THE UNIVERSITY OF MANITOBA

ISOLATION AND CHARACTERIZATION OF ACUTE PHASE GLOBULINS FROM RAT SERUM

by

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ABBREVIATIONS

DEAE-cellulose	Diethylaminoethylcellulose
CM-cellulose	Carboxymethylcellulose
tris	2-amino-2-hydroxymethylpropane-1,3-diol
NANA	N-acetylneuraminic acid
Man	Mannose
Gal	Galactose
GlcNAc	N-acetylglucosamine

NOTE ON NOMENCLATURE

The nomenclature used in this thesis to describe the various serum proteins, is that defined by electrophoretic mobility on Cellogel electrophoresis at pH 8.6; α refers to the proteins of greatest electrophoretic mobility (α_1 has greater electrophoretic mobility than α_2); β refers to the proteins of electrophoretic mobility just less than that of the α proteins; and γ refers to the proteins of slowest electrophoretic mobility (see Fig.5).

ABSTRACT

Serum, isolated from rats suffering from inflammation, was fractionated by chromatography on DEAE-cellulose. Most of the increase in protein-bound carbohydrate of serum resulting from inflammation was located in three of the fractions. Fraction 5, which accounted for 40% of the increase in protein-bound carbohydrate, gave two main bands of carbohydrate staining proteins on electrophoresis on Cellogel strips at pH 8.0. The carbohydrate staining fractions were separated by stepwise elution from columns of CM-cellulose with 0.05M acetate, pH 4.9, followed by 0.4M acetate, pH 5.1. The first buffer eluted an α_1-acid glycoprotein which was then subjected to isoelectric focusing in pH 1-3 gradients. The protein was found to be homogeneous as determined by immunological and electrophoretic examination, had an isolectric point of 2.95, a molecular weight of 43,000 and contained 40%, by weight, of carbohydrate. The second buffer eluted an α_2-macroglobulin which was obtained in a fairly pure form by gel filtration coupled with isoelectric focusing in pH 4-6 gradients. A quantitative precipitin technique employing antisera to both proteins was applied to serum from control and experimental rats. There was an increase in content of both proteins in serum from experimental animals indicating that both proteins are acute phase globulins.

INTRODUCTION

The Acute Inflammatory Response

Inflammation in mammals, caused by chemical inflammatory agents, neoplastic diseases, bacterial infections, rheumatoid arthritis and a variety of other conditions, results in an increase in the total protein-bound carbohydrate of serum arising from increases in the concentration of a variety of serum glycoproteins (Table 1). However, in spite of the well documented response of serum glycoproteins to the inflammatory stimulus, little is known about the mechanism that leads to the increased content of certain glycoproteins in serum during inflammation. With a view to obtaining some information on the mechanism and significance of the response of serum glycoproteins to inflammation, several groups of workers have studied the events that take place in the body in response to inflammation.

Glenn, Bowman and Koslowske (1) suggested that the process of inflammation should be separated into two reactions, the local reaction and the systemic reaction (Fig.1). The local reaction refers to the events occurring in the immediate area of tissue damage, whereas the systemic reaction describes events induced by the local reaction. Glenn *et al.*(1) suggested that the local

TABLE 1

Total Hexose and Hexosamine of Glycoprotein in Normal and in Some Pathological Sera

Sera from normal and diseased individuals	Total glycoprotein hexose (mg/100ml serum)	Total glycoprotein hexosamine (mg/100ml serum)	References
Normal	100	66	(11)
Advanced cancer	195 ± 25	203 ± 12	(12)
	177 ± 8.8	131.9 ± 56	(13)
Diabetes	193	153	(14)
	174 ± 24.7	145.2 ± 23	(15)
Rheumatic fever	190 ± 1.9	100.4 ± 3.5	(16)
Rheumatoid arthritis	215	-	(17)
	153 ± 3	128 ± 3	(18)
Tuberculosis	204 ± 15.6	211 ± 9	(12)
Cholera	272 ± 7	-	(19)
Pregnant women	272 ± 4	-	(20)
Acute leukemia	157.5 ± 19.8	127.5 ± 12.3	(13)
Hodgkin's disease	189.3 ± 38.9	141 ± 30.1	(13)
Lymphosarcoma	218.2 ± 32.7	142.5 ± 23.4	(13)
Multiple myeloma	255 ± 76.1	165 ± 9.5	(13)

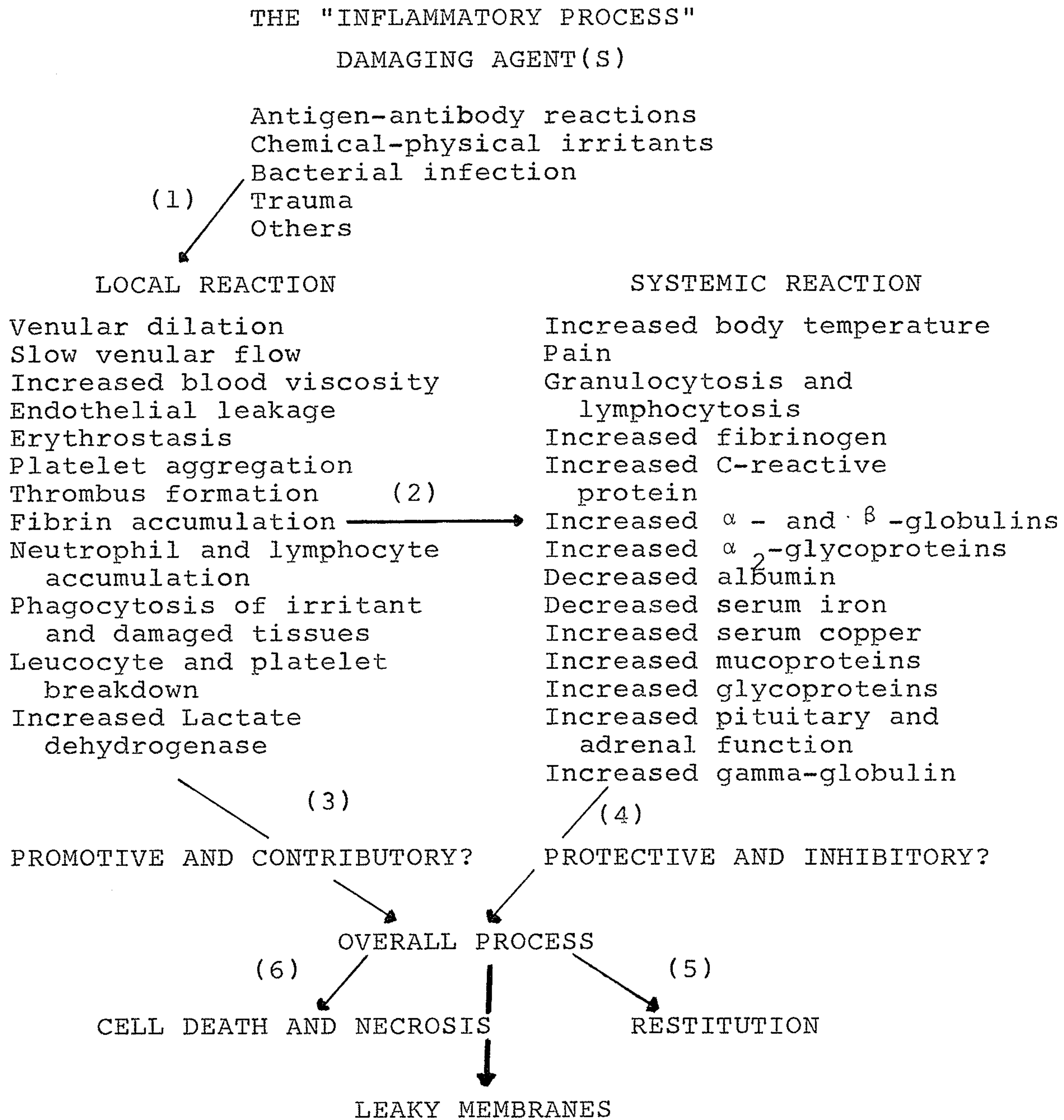

Fig.1 Schematic representation of the inflammatory process. The numbers in parenthesis describe the order in which the events are believed to occur.

events are "contributory", or events which initiate the overall process, whereas the systemic responses are "protective" or "inhibitory"; that is, events which protect the body or slow down the overall process of inflammation. The first event in the process of inflammation is believed to be venular dilation (2). This is shortly followed by platelet aggregation, the formation of thrombi and the migration of neutrophil and lymphocyte cells into the area of tissue damage (3). The process is thought to be accompanied by a release of cytoplasmic and lysosomal enzymes into the extracellular compartment of the tissue (4). It has been suggested that lysosomal enzymes, released during the inflammatory response, activate cutaneous collagenase which degrades collagen in structural components of the tissue into soluble breakdown products which then drain away from the area of tissue damage (5). The ultimate end point of the acute inflammatory process is cell necrosis resulting mainly from the local reactions (4). According to the scheme put forward by Glenn _et al_.(1),(Fig.1) the local reaction, by some unknown mechanism, is believed to induce the systemic response to inflammation. The systemic response consists primarily of increases in the levels of a variety of serum glycoproteins (Fig.1). The proteins that increase in content in serum as a

result of inflammation are normally referred to as acute phase reactants (6). The most important acute phase reactants present in human serum are orosomucoid*, ceruloplasmin, haptoglobin, transferrin, fibrinogen, α_2-macroglobulin and the γ-globulins (7). In addition to the above acute phase reactants, new serum proteins not normally present in serum, make their appearance; these include the C-reactive protein in man (8) and α_2-(acute phase) globulin in rats (9). The response of an α_1-acid glycoprotein, corresponding to orosomucoid, and the α_2-(acute phase) globulin to experimentally induced inflammation in rats has been studied extensively by several groups of workers (6,10,21,29). Darcy (10,21) found a 7-fold increase in the α_1-acid glycoprotein of rat serum in response to turpentine induced inflammation and a 20-fold increase due to growth of Walker tumor. It was originally suggested by Darcy (23) that the increase in serum α_1-acid glycoprotein was a response to substances liberated from damaged necrotic cells. The presence of α_1-acid glycoprotein in the area of tissue damage prompted the suggestion that the serum glycoprotein

* Orosomucoid is an α_1-acid glycoprotein which is the major component of the seromucoid fraction of serum. The seromucoid fraction consists of those serum proteins that are soluble in 0.6 M-perchloric acid but precipitated by 5% phosphotungstic acid.

response to inflammation resulted from the release of glycoproteins synthesized locally in the inflammed tissue (30). However, it has since been established that the liver is the site of synthesis of most of the acute phase reactants including rat α_1-acid glycoprotein and α_2-(acute phase) globulin (24,31-33). It is now believed that hormonal factors, that may be released from the site of inflammation, stimulate the liver,either to synthesize new glycoprotein found in serum, or to increase the synthesis rates of glycoproteins that are normally present in serum (9,23,34-36). This is presumably accomplished by some "switching on" and "switching off" mechanism affecting the synthesis of acute phase globulins in mammalian liver (37). The exact "switching on" and "switching off" mechanism is not understood at the present time, but is currently under active investigation. Moreover, it is still not clear what function the acute phase globulins perform in the body. It has been suggested that they act as carrier proteins either by removing small molecules from the site of tissue damage, or by carrying small molecules to the site of tissue damage for the purposes of repair (38). Clearly an understanding of the function of the acute phase reactants may have to await studies designed to determine the structure of these proteins. It is the structural

aspects of studies on acute phase globulins that this thesis mainly deals with.

Serum Glycoproteins: General aspects of structure of α_1-acid glycoproteins

Glycoproteins are normally defined as proteins that contain carbohydrate groups of relatively low molecular weight bound covalently to polypeptide chains which, by virtue of their size and amino acid composition, would be referred to as proteins (39). Such glycoproteins are present in large amounts in mammalian serum; indeed it is believed that all serum proteins with the exception of serum albumin are glycoproteins (40). Interest in serum glycoproteins has increased steadily in the past ten years, mainly due to their relationship to various pathological conditions involving inflammation (41), (also, see above). Human α_1-acid glycoprotein, one of the most extensively studied serum glycoproteins, has been found to contain an unusually high content of carbohydrate (41), (Table 2). The carbohydrate consists of the neutral sugars, mannose, galactose, and fucose (6-deoxygalactose); the amino sugar, N-acetylglucosamine and the acidic sialic acids (N-acetyl-, N-glycolyl-, and N-acetyl-O-acetylneuraminic acids). N-Acetylgalactosomine is not normally found in

TABLE 2

General properties of α_1-acid glycoprotein from various species[+]

Source	Isoelectric point	Total carbohydrate	Sialic acid	Hexose	Hexosamine	$S_{20,w}$	Molecular weight	References
Human	2.7			17.2	11.5	3.5		(58)
		38.4	10.8	14.2	12.3			(43)
	1.8			16.4	11.9	3.11	44,100	(59)
	3.5	13.7	3.3	6.8	3.6			(60)
Rat		34	10.0	15.3	8.3		35,000	(61)
	4.5-4.7	13.6	3.7	4.0	5.9		46,000	(62)
Guinea Pig			7.9	8.6	5.9			(63)
			6.2	11.1	6.4			(64)
Bovine		29.7	7.2	11.3	10.3	2.8	42,000	(65)
		41.3	16.2	12.0	11.8	3.2	49,000	(66)
Porcine		57.6	7.4	13.8	11.1	3.0	47,000	(65)
Avian		36.4	10.1	13.8	12.2	2.9	44,000	(65)
Canine			8.0	11.2	9.0	2.9	31,000	(67)
Chimpanzee	1.82	41.2	11.9	13.6	14.6	3.48	39,000	(68)

[+] In most cases carbohydrate content is expressed as a percentage of dry weight of glycoprotein.

human α_1-acid glycoprotein (42), but has been detected in α_1-acid glycoproteinsisolated from other species (41). The most commonly detected sialic acid is N-acetylneuraminic acid although N-acetyl-O-acetylneuraminic acid and N-glycolylneuraminic acid have been found in human α_1-acid glycoprotein (43,44). Table 2 gives the carbohydrate composition and some physical properties of α_1-acid glycoproteins isolated from various species.

Most of the work in recent years on human α_1-acid glycoprotein and α_1-acid glycoproteins from other species has been concerned with studies on the sequence of sugars in the oligosaccharide chains. It is generally believed that the oligosaccharide chains of human α_1-acid glycoprotein consists of fifteen to eighteen sugar residues (45) with perhaps three to seven such chains per molecule of glycoprotein (46-48). Sialic acid or fucose has always been found to occupy a terminal position on the oligosaccharide chains and is believed to be responsible for the low isoelectric points of the α_1-acid glycoproteins (49). Galactose seems to occupy the penultimate position with N-acetylglucosamine as the third sugar in the chain (41). This results in a terminal trisaccharide which appears to be common to all α_1-acid glycoproteins isolated from a variety of species. A similar terminal trisaccharide sequence has been proposed

for fetuin (50), α_2-macroglobulin (51), and thyroglobulin (52). The sequence of sugars in the inner positions of the oligosaccharide chains is not clear at the present time, although it is believed that branching occurs resulting in several of the trisaccharide units described above being located on one oligosaccharide chain (41). The complete structure of a glycopeptide, isolated from human α_1-acid glycoprotein has recently been described by Wagh et al. (45) (Fig.2).

The nature of the linkage between carbohydrate and polypeptide has also been studied extensively in recent years. Kamiyama and Schmid (48) isolated a glycopeptide from human α_1-acid glycoprotein which had aspartic acid as the sole amino acid, thus suggesting that aspartic acid was involved in the linkage between polypeptide and oligosaccharide chains in human α_1-acid glycoprotein. However, the isolation of 2-acetamido-1-β-(L-β-aspartamido)-1,2-dideoxy-D-glucose (Fig.3) from glycopeptides prepared by proteolytic digestion of human α_1-acid glycoprotein suggests that the linkage between polypeptide and oligosaccharide involves asparagine and N-acetylglucosamine (45). A similar linkage between polypeptide and oligosaccharide is believed to be present in ovalbumin (53), IgG immunoglobulin (54), and thyroglobulin (55).

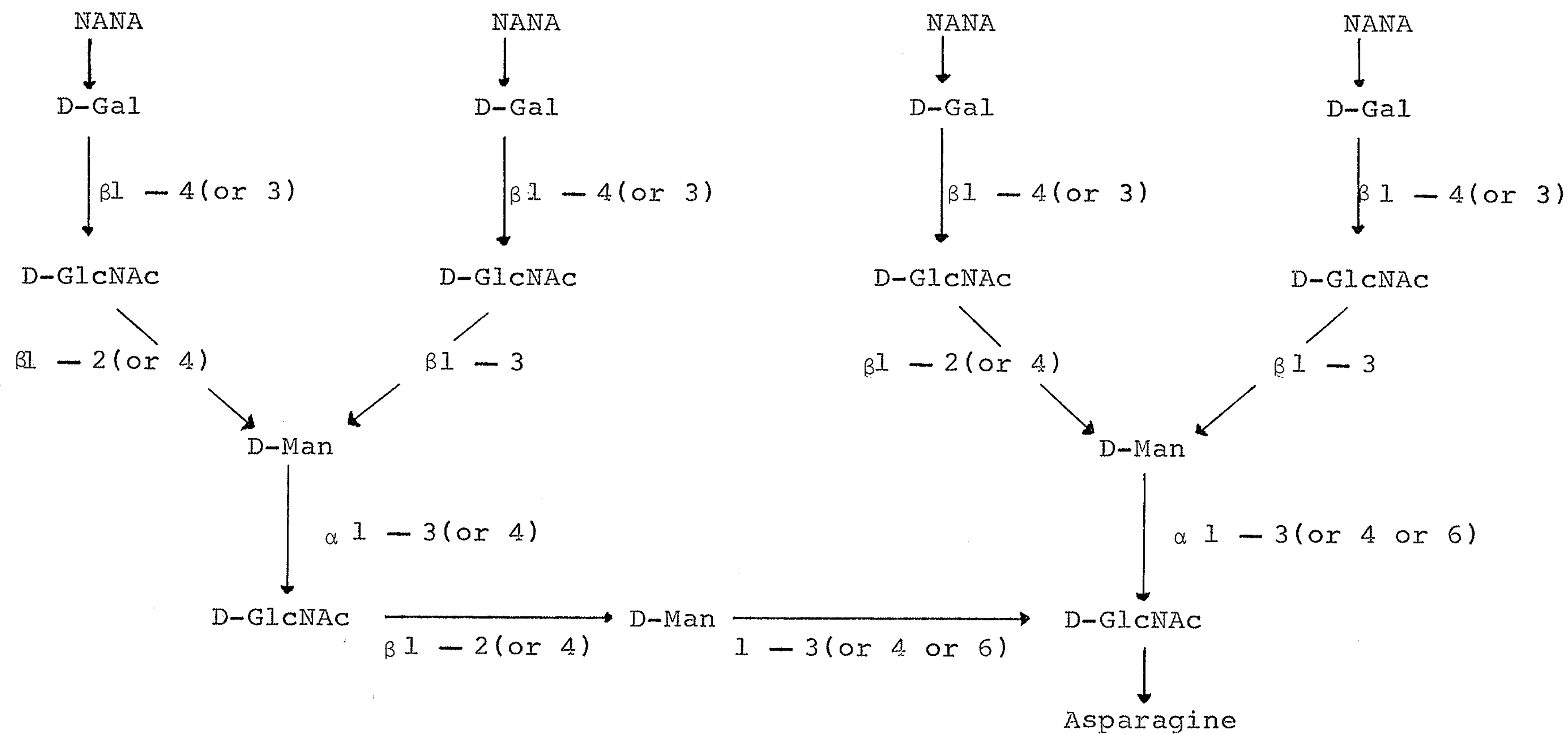

Fig.2 Proposed structure for a glycopeptide isolated from human α_1-acid glycoprotein (45).

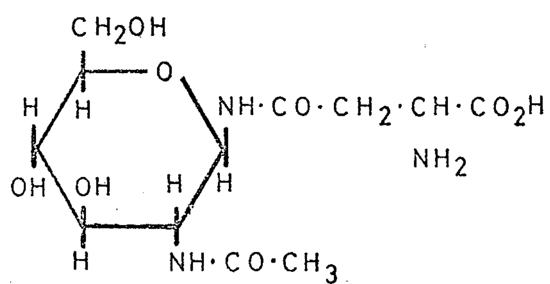

Fig. 3

2-acetamido-1-β-(L-β-aspartamido)-1,2-dideoxy-D-glucose

Many workers have studied the sequence of amino acids in glycopeptides derived from human α_1-acid glycoprotein. Sataki *et al.*(46) and Wagh *et al.*(45) both found that threonine or serine residues were either adjacent to, or near to, the asparagine residue involved in linkage with oligosaccharide in human α_1-acid glycoprotein. Serine and threonine residues have also been found in glycopeptides isolated from a variety of other serum glycoproteins of mammalian origin (56). The presence of the hydroxy-containing amino acids near to the asparagine residue involved in linkage with oligosaccharide has prompted the suggestion that these amino acids are recognized by the glycosyltransferases responsible for attachment of the first carbohydrate to the asparagine residue in the polypeptide chain (56).

Although work on the structure of the oligosaccharide chains of α_1-acid glycoproteins from rats and other species is in its infancy,it is to be expected that structural similarities to the oligosaccharide chains of human α_1-acid glycoprotein will be found.

Introduction to the work presented in this thesis

The work presented in this thesis is part of a series of studies on the response of acute phase glycoproteins of rat serum to inflammation induced experi-

mentally by subcutaneous administration of oil of turpentine. The present work is mainly concerned with the isolation and characterization of an acute phase α_1-acid glycoprotein from rat serum.

Work has already been carried out in this laboratory on the effect of turpentine induced inflammation on total serum proteins (57). These studies showed that turpentine induced inflammation resulted in an increase in total protein-bound hexose and hexosamine of rat serum reaching a maximum at 48h after administration of inflammatory agent. Serum, isolated from rats 5-96h after injection of turpentine, was fractionated by chromatography on DEAE-cellulose. Eight protein fractions were prepared and were analyzed for their content of protein-bound hexose and hexosamine. It was found that about 80% of the increase in protein-bound carbohydrate, resulting from inflammation, was located in three fractions prepared by chromatography of serum on DEAE-cellulose. Fraction 5, which accounted for 40% of the increase in protein-bound carbohydrate of serum as a result of inflammation, contained α-globulins of high electrophoretic mobility at pH 8.6. Since the high electrophoretic mobility at pH 8.6 is a characteristic property of acidic glycoproteins, fraction 5 was employed as the starting material for the isolation of an acute phase α_1-acid glycoprotein.

Animals suffering from inflammation for 48h were used as a source of serum since, under these conditions, the concentration of acute phase glycoproteins is most likely to be greatest. Serum was fractionated by chromatography on DEAE-cellulose using a scaled up method based on that described by Ashton et al.(57). Fraction 5 was further fractionated by stepwise elution from columns of CM-cellulose. Two fractions were prepared, one of which contained an α_1-acid glycoprotein which was recovered in a homogeneous form by isoelectric focusing in pH 1-3 gradients. The α_1-acid glycoprotein was characterized with respect to its physical and electrophoretic properties and was analyzed for its content of carbohydrate. The second fraction contained an α_2-macroglobulin which was isolated in a fairly pure form by a combination of preparative electrophoresis on Cellogel blocks, gel filtration on Sephadex and isoelectric focusing in pH 4-6 gradients. Antisera were prepared to both proteins and employed in a quantitative precipitin technique applied to serum from control rats and to rats suffering from inflammation. There was an increase in the content of both proteins in the serum of rats suffering from inflammation indicating that both were acute phase globulins.

EXPERIMENTAL

MATERIALS

Wherever possible, chemicals were of analytical reagent grade obtained from Fisher Scientific Co. Ltd., Toronto, Sigma Chemical Company, St.Louis, Mo.; British Drug Houses (Canada) Ltd., Toronto; The McArthur Chemical Co. Ltd., Montreal; May & Baker Ltd., Dagenham, England; Merck & Co. Ltd., Montreal; Allied Chemical Co. Ltd., Canada; Mallinckrodt Chemical Works, Montreal; J.T. Baker Chemical Co., Phillipsburg, N.S.; and Matheson, Coleman & Bell, Norwood (Cincinnati), Ohio. Other chemicals and reagents were obtained as follows:

Chromatographic and electrophoretic media

Cellogel and Celloblock, Consolidated Laboratories (Canada) Ltd., Weston, Ontario and Colab Laboratories, Inc., Chicago, Illinois; Starch-hydrolysed, Connaught Medical Research Laboratories, Toronto; Acrylamide and N,N' methylenebisacrylamide, Eastman Organic Chemicals, New York; Whatman CF11 fibrous cellulose powder, W.& R. Balston Ltd., England; Sephadex, Pharmacia (Canada)Ltd., Montreal; ampholine carrier ampholytes, LKB-Producter AB, Stockholm-Bromma, Sweden; CM-cellulose, Dowex 50 and Dowex 2, Sigma Chemical Co., St. Louis, Mo.

Proteins and sugars

D(-) Fructose, The British Drug Houses(Canada) Ltd., Toronto, bovine serum albumin (crystalline), ovalbumin, grade V, bovine γ -globulin, Cohn fraction II, horse heart cytochrome-c, type III, bovine pancreas trypsin type III, α -D(+) fucose, D(+) mannose (crystalline), D(+) galactose (crystalline), α -D(+) glucose (grade III), D(+) glucosamine HCl, D(+) galactosamine HCl, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine (crystalline), N-acetylneuraminic acid (crystalline) type IV, and N-glycolylneuraminic acid (practical grade), Sigma Chemical Co., St.Louis, Mo.

PHYSICAL MEASUREMENTS

Extinctions at 280nm were measured with a Beckman model DB spectrophotometer. Extinctions in the visible region of the spectrum were determined with a Unicam SP600 spectrophotometer, (Unicam Instruments Ltd., Cambridge). Measurements of pH were made with a radiometer model 28b pH meter, (Radiometer, Copenhagen, Denmark).

ULTRACENTRIFUGAL ANALYSIS

The sedimentation coefficient was determined on a model E analytical ultracentrifuge, (Beckman Spinco,

California). A Sedimentation velocity experiment was carried out on a protein solution (5mg/ml) in 0.02M phosphate buffer, pH 8.0, at 60,000 r.p.m. and 20°. A double sector cell was used with solvent in the second compartment. The sedimentation coefficent of the protein was calculated according to the method of Schachman (58).

ULTRAFILTRATION

The procedure used was based on that of Sober, Gutter, Wyckoff, and Peterson (69). Samples were reduced to small volumes by ultrafiltration through dialysis tubing immersed in an evacuted chamber containing about 3 litres of distilled water or appropriate dialysis medium.

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 (70). Dowex 2 was first converted to the hydroxyl form by washing with 6N NaOH and then with water. It was converted to the formate form by washing successively with 2N HCl, water and 2M sodium formate until the effluent gave a negative reaction for chloride (71). Excess

formate was removed by washing with water. Dowex 50 was converted to the hydrogen form by washing successively with 2N NaOH, water, 2N HCl and water; the procedure was repeated.

ANIMALS

Male hooded rats of 300-350g body weight were purchased from The Quebec Breeding Farm Inc., St.Eustache, Quebec. Rats were maintained on a diet of Purina laboratory chow and tap water and were starved for 16h prior to sacrifice.

PREPARATION OF SERUM

Inflammation was induced by subcantaneous injection of 0.5ml turpentine per 100g body weight into the dorsolumbar region (72). After 48h rats were lightly anaesthetized with ether and blood collected by severing the jugular veins. Blood was allowed to clot for 2-3h at room temperature and serum prepared by removing the clot by centrifugation at 2000 r.p.m. for 10 min. Serum was stored at -20° until required for use.

PREPARATION OF SERUM PROTEINS

Chromatography on DEAE-cellulose

Six fractions were prepared from serum by stepwise elution from columns of DEAE-cellulose by a scaled-up modification of the method described by Ashton *et al.* (57). The exchanger was prepared according to the method of Peterson and Sober (73). Before use the exchanger was washed twice with 0.5M NaOH - 0.5M NaCl and then with water until neutral. The exchanger was then suspended in 0.02M sodium phosphate buffer, pH 8.0, for 24h. Columns containing a bed of exchanger, 2.5cm x 45cm, were prepared using columns of constant-bore tubing supplied by Pharmacia (Canada) Ltd. Columns were packed and run under a hydrostatic pressure of 30cm; sodium phosphate buffer, pH 8.0, was allowed to pass through the columns for 30h at 2° before use. All subsequent procedures were carried out at 2°.

For each experiment serum from ten rats (30-35ml total volume) was dialysed against 0.02M sodium phosphate buffer, pH 8.0, and then applied to a column of DEAE-cellulose. The column was eluted with 0.02M sodium phosphate buffer, pH 8.0, and then with a series of 0.02M sodium phosphate buffers containing; 30mM NaCl, pH 7.6; 45mM NaCl, pH 7.0; 70mM NaCl, pH 6.6; 100mM NaCl, pH 6.0;

and 300mM NaCl, pH 5.0; 350-400ml of each buffer were passed through the column. The flow rate was 10ml/h and fractions of 3.0ml were collected. Protein was detected by measuring extinctions at 280nm. Appropriate fractions were pooled, concentrated to about 5ml by ultrafiltration and freeze-dried.

Chromatography on CM-cellulose

CM-cellulose was regenerated before use by washing twice with 0.25M NaOH - 0.25M NaCl and then with water until neutral. The exchanger was suspended in 0.05M sodium acetate buffer, pH 4.9, for 24h. Columns were packed and run as described above for DEAE-cellulose, but with 0.05M sodium acetate buffer, pH 4.9.

Fraction 5, (250-300mg) prepared by chromatography of serum on DEAE-cellulose (protein eluting from DEAE-cellulose column with 100mM sodium phosphate buffer, pH 6.0, see Results section) was dissolved in 5ml of 0.05M sodium acetate buffer, pH 4.9, and applied to a CM-cellulose column. The column was eluted with 300ml of 0.05M sodium acetate buffer, pH 4.9, and then with 300ml of 0.40M sodium acetate buffer, pH 5.1. The flow rate was 10ml/h and fractions of 3.0ml were collected. Protein was detected by measuring extinctions at 280nm. Appropriate fractions were pooled, concentrated to about

5ml by ultrafiltration and freeze-dried.

Gel filtration on sephadex

Gel filtration on sephadex was employed in two ways; firstly for the preparation of an α_2-macroglobulin and secondly to determine the approximate molecular weight of α_1-acid glycoprotein by a method based on that described by Andrews (74).

For the preparation of an α_2-macroglobulin, sephadex G-200 was allowed to swell in 1% NaCl for 3-4 days, packed into a 1.5cm x 90cm column and 0.02M sodium phosphate buffer, pH 7.0, allowed to pass through for 2-3 days. The sample of protein (10-15mg protein; see Results section) dissolved in 1-2ml buffer was applied to the column and eluted with sodium phosphate buffer, pH 7.0. The flow rate was 4ml/h and fractions of 2ml were collected. Protein was detected by measuring extinctions at 280nm. Appropriate fractions were pooled, concentrated by ultrafiltration and freeze-dried.

For determination of the approximate molecular weight of an α_1-acid glycoprotein a 1.5cm x 90cm column of sephadex G-150 was prepared as described above. A mixture of 5mg of each of bovine serum albumin, ovalbumin, γ-globulins and cytochrome-c dissolved in 1ml buffer was applied to the column and eluted with buffer

under a hydrostatic head of 10cm. The flow rate was 3ml/h and fractions of 1ml were collected. Protein was detected by measuring extinctions at 280nm. A sample of 2ml of an α_1-acid glycoprotein was then applied to the column and eluted as described above. An approximate molecular weight for the α_1-acid glycoprotein was determined according to the method of Andrews (74).

ELECTROPHORETIC METHODS

Cellogel

Electrophoresis was carried out on 5.0cm x 20cm, or 2.5cm x 18cm strips of Cellogel (gelatinized cellulose acetate) according to the procedure of Kohn (75). The buffer contained 8.3mM-diethylbarbituric acid 41.6mM-sodium diethylbarbiturate, pH 8.6. For analytical electrophoresis on Cellogel strips 3-5µl of rat serum or a 5% solution of protein were applied per centimeter width. A potential of 140V was applied for 5h at room temperature. Strips were stained for protein with Naphthalene Black 10B (76) and for carbohydrate by the periodic acid-Schiff technique (77). In some experiments strips stained for carbohydrate were subsequently stained for protein using the method described above.

For preparative electrophoresis blocks of Cellogel

(3.5cm x 12cm x 0.3cm) were employed. Samples containing approximately 20mg of protein in 0.1ml were applied on a 4.5cm origin located 4cm from the cathode. A potential of 140V was applied for 3h. Narrow strips cut from each edge of the Cellogel block were stained for protein or carbohydrate as described above. The section containing the protein to be isolated was removed, cut into small pieces and eluted by shaking with 3 x 5ml water over about 4-5h at 2°. Solid material was removed by centrifuging at 2000 r.p.m. for 5 min.; the supernatant was dialyzed against water and freeze-dried.

Starch Gel

The horizontal procedure of Smithies (76) was followed. The gels were formed from a mixture of 11gm of starch-hydrolysed and 100ml of a buffer containing 76mM Tris-5mM citric acid, pH 9.0. The gels were allowed to form in troughs measuring 18.5cm x 5.0cm x 0.6cm. The samples (0.5 - 1.0mg protein) were applied on Whatman 3MM filter paper inserted into a slit in the gel located 5cm from the cathode. The buffer in the electrode and bridge compartments of the tank contained 300mM boric acid - 50mM sodium hydroxide, pH 8.2. Whatman 3MM filter paper was used as a bridge between the electrode compartment and the gel. A potential of 170V was applied for 5h

at 2°. Gels were stained for protein with Naphthalene Black 10B, (76).

Disc electrophoresis

Disc electrophoresis was employed in two ways; first as an analytical technique for the examination of various protein fractions, and secondly to determine the molecular weight of α_1-acid glycoprotein by a method based on that described by Weber and Osborn (78).

For use as an analytical technique, the system of Ornstein and Davis (79) was followed. The gel was formed in a buffer containing 377mM tris, 25mM HCl, pH 8.9. The electrode compartment of the tank contained 380mM glycine, 50mM tris, pH 8.2. A solution of protein (10-50μg), containing 5% bromophenol blue, was layered on the top of the gels which were then transferred to the electrophoresis tank. A current of 4ma per gel column was applied until the bromophenol blue had migrated to the bottom of the gel; this required 1-2h. The gels were stained for protein with Naphthalene Black 10B in methanol-water-acetic acid (50:50:10,v/v) as described by Smithies (66) and for carbohydrate by the periodic acid-Schiff method described by Clarke (80).

For determination of the molecular weight of an α_1-acid glycoprotein a 10% acrylamide gel was prepared

in a buffer which contained 0.2M sodium phosphate, pH 7.0, and 0.2% sodium dodecyl sulphate. The electrode compartment of the tank contained the buffer at half the above strength. The proteins (0.2-0.6mg/ml of trypsin, ovalbumin, bovine serum albumin and cytochrome-c), were allowed to stand at room temperature for 2h in a buffer containing 1% sodium dodecyl sulphate, 1% β-mercapto-ethanol and 0.01M sodium phosphate, pH 7.0. To 10-50μl of these protein solutions were added 3μl of 0.05% bromophenol blue in water, 1 drop glycerol, 5 μl of β-mercaptoethanol, and 50 μl of 0.01M sodium phosphate buffer, pH 7.0. The protein solutions were applied to gels as described above and electrophoresis performed at a constant current of 8ma per gel column until the bromophenol blue had moved 80%-90% down the length of the gel column; this required about 1-2h.

The length of the gel and distance that the bromophenol blue had migrated were measured before staining with Naphthalene black. The gel length and the distance of migration of the proteins were measured after staining and a value for the molecular weight of α_1-acid glycoprotein calculated as described by Weber and Osborn (78).

ISOELECTRIC FOCUSING

Isoelectric focusing was performed by a method based on that described by Vesterberg and Svensson (81). A 110ml isoelectric focusing column (LKB 8100-10) and a gradient mixing device (LKB 8121) - (LKB Producter AB, Stockholm-Bromma, Sweden)were used in all experiments.

Experiments were performed with pH 3-10, pH 3-6 and pH 4-6 ampholine carrier ampholytes or with a pH 1-3 gradient system. The following method was used with ampholine carrier ampholytes. A dense electrode solution containing 0.2ml sulphuric acid, 12g sucrose and 14ml distilled water was added to the anode at the bottom of the column. A sucrose gradient containing the carrier ampholytes was slowly introduced into the column using the LKB gradient mixing device. The dense gradient solution contained 28g sucrose, 1.9ml ampholine carrier ampholytes and 42ml water. The light gradient solution contained 0.6ml ampholine carrier ampholyte in water. The final concentration of carrier ampholytes in the gradient was 1%,(w/v). The light electrode solution, containing 0.1g sodium hydroxide and 10ml water, was added to the cathode at the top of the sucrose gradient. To prevent exposure of the protein to sulphuric acid at the anode, the sample (10-20mg protein) was added to the gradient solutions after the gradient solution half filled

the isoelectric focusing column. All procedures were performed at 2° with water at 2° circulating through the cooling jacket of the column.

After isoelectric focusing was completed the valve at the bottom of the column was closed and the column was eluted at the rate of 1-2ml/min.; 1ml fractions were collected. The pH and extinctions at 280nm of each sample were determined.

For isoelectric focusing in pH 1-3 gradients a method based on the modification of Pettersson (82) was used, (Vesterberg, personal communication). The dense electrode solution contained 0.15ml sulphuric acid, 16ml glycerol and 4ml distilled water. The dense gradient solution contained 0.1gm monochloroacetic acid, 0.1gm phosphoric acid, 0.1gm dichloroacetic acid, 35ml glycerol and 20ml water. The light gradient solution contained 0.1gm acetic acid, 0.1gm formic acid, 0.1gm citric acid, 0.05gm glutamic acid, 0.05g aspartic acid, 0.3ml pH 5-8 ampholine carrier ampholyte and 60ml water. The sample of protein was added to the gradient solutions after the gradient solution half filled the column. When the column was filled the light electrode solution, consisting of 0.1g sodium hydroxide and 10ml water, was added to the cathode at the top of the column. The column was emptied and fractions were collected as described above.

For isoelectric focusing in pH 3-10 gradients a potential of 300V was applied for 72h after which time the current dropped from 10ma to 1ma. In the case of isoelectric focusing in pH 3-6 and pH 4-6 gradients a potential of 400V was applied for 72h and the current dropped from 8ma to 1ma. For isoelectric focusing in pH 1-3 gradients the potential was slowly increased from 150V to a maximum of 400V and isoelectric focusing carried out for 120-144h. The current slowly dropped from 15ma to 1ma.

IMMUNOLOGICAL METHODS

Antisera were prepared in rabbits by F.E. Ashton using the method described by Jamieson (39).

Double diffusion analysis

The procedure employed was based on that of Ouchterlony (83). The medium used contained 1.25% (w/v) Nobel agar, 0.15M NaCl and 0.01% thimersal. Wells were cut with a cork borer.

Precipitin technique

Quantitative precipitin curves were prepared by a method based on that described by Jamieson (39). Mixtures (total volume 0.45ml) were prepared containing up to 100μg

of α_2-macroglobulin or 25µg of α_1-acid glycoprotein; 0.15M NaCl, 1mM sodium azide, and 4.7% (w/v) Dextran 80; 50µl of anti α_2-macroglobulin or 0.25ml anti α_1-acid glycoprotein were added to solutions containing their corresponding antigens. Mixtures were incubated at 37° for 45 min. in the case of α_2-macroglobulin and for 2h in the case of α_1-acid glycoprotein and then allowed to stand for 48h at 2°. The precipitates formed upon incubation were collected by centrifuging at 2000 r.p.m. for 10 min. and then washed with 0.3ml of 0.15M NaCl containing 4% Dextran 80, and then with 3 x 0.3ml 0.15M NaCl. All washing procedures were performed at 2°. The precipitates were then dissolved in 1.0ml of 0.1N NaOH and suitable volumes removed for determination of protein. When the technique was applied to samples of serum from control rats and rats suffering from inflammation for 4-96h, 80 fold dilutions of serum were reacted with anti-α_1-acid glycoprotein and anti α_2-macroglobulin as described above.

CHEMICAL ANALYSES

Substances used to standardize quantitative procedures were allowed to stand over $CaCl_2$ *in vacuo* for about 16h before use.

Protein

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (84) but with modified reagents and volumes as described by Miller (85). Crystalline bovine serum albumin was used as standard.

Hydrolysis of protein with H_2SO_4 for preparation of sialic acid fraction

For qualitative and quantitative analysis of sialic acid a solution containing 1-2mg α_1-acid glycoprotein in 2.0ml of 0.05N H_2SO_4 was heated in a glass stoppered tube at 90° for 60 min. (86). The hydrolysate was applied to a 1.05cm x 7.0cm column of Dowex 2 (formate form). The tube was washed with 2 x 0.5ml water and the washings were applied to the column. The column was then eluted with a further 2 x 2.5ml of water and the washings discarded. The column was finally eluted with a total of 15ml of 0.3N - formic acid and the effluent collected in a pear shape flask. In order to avoid concentrating the formic acid which might cause destruction of sialic acid, the effluent was first concentrated to about one-third volume in a rotary evaporator and then diluted back to the original volume with water. The process was repeated. Finally the effluent was evaporated to dryness *in vacuo*

at 35°. The residue is referred to as the sialic acid fraction.

<u>Hydrolysis of protein with Dowex 50-HCl for preparation of neutral sugar and hexosamine fractions</u>

For qualitative and quantitative analysis of neutral sugars and hexosamines α_1-acid glycoprotein was first hydrolyzed with Dowex 50 and HCl by a method based on that described by Simkin, Skinner and Seshadri (86). Dowex 50 (H^+ form) was washed with 5 volumes of 0.46N HCl and then suspended in an equal volume of 0.46N HCl. The protein (1-2mg) dissolved in 0.25ml water was mixed with 0.6ml of the Dowex 50 (H^+ form) - 0.46N HCl resin suspension and placed in 1cm x 14cm bomb tubes. The tubes were sealed and heated in an oven at 100° for 30-36h. The tubes were positioned at an angle of 45° and rotated several times during hydrolysis.

For fractionation of hydrolysates an arrangement was made such that the filtrate from a small funnel fitted with a glass wool plug could pass through a 0.5cm x 7.0cm column of Dowex 50 (H^+ form) and the effluent from the latter could pass directly through a similar column containing Dowex 2 (formate form). The hydrolysis mixture was transferred to the small funnel and the bomb tube washed with 5 x 0.5ml water and the washings transferred

to the funnel. The filtrates were allowed to pass successively through the Dowex 50 and Dowex 2 columns. The resin in the funnel was then washed with a total of 12.5ml water applied in 1-2ml volumes, each wash being allowed to flow through both columns before the next wash was applied. 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 had stopped it was clipped off and the resin in the funnel containing the glass wool plug was washed successively with 0.25ml of 2N HCl, 2 x 0.25ml water and 0.25ml of 2N HCl and the filtrates allowed to pass into 1.0ml water which was applied to the top of the Dowex 50 column. When all the washings from the funnel had collected on the top of the Dowex 50 column the clip was opened and the liquid allowed to pass through the column; the effluent was discarded. The Dowex 50 column was then eluted with 3ml of 2N HCl (87); the effluent was collected and evaporated to dryness in vacuo at 35°. The residue is referred to as the hexosamine fraction.

Controls in which the assay solution for hydrolysis was replaced with 0.25ml of a solution containing 1-2mg bovine serum albumin and 50-100 μg of glucosamine HCl or hexose (an equimolar mixture of galactose and mannose)

were included in all determinations (86).

Sialic acid fraction

The sialic acid fraction was examined by paper chromatography using Whatman No.1. Butan-1-ol-acetic acid-water, (4:1:5,v/v) and butan-1-ol-pyridine-water, (6:4:3,v/v) were employed as solvents. Downward development at room temperature was employed for all chromatographic procedures. Sialic acid was detected by spraying with the periodate-thiobarbituric system of Warren (88).

Sialic acid was determined by the periodate-thiobarbituric acid method of Aminoff (89). The standard curve was scaled down to a maximum of 25 μg sialic acid. N-Acetylneuraminic acid was used as standard.

Neutral sugar fraction

The neutral sugar fraction was examined by paper chromatography 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-acetic acid-water (4:1:5,v/v) as solvent. Hexoses were detected by spraying with aniline phthalate reagent (90).

Total hexose was determined by the orcinol method of Winzler (12) with an equimolar mixture of galactose

and mannose as standard. The method was scaled down to a total volume of 4.75ml.

Hexosamine fraction

The hexosamine fraction was examined by paper chromatography on Whatman No.1 using butan-1-ol-acetic acid-water, (4:1:5,v/v) as the solvent. Hexosamine was detected by spraying with Ehrlichs reagent as described by Partridge (91).

Hexosamine was determined by the method of Rondle and Morgan (92) with D-glucosamine HCl as standard.

RESULTS

PREPARATION OF α_1-ACID GLYCOPROTEIN AND α_2-MACROGLOBULIN FROM RAT SERUM

Serum, isolated from rats 48h after administration of turpentine, was fractionated by stepwise elution from columns of DEAE-cellulose using a scaled up method based on that described by Ashton *et al*. (57). A typical elution profile is shown in Fig.4. Fractions 1,4 and 5 contained, between them, glycoproteins that were responsible for about 80% of the increase in total protein-bound carbohydrate of serum as a result of inflammation (57). Fraction 5, which accounted for 40% of the increase in total protein-bound carbohydrate of serum (57), contained serum albumin together with two main bands of α -globulins that stained strongly for carbohydrate when examined by electrophoresis on cellogel strips at pH 8.6,(Fig.5). The carbohydrate staining proteins had electrophoretic mobilities less than that of serum albumin (Fig.5).

In order to isolate the carbohydrate-containing proteins present in fraction 5 a stepwise elution procedure from columns of CM-cellulose was employed. Two fractions were obtained (Fig. 6). Fraction 5A which eluted with the 0.05M acetate buffer, pH 4.9, contained carbohydrate staining proteins with electrophoretic

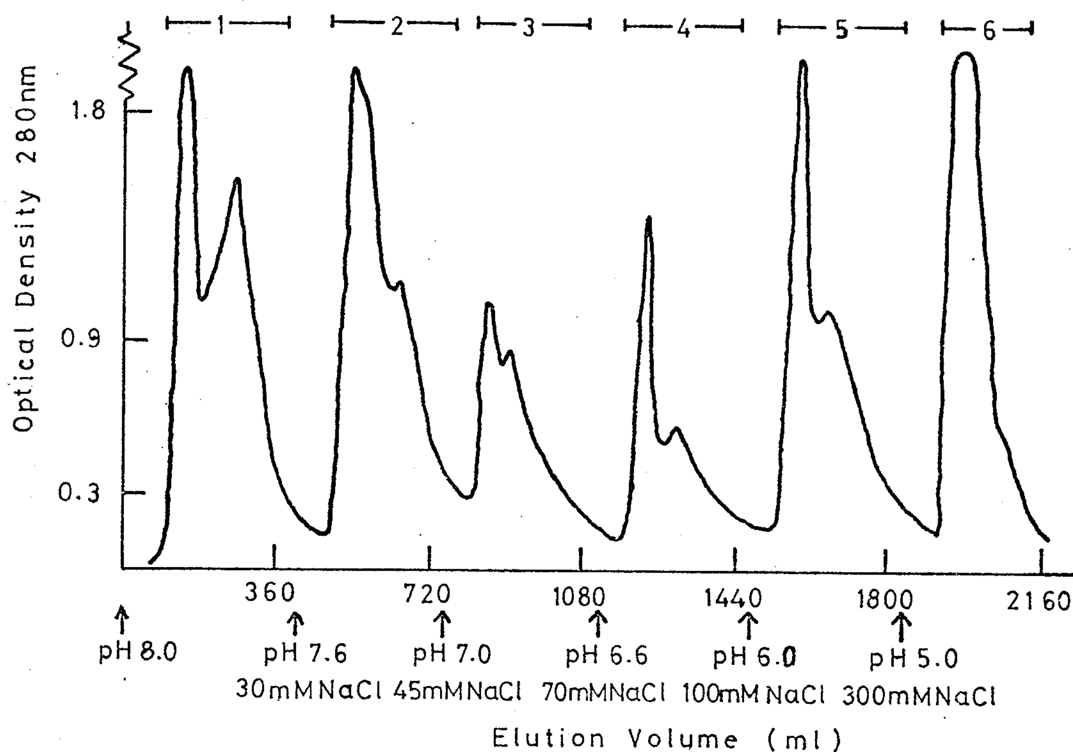

Fig.4

Stepwise elution chromatography on a 45cm x 2.5cm column of DEAE-cellulose of 30ml serum isolated from rats 48h after injection of turpentine. The column was eluted with 0.02M sodium phosphate buffer, pH 8.0, and then with a series of 0.02M sodium phosphate buffers of decreasing pH and increasing concentrations of NaCl. Arrows indicate the positions at which the buffers were applied. The fractions were pooled as indicated.

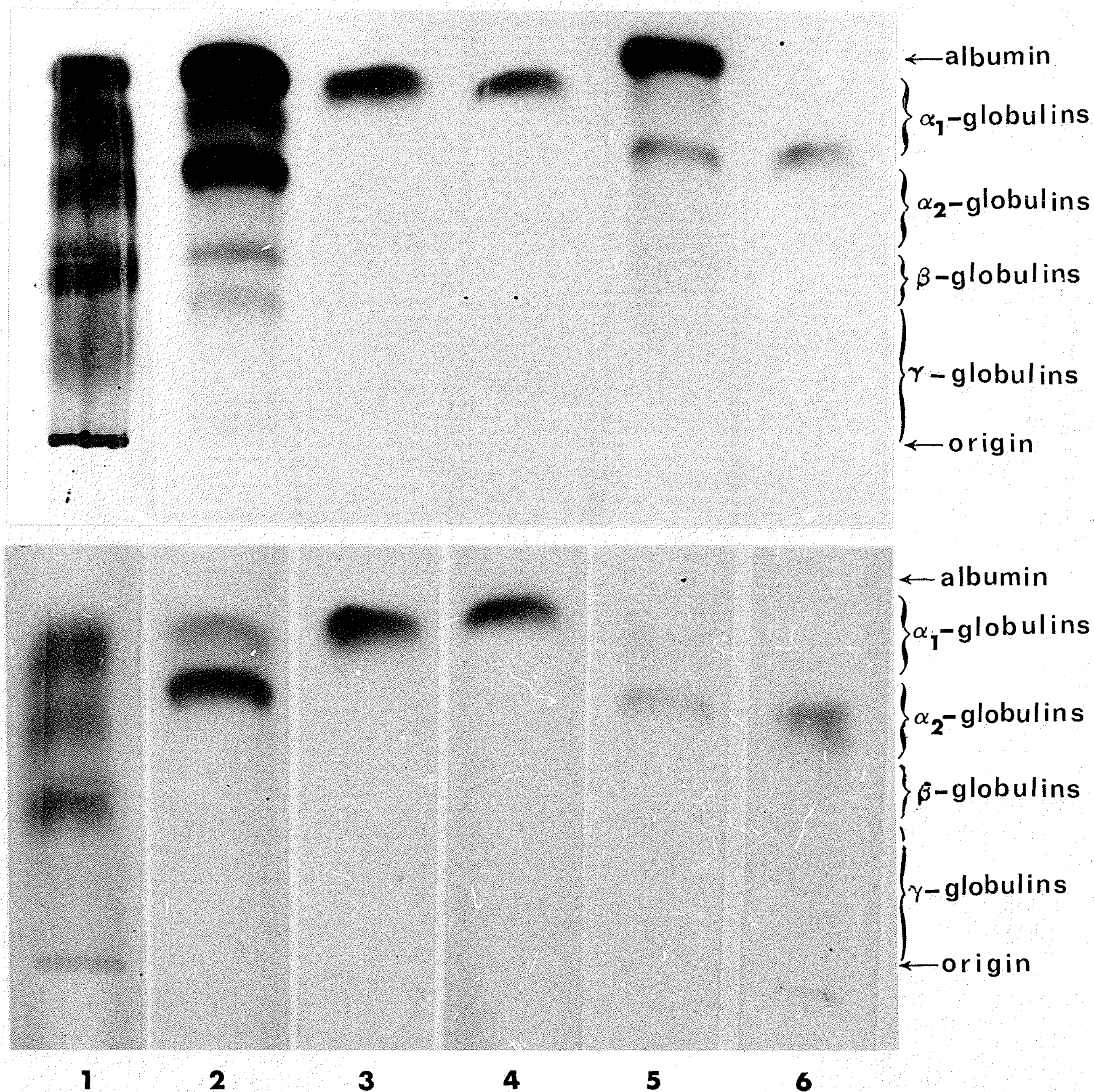

Fig.5

Electrophoresis on Cellogel strips at pH 8.6 of 1, serum from rats 48h after injection of turpentine; 2, fraction 5; 3, fraction 5A; 4, α_1-acid glycoprotein; 5, fraction 5B; 6, α_2-macroglobulin. Bottom, cellogel strips stained for carbohydrate; top, cellogel strips stained for protein.

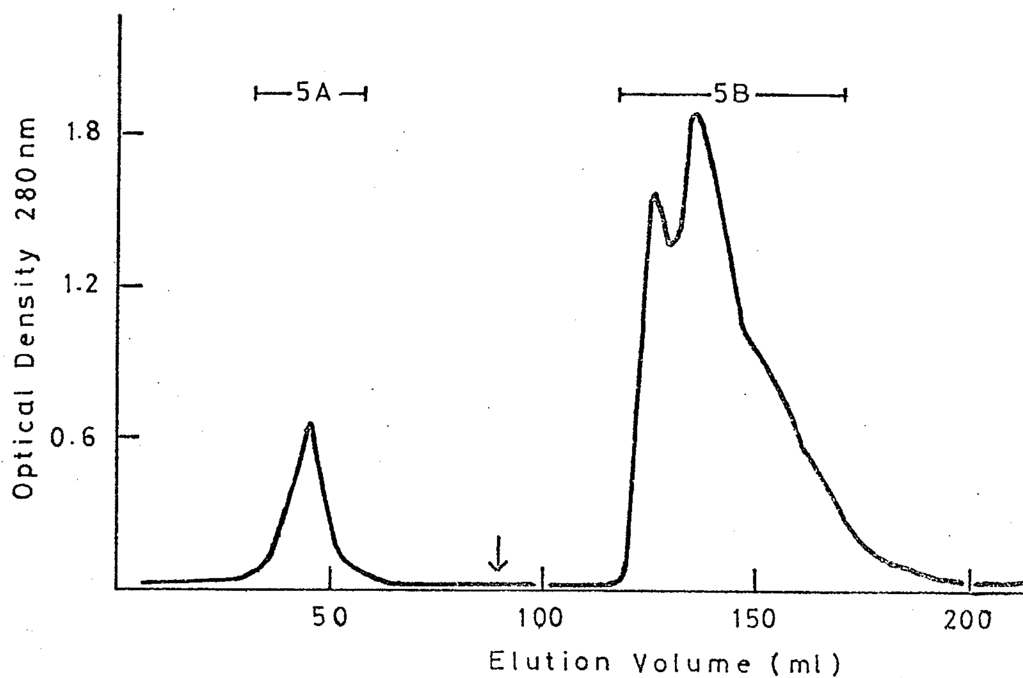

Fig.6

Stepwise elution chromatography of 100mg of fraction 5 on a 45cm x 2.5cm column of CM-cellulose. The column was eluted initially with 0.05M acetate buffer, pH 4.9. The arrow indicates the position at which a 0.40M acetate buffer, pH 5.1 was applied. The fractions were pooled as indicated.

mobilities just less than that of serum albumin (Fig. 5). Fraction 5B, which eluted with the 0.40M acetate buffer, pH 5.1, contained serum albumin together with the second band of carbohydrate-staining proteins (Fig.5).

Fraction 5A was further fractionated by the technique of isoelectric focusing in pH 1-3 gradients using a modification of the method of Pettersson (82). A typical elution profile is shown in Fig.7 . A sharp peak of E_{280}-positive material was obtained at an isoelectric point of pH 2.95. This fraction was collected and is referred to as the α_1-acid glycoprotein. The α_1-acid glycoprotein moved as a single band when examined by electrophoresis on Cellogel strips at pH 8.6, (Fig.5), stained strongly for carbohydrate and corresponded to the carbohydrate-staining band of greatest electrophoretic mobility present in fraction 5A, (Fig.5).

Fraction 5B was fractionated by preparative electrophoresis on Cellogel blocks at pH 8.6. Two fractions were prepared, one containing serum albumin, and the other containing the carbohydrate-staining proteins present in fraction 5B. The carbohydrate-containing fraction was applied to a column of Sephadex G-200 and eluted with 0.02M phosphate buffer, pH 7.0. A large peak of E_{280}-positive material eluted near the void volume of the column (Fig.8). This material, called fraction $5B_1$, was

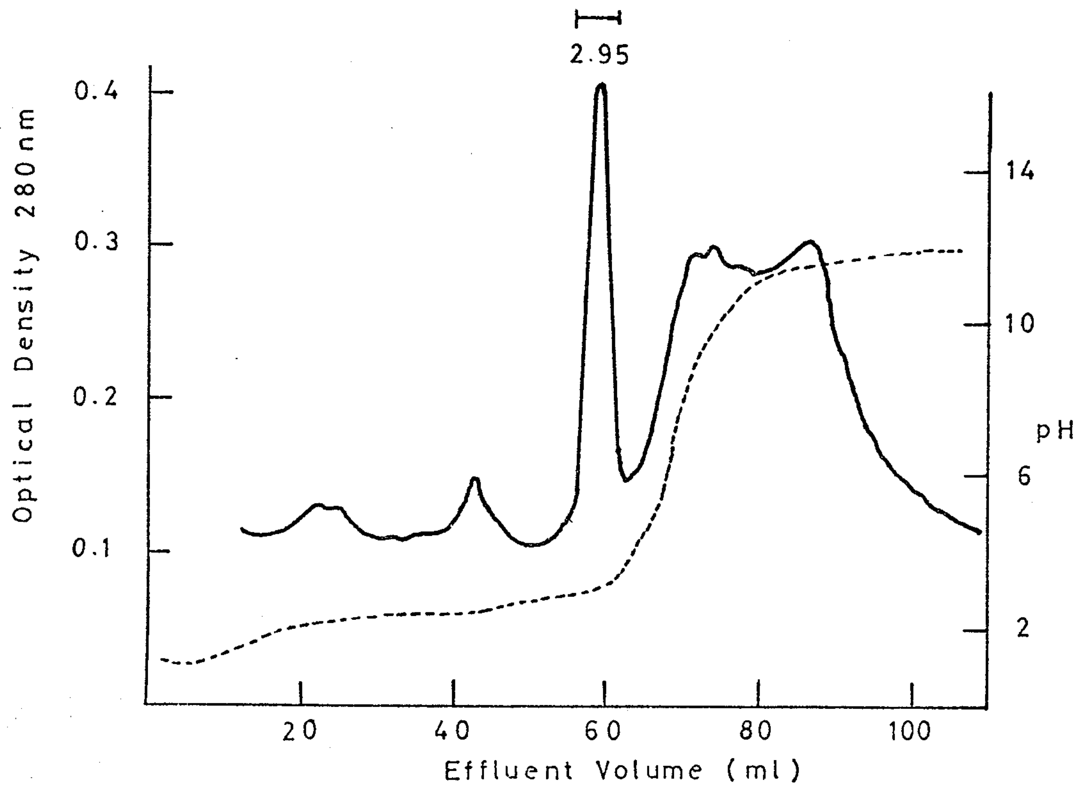

Fig.7

Fractionation of 13mg of fraction 5A, by iso-electric focusing in a pH 1-3 gradient; solid line, optical density; broken line, pH. The fraction at pH 2.95 was pooled as indicated.

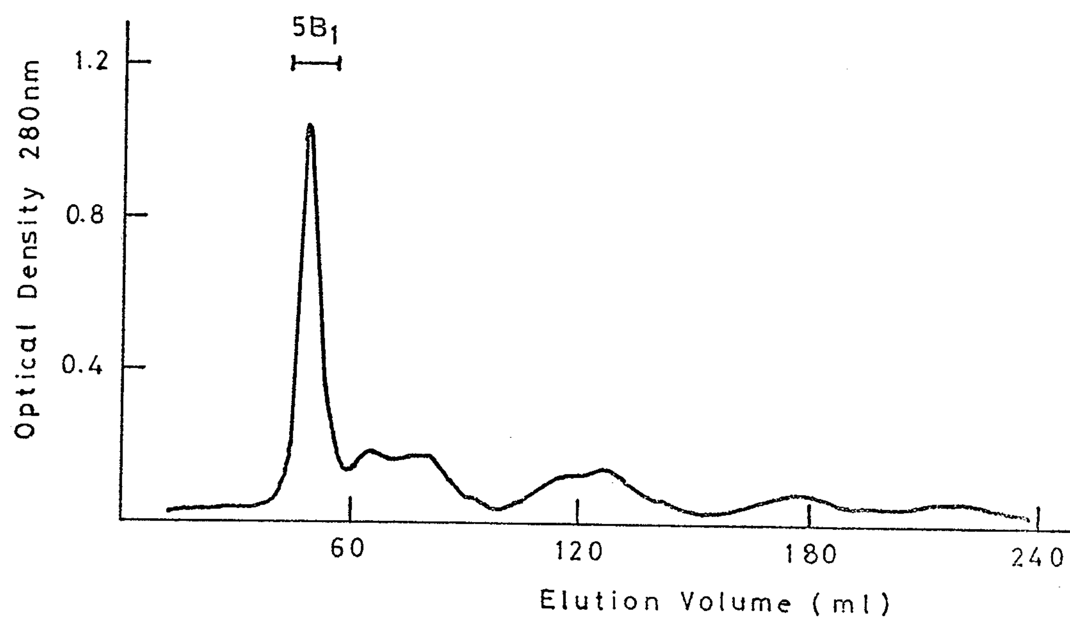

Fig.8

Gel filtration of the carbohydrate staining band of fraction 5B on a 90cm x 1.5cm column of Sephadex G-200. The column was eluted with 0.02M phosphate buffer, pH 7.0. The fraction was pooled as indicated.

collected and was further fractionated by isoelectric focusing in pH 4-6 gradients (Fig.9). The material eluted as a series of three overlapping peaks; the major peak corresponding to an isoelectric point of pH 4.6. A narrow fraction from the major peak was collected and is referred to as the α_2-macroglobulin. The α_2-macroglobulin moved as a single band when examined by electrophoresis on Cellogel strips at pH 8.6, stained strongly for carbohydrate and corresponded in electrophoretic mobility to the main carbohydrate-staining band present in fraction 5B (Fig.5).

CHARACTERIZATION OF α_1-ACID GLYCOPROTEIN AND α_2-MACROGLOBULIN

In order to determine if the α_1-acid glycoprotein and the α_2-macroglobulin were homogeneous, both proteins were examined electrophoretically and immunologically. The α_1-acid glycoprotein was also examined in the analytical ultracentrifuge.

Examination of the α_1-acid glycoprotein by electrophoresis on starch gel using the discontinuous buffer system of Smithies (76) gave only one band that stained for protein (Fig.10). The α_1-acid glycoprotein moved slightly ahead of serum albumin and corresponded in electrophoretic mobility to the main protein-staining

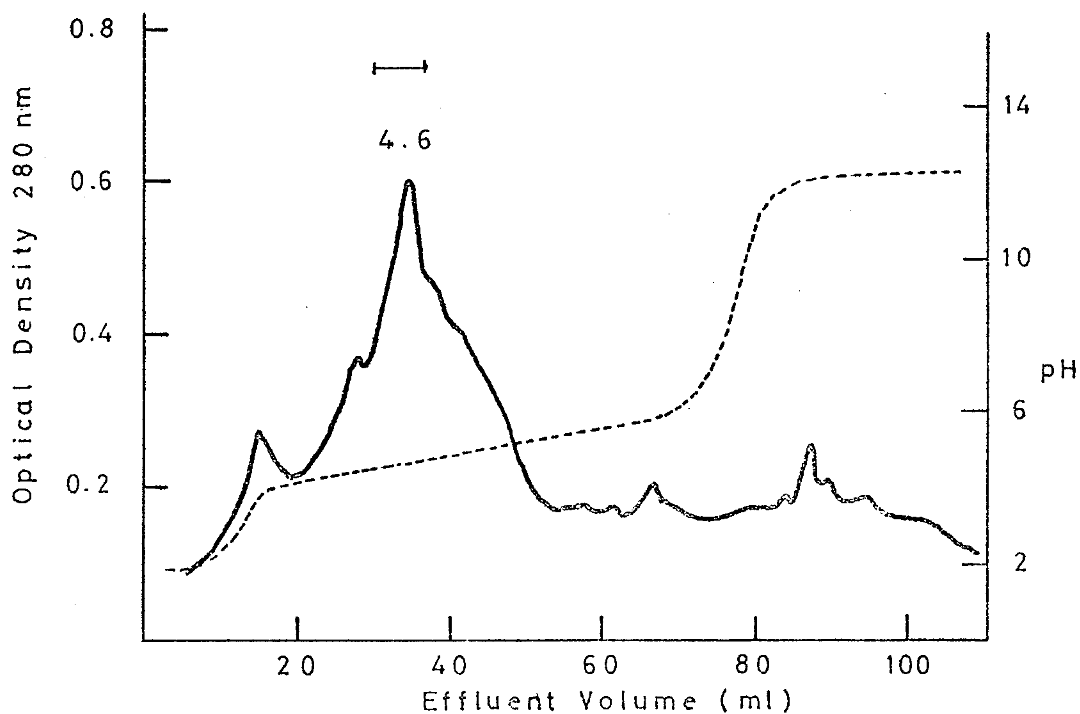

Fig.9

Fractionation of 20mg of fraction $5B_1$ by isoelectric focusing in a pH 4-6 gradient; solid line, optical density; broken line, pH. The fraction was pooled as indicated.

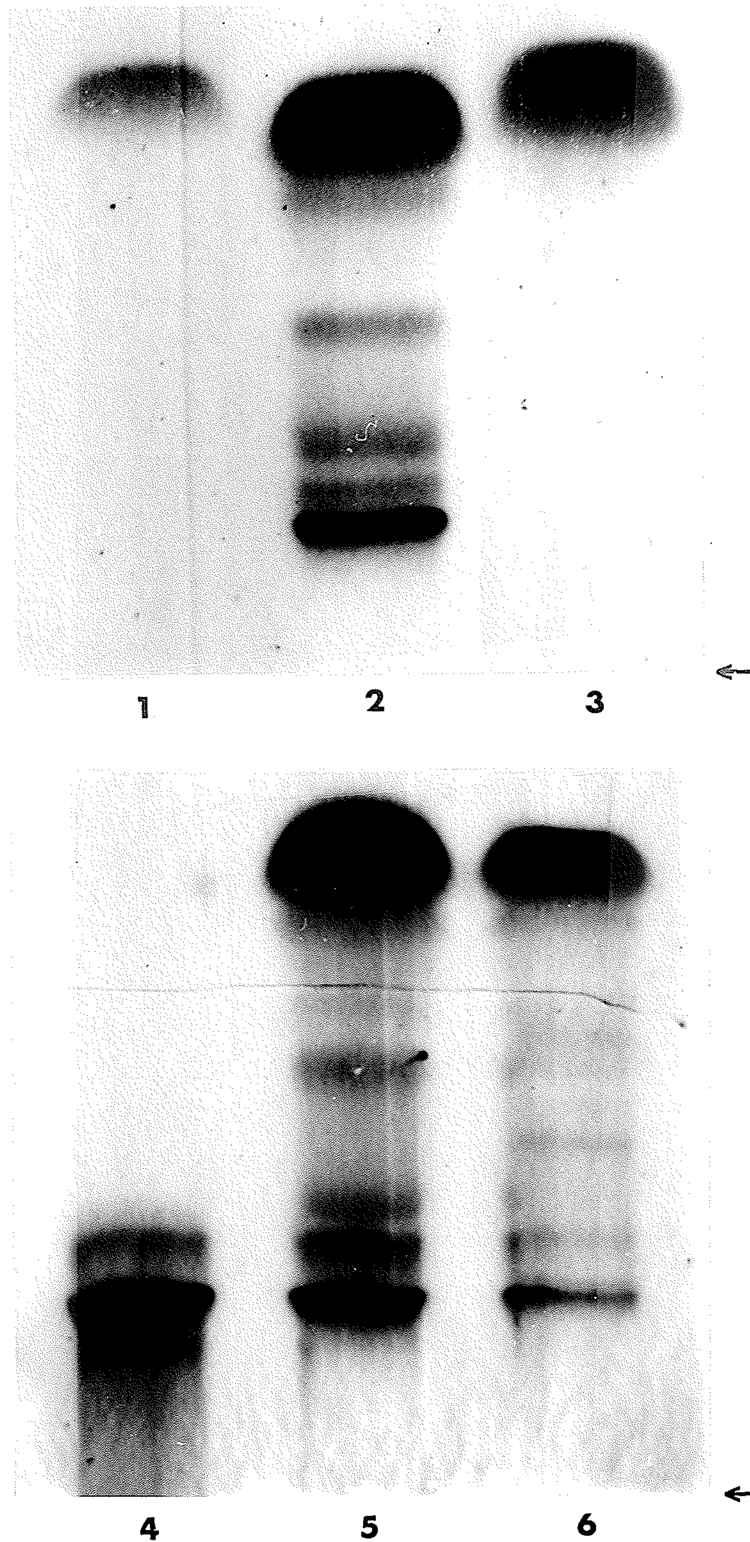

Fig.10

Electrophoresis of protein fractions on starch gel at pH 8.0. Top photograph; 1, α_1-acid glycoprotein; 2, fraction 5; 3, fraction 5A. Bottom photograph; 4, α_2-macroglobulin; 5, fraction 5; 6, fraction 5B. The arrows indicate the point of application of samples.

band present in fraction 5A (Fig. 10). Fraction 5A also contained some minor components of slower electrophoretic mobilities than the α_1-acid glycoprotein (Fig. 10). Disc electrophoresis of the α_1-acid glycoprotein on acrylamide gels at pH 8.9 also gave one band that stained both for protein and for carbohydrate and was located just ahead of the band corresponding to serum albumin (Fig. 11). The α_1-acid glycoprotein corresponded in electrophoretic mobility to the main carbohydrate-staining band present in fractions 5 and 5A (Fig. 11).

The α_1-acid glycoprotein was examined by double diffusion analysis in agar gel employing antisera to fraction 5A. One precipitin line was obtained which showed a reaction of immunological identity with a line obtained from rat serum, fraction 5 and fraction 5A (Fig. 12). Examination of the α_1-acid glycoprotein in the analytical ultracentrifuge at pH 8.0 gave one symmetrical peak (Fig. 13). On the basis of the above criteria the α_1-acid glycoprotein was considered to be a homogeneous protein.

The α_2-macroglobulin was examined in a similar manner to the α_1-acid glycoprotein described above. Electrophoresis of the α_2-macroglobulin on starch gel gave one major band and two minor bands with electro-

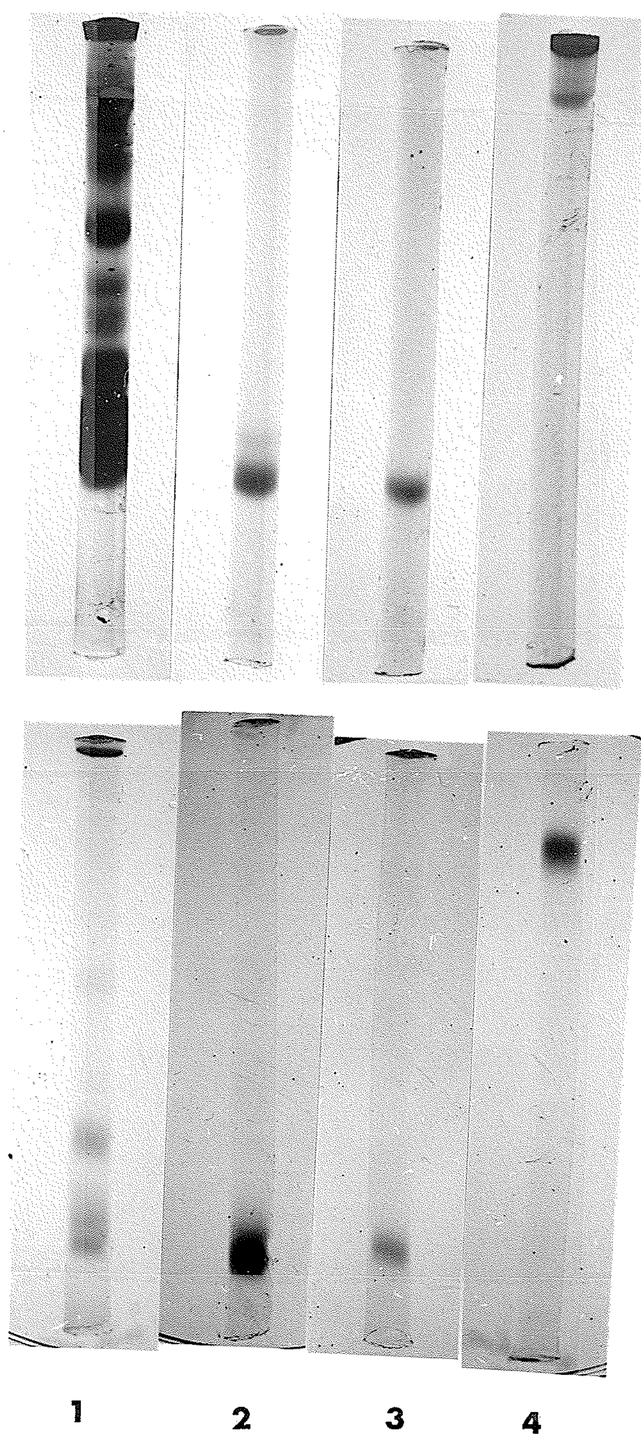

Fig.11

Disc electrophoresis on acrylamide gels at pH 8.9 of 1, fraction 5; 2, fraction 5A; 3, α_1-acid glycoprotein; 4, α_2-macroglobulin. Bottom, acrylamide gels stained for carbohydrate; top, acrylamide gels stained for protein.

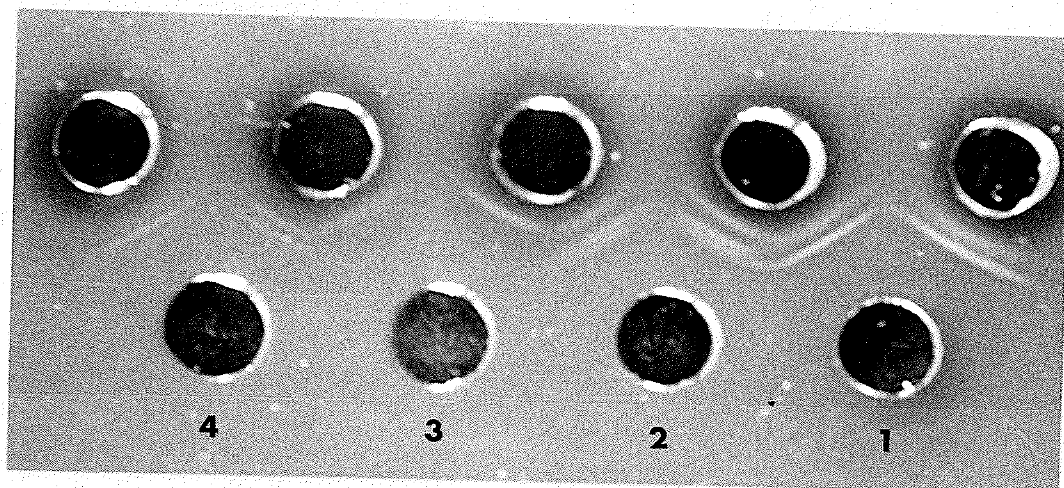

Fig.12

Double diffusion analysis of 1, serum; 2, fraction 5A; 3, α_1-acid glycoprotein; 4, fraction 5 with anti-fraction 5A. All the wells in the top of the picture contained anti-fraction 5A. Antigens contained 1mg/ml of protein, except for serum, which was diluted 1:4(v/v) with 0.15M NaCl.

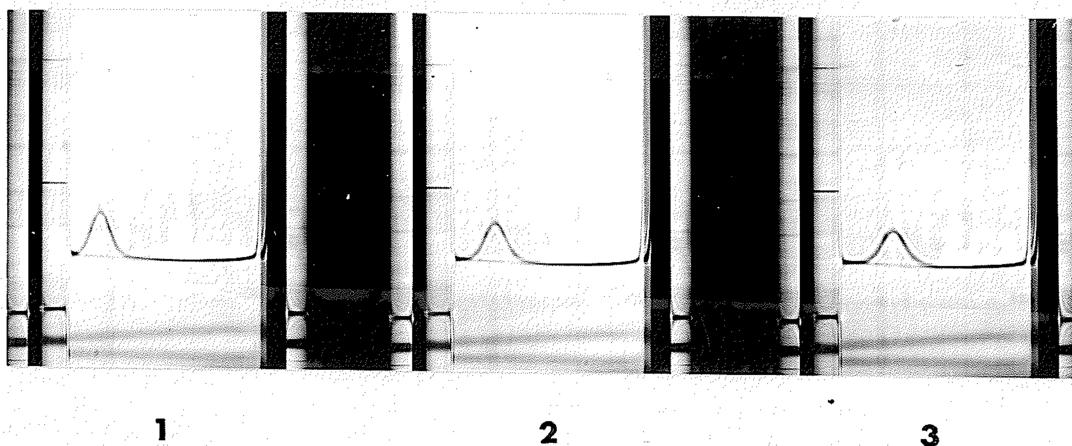

Fig.13

Sedimentation pattern at 60,000 r.p.m. and 20° of 5mg/ml of rat α_1-acid glycoprotein in a 0.02M sodium phosphate buffer, pH 8.0. Picture 1 was taken 50 min after zero time and picture 2 and 3 were taken at 16 min intervals thereafter.

phoretic mobilities slightly greater and slightly less than the major band (Fig. 10). The major band from the α_2-macroglobulin fraction corresponded in electrophoretic mobility to a strongly staining macroglobulin present in fraction 5 and 5B (Fig. 10). Disc electrophoresis of the α_2-macroglobulin also gave one major band with some minor bands of slower electrophoretic mobilities(Fig. 11). The main band from the α_2-macroglobulin fraction stained both for protein and carbohydrate and corresponded in electrophoretic mobility to a band present in fraction 5 (Fig. 11).

The α_2-macroglobulin was also examined by double diffusion analysis employing antiserum to α_2-macroglobulin. One precipitin line was obtained with the α_2-macroglobulin which gave a reaction of immunological identity with a line obtained from serum,fraction 5 and fraction 5B (Fig. 14). However, at least two or three additional lines were obtained from serum, fraction 5 and fraction 5B (Fig. 14).

CHEMICAL ANALYSES

Carbohydrate composition

The α_1-acid glycoprotein was analyzed qualitatively and quantitatively for neutral sugars, hexosamines and

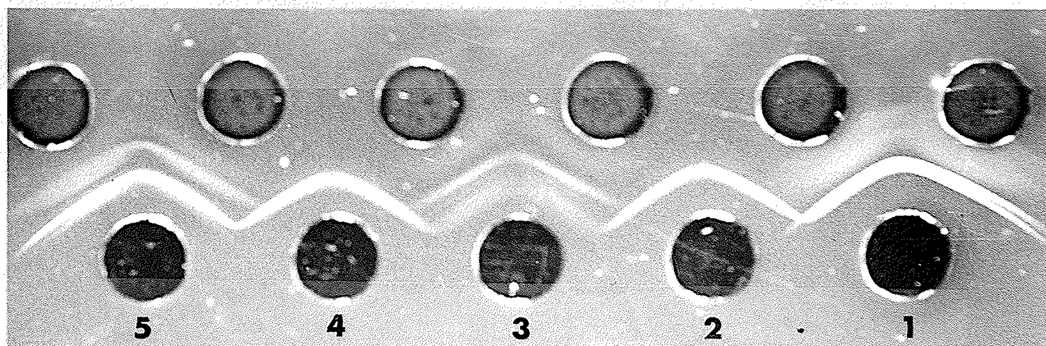

Fig.14

Double diffusion analysis of 1, serum; 2, α_2-macroglobulin; 3, fraction 5; 4, α_2-macroglobulin; 5, fraction 5B with anti α_2-macroglobulin. All the wells in the top of the picture contained anti α_2-macroglobulin. Antigens contained 1mg/ml of protein, except for serum, which was diluted 1:20 (v/v) with 0.15M NaCl.

sialic acids. Paper chromatography showed that the neutral sugar fraction contained only galactose and mannose; only glucosamine was detected in the hexosamine fraction and N-acetylneuraminic acid in the sialic acid fraction.

Quantitative analyses indicated that the α_1-acid glycoprotein contained 40.6% by weight of carbohydrate; the carbohydrate consisted of 14.8% hexose, 13.2% hexosamine and 12.6% sialic acid (values are given in g sugar per 100g dry weight of glycoprotein). Recoveries, as determined by including appropriate sugar standards during hydrolysis and fractionation (see Experimental section), were 95% for hexose, 90% for sialic acid, but only 70% for hexosamine. The values given above for the carbohydrate composition of α_1-acid glycoprotein represent mean values from analyses of four separate preparations of α_1-acid glycoprotein; the values have been corrected for loss of sugars during hydrolysis.

Determination of molecular weight

The molecular weight of the α_1-acid glycoprotein was determined by disc electrophoresis on acrylamide gels in presence of sodium dodecyl sulphate by a method based on that described by Weber and Osborn (78). The results shown in Fig.15 indicate a molecular weight of

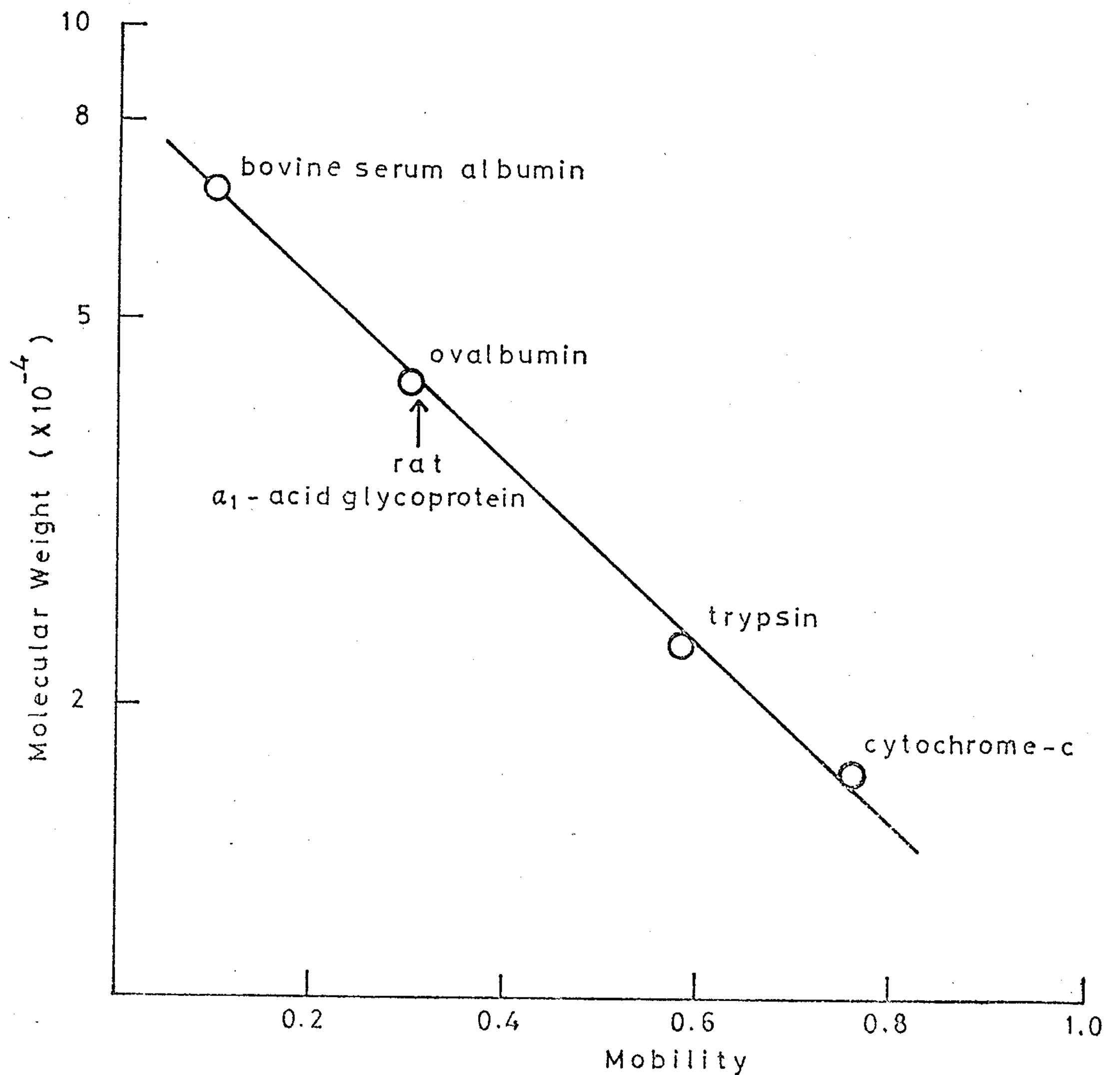

Fig.15

Determination of the molecular weight of rat α_1-acid glycoprotein by disc electrophoresis in presence of sodium dodecyl sulphate. The arrow indicates the mobility of rat α_1-acid glycoprotein (0.310); the extrapolated value for the molecular weight is 43,000 (for details see Text).

43,000 for the α_1-acid glycoprotein. However, when the α_1-acid glycoprotein was eluted from a column of Sephadex G-150 and the molecular weight calculated according to the method of Andrews (74) a value of 76,000 was obtained (Fig. 16).

The sedimentation coefficient ($S_{20,w}$) determined by the method of Schachman (58) was 2.86S. The value for the partial specific volume of the α_1-acid glycoprotein required to calculate $S_{20,w}$ was taken as 0.676. This was the partial specific volume of chimpanzee α_1-acid glycoprotein (68) which has a similar carbohydrate composition and similar molecular weight (as determined by disc electrophoresis) as the rat α_1-acid glycoprotein examined in the present studies.

<u>Quantitative precipitin technique</u>

In order to determine if the α_1-acid glycoprotein and α_2-macroglobulin were acute phase globulins, antisera were prepared to both proteins. A quantitative precipitin technique was employed to determine the amount of both glycoproteins present in rat serum from control animals and in rat serum from animals at various times after administration of turpentine (see Experimental section).

Figures 17 and 18 show quantitative precipitin

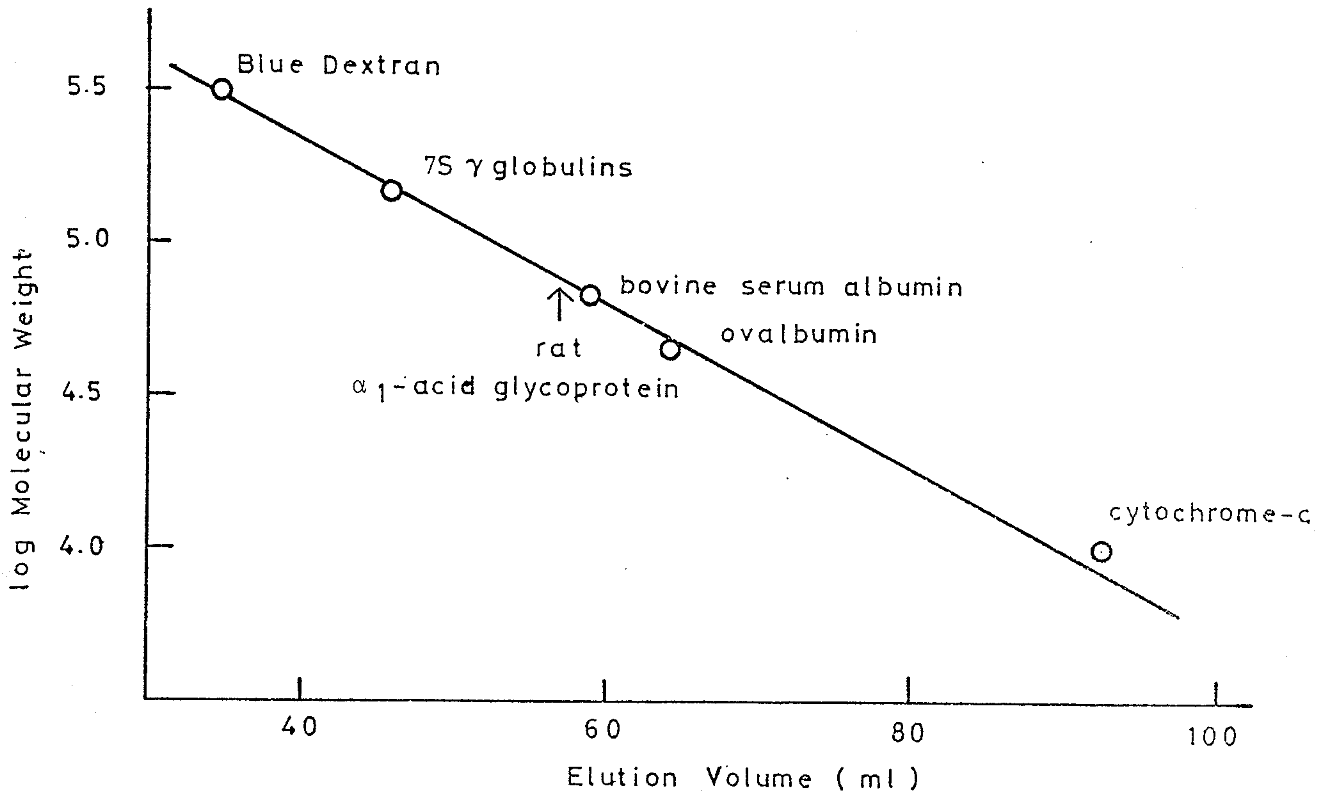

Fig.16

Determination of the molecular weight of rat α_1-acid glycoprotein by gel filtration on Sephadex G-150. The proteins indicated were applied to the column and eluted with 0.02M sodium phosphate buffer, pH 7.0. The figure shows the plot of log molecular weight against elution volume. The molecular weight of the α_1-acid glycoprotein was estimated from its elution volume (for details see Text).

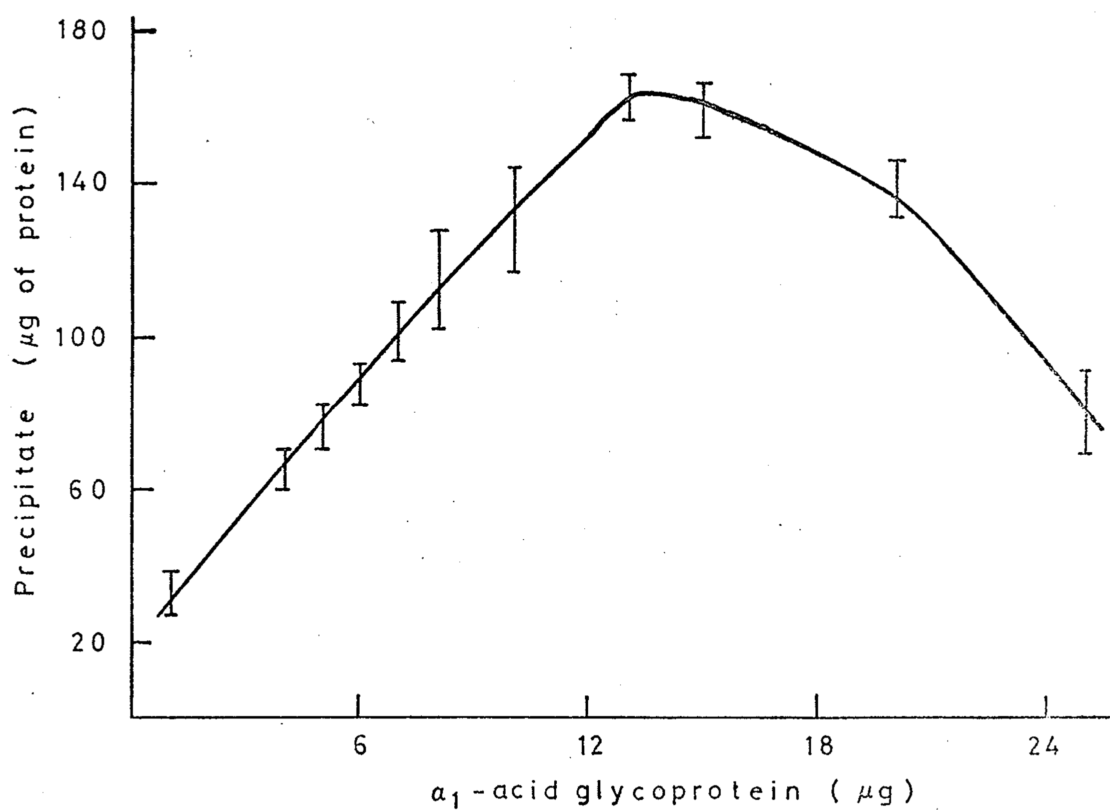

Fig.17

Quantitative precipitin curve for α_1-acid glycoprotein when titrated with 0.25ml anti α_1-acid glycoprotein. Each bar represents results from 4-8 experiments.

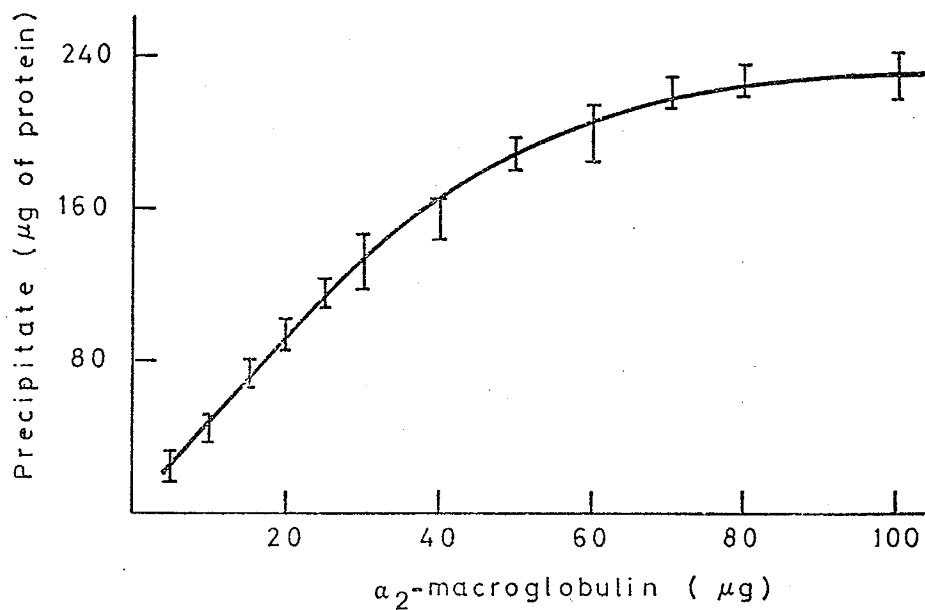

Fig.18

Quantitative precipitin curve for α_2-macroglobulin when titrated with 0.05ml anti α_2-macroglobulin. Each bar represents results from 4-8 experiments.

curves for α_1-acid glycoprotein and α_2-macroglobulin, respectively, when titrated with their corresponding antisera. The content of both glycoproteins in serum from control and experimental rats was determined by titrating serum samples with antisera in a region of antibody excess. The amount of both glycoproteins in serum samples was calculated from the amount of precipitate collected using the precipitin curves in Figs. 17 and 18 as standard curves. The results, shown in Figs. 19 and 20 for the content of both glycoproteins in serum from control and experimental animals, indicate that there is an increase in content of both proteins in serum as a result of inflammation reaching a maximum at 48-72h after injection of turpentine.

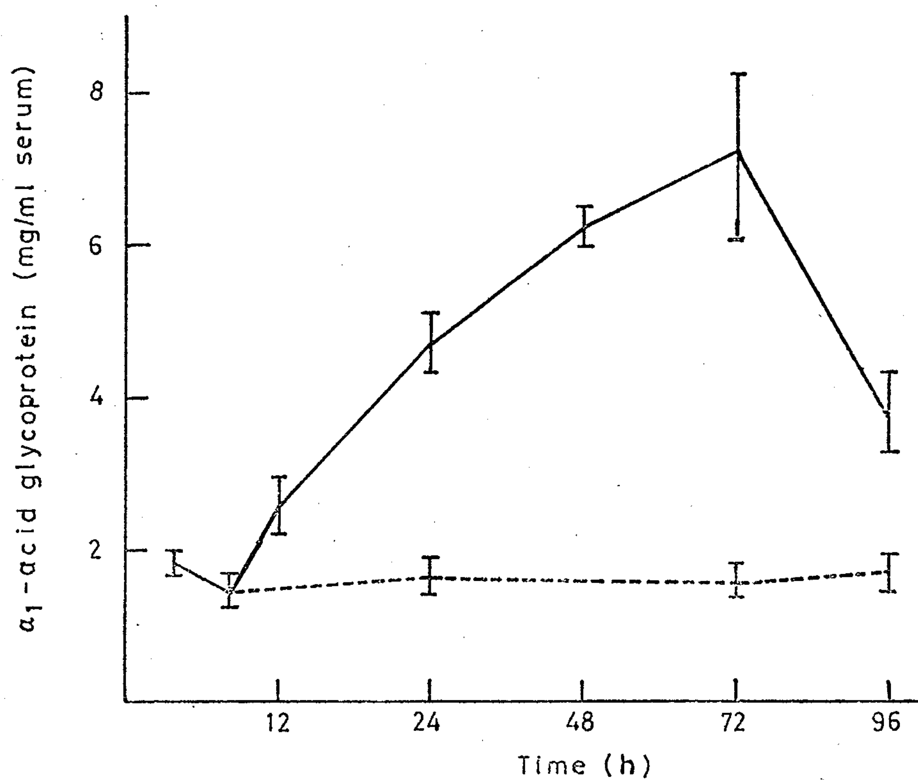

Fig.19

The effect of turpentine induced inflammation on the content of α_1-acid glycoprotein of rat serum. Solid line; results from experimental animals; broken line, results from control animals. Each bar represents results from 4-8 separate serum samples.

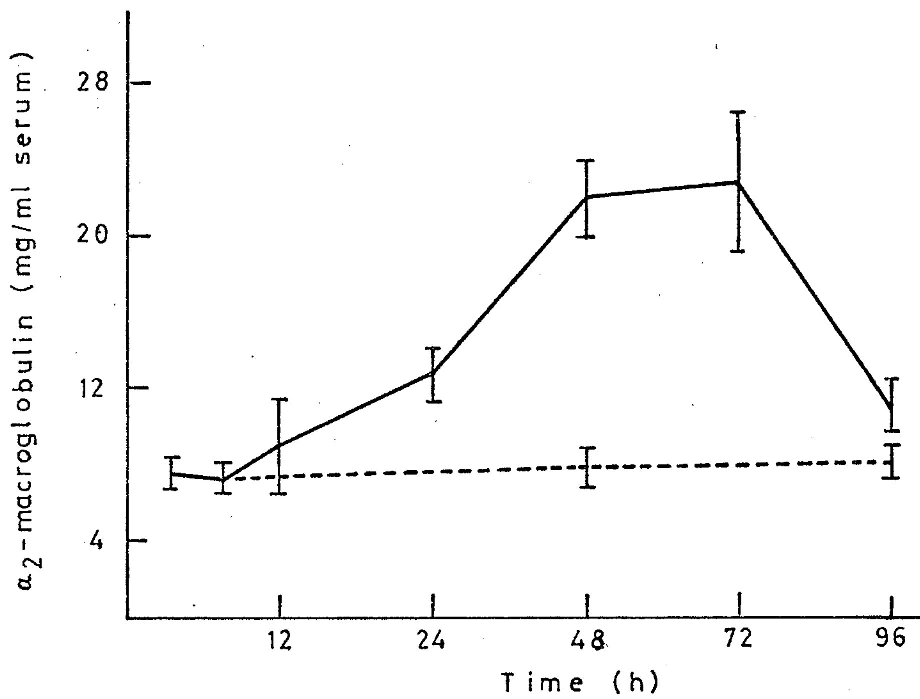

Fig.20

The effect of turpentine induced inflammation on the content of α_2-macroglobulin of rat serum. Solid line, results from experimental animals; broken line, results from control animals. Each bar represents results from 4-8 separate serum samples.

DISCUSSION

PREPARATION AND CHARACTERIZATION OF α_1-ACID GLYCOPROTEIN

The α_1-acid glycoprotein isolated in the present studies was prepared from rat serum by a combination of chromatography on DEAE- and CM-cellulose and isoelectric focusing in pH 1-3 gradients. Many other workers have employed the above chromatographic procedures for the isolation of α_1-acid glycoproteins from mammalian serum (66,93,94), but in most cases the material isolated was heterogeneous as judged by examination with immunological and electrophoretic techniques. The technique of isoelectric focusing was, therefore, employed in the present work, in addition to chromatography on DEAE- and CM-cellulose, in the hope of isolating a homogeneous α_1-acid glycoprotein. Isoelectric focusing was used by Gordon and Louis (62) for the isolation of an α_1-acid glycoprotein from rat serum. However, the material prepared by Gordon and Louis (62) had an isoelectric point of 4.5 - 4.6; hence commercially supplied ampholine carrier ampholytes were used in the preparative procedure. Isoelectric focusing of fraction 5A in pH 3-6 gradients initially indicated that the isoelectric point of the α_1-acid glycoprotein present in this fraction was less than pH 3.0. Therefore, a method had to be found to

produce stable gradients of pH which were less than pH 3.0. Such a method was suggested by Vesterberg (personal communication; see also Pettersson (82). The method involved the use of a mixture of acids with pK_a values less than 4.75. When these were included in the gradient solutions, in place of carrier ampholytes, a stable, fairly linear gradient, ranging from pH 1-3, was obtained (Fig.7). A similar procedure has recently been employed for the preparation of chimpanzee α_1-acid glycoprotein which has an isoelectric point of pH 1.82 (68). Isoelectric focusing of fraction 5A in pH 1-3 gradients gave one sharp peak at pH 2.95. This fraction was collected and was examined in the analytical ultracentrifuge, by electrophoresis on starch and acrylamide gels, and by double-diffusion analysis in agar gels employing antiserum to fraction 5A. One band was obtained on electrophoresis, with both media employed, and only one sedimentation peak was observed in the analytical ultracentrifuge. The immunological studies, however, were not so conclusive, mainly because the precipitin line obtained was rather faint; nevertheless, immunological correspondence was demonstrated between the precipitin line obtained from the α_1-acid glycoprotein and a line obtained from rat serum and from fraction 5A. Since glycoproteins of the type under examination are known to give rise to poor

antisera (95), the faintness of the precipitin line may be explained by the presence of a low content of antibody components to α_1-acid glycoprotein in the antiserum.

Other workers have reported that α_1-acid glycoproteins have a tendency to show microheterogeneity resulting in several bands when examined by electrophoresis on acrylamide gels (61,96,97). Microheterogeneity is thought to be due to minor structural variations within the carbohydrate prosthetic groups of different molecules. Gordon and Louis (62) have also suggested that highly purified α_1-acid glycoproteins have a tendency to polymerize, or become modified during isolation. Although microheterogeneity was not found in the α_1-acid glycoprotein isolated in the present studies, it cannot be ruled out, especially in view of the difficulties encountered in obtaining a sharp, single line on double diffusion analysis in agar gels.

The studies discussed above strongly suggested that the α_1-acid glycoprotein was homogeneous; therefore, the protein was characterized with respect to its' carbohydrate composition and physical properties. Table 3 gives the results obtained, together with similar data from the only other rat α_1-acid glycoproteins that have, so far, been well characterized. The isoelectric point of the α_1-acid glycoprotein isolated in the present studies is lower than

TABLE 3

General properties of rat α_1-acid glycoproteins*

Isoelectric point	$S_{20,w}$	Molecular weight	Hexose	Hexosamine	Sialic acid	Total carbohydrate	References
2.95 [1]	2.8S [5]	43,000 [2]	14.8	13.2	12.6	40.6	
4.5 - 4.6 [1]	3.5S [5]	45,000 [3]	4.0	5.9	3.7	13.6	(62)
not given		35,000 [4]	15.3	8.3	10.0	34.0	(61)

* All values are expressed in terms of g sugar/g dry weight glycoprotein.

[1] Determined from results of isoelectric focusing

[2] Disc electrophoresis in acrylamide gels presence of sodium dodecyl sulphate (74)

[3] Archibald approach to sedimentation equilibrium

[4] Sedimentation velocity technique

[5] Protein concentration, 0.5%

that of the protein described by Gordon and Louis (62); but the latter material is clearly a different protein in view of its' lower carbohydrate content. The carbohydrate content of the α_1-acid glycoprotein described by Kawasaki et al.(61) is similar to that of the protein isolated in the present studies, but the protein isolated by Kawasaki et al.(61) has a lower molecular weight, and no isoelectric point was reported. The molecular weight of the α_1-acid glycoprotein isolated in the present work was taken as 43,000, although a value of 76,000 was suggested by gel filtration on sephadex. However, glycoproteins with a high content of carbohydrate are known to behave abnormally on sephadex, possibly because of the expanded nature of molecules of this type (74). Kawasaki et al.(61), for example, reported a value of 79,000 for the molecular weight of their α_1-acid glycoprotein using gel filtration on sephadex. It appears from Table 3 that the α_1-acid glycoprotein isolated in the present studies is different from that isolated by Gordon and Louis (62), but has similarities to that isolated by Kawasaki et al.(61). Since Kawasaki et al.(61) isolated their protein from albino Wistar rats, it may be that the differences, particularly in molecular weight and hexosamine content, may be explained on the basis of species differences. Furthermore, the content of hexo-

samine in the α_1-acid glycoprotein isolated in the present work, may be subject to slight error because of the low recovery of hexosamine observed in the analytical procedures. Loss of hexosamine has been reported by other workers and is believed to occur during hydrolysis of glycoproteins in presence of Dowex 50 resin (86).

PREPARATION AND CHARACTERIZATION OF α_2-MACROGLOBULIN

The α_2-macroglobulin isolated in the present studies was prepared from rat serum by a combination of chromatography on DEAE- and CM-cellulose, preparative electrophoresis on Cellogel blocks, gel filtration on sephadex G-200 and isoelectric focusing in pH 4-6 gradients. Preparative electrophoresis on Cellogel was employed in order to separate serum albumin from the carbohydrate containing proteins present in fraction 5B. Chromatography on cellulose ion-exchangers and gel filtration on sephadex have been used by other workers for the preparation of α_2-macroglobulins from mammalian serum (98,99), but, in most cases, the material isolated was heterogeneous. Therefore, isoelectric focusing was employed as a final step in the preparative procedure in the hope of isolating a homogeneous protein. Isoelectric focusing in pH 4-6 gradients, however, did not result in a single, sharp peak as was found in the case of the

α_1-acid glycoprotein. Instead, three overlapping peaks were observed which could not be completely resolved, even when experiments were carried out with low concentrations of protein. A narrow fraction from the main peak was, therefore, collected and examined by electrophoretic and immunological techniques in a similar manner to that described for the α_1-acid glycoprotein. Electrophoresis of the α_2-macroglobulin on starch and acrylamide gels gave one major band and, at least, two other bands. However, examination of the α_2-macroglobulin by double diffusion analysis in agar gel, employing antiserum to α_2-macroglobulin, gave only one precipitin line, although additional lines were observed on reaction of serum, fraction 5 and fraction 5A with the same antiserum. The electrophoretic and immunological studies clearly indicated that the α_2-macroglobulin was not a homogeneous protein. However, the observation that the α_2-macroglobulin preparation gave only one precipitin line on reaction with its' antiserum, suggests that the contaminating proteins are relatively minor components. The α_2-macroglobulin preparation is currently being subjected to a further fractionation procedure, involving electrofocusing in pH 4-5 gradients, in the hope of obtaining a homogeneous protein for study of physical properties and carbohydrate composition.

DETERMINATION OF CONTENT OF α_1-ACID GLYCOPROTEIN AND α_2-MACROGLOBULIN IN RAT SERUM

The content of the α_1-acid glycoprotein and α_2-macroglobulin in serum from control rats and rats suffering from inflammation for 5-96h was determined by applying a quantitative precipitin technique to samples of serum. Quantitative precipitation of an antigen from a mixture of proteins, such as is found in serum, is obtained only if a monospecific antiserum is employed in a region of antibody excess (100). The antiserum* prepared against α_1-acid glycoprotein was apparently monospecific as judged by immunological analysis, and complete precipitation would, most likely, have been obtained during the quantitative precipitin technique. Complete precipitation of antigen, however, has still to be confirmed using α_1-acid glycoprotein which has been labelled, either with a fluorescent dye, or a radioactive isotope.

* Antiserum to α_1-acid glycoprotein was prepared by F.E.Ashton and became available while this thesis was being completed. Although antiserum to α_1-acid glycoprotein was not used during characterization of α_1-acid glycoprotein (see Results section), it was shown to be monospecific as judged by double diffusion analysis against rat serum, fraction 5 and fraction 5A. The precipitin curves described in this thesis were prepared by F.E.Ashton and are included to show that the α_1-acid glycoprotein and α_2-macroglobulin are acute phase globulins.

The content of α_1-acid glycoprotein and α_2-macroglobulin increased about four-fold as a result of inflammation, reaching a maximum at 48-72h after injection of turpentine, indicating that both proteins are acute phase globulins. In view of the high carbohydrate content of the α_1-acid glycoprotein, it is likely that it makes a significant contribution to the increase in total protein-bound carbohydrate of serum resulting from inflammation. The content of the α_1-acid glycoprotein in normal serum (i.e. 1.5mg/ml, see Fig.19) agrees with a value reported by Weimer and Benjamin (9) for the content of their seromucoid fraction in rat serum (i.e. 1.5mg/ml); a five-fold increase in their protein was observed as a result of inflammation. The protein isolated by Weimer and Benjamin (9), however, was not well characterized, although it probably contained an acidic glycoprotein similar to the α_1-acid glycoprotein studied in the present work. It is unfortunate that there is no information on the content in rat serum of α_1-acid glycoproteins isolated by other workers, especially the α_1-acid glycoprotein studied by Kawasaki *et al.*(61), since it closely resembles the protein isolated in the present studies.

The results obtained in the quantitative precipitin technique with antiserum to α_2-macroglobulin are likely

to be inaccurate since, as previously discussed, the antiserum was not monospecific. Hence precipitates obtained from serum samples on addition of antiserum to α_2-macroglobulin probably consisted of complexes formed between antibody components to the major and minor components present in the α_2-macroglobulin fraction. The heterogeneity of the system may be an explanation for the apparent high content of α_2-macroglobulin in normal rat serum (i.e. 7mg/ml, see Fig.20). The concentration of α_2-macroglobulin in rat serum has not been reported, but values for the content of a similar protein in human serum are seldom greater than 3.0mg/ml (see, e.g. 101).

Work in the future will involve the preparation of glycopeptides from the α_1-acid glycoprotein and α_2-macroglobulin for structural studies, particularly with respect to the sequence of sugars in the oligosaccharide chains. It is hoped that a study of structure may throw some light on the problem of assigning a biochemical function to the glycoproteins in question, and other glycoproteins that respond to induced inflammation. Concurrently with structural work, studies are being carried out in the laboratory on the mechanism of biosynthesis of the α_1-acid glycoprotein and α_2-macroglobulin and how their biosynthesis is influenced by various hormones and anti-inflammatory agents.

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