analysis it was difficult to suggest a structure for the oligosaccharide chains of form 2. However, by taking the carbohydrate analysis of form 2 isolated from the rough fraction at face value, and assuming the presence of five oligosaccharide chains per molecule of α_1-AGP as before, the most likely structure in this form would be $Man_3GlcNAc_1$ which is close to the common core structure $Man_3GlcNAc_2$ for complex and high mannose oligosaccharides. Since form 2 clearly contains less carbohydrate than form 1 it would appear that differences in carbohydrate composition between the two forms is responsible for the presence of two bands on SDS PAGE.

The improved separation procedure developed for isolation of intracellular α_1-AGP was also applied to ultrasonic extracts of smooth and Golgi fractions. The preparations of intracellular α_1-AGP from these fractions were similar to forms isolated from the rough fractions when examined by basic and SDS PAGE. This was somewhat surprising since the α-mannosidases which remove mannose residues from high mannose forms of glycoproteins have been reported to be associated mainly with Golgi fractions (100,101). In addition, it has been known for some time that the glycosyl transferases which are responsible for synthesis of the terminal triplet sugars -GlcNAc-Gal-NeuAc are largely present in the Golgi complex (103,104). Thus, it might be expected that a variety of forms of α_1-AGP containing larger amounts of GlcNAc and galactose might be detected, particularly in the Golgi fraction. Forms of α_1-AGP containing some sialic acid would likely not be found since the fractionation procedure would eliminate them at the CM-cellulose chromatography step. However, electrophoretic analyses of extracts of smooth and Golgi fractions suggested that forms other than the high and low mannose forms described above, were either not present, or were present in such small amounts as to escape detection. Although carbohydrate analysis of form 2 was again subject to variability there was no indication that there was any significant increase in galactose in this form isolated from Golgi fractions. These results would suggest that

the two major forms of α_1-AGP that exist intracellularly are a high mannose form containing oligosaccharides which are largely unprocessed with respect to mannose, and a second form which contains oligosaccharide chains which are largely processed with respect to mannose. The existence of these two forms would indicate that the form containing nine mannose residues per oligosaccharide chain represents a major intracellular form, but that there is rapid processing to a form containing three mannose residues. The results further suggest that once this structure is formed and GlcNAc transferase I has acted, the subsequent reactions leading to completed chains and secretion of the final molecules are quite rapid.

The presence of a high mannose form of α_1-AGP in all three fractions was not unexpected if, as is likely, the high mannose form is processed mainly in the Golgi complex (100). However, the presence of a low mannose form in the rough fraction was surprising since it would imply that some processing occurred at an early stage of secretion. The presence of the low mannose form in the rough fraction could be explained if some processing occurred during the homogenization and fractionation steps. When the cell is disrupted the processing enzymes would be released into the medium and might become associated with rough fractions and cause some oligosaccharide processing. A more likely explanation would be that mannose processing occurred in later steps,

possibly during fractionation. An attempt was made to minimize this effect by the addition of the mannosidase inhibitor, L-mannonic acid-γ-lactone. Although lactone was present during early stages of fractionation, it was not added again at later stages of the separation procedure because it was felt it might interfere in the ConA-Sepharose chromatography step. Another explanation for the presence of the low mannose form in preparations from rough fractions is that it is derived from Golgi membranes. Since it is known that these membranes are easily disrupted by homogenization it is possible that, under the conditions used for preparation of the rough membrane fraction, the low mannose form simply redistributes itself between the other fractions. A detailed kinetic study involving labelling of the two forms would be needed in order to settle this question.

The demonstration, in this study, of the existence of intracellular high mannose forms of α_1-AGP in rat liver was initially reported in 1979 (168) and has subsequently been confirmed by Nagashima et al.(169) who isolated an intracellular form of α_1-AGP from total liver homogenate which also had a significantly higher hexose content and lower GlcNAc content than serum α_1-AGP. In our initial report, intracellular forms of α_1-AGP had been isolated from only the rough fraction and in the report by Nagashima et al.(169) intracellular α_1-AGP was isolated from total liver homogenate not subcellular fractions thereof. Isolation

of intracellular α_1-AGP from each of the rough, smooth and Golgi fractions was, however, subsequently performed in our study and carbohydrate analyses, although subject to variability, indicated that the high mannose form 1 of the Golgi fraction contained slightly fewer mannose residues that the same form isolated from rough and smooth fractions. This is what would be expected, since the mannosidases capable of removing mannose from high mannose oligosaccharides are known to be present in the Golgi. However, the presence of different oligosaccharide structures on form 1 isolated from Golgi compared with form 1 isolated from rough and smooth fractions was unexpected, since analyses on SDS PAGE indicated that form 1 isolated from all three subcellular fractions were the same. A similar apparent anomaly has been observed for intracellular forms of α_1-antitrypsin and thyroglobulin. Hercz *et al.*(170,171) isolated and characterized intracellular forms of α_1-antitrypsin which were shown to contain high mannose oligosaccharide chains with three types of structures, $Man_7GlcNAc_2$, $Man_6GlcNAc_2$ and $Man_5GlcNAc_2$. Electrophoretic analysis of these intracellular forms with varying mannose content on SDS PAGE, however, showed only a single discrete band. Spiro's group (172,173) reported the isolation and characterization of intracellular forms of another secretory glycoprotein, thyroglobulin. Electrophoretic examination on SDS PAGE of the intracellular forms isolated from each of the rough, smooth

and Golgi fractions showed the same two discrete band patterns observed for intracellular α_1-AGP in our study. Although they did not analyze the individual forms for carbohydrate, they did suggest that thyroglobulin was glycosylated by the en bloc transfer of a high mannose oligosaccharide. They also suggested that at various stages of the secretory process from the RER to the Golgi complex, thyroglobulin contained high mannose oligosaccharide structures ranging from $Glc_3Man_9GlcNAc_2$- to $Man_5GlcNAc_2$-, as well as some oligosaccharides which contained some galactose, fucose and additional GlcNAc. Thus, it is possible that, although intracellular forms of α_1-AGP isolated from the different subcellular fractions appear to be similar on SDS PAGE, each form contains oligosaccharides of slightly varying composition depending on the subcellular fraction it was isolated from. The carbohydrate analyses suggested the possibility that the high mannose form 1 contained oligosaccharides with structures ranging from a $Man_9GlcNAc_2$-type in the rough fraction to a $Man_5GlcNAc_2$-type in the Golgi fraction, whereas form 2 contained oligosaccharides which appear to contain mainly the core-type structure, $Man_3GlcNAc_2$.

The structural differences between forms 1 and 2 indicated from the carbohydrate analyses were supported by the immunodiffusion analyses (Fig. 22). The formation of an immunoprecipitin spur between the two forms of intracellular α_1-AGP indicated that, although both forms were immunologically similar to serum α_1-AGP,

form 1 contained an antigenic determinant not present in form 2. Since antibodies produced by glycoproteins are usually directed primarily to the polypeptide moiety, the production of the immunological spur of partial identity was not expected to be due to a difference in carbohydrate. However, an immunological reaction specifically for high mannose oligosaccharides has been observed. Nakajima et al.(174) reported that both α-1,2 mannose and α-1,3 mannose were involved in antigenic determinants and that the antibodies cross reacted with glycopeptide containing mannose oligosaccharides from various sources. In addition, Monaco and Andreoli (175) reported that the terminal non-reducing sugars of thyroglobulin were involved in formation of an immunological spur of partial identity like that observed in our study for intracellular α_1-AGP. Thus, the spur produced by the two forms of intracellular α_1-AGP appears to be another indication that the difference between the forms is that form 1 contains high mannose oligosaccharides and form 2 contains low mannose oligosaccharides. The absence of a spur formation in Fig. 18 which compared trypsin treated with untreated intracellular α_1-AGP, would be expected since both samples contained form 1 and were compared with precipitin lines formed by serum α_1-AGP against which the antiserum was raised.

Amino acid analyses indicated that the two intracellular forms of α_1-AGP isolated from each of the three subcellular fractions of rat liver did not differ significantly in amino acid

composition. The analyses, however, indicated that all forms of intracellular α_1-AGP contained about 29 additional amino acids not present in serum α_1-AGP. This suggested the possibility of the presence of a polypeptide extension in rat liver α_1-AGP like that reported by Judah's group for albumin (61,69,134). A study to determine the N-terminal sequence of both serum α_1-AGP and intracellular α_1-AGP was attempted in order to clearly establish whether or not the additional amino acid residues observed in intracellular α_1-AGP were due to an N-terminal extension. In this study it was shown that rat serum α_1-AGP contained the same N-terminal cyclized pyroglutamate found on human α_1-AGP (156), but following enzymatic removal of this residue from rat serum α_1-AGP, the amino acid sequence of the 19 N-terminal residues was determined (Fig. 28). Although the amino acid residues at positions 7 and 16 could not be determined, this sequence was identical with respect to the amino acid sequence of the 14 N-terminal residues of rat α_1-AGP reported by Nagashima *et al.*(176) as well as the sequence recently reported by Ricca and Taylor (158, also see Appendix I). It appears that all three groups are studying the same protein. The sequence reported by Ricca and Taylor (158) showed that the amino acids in positions 7 and 16 were asparagine residues in a sequon; hence, they contained covalently linked oligosaccharide chains which prevented the identification of the asparagine by sequence analysis.

Comparison of the N-terminal amino acid sequence of the rat α_1-AGP determined in our study with the corresponding segment of the human α_1-AGP, reported by Schmid et al.(156) showed that of the N-terminal 19 residues, the amino acids in position 1, 3, 6 and 7 were identical, and if a gap was introduced following position 10 of the human sequence the amino acids in position 13-16, 18 and 19 would also be identical. A comparison of the entire inferred amino acid sequence of rat α_1-AGP reported by Ricca and Taylor (158) showed a 44% identity with the sequence of human α_1-AGP reported by Schmid et al.(156).

In this study to obtain amino acid sequence information of rat serum α_1-AGP, CNBr peptides of the protein were prepared and isolated for N-terminal sequence analysis. Gel permeation chromatography of the mixture of CNBr peptides indicated the presence of four peptides which would be the number expected, since the amino acid analyses indicated the presence of three methionine residues. N-terminal sequence analysis of CNBr-4 indicated that this peptide was derived from the N-terminus of α_1-AGP and, in addition, confirmed the amino acid sequence of seven of the first nine residues from the N-terminus. Additional confirmation that CNBr-4 was the N-terminal peptide was obtained by comparison of its amino acid composition with the composition of the N-terminal CNBr peptide predicted from the sequence reported by Ricca and Taylor (Table 9).

Table 9

Amino acid composition of CNBr-4 and N-terminal CNBr peptide of Ricca and Taylor rat α_1-AGP

Residue	residues/mole	
	CNBr-4[a]	Ricca & Taylor[b]
Asx	4.3	4
Thr	2.5	3
Ser	1.3	1
Glx	3.5	3
Pro	2.1	3
Gly	2.0	1
Ala	1.4	1
Val	1.0	-
Met	-	-
Ile	2.4	3
Leu	3.2	3
Tyr	0.4	1
Phe	1.2	1
His	0.6	-
Lys	2.5	2
Arg	0.9	-

[a] Composition was determined by assuming that CNBr-4 contained the same total number of amino acid residues as the N-terminal CNBr peptide predicted from amino acid sequence of rat α_1-AGP reported by Ricca and Taylor (158).

[b] Composition was determined from the amino acid sequence of rat α_1-AGP reported by Ricca and Taylor (158).

Nagashima et al.(169), who reported on an intracellular form of α_1-AGP subsequent to the initial report (168) of the work presented in this thesis, were unable to detect an amino acid extension as observed in our study. The possibility that rat α_1-AGP is synthesized with an N-terminal polypeptide extension is, however, supported by the complete nucleotide sequence of rat α_1-AGP mRNA reported by Ricca and Taylor (158). The amino acid sequence which is presented in Appendix I was determined from DNA sequence analysis of molecular cloned complementary DNA (cDNA) synthesized from α_1-AGP mRNA. Comparison of this sequence with that established in the present work and with the sequence reported by Nagashima et al.(176), suggests that the Gln residue in position 19 of the Ricca and Taylor sequence is the N-terminal residue of serum α_1-AGP. The additional 18 residues reported by Ricca and Taylor would most likely represent a signal sequence. These 18 residues have the sequence Met-Ala-Leu-His-Met-Val-Leu-Val-Val-Leu-Ser-Leu-Leu-Pro-Leu-Leu-Glu-Ala which contains two methionine residues. As mentioned in the Introduction, methionine initiates protein synthesis in eukaryotic systems, so the primary translation product under in vivo conditions could contain an extension with all 18 amino acids, or one which contains only 14 amino acids. Although sequence information on the precursor forms of α_1-AGP described in the present work could not be obtained, information on amino acid composition was available. Table 10

Table 10

Amino acid composition of α_1-AGP from serum and liver

	residues/mole α_1-AGP					
	Rat					
	Thesis[a]		Nagashima[b]		Ricca & Taylor[c]	
Residue	Serum	Liver	Serum	Liver	Serum	Liver
Asx	22.1	23.0	25	26	21	21
Thr	16.6	15.2	21	20	16	16
Ser	13.2	16.6	9	9	6	7
Glx	27.7	26.5	35	37	28	29
Pro	9.5	13.9	} 10	} 10	8	9
Cys	-	-			3	3
Gly	11.7	18.6	11	14	8	8
Ala	13.2	16.8	12	12	10	12
Val	12.6	15.0	14	14	10	13
Met	3.0	3.0	3	3	3	5
Ile	10.0	9.0	11	12	10	10
Leu	16.5	20.4	22	20	17	24
Tyr	5.6	6.1	6	6	5	5
Phe	11.0	11.0	15	15	12	12
His	4.2	5.8	4	4	3	4
Lys	16.5	17.0	22	21	19	19
Arg	6.0	8.8	6	6	5	5
Trp	3.9	ND[d]	ND	ND	3	3

[a] Amino acid composition of serum α_1-AGP and intracellular forms of α_1-AGP isolated from the rough membrane fractions of rat liver (from Table 4 of this thesis)

[b] Amino acid compositions reported by Nagashima et al.(169)

[c] Amino acid composition determined from the sequence of rat α_1-AGP reported by Ricca and Taylor (158)

[d] ND, not determined

shows the amino acid compositions for α_1-AGP isolated from serum and rough fractions in the present work together with similar information recently published by Nagashima et al.(169); the data is compared with an amino acid composition for the serum α_1-AGP and a primary translation product containing 18 extra amino acids based on the work of Ricca and Taylor (158). As can be seen in Table 10, the amino acid composition reported in the present studies and that reported by Nagashima et al.(169) is similar, except for a higher content of serine and a lower content of glutamate obtained in the analysis of α_1-AGP in this thesis. Based on the data provided by Ricca and Taylor there is very good agreement of amino acid composition with the α_1-AGP analyzed in this thesis, except for a higher content of serine. Analysis of the liver form of α_1-AGP isolated in the present work was clearly different from that reported by Nagashima et al.(169) which had a composition close to that of serum α_1-AGP. However, the amino acid composition of the liver form of α_1-AGP presented in this thesis had some similarities to the amino acid composition of a primary translation product of the α_1-AGP mRNA sequenced by Ricca and Taylor(158). This is shown in Table 11 where the additional amino acid residues found in the analysis of intracellular forms of α_1-AGP isolated from the rough fraction are compared with the 18 residues present as an N-terminal extension in the Ricca and Taylor sequence. Although the amino acid residues

present in the extension sequence of Ricca and Taylor are not identical to the extra complement of amino acids found in the present work, it is interesting to note that except for arginine, glycine and methionine the same amino acid residues are involved; in addition, both columns of Table 11 show high levels for leucine, a common amino acid in N-terminal extensions. It appears that the values obtained for glycine in α_1-AGP in our study and that reported by Nagashima et al.(169) are considerably higher than the values obtained from Ricca and Taylor's sequence (see Table 10); this suggests that the values obtained for glycine are not as reliable as values obtained for other amino acid residues.

The N-terminal polypeptide extension reported by Ricca and Taylor (158) is similar to the characteristic signal sequence found for many secretory proteins (52). The extension has a central core which contains mainly hydrophobic amino acids with a charged amino acid residue near the N-terminus (Histidine). The majority of signal peptides studied so far are, however, believed to be cleaved during translation. Since the ultrasonic procedure used in our study mainly extracts polypeptides released into the intracisternal space, the peptide extension reported by Ricca and Taylor (158) would not be expected to be present on the intracellular forms of α_1-AGP isolated from the subcellular fractions of rat liver. However, at least one example where the signal peptide is not removed during translation across the RER membrane has been

Table 11

Amino acid composition differences between serum and intracellular forms of rat α_1-AGP

Residue	residues/mole	
	Friesen & Jamieson[a]	Ricca & Taylor[b]
Ser	3	1
Glx	-	1
Pro	4	1
Gly	7	-
Ala	4	2
Val	2	3
Met	-	2
Leu	4	7
His	2	1
Arg	3	-
Total	29	18

[a] The additional amino acid residues found in intracellular forms of α_1-AGP isolated from the rough membrane fraction of rat liver not found in serum α_1-AGP; observed in the present study (From Table 10).

[b] The additional amino acid residues expected to be found on the primary translation product of α_1-AGP mRNA, but not observed in rat serum α_1-AGP; reported by Ricca and Taylor (158).

reported (60). Ovalbumin has been shown to retain its signal sequence to maturity. Thus, it is possible that rat α_1-AGP could also retain the signal sequence through the translation process and during transport throughout the process of secretion from the RER to the Golgi. It could be argued, however, that in the ovalbumin case, the signal sequence was contained within the internal portion of the polypeptide rather than an N-terminal sequence like that reported for rat α_1-AGP according to Ricca and Taylor (158).

The second part of the study to determine if intracellular α_1-AGP contained an N-terminal amino acid extension required the determination of the amino acid sequence of the N-terminus of intracellular α_1-AGP. Automated N-terminal Edman degradation sequence was attempted before and after treatment of the intracellular α_1-AGP with the enzyme, pyroglutamate amino peptidase, which was used to remove the blocking N-terminal residue from serum α_1-AGP. In both cases, the yields of thiazolinone derivatives were extremely low, and despite repeated efforts to sequence intracellular α_1-AGP isolated from rough membrane fractions, determination of N-terminal amino acid sequence was not achieved.

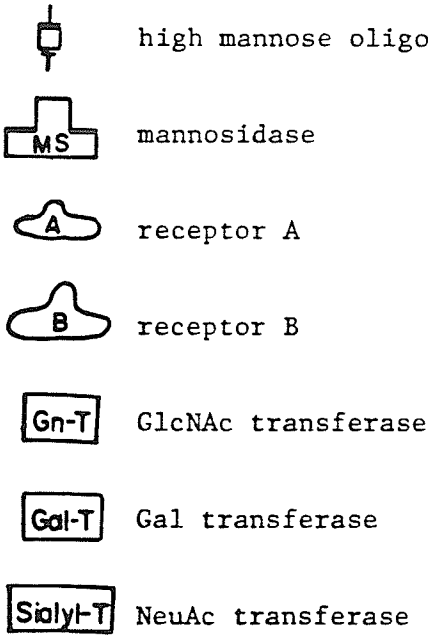
It is clear from the above discussion that the existence of a peptide extension on the N-terminus of intracellular α_1-AGP is far from conclusive. The additional amino acids found in intracellular α_1-AGP only partially resemble those predicted

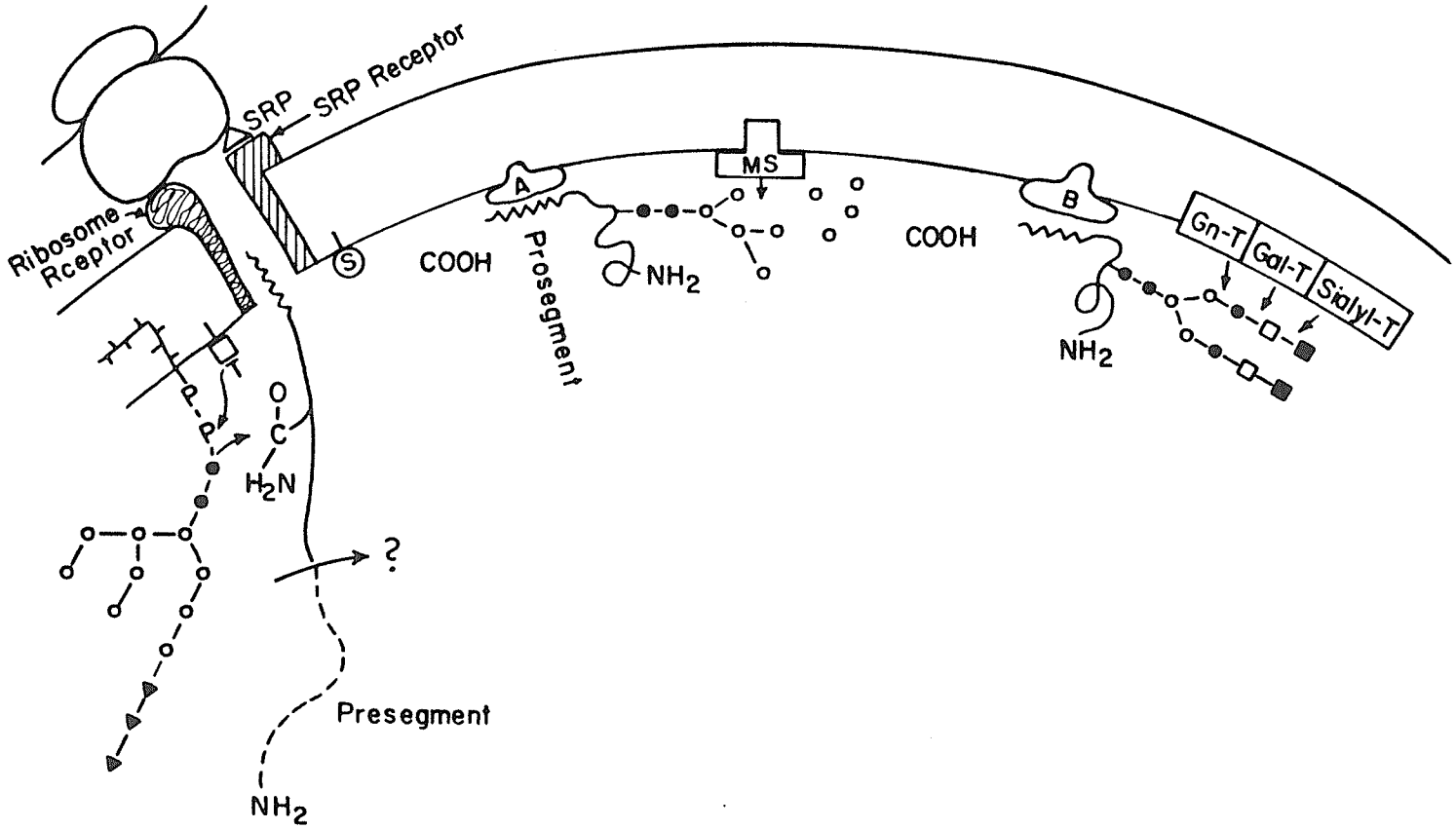
from Ricca and Taylor's sequence. In addition, neither arginine nor lysine residues were present in the N-terminal extension predicted from the Ricca and Taylor sequence which makes it difficult to explain why trypsin would remove such a sequence from an intracellular form of α_1-AGP as was found in this study. One possible explanation is that the extra complement of amino acids is present as a peptide extension in some other part of the polypeptide structure of α_1-AGP. The other obvious site at which a peptide could be located would be on the C-terminus. There is some evidence in the literature that C-terminal peptide extensions exist on some proteins like glucagon (161,177), but there is no evidence that such extensions are present on liver secreted glycoproteins, like α_1-AGP. Further work, which could involve the determination of the C-terminal amino acid sequence of both intracellular and serum α_1-AGP, is required to arrive at a satisfactory explanation for the presence of the additional amino acid residues in intracellular forms of α_1-AGP.

The present work has established that a major form of α_1-AGP formed *in vivo* is a high mannose form with oligosaccharide chains which appear to be largely processed with respect to glucose, but unprocessed with respect to mannose. Another major form of α_1-AGP also appears to exist *in vivo*, and although it has been less well characterized, it appears to be a low mannose form containing perhaps three mannose residues per oligosaccharide chain. All intracellular forms isolated contain an additional peptide which is

easily removed by limited treatment with trypsin, although it is far from clear where this peptide is located in the molecule. These findings have been incorporated into a model to describe the events that may occur during the secretory process of α_1-AGP in the channels of the endoplasmic reticulum and Golgi complex (Fig. 35). It should be emphasized that the purpose of this model is to serve as a basis for future studies on aspects of synthesis and secretion of α_1-AGP from the liver cell. According to the model the polypeptide of α_1-AGP is synthesized with a signal peptide and possibly also a C-terminal extension, although as discussed above there is little evidence to support this latter idea. After translation, oligosaccharide is transferred to nascent polypeptide as a post-ribosomal event. This is suggested since the weight of evidence on initial glycosylation of α_1-AGP in our laboratory (91) supports this idea. It is likely that the signal sequence is rapidly removed, although the possibility of the continued existence of the N-terminal extension cannot be completely excluded. Although the existence of a C-terminal extension is questionable, it is shown in the model as performing the function of anchoring a hydrophilic glycoprotein to the luminal face of the channels of the endoplasmic reticulum and Golgi via two binding receptors A and B. This would allow ordered oligosaccharide processing and synthesis of terminal sugars by membrane located processing glycosidases and glycosyl transferases.

Fig. 35 Proposed model for the translation and glycosylation of rat α_1-AGP. Rat α_1-AGP is translated by membrane-bound ribosomes with an N-terminal signal sequence which may, or may not, be removed during translation. Post-ribosomal glycosylation of the polypeptide occurs by the transfer of a high mannose oligosaccharide from a lipid carrier. This model also proposes the existence of a C-terminal extension which performs the function of anchoring the polypeptide to the luminal face of the ER and Golgi to allow oligosaccharide processing and synthesis of the terminal sugars by membrane located glycosidases and glycosyl transferases.




SRP
SRP Receptor
Ribosome Rceptor
S
COOH
Prosegment
NH2
A
MS
COOH
B
Gn-T
Gal-T
Sialyl-T
NH2
P
P
O
C
H2N
?
Presegment
NH2

Thus the model predicts the existence of a C-terminal extension and a binding site for α_1-AGP in the membranes of the endoplasmic reticulum and Golgi complex. Although a study of the existence of a C-terminal extension and binding sites together with further work on the low mannose form of α_1-AGP is necessary, the work presented in this thesis has contributed to our knowledge and understanding of the mechanism of assembly and secretion of a specific, well characterized, liver synthesized serum glycoprotein. In addition, the work has provided "food for thought" on the future direction that research on intracellular forms of α_1-AGP should take.

Proposal for future work on the study of intracellular forms of rat α_1-AGP and the biosynthetic pathway of secretory glycoproteins

The development of a procedure for the isolation of intracellular forms of α_1-AGP from liver made possible the analysis of some of the structures that exist *in vivo*. These structures are undergoing processing reactions with respect to polypeptide and carbohydrate moieties to fashion the final molecule. The processing steps coupled with synthesis of terminal sugar moieties collectively represent post transcriptional control reactions and are of special interest for the study of the synthesis of acute phase reactants, like α_1-AGP, where synthesis rates are increased tremendously during inflammation. Thus, future studies should be directed towards aspects of post transcriptional control. One study would involve the determination of the intracellular site of removal of mannose during processing. This could be approached by using high mannose forms, labelled with D-[^{14}C]-mannose, as a substrate for mannosidases present in each of the rough, smooth and Golgi fractions. The subcellular fraction responsible for the removal of mannose would release radiolabelled mannose which could be monitored as radioactivity in the non-protein fraction. The low mannose form isolated in this work should also function as a substrate for some of the glycosyl transferases; thus, if sufficient quantities could be isolated it could be employed in conjunction with rough, smooth and Golgi fractions to determine if the terminal

triplet of sugars could be added to the molecule. As mentioned above, the presence of the low mannose form in the rough fraction was unexpected and it was suggested that this may have occurred due to contamination from Golgi material. This could be tested by adding radiolabelled intracellular forms 1 and 2 to liver homogenate before subcellular fractionation. Measurement of the radioactivity associated with the low mannose form 2 on SDS PAGE isolated from this homogenate would indicate whether the material was derived from the intracellular space of the rough fraction, or from the radiolabelled material added to the cytoplasmic medium.

The question of the extra complement of amino acids in intracellular α_1-AGP is unresolved. Further work would involve N-terminal sequence analyses on larger quantities of material or, perhaps, a more sensitive sequence analysis technique. In addition, other approaches to removing N-terminal blocking groups should be used. For example, some proteins are known to be blocked by acyl groups which can be removed by commercially available acylase. An acylase should be tried along with a more extensive treatment with pyroglutamate amino peptidase. However, this approach would not serve to reveal a C-terminal extension so it should be carried out in conjunction with a study of the C-terminal sequence of both serum and intracellular α_1-AGP. This study could be coupled with one aimed at isolating the peptide or peptides released from intracellular α_1-AGP by limited tryptic treatment.

This study could also be extended to an examination of the CNBr peptides prepared from serum α_1-AGP with those prepared from intracellular α_1-AGP in order to determine if the N-terminal or C-terminal peptide of the intracellular α_1-AGP is larger than the corresponding peptide from serum α_1-AGP.

APPENDIX I

Fig. 36 Amino acid sequence of rat α_1-AGP determined by Ricca and Taylor (158) from cDNA of rat α_1-AGP mRNA. From the amino acid sequence of the N-terminal 19 residues of rat serum α_1-AGP determined in our study, together with the N-terminal sequence of a rat α_1-AGP reported by Nagashima et al.(176), it was assumed that the Gln in position 19 of the Ricca and Taylor sequence is the N-terminal residue of serum α_1-AGP and the N-terminal 18 residues extension is a "signal" sequence.

10 20

Met-Ala-Leu-His-Met-Val-Leu-Val-Val-Leu-Ser-Leu-Leu-Pro-Leu-Leu-Glu-Ala-Gln-Asn-

30 40

Pro-Glu-Pro-Ala-Asn-Ile-Thr-Leu-Gly-Ile-Pro-Ile-Thr-Asn-Glu-Thr-Leu-Lys-Trp-Leu-

50 60

Ser-Asp-Lys-Trp-Phe-Try-Met-Gly-Ala-Ala-Phe-Arg-Asp-Pro-Val-Phe-Lys-Gln-Ala-Val-

70 80

Gln-Thr-Ile-Gln-Thr-Glu-Tyr-Phe-Tyr-Leu-Thr-Pro-Asn-Leu-Ile-Asn-Asp-Thr-Ile-Glu-

90 100

Leu-Arg-Glu-Phe-Gln-Thr-Thr-Asp-Asp-Gln-Cys-Val-Tyr-Asn-Phe-Thr-His-Leu-Gly-Val-

110 120

Gln-Arg-Glu-Asn-Gly-Thr-Leu-Ser-Lys-Cys-Ala-Gly-Ala-Val-Lys-Ile-Phe-Ala-His-Leu-

130 140

Ile-Val-Leu-Lys-Lys-His-Gly-Thr-Phe-Met-Leu-Ala-Phe-Asn-Leu-Thr-Asp-Glu-Asn-Arg-

150 160

Gly-Leu-Ser-Phe-Tyr-Ala-Lys-Lys-Pro-Asp-Leu-Ser-Pro-Glu-Leu-Arg-Lys-Ile-Phe-Gln-

170 180

Gln-Ala-Val-Lys-Asp-Val-Gly-Met-Asp-Glu-Ser-Glu-Ile-Val-Phe-Val-Asp-Trp-Thr-Lys-

190 200

Asp-Lys-Cys-Ser-Glu-Gln-Gln-Lys-Gln-Gln-Leu-Glu-Leu-Glu-Lys-Glu-Thr-Lys-Lys-Glu-

Thr-Lys-Lys-Asp-Pro

ACKNOWLEDGEMENTS

I wish to express my thanks to Dr. J.C. Jamieson for his valuable guidance during the course of these studies and for helpful advice and suggestions throughout the preparation of this thesis. I would also like to thank Dr. H.W. Duckworth and W.D. Buchannon for their helpful suggestions, encouragement and assistance with the work throughout this study.

I wish to thank B.M.R.N.J. Woloski, H.A. Kaplan, and A. Bell for their assistance and discussions relating to this project as well as Mrs. L. Cote for typing this thesis.

Finally, I am indebted to the Department of Chemistry, Faculty of Graduate Studies and the Winnipeg Rh Institute for providing me with the opportunity to carry out this project and to the two people without whose support and continual encouragement the completion of this project would have been impossible: my wife, Lee M. Friesen and the Director of the Winnipeg Rh Institute, Dr. J.M. Bowman.

REFERENCES

1. Blix, G., Tiselius, A. and Svensson, H. (1941) J.Biol.Chem. 137, 485-494.

2. Svedberg, T. and Pederson, K.O. (1940) in the Ultracentrifuge, Oxford University Press, New York.

3. Sober, H.A. and Peterson, E.A. (1958) Fed.Proc. 17, 1116-1126.

4. Porath, J. and Flodin, P. (1959) Nature, London 183, 1657-1659.

5. Grabar, P. and Williams, C.A. (1955) Biochim.Biophys.Acta 17, 67-74.

6. Vesterberg, O. and Svensson, H. (1966) Acta Chem.Scand. 20, 820-834.

7. Gottschalk, A. (1972) in Glycoproteins, Vol.5 (Gottschalk, A., ed.) pp.1-23, Elsevier Publishing Co., New York.

8. Lennarz, W.J. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W.J., ed.) pp.VII - VIII, Plenum Press, New York.

9. Schwick, H.G., Heide, K. and Haupt, H. (1977) in The Glycoconjugates, (Horowitz, M.I. and Pigman, W., ed.) Vol.I, pp.261-321, Academic Press, New York.

10. Putnam, F.W. (1975) in The Plasma Proteins, (Putnam, F.W., ed.) Vol.I, pp.1-55, Academic Press, New York.

11. Koj, A. (1974) in Structure and Function of Plasma Proteins (Allison, A.C., ed.) pp.73-125, Plenum Press, New York.

12. Winzler, R.J. (1965) in The Amino Sugars, (Balazos, E.A. and Jeanloz, W., ed.) Vol.IIa, pp.337-352, Academic Press, New York.

13. Gordon, A.H. (1970) in Plasma Protein Metabolism (Rothschild, M.A. and Waldmann, T., ed.) pp.351-368, Academic Press, New York.

14. Glenn, E.M., Bowman, B.J. and Koslowski, T.C. (1968) Biochem. Pharmac.Supplement, pp.27-38, Pergamon Press, Great Britain.

15. Kulka, J.P. (1964) Ann.N.Y.Acad.Sci. 116, 1018-1044.

16. Gorog, P. and Kovacs, I.B. (1969) in Inflammation Biochemistry and Drug Interaction (Bertelli, A. and Houck, J.C., ed.) pp.197-203, Excerpta Medica Foundation, Amsterdam.

17. Houck, J.C., Johnston,J. and Jacob, R.A. (1968) Biochem. Pharmac.Supplement, pp.19-26, Pergamon Press, Great Britain.

18. Weissmann, G. and Uhr, J.W. (1968) Biochem.Pharmac.Supplement, pp.5-18, Pergamon Press, Great Britain.

19. Weissmann, G. (1966) Arth.Rheum. 9, 834-840.

20. Darcy, D.A. (1965) Brit.J.Exptl.Path. 46 155-163.

21. Balegno, H.F. and Neuhaus, O.W. (1970) Life Science 9, Pt.II, 1039-1044.

22. Jamieson, J.C. and Ashton, F.E. (1973) Can.J.Biochem. 51, 1034-1045.

23. John, D.W. and Miller, L.L. (1969) J.Biol.Chem. 244, 6134-6142.

24. Beisel, W.R. (1975) Ann.Rev.Med. 26, 9-20.

25. Eddington, C.L., Upchurch, H.F. and Kampschmidt, R.F. (1971) Proc.Soc.Exp.Biol.Med. 136, 159-164.

26. Cohen, S. (1981) Fed.Proc. 40, 50-55.

27. Lazar, G. and Karady, S. (1967) Acta Physiol.Acad.Sci.Hung. 31, 77-82.

28. Beeson, P.B. (1948) J.Clin.Invest. 27, 524-526.

29. Wannemacher, R.W., Pekarek, R.S., Thompson, W.L., Curnow, T., Beall, F.A., Zenser, T.V., DeRubertis, R.F. and Beisel, W.R. (1975) Endocrinology, 96, 651-661.

30. Woloski, B.M.R.N.J. (1980) M.Sc.Thesis, University of Manitoba.

31. Jamieson, J.C., Kutryk, M., Woloski, B.M.R.N.J. and Kaplan, H.A. (1982) Biochem.Med. (in press).

32. Atryzek, V. and Fausto, N. (1979) Biochemistry 18, 1281-1287.

33. Haugen, T.H., Hanley, J.M. and Heath, E.C. (1981) J.Biol.Chem. 256, 1055-1057.

34. Ricca, G.A., Hamilton, R.W., McLean, J.W., Conn, A., Kalinyak, J.E. and Taylor, J.M. (1981) J.Biol.Chem. 256, 10362-10368.

35. Sarcione, E.J. (1970) in Plasma Protein Metabolism (Rothschild, M.A. and Waldmann, T., ed.) pp.369-382, Academic Press, New York.

36. Barnabei, O. and Sereni, F. (1964) Biochim.Biophys.Acta 91, 239-247.

37. Stackhouse, H.L., Chetsanga, C.J. and Tan, C.H. (1968) Biochim. Biophys.Acta 155, 159-168.

38. Weissbach, H. and Ochoa, S. (1976) Ann.Rev.Biochem. 45, 191-216.

39. Revel, M. and Groner, Y. (1978) Ann.Rev.Biochem. 47, 1079-1126.

40. Shafritz, D.A. (1977) in Molecular Mechanism of Protein Biosynthesis (Weishack, H. and Pestka, S., ed.) 556-602, Academic Press, New York.

41. Greenberg, G.R. (1975) J.Cell Biol. 64, 269-288.

42. Siekevitz, P. and Palade, G.E. (1960) J.Biophys.Biochem.Cytol. 7, 619-630.

43. Shore, G.C. and Tata, J.R. (1977) Biochim.Biophys.Acta 472, 197-236.

44. Peters, T. (1977) Clin.Chem. 23, 5-12.

45. Palade, G.E. (1975) Science 189, 347-358.

46. Redman, C.M. and Cherian, M.G. (1972) J.Cell Biol. 52, 231-245.

47. Kreil, G. (1981) Ann.Rev.Biochem. 50, 317-348.

48. Jamieson, J.C. and Ashton, F.E. (1973) Can.J.Biochem. 51, 1281-1291.

49. Blobel, G. and Dobberstein, B. (1975) J.Cell Biol. 67. 835-851.

50. Prehn, S., Nurnberg, P. and Rapaport, T.A. (1981) FEBS Letters 123, 79-84.

51. von Heijne, G. (1980) Biochem.Biophys.Res.Commun. 93, 82-86.

52. Sabatini,D.D., Kreibich, G., Morimoto,T. and Adesnik, M. (1982) J.Cell Biol. 92, 1-22.

53. Walter, P., Ibrahimi, I. and Blobel, G. (1981) J.Cell Biol. 91, 545-550.

54. Walter, P. and Blobel, G. (1981) J.Cell Biol. 91, 551-556.

55. Walter, P. and Blobel, G. (1981) J.Cell Biol. 91, 557-561.

56. Palmiter, R.D., Gagnon, J. and Walsh, K.A. (1978) Proc.Natl. Acad.Sci. U.S.A. 75, 94-98.

57. Lingappa, V.R., Devillers-Thiery, A. and Blobel, G. (1977) Proc.Natl.Acad.Sci. U.S.A. 74, 2432-2436.

58. Strauss, A.W., Bennett, C.D., Donohue, A.M., Rodkey, J.A. and Alberts, A.W. (1977) J.Biol.Chem. 252, 6846-6855.

59. Cohn, D.V. and MacGregor, R.R. (1981) Endocrine Reviews 2, 1-26.

60. Lingappa, V.R., Lingappa, J.R. and Blobel, G. (1979) Nature 281, 117-121.

61. Quinn, P.S., Gamble, M. and Judah, J.D. (1975) Biochem.J. 146, 389-393.

62. Bornstein, P. and Sage, H. (1980) Ann.Rev.Biochem. 49, 957-1003.

63. Kassell, B. and Kay, J. (1973) Science 180, 1022-1027.

64. Jackson, C.M. (1980) Ann.Rev.Biochem. 49, 765-811.

65. Edwards, J., Fleischer, B., Dryburgh, H., Fleischer, S. and Schreiber, G. (1976) Biochem.Biophys.Res.Commun. 72, 310-318.

66. Ikehara, Y., Oda, K. and Kato, K. (1976) Biochem.Biophys.Res. Commun. 72, 319-326.

67. Schreiber, G. and Urban, J. (1978) Rev.Physiol.Biochem. Pharmacol. 82, 28-90.

68. Schreiber, G., Urban, J. and Edwards, J. (1976) J.Theor.Biol. 60, 241-245.

69. Judah, J.D. and Quinn, P.S. (1976) Trends Biochem.Sci. 1, 107-109.

70. Steiner, D.F., Kemmler, W., Tager, H.S. and Peterson, J.D. (1974) Fed.Proc. 33, 2105-2115.

71. Marshall, R.D. and Neuberger, A. (1964) Biochemistry 3, 1596-1600.

72. Kornfeld, R. and Kornfeld, S. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W.J., ed.) pp.1-34, Plenum Press, New York.

73. Liu, T., Stetson, B., Turco, S.J., Hubbard, S.C. and Robbins, P.W. (1979) J.Biol.Chem. 254, 4554-4559.

74. Parodi, A.J. and Leloir, L.F. (1979) Biochim.Biophys.Acta 559, 1-37.

75. Struck, D.K. and Lennarz, W.J. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W.J., ed.) pp.35-83, Plenum Press, New York.

76. Behrens, N.H. and Leloir, L.F. (1970) Proc.Natl.Acad. Sci. U.S.A. 66, 153-159.

77. Wolf, G., Kiorpes, T.C., Masushige, S., Schreiber, J.B., Smith, M.J. and Anderson, R.S. (1979) Fed.Proc. 38, 2540-2543.

78. Richards, J.B. and Hemming, F.W. (1972) Biochem.J. 130, 77-93.

79. Quill, H. and Wolf, G. (1981) Ann.N.Y.Acad.Sci. 359, 331-344.

80. Parodi, A.J., Behrens, N.H., Leloir, L.F., Carminatti, H. (1972) Proc.Natl.Acad.Sci. U.S.A. 69, 3268-3272.

81. Hubbard, S.C. and Ivatt, R.J. (1981) Ann.Rev.Biochem. 50, 555-583.

82. Sharon, N. and Lis, H. (1981) in Special Report, Chemical and Engineering News, March 30, pp.21-44.

83. Marshall, R.D. (1974) Biochem.Soc.Symp. 40, 17-26.

84. Ronin, C., Bouchilloux, S., Granier, C. and van Rietschoten, J. (1979) FEBS Letters 96, 179-182.

85. Pless, D.D. and Lennarz, W.J. (1977) Proc.Natl.Acad.Sci. U.S.A. 74, 134-138.

86. Katz, F.N., Rothman, J.E., Lingappa, V.R., Blobel, G. and Lodish, H.F. (1977) Proc.Natl.Acad.Sci. U.S.A. 74, 3278-3282.

87. Lingappa, V.R., Lingappa, J.R., Prasad, R., Ebner, K.E. and Blobel, G. (1978) Proc.Natl.Acad.Sci. U.S.A. 75, 2338-2342.

88. Hanover, J.A. and Lennarz, W.J. (1980) J.Biol.Chem. 255, 3600-3604.

89. Kiely, M.L., McKnight, G.S. and Schimke, R.T. (1976) J.Biol. Chem. 251, 5490-5495.

90. Bergman, L.W. and Kuehl, W.M. (1978) Biochemistry 16, 4490-4497.

91. Jamieson, J.C. (1977) Can.J.Biochem. 55, 408-414.

92. Snider, M.D., Sultzman, L.A. and Robbins, P.W. (1981) Cell 21, 385-392.

93. Hanover, J.A. and Lennarz, W.J. (1979) J.Biol.Chem. 254, 9237-9246.

94. Turco, S.J., Stetson, B., Robbins, P.W. (1977) Proc.Natl.Acad.Sci. U.S.A. 74, 4411-4414.

95. Spiro, M.J., Spiro, R.G. and Bhoyroo, V.D. (1979) J.Biol.Chem. 254, 7668-7674.

96. Grinna, L.S. and Robbins, P.W. (1980) J.Biol.Chem. 255, 2255-2258.

97. Ugalde, R.A., Staneloni, R.J. and Leloir, L.F. (1979) Biochem. Biophys.Res.Commun. 91, 1174-1181.

98. Michael, J.M. and Kornfeld, S. (1980) Arch.Biochem.Biophys. 199, 249-258.

99. Elting, J.J., Chen, W.W. and Lennarz, W.J. (1980) J.Biol.Chem. 255, 2325-2331.

100. Tabas, I. and Kornfeld, S. (1979) J.Biol.Chem. 254, 11655-11663.

101. Tulsiani, D.R.P., Hubbard, S.C., Robbins, P.W. and Touster, O. (1982) J.Biol.Chem. 257, 3660-3668.

102. Forsee, W.T. and Schutzbach, J.S. (1981) J.Biol.Chem. 256, 6577-6582.

103. Schachter, H. and Roseman, S. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W.J., ed.) pp.85-160, Plenum Press, New York.

104. Harpaz, N. and Schachter, H. (1980) J.Biol.Chem. 255, 4885-4893.

105. Tabas, I. and Kornfeld, S. (1978) J.Biol.Chem. 253, 7779-7786.

106. Wilson, J.R., Williams, D. and Schachter, H. (1976) Biochem.Biophys.Res.Commun. 72, 909-916.

107. Carey, D.J. and Hirschberg, C.B. (1980) J.Biol.Chem. 255, 4348-4354.

108. Carey, D.J. and Hirschberg, C.B. (1981) J.Biol.Chem. 256, 989-993.

109. Montreuil, J. (1975) Pure Appl.Chem. 42, 431-477.

110. Paulson, J.C., Rearick, J.I. and Hill, R.L. (1977) J.Biol.Chem. 252, 2363-2371.

111. Schachter, H. and Roden, L. (1973) in Metabolic Conjugation and Metabolic Hydrolysis (Fishman, W.H., ed), Vol.III, pp.1-149, Academic Press, New York.

112. Munro, J.R., Narasimham, S., Wetmore, S., Riordan, J.R. and Schachter, H. (1975) Arch.Biochem.Biophys. 169, 269-277.

113. Takasaki, S., Ikehira, H. and Kobata, A. (1980) Biochem. Biophys.Res.Commun. 92, 735-742.

114. Fournet, B., Montreuil, J., Stecker, G., Dorland, L., Haverkamp, J., Vliegenthart,J.F.G., Binette, J.P. and Schmid, K. (1978) Biochemistry 17, 5206-5214.

115. Tabas, I. and Kornfeld, S. (1980) J.Biol.Chem. 255, 6633-6639.

116. Sly, W.S. (1979) in Structure and Function of Gangliosides; Advances in Experimental Biology and Medicine (Svennerholm, L., Mandel, P., Dreyfus, H. and Urban, P.F., ed.) Vol.125, pp.433-451, Plenum Press, New York.

117. Jamieson, J.C., Friesen, A.D., Ashton, F.E. and Chou, B. (1972) Can.J.Biochem. 50, 856-870.

118. Sober, H.A., Gutter, F.J., Wycoff, M.M. and Peterson, E.A. (1956) J.Am.Chem.Soc. 78, 756-763.

119. Montreuil, J. and Scheppler, N. (1959) Bull.Soc.Chim.Biol. (Paris) 41, 13-28.

120. Svennerholm, L. (1958) Acta Chem.Scand. 12, 547-554.

121. Ornstein, L. (1964) Ann.N.Y.Acad.Sci. 121, 321-349.

122. Davis, B.J. (1964) Ann.N.Y.Acad.Sci. 121, 404-427.

123. Weber, K. and Osborn, M. (1969) J.Biol.Chem. 244, 4406-4412.

124. Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606-2617.

125. Simkin, J.L., Seshadri, H.S. and Skinner, E.R. (1964) Nature, London 202, 702-703.

126. Ouchterlony, O. (1953) Acta Pathol.Microbiol.Scand. 32, 231-236.

127. Hunter, J.R. (1959) Nature, London 183, 1283-1284.

128. Dallner, G. (1963) Acta Path.Microbiol.Scand. 166,(Suppl.) 1-94.

129. Schachter, H., Jabbal, I., Hudgin, R.L., Pinteric, L., McGuire, E.J. and Roseman, S. (1970) J.Biol.Chem. 245, 1090-1100.

130. Simkin, J.L. and Jamieson, J.C. (1967) Biochem.J. 103, 153-164.

131. Weinfeld, H. and Tunis, M. (1960) J.Biol.Chem. 235, 1668-1672.

132. Maung, M., Baker, D.G. and Murray, R.K. (1968) Can.J.Biochem. 46, 477-481.

133. Simkin, J.L., Skinner, E.R. and Seshadri, H.S. (1964) Biochem.J. 90, 3i6-330.

134. Judah, J.D., Gamble, M. and Steadman, J.H. (1973) Biochem.J. 134, 1083-1091.

135. Lowry, O.H. Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J.Biol.Chem. 193, 265-275.

136. Miller, G.L. (1959) Anal.Chem. 31, 964.

137. Bradford, M.M. (1976) Anal.Biochem. 72, 248-254.

138. Edelhoch, H. (1967) Biochemistry 6, 1948-1954.

139. Sreekrishna, K., Jones, C.E., Guetzow, K.A., Prasad, M.R. and Joshi, V.C. (1980) Anal.Biochem. 103, 55-57.

140. Talbot, D.N. and Yphantis, D.A. (1971) Anal.Biochem. 44, 246-253.

141. Torello, L.A., Yates, A.J. and Thompson, D.K. (1980) J.Chromat. 202, 195-209.

142. Aminoff, D. (1961) Biochem.J. 81, 384-392.

143. Boas, N.F. (1953) J.Biol.Chem. 204, 553-563.

144. Winzler, R.J. (1955) Method.Biochem.Anal. 2, 279-311.

145. Rondle, C.J.M. and Morgan, W.T.J. (1955) Biochem.J. 61, 586-589.

146. Li, Y-T. and Shetlar, M.R. (1964) Arch.Biochem.Biophys. 108, 301-313.

147. Gordon, H.T., Thornburg, W.T. and Werum, L.M. (1956) Anal.Chem. 28, 849-855.

148. Li, Y-T. (1966) J.Biol.Chem. 241, 1010-1012.

149. Partridge, S.M. and Westall, R.G. (1948) Biochem.J. 42, 238-250.

150. Pritchard, D.G. and Niedermeier, W. (1978) J.Chromat. 152, 487-494.

151. Podell, D.N. and Abraham, G.N. (1978) Biochem.Biophys. Res.Commun. 81, 176-185.

152. Niall, H.D. (1973) Methods Enzymol. 270, 942-1010.

153. Niall, H.D. (1977) in The Proteins, (Neurath, H. and Hill, R.L., eds.) 3rd edition, Vol.3, pp.179-238, Academic Press, New York.

154. Pisano, J.J. and Bronzert, T.J. (1972) Anal.Biochem. 45, 43-59.

155. Steers, E., Craven, G.R., Anfinsen, C.B. and Bethune, J.L. (1965) J.Biol.Chem. 240, 2478-2484.

156. Schmid, K., Kaufmann, H., Isemura, S., Bauer, F., Emura, J., Motoyama, T., Ishiguro, M. and Nanno, S. (1973) Biochemistry 12, 2711-2724.

157. Ikenaka, T., Ishiguro, M., Emura, J., Kaufmann, H., Isemura, S., Bauer, W. and Schmid, K. (1972) Biochemistry 11, 3817-3829.

158. Ricca, G.A. and Taylor, J.M. (1981) J.Biol.Chem. 256, 11199-11202.

159. Hubbard, S.C. and Robbins, P.W. (1979) J.Biol.Chem. 254, 4568-4576.

160. Judah, J.D. and Nicholls, M.R. (1971) Biochem.J. 123, 643-648.

161. Tager, H.S. and Steiner, D.F. (1973) Proc.Natl.Acad.Sci. U.S.A. 70, 2321-2325.

162. Steiner, D.F., Quinn, P.S., Chan, S.J., Marsh, J. and Tager, H.S. (1980) Ann.N.Y.Acad.Sci. 343, 1-16.

163. Geller, D.M., Judah, J.D. and Nicholls,M.R. (1972) Biochem.J. 127, 865-874.

164. Bettelheim, F.A. (1977) in The Glycoconjugates (Horowitz, M.I. and Pigman, W., ed.) Vol.1, pp.111-128, Academic Press, New York.

165. Yoshima, H., Matsumoto, A., Mizuochi, T., Kawasaki, T. and Kobata, A. (1981) J.Biol.Chem. 256, 8476-8484.

166. Li, E., Tabas, I. and Kornfeld, S. (1978) J.Biol.Chem. 253, 7762-7770.

167. Rearick, J.I., Fujimoto, K. and Kornfeld, S. (1981) J.Biol.Chem. 256, 3762-3769.

168. Friesen, A.D. and Jamieson, J.C. (1979) Abstracts XI International Congress of Biochemistry, Toronto, p.170.

169. Nagashima, M., Urban, J. and Schreiber, G. (1980) J.Biol.Chem. 255, 4951-4956.

170. Hercz, A., Katona, E., Cutz, E., Wilson, J.R. and Barton, M. (1978) Science 201, 1229-1232.

171. Hercz, A. and Harpaz, N. (1980) Can.J.Biochem. 58, 644-648.

172. Spiro, M.J. (1973) J.Biol.Chem. 248, 4446-4460.

173. Godelaine, D., Spiro, M.J. and Spiro, R.G. (1981) J.Biol.Chem. 256, 10161-10168.

174. Nakajima, H., Itoh, N., Kawasaki, T. and Yamashina, I. (1979) J.Biochem. 85, 209-216.

175. Monaco, F. and Andreoli, M. (1976) Acta Endocr. 83, 93-98.

176. Nagashima, M., Urban, J. and Schreiber, G. (1981) J.Biol.Chem. 256, 2091-2093.

177. Tager, H.S., Steiner, D.F. and Patzelt, C. (1981) in Methods in Cell Biology (Hand, A.R. and Oliver, C., ed.,) Vol. 23, pp.73-88, Academic Press, New York.