Physicochemical and Biological Properties of Glycoproteins Isolated from the Plasma of Different Species of Animals

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1. Glycoproteins were isolated from the plasma of sheep, goat, cow, buffalo and monkey. They were homogeneous by electrophoresis; on ultracentrifugation, a faster-sedimenting fraction, to an extent of 5–8% only, was observed in each case. 2. Similar physical properties were exhibited by these glycoproteins and they each have a molecular weight of about 105 000. 3. In chemical composition, differences have been observed and the glycoproteins can be classified into three groups: (a) sheep and goat glycoproteins; (b) cow and buffalo glycoproteins; (c) monkey glycoprotein. Glucose, galactosamine and *N*-terminal amino acid were absent from these proteins. 4. These glycoproteins were trypsin inhibitors and prolonged the clotting time of plasma.

A few carbohydrate-containing proteins have been successfully isolated in a homogeneous form and their properties characterized (Weimer, Mehl & Winzler, 1950; Blix, Svennerholm & Werner, 1952; Schmid, 1953*a,b*; Gottschalk & Graham, 1958; Johansen, Marshall & Neuberger, 1961; Spiro, 1960; Neuberger & Papkoff, 1963; Simkin, Skinner & Seshadri, 1964). The remarkable feature of these complexes is their high degree of structure, tissue and species specificity. A comparative study of these complexes isolated from different sources is essential for elucidating their composition and function. In our Laboratory, Das (1962) isolated and partially characterized a glycoprotein from sheep plasma. The present paper reports the isolation, physicochemical and biological properties of glycoproteins from the plasma of sheep, goat, cow, buffalo and monkey.

MATERIALS AND METHODS

Materials. DEAE-cellulose was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. The anion-exchanger was regenerated according to the method of Peterson & Sober (1956) before the column was packed. The regenerated DEAE-cellulose had 0.97 m-equiv. of ionizable groups/g. Sephadex G-25 was a product of Pharmacia, Uppsala, Sweden. Trypsin (3× crystallized) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. The other chemicals were obtained commercially from British Drug Houses Ltd., Poole, Dorset, or E. Merck, Darmstadt, Germany, and were of analytical-reagent grade.

Sheep and goat blood were collected from the slaughter-house and cow and buffalo blood from the Mysore Serum Institute, Hebbal, Bangalore, India. Monkeys were bled in the animal house.

Isolation of glycoproteins. The following procedure, which is a modification of the method of Weimer *et al.* (1950), was adopted for the fractionation of the glycoprotein from plasma of different animals. Freshly drawn blood (1.5 l.), containing 0.2% of oxalate, was centrifuged and the plasma cooled to 0°. Unless otherwise stated, all further operations were carried out at 0–5°. Sodium acetate was added to the plasma to give a final concentration of 0.1 M. An equal volume of a saturated solution of ammonium sulphate was added and the plasma kept in the cold for 16 hr. The precipitate obtained on centrifugation at 10 000*g* for 30 min. was discarded. The supernatant solution was adjusted to pH 4.8 by adding *N*-HCl and kept in the cold for 18 hr. The precipitate was removed by centrifugation at 10 000*g* for 30 min.; the supernatant solution was adjusted to pH 3.5 with *N*-HCl and allowed to stand in the cold for 16 hr. To the clear supernatant solution obtained on recentrifugation at 10 000*g* for 30 min. was added solid ammonium sulphate to give 100% saturation. The mixture was stirred well and left in the cold for 48 hr. The precipitate obtained on centrifugation at 10 000*g* for 30 min. was dissolved in 25 ml. of water. This fraction containing the glycoprotein along with some impurities was further purified by chromatography on DEAE-cellulose, after being desalted on a Sephadex G-25 column.

Desalting on Sephadex G-25 column. The ammonium sulphate fraction (25 ml.) was added to a column (40 cm. × 3 cm.) of Sephadex G-25 prepared in water. The column was eluted with water and 10 ml. fractions were collected. The eluents were analysed for salts by using Philips conductivity-measuring bridge PR9500 and for protein by the biuret

method (Gornall, Bardawill & David, 1949). The protein fraction, essentially free of salts, is eluted between 125 and 150 ml. The pooled eluents were freeze-dried in a VirTis refrigerated freeze-drier.

Chromatography on DEAE-cellulose. Instead of electrophoresis on agar gel for the isolation in a homogeneous form as carried out by Das (1962), the glycoprotein fraction was applied to a DEAE-cellulose column (35 cm. $\times$ 1.5 cm.) equilibrated with 50 mM-phosphate buffer, pH 6.0. The optimum load for each column was 125 mg. of protein. The column was developed with 50 mM-phosphate buffer, pH 6.0, containing NaCl (0.1M), and 5 ml. fractions were collected. The elution pattern is shown in Fig. 1. Protein was estimated by measuring the extinction at 280 m μ . It is evident from Fig. 1 that a clear separation of the glycoprotein from other protein impurity is obtained. The glycoprotein fraction was dialysed against repeated changes of water (4l.) and freeze-dried. The glycoprotein was positive to the Molisch and biuret tests and negative to the hexuronic acid test. From 1l. of plasma, approx. 150 mg. of purified glycoprotein is obtained.

Agar-gel electrophoresis. Agar-gel electrophoresis was carried out as described by Giri (1956). The following buffers of ionic strength 0.05 were used: (a) sodium veronal-sodium acetate-HCl, pH 8.6; (b) sodium phosphate, pH 6.0; sodium acetate-HCl, pH 3.6. A potential of 250 v was applied for 6 hr. at 22–23°. The plates were stained with Amido Black.

Moving-boundary electrophoresis. The glycoproteins were analysed by free-boundary electrophoresis in a Kern model LK 30 micro-electrophoresis apparatus fitted with a modified Jamin interference optical system supplied by Kern and Co., Aarau, Switzerland. The glycoprotein sample was dialysed against two changes of the buffer (200 ml. each time) to be used in the analysis; the second change of buffer was used to fill the electrode vessel. The glycoproteins

were analysed in the following buffers of ionic strength 0.1: sodium acetate-HCl, pH 3.3 and 4.0; sodium acetate-acetic acid, pH 4.35 and 5.4; sodium phosphate, pH 6.0 and 6.7; sodium veronal-sodium acetate-HCl, pH 8.6. The mobilities were evaluated by measuring the distance travelled by the mean position of the protein fraction and normalized to 25° by applying the correction for viscosity and temperature (Johnson & Shooter, 1948).

All extinction measurements were made in a Beckman model DU spectrophotometer. Refractive index was measured in Abbey's refractometer, and optical rotation was determined in a Bellingham and Stanley model D polarimeter.

Ultracentrifugal analyses. Ultracentrifugal analysis of the glycoproteins was carried out in a Spinco model E analytical centrifuge at 20–22° in 0.1M-sodium acetate-acetic acid buffer, pH 4.55. Sedimentation velocity was determined at 59780 rev./min. in the analytical rotor An-D. Molecular weight was determined by the method of Klainer & Kegeles (1955). The synthetic-boundary cell described by Kegeles (1952) was used for the determination of concentration of protein. A standard 4°-sector cell was used for the Archibald run and carbon tetrachloride was used to provide the bottom meniscus.

Carbohydrate analysis. Total hexoses were determined by the method of Winzler (1955), by using the orcinol-H₂SO₄ reagent. Individual hexoses were determined by the procedure of Spiro (1960). A 20 mg. sample of glycoprotein was hydrolysed in N-H₂SO₄ for 8 hr. in a sealed tube at 100°. The hydrolysate was neutralized with Ba(OH)₂ and the pH adjusted to 5.0 with dilute HCl. The precipitated BaSO₄ was removed on a sintered-glass funnel and washed with water. The filtrate and the washings were pooled and concentrated *in vacuo* to a volume of 2–3 ml. The concentrate was passed successively through a Dowex 50 (X8; H⁺ form) column (5 cm. $\times$ 0.8 cm.) and a Dowex 1 (X8; formate form) column (5 cm. $\times$ 0.8 cm.). The eluate (20 ml.) was concentrated to dryness *in vacuo*, dissolved in 1 ml. of water and subjected to paper chromatography by the method of Pridham (1956). Chromatograms were irrigated with butan-1-ol-pyridine-water (6:4:3, by vol.) solvent system for 15 hr. The air-dried chromatograms were sprayed with *p*-anisidine-HCl reagent (1 g. of *p*-anisidine hydrochloride in 10.0 ml. of methanol made up to 100 ml. with butan-1-ol; to this solution 0.1 g. of Na₂S₂O₄ was added) and heated for 10 min. at 130°. The spots were cut out and eluted with stannous chloride-methanol solution. The extinction at 400 m μ was measured after 30 min.

Total hexosamines were determined by the method of Boas (1953), and individual hexosamines were determined by the method of Gardell (1953). For these determinations, the glycoproteins were hydrolysed in 6N-HCl for 8 hr. in a boiling-water bath.

Sialic acid was estimated by hydrolysing the glycoprotein at 80° for 2 hr. with 0.1N-H₂SO₄. The hydrolysate was processed by passing through Dowex 1 (X10) (Whitehouse & Zilliken, 1960) before estimation by the method of Svennerholm (1956). Fucose was estimated by the method of Dische & Shettles (1948), by using the cysteine-H₂SO₄ reagent. Uronic acid was tested by the carbazole method (Dische, 1947).

Protein analysis. Nitrogen was estimated by the micro-Kjeldahl method and polypeptides by the biuret method, with bovine serum albumin as standard.

Fig. 1. Chromatography of impure glycoprotein fraction on a DEAE-cellulose column (35 cm. $\times$ 1.5 cm.). Elution was brought about by 50 mM-sodium phosphate buffer, pH 6.0, containing NaCl (0.1M). Fractions of volume 5 ml. were collected. The glycoprotein (○) is separated from the protein impurity (▲).

Amino acids were analysed by the 1-fluoro-2,4-dinitrobenzene method of Levy (1954), by using toluene-pyridine-2-chloroethanol-0.8N-ammonia (10:3:9:9, by vol.) in one direction and 1.5M-phosphate buffer, pH 6.0, in the other for the separation of ether-soluble DNP-amino acids. DNP-arginine and DNP-histidine in the aqueous extract of the hydrolysate were separated by descending chromatography for 20 hr. in 2-methylbutan-2-ol saturated with 50 mM-phthalate buffer, pH 6.0 (Blackburn & Lowther, 1951). Tryptophan was analysed by treating the glycoprotein with *N*-bromosuccinimide (Witkop, 1961). The tyrosine/tryptophan ratio was determined by the method of Benze & Schmid (1957).

N-Terminal amino acids were determined according to the method of Sanger (1945).

Biological activity. Anti-tryptic activity was assayed by the method of Kunitz (1947). The reaction mixture consisted of 0.3 ml. of trypsin solution (6 μ g.) in 2.5 mM-HCl, and various concentrations of glycoprotein solution in water, warmed at 35° for 2 min.; 1 ml. of 1% (w/v) casein in phosphate buffer, pH 7.6, was added to give a total volume of 2.0 ml., and the mixture incubated for 20 min. at 35°. Then 3 ml. of 5% (w/v) trichloroacetic acid was added to stop the reaction. The solution was mixed well and allowed to stand at room temperature (22–23°) for 1 hr. After removal of the precipitate by centrifugation at 5000g for 10 min., the extinction for the breakdown products was measured at 280 m μ .

The one-stage clotting method of Quick (1935) was used to determine the effect of glycoprotein on clotting time. The reaction mixture consisted of 0.2 ml. of plasma, 0.2 ml. of 1% (w/v) glycoprotein solution in 67 mM-sodium phosphate buffer, pH 7.2, and 0.2 ml. of 25 mM-CaCl₂ incubated for 2 min. at 37°. Then 0.2 ml. of 4.0% (w/v) sheep-brain thromboplastin solution (thromboplastin solution prepared so as to give a clotting time of 16 sec. with normal sheep plasma) was added and the time taken for the appearance of the clot was noted.

RESULTS

Electrophoretic analysis. The glycoproteins when subjected to agar-gel electrophoresis and moving-boundary electrophoresis were found to be homogeneous. The agar-gel electrophoretograms of sheep plasma glycoprotein at pH 3.6, 6.0 and 8.6 are presented in Fig. 2. From this Figure it is evident that sheep plasma glycoprotein moves as a single band at the pH values tested. The electrophoretic behaviour of the four other glycoproteins at these pH values was similar to that observed for sheep plasma glycoprotein. When similar experiments were carried out in the following buffers, sodium acetate-hydrochloric acid, pH 3.3 and 4.0, sodium acetate-acetic acid, pH 4.35 and 5.4, sodium phosphate, pH 6.0 and 6.7, and sodium veronal-sodium acetate-hydrochloric acid, pH 8.6, in the Kern micro-electrophoretic apparatus, all the glycoproteins behaved as homogeneous proteins. Typical schematic moving-boundary electro-

Fig. 2. Electrophoresis of the sheep plasma glycoprotein on agar gel in: (a) acetate-HCl buffer, pH 3.6; (b) phosphate buffer, pH 6.0; (c) veronal-acetate-HCl buffer, pH 8.6. The electrophoreses were done at 22–23° at 250 v for 6 hr. The arrow indicates the point of application of the sample.

Fig. 3. Moving-boundary electrophoresis of sheep plasma glycoprotein in: (a) acetate buffer, pH 5.4 (45 v for 90 min.); (b) phosphate buffer, pH 6.7 (45 v for 60 min.); (c) veronal-acetate-HCl buffer, pH 8.6 (45 v for 20 min.). The schematic diagrams represent the ascending patterns on the right and descending ones on the left.

phoretic diagrams of sheep plasma glycoprotein at pH 5.4, 6.7 and 8.6 are presented in Fig. 3.

The mobilities of the glycoproteins at different pH values indicated in Table 1 are the averages of ascending and descending mobilities, determined by moving-boundary electrophoresis. These results show that the glycoproteins isolated from different sources have similar mobilities and their isoelectric points are between pH 4.6 and 4.8 (Table 2). A typical mobility diagram of sheep plasma glycoprotein is shown in Fig. 4.

Ultracentrifugal analysis. The ultracentrifugal schematic patterns of sheep plasma glycoprotein in 0.1M-sodium acetate-acetic acid buffer, pH 4.55,

Table 1. *Mobilities of glycoproteins from plasma of sheep, goat, cow, buffalo and monkey in different buffer systems*

Buffers of ionic strength 0.1 were used. The 1% (w/v) glycoprotein solutions were dialysed before the electrophoretic run. Mobilities and temperature have been normalized to 25° by applying the correction for viscosity and temperature. The values given represent the averages of mobilities of ascending and descending boundaries.

Buffer	pH	10 ⁵ × Mobility (cm. ² v ⁻¹ sec. ⁻¹)				
		Sheep glycoprotein	Goat glycoprotein	Cow glycoprotein	Buffalo glycoprotein	Monkey glycoprotein
Sodium acetate-HCl	3.3	8.071	7.849	8.014	8.092	8.038
Sodium acetate-HCl	4.0	6.114	6.028	5.147	5.402	5.564
Sodium acetate-acetic acid	4.35	1.004	0.923	1.072	1.036	1.038
Sodium acetate-acetic acid	5.4	3.607	3.687	3.629	3.694	3.602
Sodium phosphate	6.0	4.416	4.418	4.271	4.360	4.306
Sodium phosphate	6.7	8.267	8.313	8.267	8.308	8.272
Sodium veronal-acetate-HCl	8.6	12.860	12.740	12.845	12.730	12.980

Table 2. *Physical properties of glycoproteins isolated from plasma of sheep, goat, cow, buffalo and monkey*

The optical-rotation measurements were carried out with 1% (w/v) glycoprotein solutions in water. The error of the measurements of specific optical rotation in each case is 2.

	Sheep glycoprotein	Goat glycoprotein	Cow glycoprotein	Buffalo glycoprotein	Monkey glycoprotein
Isoelectric point (pH)	4.6	4.6	4.7	4.7	4.8
$E_{1\text{cm.}}^{1\%}$ at 279 m μ	10.2	10.2	10.4	10.3	10.4
$S_{20,w}$ (s)	4.67	4.73	4.65	4.64	4.78
Molecular weight	10.6×10^4	10.6×10^4	10.4×10^4	10.6×10^4	10.7×10^4
Refractive index increment ($\Delta n/\Delta w$) (g. of protein/l.)	1.90×10^{-4}	1.88×10^{-4}	1.89×10^{-4}	1.90×10^{-4}	1.86×10^{-4}
Specific optical rotation ($[\alpha]_D^{25}$)	-60°	-58°	-60°	-60°	-59°

Fig. 4. Plot of mobility against pH for sheep plasma glycoprotein indicating the isoelectric point at pH 4.6. The mobility values are the averages of ascending and descending mobilities.

Fig. 5. Sedimentation of sheep plasma glycoprotein in 0.1M-acetate buffer, pH 4.55, at 59780 rev./min. in the Spinco model E analytical centrifuge. The diagrams were drawn from photographs taken after 20 min., with diaphragm angle 75° (a), and at 60 min., with diaphragm angle 55° (b), after the start of the experiment. The smaller peak in (b) represents about 5% of the solute. The arrow shows the direction of sedimentation.

are presented in Fig. 5. It is clear that the glycoprotein is 95% homogeneous and a heavier-sedimenting contaminant to the extent of 5% is present. The ultracentrifugal behaviour of glycoproteins of

Table 3. *Carbohydrate composition of glycoproteins isolated from plasma of sheep, goat, cow, buffalo and monkey*

Values are expressed in $\mu\text{moles}/0.1\mu\text{mole}$ of glycoprotein. The numbers of determinations are given in parentheses.

	Composition ($\mu\text{moles}/0.1\mu\text{mole}$)				
	Sheep glycoprotein	Goat glycoprotein	Cow glycoprotein	Buffalo glycoprotein	Monkey glycoprotein
Total hexoses (6)	2.86	2.92	6.77	6.76	5.83
Galactose (6)	1.77	1.91	3.46	3.55	2.59
Mannose (6)	1.11	1.02	3.27	3.23	3.31
Glucose	—	—	—	—	—
Total hexosamine (6)	4.22	4.25	5.82	5.81	7.53
Glucosamine (4)	4.16	4.22	5.65	5.68	7.44
Galactosamine	—	—	—	—	—
N-Acetylneuraminic acid (6)	1.51	1.65	2.81	2.75	4.39
Fucose (6)	0.67	0.64	0.38	0.41	1.36

Table 4. *Nitrogen and polypeptide contents and amino acid composition of glycoproteins isolated from plasma of sheep, goat, cow, buffalo and monkey*

Nitrogen was determined in the hydrolysate used for amino acid analysis. A 10 mg. sample of protein was hydrolysed at 105° for 22 hr. Polypeptide determinations were done by the biuret method by taking albumin as a standard (with 100% peptides). The tyrosine/tryptophan ratio was determined according to the method of Bencze & Schmid (1957). The values are the averages of six determinations.

	Sheep glycoprotein	Goat glycoprotein	Cow glycoprotein	Buffalo glycoprotein	Monkey glycoprotein
Nitrogen content (g./100 g.)	12.81	12.67	10.80	10.95	9.52
Polypeptide content (g./100 g.)	77.84	78.21	70.40	70.68	63.80
Amino acid composition ($\mu\text{moles}/0.1\mu\text{mole}$ of glycoprotein)					
Asp	5.90	5.78	5.35	5.26	4.28
Glu	9.05	8.84	5.96	6.04	2.98
Thr	3.31	3.15	2.98	3.04	2.58
Ser	3.64	3.41	2.35	2.44	1.70
Pro	2.05	1.96	2.09	2.02	1.84
Ala	3.55	3.40	3.86	3.97	3.63
Gly	1.87	1.85	1.62	1.56	1.45
Val	4.28	4.23	3.90	3.95	4.34
Leu	6.35	6.43	5.99	5.86	5.75
Phe	2.54	2.64	2.49	2.55	2.39
Tyr	2.18	2.30	2.34	2.28	2.29
Met	0.61	0.58	0.54	0.47	0.55
Lys	6.01	5.97	5.31	5.45	5.09
Arg	3.59	3.67	2.49	2.38	2.82
His	1.34	1.45	0.99	1.08	1.15
Trp	1.49	1.56	1.66	1.56	1.60
Tyrosine/tryptophan ratio	1.46	1.47	1.41	1.45	1.43

other species was similar to that obtained from sheep plasma.

The glycoproteins showed $S_{20,w}$ between 4.64 and 4.78s and the average molecular weight in each case was between 10.4×10^4 and 10.7×10^4 (Table 2).

The other physical properties of the glycoproteins of various species were also similar. The $[\alpha]_D^{24}$ was about -60° and the glycoproteins exhibited absorption maxima at $279m\mu$ with $E_{1\text{cm}}^{1\%}$ values between 10.2 and 10.4. The refractive index

increments of the glycoproteins varied between 1.86×10^{-4} and 1.90×10^{-4} .

The glycoproteins were not precipitated when boiled in water for 10 min. or when dissolved in the following solutions: 0.2M-sulphosalicylic acid, 0.6M-perchloric acid, m-phosphoric acid, 2N-hydrochloric acid or 0.2M-lead acetate, with or without ethanol (15%, v/v) or urea (8M). However,

they were precipitated by 5% (w/v) phosphotungstic acid in 2N-hydrochloric acid, 10% (v/v) trichloroacetic acid and on saturation with ammonium sulphate.

Chemical analysis. The carbohydrate contents of the glycoproteins are presented in Table 3. The glycoproteins show considerable variations in the total hexose, hexosamine, sialic acid and fucose contents. The total hexose content of sheep and goat glycoproteins is about $2.9 \mu\text{moles}$, of cow and buffalo glycoproteins about $6.8 \mu\text{moles}$, and of monkey glycoprotein about $5.8 \mu\text{moles}/0.1 \mu\text{mole}$. All the glycoproteins contain galactose and mannose as the neutral sugar components, and glucose is totally absent. Hexosamine is present as glucosamine, whereas galactosamine is absent. The total hexosamine content of sheep and goat glycoproteins is about $4.2 \mu\text{moles}$, of cow and buffalo glycoproteins $5.8 \mu\text{moles}$, and of monkey glycoprotein $7.5 \mu\text{moles}/0.1 \mu\text{mole}$. The sialic acid content of sheep and goat glycoproteins is about $1.5 \mu\text{moles}$, of cow and buffalo glycoproteins $2.8 \mu\text{moles}$, and of monkey glycoprotein $4.4 \mu\text{moles}/0.1 \mu\text{mole}$, and showed a group relationship among the glycoproteins. Fucose also showed variation in composition and the content of sheep and goat glycoproteins is $0.6 \mu\text{mole}$, of cow and buffalo glycoproteins $0.4 \mu\text{mole}$, and of monkey glycoprotein $1.3 \mu\text{moles}/0.1 \mu\text{mole}$.

The glycoproteins also showed differences in their nitrogen and polypeptide contents (Table 4). The amino acid compositions of the glycoproteins presented in Table 4 indicate very little difference in the contents of threonine, alanine, proline, glycine, valine, leucine, phenylalanine, tyrosine, methionine, lysine, histidine and tryptophan. However, there were differences in the contents of aspartic acid, glutamic acid, serine and arginine between species. The tyrosine/tryptophan ratios

Fig. 6. Effect of inhibition of tryptic activity by glycoproteins. The reaction mixture consisted of $6 \mu\text{g.}$ of trypsin in 0.3 ml. of 2.5 mM-HCl , various concentrations of glycoprotein in water and 1 ml. of 1% (w/v) casein in sodium phosphate buffer, $\text{pH } 7.6$; the total volume was 2.0 ml. Incubation was carried out for 20 min. at 35° . ■, Sheep plasma glycoprotein; □, goat plasma glycoprotein; ●, cow plasma glycoprotein; ○, buffalo plasma glycoprotein; △, monkey plasma glycoprotein.

Table 5. Effect on coagulation by plasma glycoproteins isolated from plasma of sheep, goat, cow, buffalo and monkey

The reaction mixture consisted of 0.2 ml. of plasma, 0.2 ml. of 1% (w/v) glycoprotein solution in $67 \text{ mM-sodium phosphate buffer}$, $\text{pH } 7.2$, and 0.2 ml. of 25 mM-CaCl_2 . Then 0.2 ml. of sheep-brain thromboplastin solution (previously prepared so as to give a normal clotting time of 16 sec.) was added and the time taken for the appearance of clot was noted. The tests were carried out at 35° . The values are the averages of six determinations.

	Normal clotting time (sec.)	Clotting time (sec.) with				
		Sheep glycoprotein	Goat glycoprotein	Cow glycoprotein	Buffalo glycoprotein	Monkey glycoprotein
Sheep plasma	16.1	36.0	35.8	36.9	36.0	37.0
Goat plasma	16.6	36.1	35.2	35.8	36.2	36.5
Cow plasma	16.2	37.6	36.7	37.2	36.2	36.7
Buffalo plasma	16.2	37.2	36.2	35.5	36.4	37.1
Monkey plasma	16.4	36.4	36.6	35.7	35.9	36.9

as determined by two independent methods are in agreement.

None of these glycoproteins revealed the presence of an *N*-terminal amino acid, although ϵ -lysine was identified in the aqueous phase of DNP-protein hydrolysate.

Biological activity. The effect of the glycoproteins on tryptic activity is shown in Fig. 6. All the glycoproteins exhibit inhibitory effects on trypsin to varying degrees. Sheep and goat glycoproteins had similar inhibitory effects, as also did cow and buffalo glycoproteins; the inhibitory effect of monkey glycoprotein differed slightly from those of the other two groups.

The effects of the glycoproteins on the plasma clotting time are shown in Table 5. All the glycoproteins markedly enhanced the clotting time and do not exhibit species specificity to the plasma used in studying the clotting time.

DISCUSSION

Das (1962) isolated a carbohydrate-containing mucoprotein from plasma of sheep that had the mobility of α_1 -globulin. In the present investigation, glycoproteins have been isolated from the plasma of sheep, goat, cow, buffalo and monkey by a modification of the procedure of Weimer *et al.* (1950). The glycoproteins were shown to be homogeneous by agar-gel electrophoresis and moving-boundary electrophoresis; however, ultracentrifugal analysis revealed the presence of a small amount (5–8%) of a faster-sedimenting fraction ($S_{20,w}$ about 7.5s) in each case. The sedimentation-velocity determinations at different protein concentrations indicated that the faster-sedimenting fraction was an impurity and not a polymer of the glycoprotein. All the isolated glycoproteins had similar mobilities in different buffers and the isoelectric points were about pH 4.7. The glycoproteins have $S_{20,w}$ 4.7s. These glycoproteins have an average molecular weight of 105 000, whereas other homogeneous glycoproteins, orosomucoid and submaxillary-gland mucoproteins, have molecular weights of about 45 000. The glycoproteins have similar solubility characteristics in that they do not precipitate in perchloric acid but do so in 5% phosphotungstic acid. They are positive to the Molisch and biuret tests and negative to the hexuronic acid test. Similar optical rotations and refractive index increments are observed for these glycoproteins.

In spite of considerable similarity in physical properties, these glycoproteins show divergence in chemical composition. The glycoproteins can be classified into three groups on the basis of chemical composition and inhibition of tryptic activity: (1) sheep and goat glycoproteins; (2) cow and buffalo glycoproteins; (3) monkey glycoprotein.

Galactose, mannose, sialic acid, glucosamine and fucose are the main carbohydrate constituents; glucose and galactosamine are absent. The carbohydrate composition varies quantitatively between groups, both in the amounts and in the galactose/mannose, total hexoses/sialic acid, total hexoses/fucose and fucose/sialic acid ratios, indicating that the glycoproteins belonging to the three groups differ in carbohydrate composition. Variations between the groups and similarity within them in aspartic acid, glutamic acid, serine and arginine contents are also observed. The absence of an *N*-terminal residue indicates the possibility of carbohydrate residues being linked with the *N*-terminal amino acid.

The acid nature of fetuin (Spiro, 1960) and acid glycoprotein (Yamashina, 1956; Popenoe & Drew, 1957) has been attributed to their sialic acid contents. Experimental evidence for this postulate has been obtained by the selective removal of sialic acid from protein, either by mild acid hydrolysis or by enzymic hydrolysis, resulting in an increase of isoelectric point and also a shift in the titration curves. Spiro (1960) also observed a loss of an approximately equivalent number of strongly acid groups on the removal of sialic acid from fetuin. In the present studies, it is surprising that, in spite of differences in sialic acid concentration among the glycoproteins, the isoelectric point remains the same. The acidity due to sialic acid might possibly be neutralized by positive charges on the protein to maintain the isoelectric point at pH 4.5.

Bundy & Mehl (1959) isolated and partially characterized an α_1 -trypsin inhibitor from human plasma. The glycoproteins that have been isolated from plasma of different animals also inhibit tryptic activity and show similar group relationship in chemical composition.

Nilsson & Yamashina (1958) and, in our Laboratory, Das & Rao (1961) reported a prolongation of clotting time by glycoproteins and the anticoagulant effect has been attributed to an inhibition of transformation of prothrombin into thrombin. The glycoproteins isolated from different sources of plasma prolong the clotting time to the same extent; they do not show any species specificity for the plasma used for clotting studies and could be freely cross-matched.

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