Partial Characterization of the Sialic Acid-Free Forms of α_1-Acid Glycoprotein from Human Plasma

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Some of the properties of sialic acid-free α_1-acid glycoprotein prepared by mild acid hydrolysis (pH 1·6 at 80° for 1 hr.) were compared with those of neuraminidase-treated α_1-acid glycoprotein. Chemically, the former contained less fucose (15%) and amide (2%) residues. Physicochemically, it had undergone certain changes primarily pertaining to the secondary structure, so that the specific optical rotation was more negative than that of the latter. A further expression of this change is probably the difference in the pH range of the resolution into two bands on electrophoresis. The resolution of the glycoprotein prepared by mild acid hydrolysis seems to be extended to more acidic pH values both by starch-gel and free moving-boundary electrophoresis. On ultracentrifugation both preparations appeared homogeneous and sedimented with a rate of 3s. Removal of sialyl residues at different pH values, in the range 1–7, showed that 2 moles of sialic acid/mole of protein are very strongly bound. The two variants of α_1-acid glycoprotein were isolated from pooled sialic acid-free α_1-acid glycoprotein by preparative starch-gel electrophoresis and from selected blood of normal adults by fractionation by solubility and chromatography. Ultracentrifugal and starch-gel electrophoretic analyses at pH 5, with incubation times of 1 or 24 hr., demonstrated that no dissociation–association equilibrium (constant sedimentation coefficient and molecular weight) or isomerization (constant apparent electrophoretic mobilities) exist between the two variants. Therefore these variants are not sub-units of native α_1-acid glycoprotein but represent modifications of naturally occurring proteins. Further, it was shown that the difference in the electrophoretic mobilities between the two variants was not due to any difference in amide content. Immunochemically, the two variants share the same determinants.

α_1-Acid glycoprotein (orosomucoid) (Weimer, Mehl & Winzler, 1950; Schmid, 1953; Gottschalk & Graham, 1966; Jeanloz, 1966) of pooled normal human plasma has been shown to be heterogeneous on starch-gel electrophoresis. Near its isoelectric point (pH 2·7) this protein resolves into several polymorphic forms (Schmid, Binette, Tokita, Moroz & Yoshizaki, 1964), and after removal of its sialyl residues two main bands were noted near pH 5, the isoelectric point of the modified glycoprotein (Schmid, Tokita & Yoshizaki, 1965). The proteins present in these two bands appear to represent genetically determined variants (Schmid *et al.* 1965; Tokita, Burke, Yoshizaki & Schmid, 1966).

The aim of the present study was to describe certain chemical and physicochemical properties of the sialic acid-free α_1-acid glycoprotein, to separate the two variants of this protein from each other and to determine some of their chemical and physicochemical properties.

Part of this study has been presented at meetings of the American Chemical Society (Schmid & Fricke, 1964; Schmid, 1966).

MATERIALS AND METHODS

Preparative procedures

α_1-Acid glycoprotein. α_1-Acid glycoprotein was isolated from Cohn fraction VI of pooled outdated normal human plasma by a method described previously (Schmid & Bürgi, 1961; Bürgi & Schmid, 1961).

For the removal of its sialyl residues, native α_1-acid glycoprotein was subjected to mild acid hydrolysis (pH 1·6 at 80° for 1 hr.) (Zilliken, Brown & Gyorgy, 1955). The hydrolysate was immediately cooled to room temperature, neutralized with 0·1 M-Na_2HPO_4, dialysed and freeze-dried. The resulting product is referred to in this paper as preparation A. Sialic acid was also removed with a highly purified

and concentrated neuraminidase, free of proteolytic and other hydrolytic activities (Cassidy, Jourdian & Roseman, 1965). The enzymically modified glycoprotein is referred to here as preparation B.

Individual α_1-acid glycoprotein was isolated from sera of normal adults as described previously (Schmid *et al.* 1964; Tokita *et al.* 1966). The sialyl residues were cleaved off by mild acid hydrolysis and by neuraminidase as indicated above. The preparations obtained were subjected to starch-gel electrophoresis at pH 5·1, permitting determination of the types of variants of α_1-acid glycoprotein (Schmid *et al.* 1965). About 1–2 mg. of modified protein was sufficient for each analysis (Schmid *et al.* 1965).

Preparative starch-gel electrophoresis. Preparative starch-gel electrophoresis employed for the separation of the α_1-acid glycoprotein variants from one another was carried out at 2° in acetate buffer, pH 5·1 and *I* 0·02. A slot (1·5 mm. × 50 mm. × 18 mm.) was cut in the gel (28·0 cm. × 19·8 cm. × 1·8 cm.) and filled with a solution of 200–350 mg. of sialic acid-free α_1-acid glycoprotein in 1·5 ml. of the same buffer and mixed with partially hydrolysed starch. The electrophoresis was carried out at 550 v and 170 mA for 72 hr. at 4°. The determination of the position of the protein in the starch gel, the elution of the protein from this gel, the digestion of the soluble starch and the isolation by chromatography on DEAE-cellulose at pH 5·0 of the protein from Taka amylase A and the digestion products were carried out essentially as described by Ikenaka, Gitlin & Schmid (1965).

Analytical techniques

The homogeneity of the protein preparations was determined with a Spinco model E ultracentrifuge (the partial specific volume of the native α_1-acid glycoprotein was taken as 0·675 and that of the sialic acid-free glycoprotein as 0·693) equipped with an R.T.I.C. type temperature controller and by starch-gel electrophoresis carried out over the pH range 2·5–10·5 with phosphate, formate, acetate, arsenate, diethylbarbiturate, borate and glycine buffers, all *I* 0·02. The size of the starch gel, the applied voltage and the resulting current were as indicated by Schmid *et al.* (1964, 1965). Free moving-boundary electrophoresis (Perkin–Elmer electrophoresis apparatus, model no. 38) and paper electrophoresis were performed by standard techniques with the following buffers (all *I* 0·1): pH 2·5, phosphate; pH 3·5, formate; pH 4·0 and 5·1, acetate; pH 6·1, cacodylate; pH 8·6, citrate–diethylbarbiturate; pH 10·5, glycine.

The method of Lowry, Rosebrough, Farr & Randall (1951) was employed for the measurement of the polypeptide moiety. Crystallized bovine serum albumin was used as standard.

The orcinol technique served for the determination of the neutral sugars (Sørensen & Haugaard, 1933).

The sialic acid content was measured by the Warren (1959) method. To ascertain complete cleavage of this acid the protein was hydrolysed as a routine with 0·1 N-H_2SO_4 at 80° for 1 hr. In an additional series of experiments samples of a 0·1% solution of the native glycoprotein were adjusted with 0·05 M-Na_2HPO_4 or 0·05 N-HCl to pH values in the range 1–7 and heated at 80° for exactly 1 hr. After immediate cooling the liberated sialic acid of the hydrolysates was determined. After dialysis and freeze-drying of samples of these hydrolysates, the content of remaining sialyl residues was measured and starch-gel electrophoresis carried out at pH 5·1.

The specific optical rotation was measured at room temperature with a Bendix NPL automatic polarimeter (Type 143A) at 546 mμ in 10 cm. tubes. Sucrose was utilized to standardize this apparatus. Samples of glycoprotein stock solutions were adjusted to pH values in the range 2–12 and diluted to a concentration of about 0·3%. The exact protein concentration was determined from extinction measurements and the extinction coefficient of 8·93 (Schmid, 1953) of native α_1-acid glycoprotein. Hence the specific optical rotation values of the modified α_1-acid glycoprotein purposely refer to the same polypeptide content as that of native glycoprotein, rather than to the total weight.

For the immunochemical analysis (Debray-Sachs, Antoine & Fine, 1961; Grabar, 1958) of the α_1-acid glycoprotein variants, sialic acid-free α_1-acid glycoprotein was first resolved into its components by starch-gel electrophoresis at pH 5·1. The gel was then cut into strips, which were placed in a Petri dish and embedded in 2% agar dissolved in diethylbarbiturate buffer, pH 8·6 and *I* 0·1. Appropriate slots were cut and filled with a specific goat antiserum active against α_1-acid glycoprotein (Behringwerke, Marburg/Lahn, Germany). After standing in the cold for 36 hr., the precipitin lines were photographed.

For the determination of amide nitrogen (Conway & Byrne, 1933; Ballentine, 1957) the protein preparations were hydrolysed in sealed tubes under N_2 with 2 N-HCl at 100° for 3 hr. Each hydrolysate was then adjusted to about pH 3·5 with 5 N-NaOH, with methyl orange as indicator. After concentration in an evaporator they were transferred to standard micro Conway vessels. The centre well contained 1 ml. of 0·02 N-H_2SO_4. After 10 ml. of saturated K_2CO_3 had been added to the transferred hydrolysate, the isothermal diffusion of NH_3 was allowed for 6–7 hr. in the cold (Spiro & Spiro, 1962; Marshall & Neuberger, 1960; Chibnall, 1946). Titration of the excess of acid was done with 0·01 N-NaOH to the grey point of the Tashiro indicator (bromocresol green and methyl red, 1:1). As controls the following compounds were employed: $(NH_4)_2SO_4$, α-asparagine, fetuin, lysozyme, sialic acid and glucosamine. Further, all preparations were analysed for preformed NH_3 and for possible release of NH_3 by mild acid hydrolysis selected for cleavage of the sialyl residues from the native glycoprotein. For the latter experiments 200 mg. of protein was used for each determination so that the minute amounts of NH_3 released could be measured with high accuracy.

RESULTS

Sialic acid-free α_1-acid glycoprotein. Preparation A was recovered in a yield of 85% in terms of the weight or 96% in terms of the polypeptide moiety of the native protein. This preparation contained approx. 0·5% of sialic acid. As judged by chromatography of the neutralized and concentrated dialysate of the hydrolysate, a considerable portion (15%) of the fucose had also been cleaved off. Preparation B also contained approx. 0·05% of sialic acid. However, no other sugars had been liberated.

On starch-gel electrophoresis at pH 5·1, prepara-

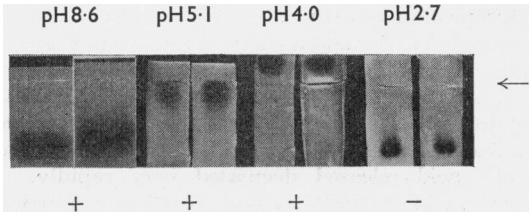

Fig. 1. Starch-gel electrophoresis at various pH values of pooled sialic acid-free α_1-acid glycoprotein prepared by mild acid hydrolysis (preparation A) and by neuraminidase (preparation B). The pH values at which these two preparations were analysed are given on top of each set of patterns. The left pattern of each set represents preparation A and that on the right preparation B. The direction of the electrophoretic migration is indicated by the anodic (+) and cathodic (−) side of the gels respectively. The slot of application of the protein is indicated by the arrow.

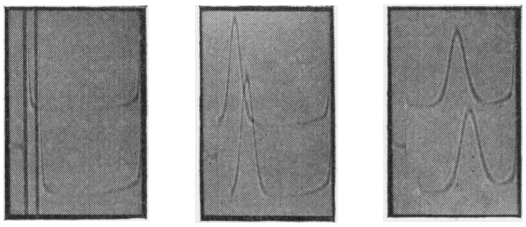

Fig. 2. Ultracentrifugal analysis of preparation A (upper curves, cell with wedged window) and preparation B (lower curves, normal cell) in sodium acetate buffer, pH 5·0 and I 0·1. These pictures were taken after 7 min. (*a*), 60 min. (*b*) and 240 min. (*c*) of centrifugation at 56 100 rev./min. The direction of sedimentation is from left to right.

tion A separated into the expected two bands, which exhibited the same apparent electrophoretic mobilities as those of preparation B, and a faster-moving minor component (component 3) (Fig. 1). Between pH 4 and pH 6 both preparations appeared heterogeneous to various degrees. However, preparation A also appeared heterogeneous at pH 3·5. No separation was observed at the isoelectric point of the native glycoprotein (pH 2·7). At pH 8·6 in borate buffer or at pH 8·0 in arsenate buffer again no resolution was noted. Further, on disk electrophoresis at pH 8·6 these preparations also appeared monodisperse.

On ultracentrifugal analysis carried out over the pH range 2·5–10·5 both preparations revealed symmetrical refractive-index gradient curves (Fig. 2) and sedimentation coefficients of 2·7 s when 1% protein solutions were analysed. Equal distances of the peaks of A and B from their respective menisci

Table 1. *Sedimentation coefficients of preparations A and B at various* pH *values*

pH	$S^{1\%}_{20,w}$ (s) Prep. A	Prep. B
2·5	2·8*	2·5
3·5	2·5*	2·7
5·0	2·7	2·7
6·8	—	2·7
10·5	2·5	2·5

* A faster-moving component was observed in these analyses.

were observed (Fig. 2). No fast or slowly sedimenting material was discerned, except when preparation A was analysed at pH 3·5 and 2·5 (Table 1).

Free moving-boundary electrophoresis of preparations A and B at pH 5·0 in sodium acetate buffer, I 0·1, effected a resolution into two major components after 6 hr. (Fig. 3*a*). The electrophoretic mobilities of the two main components of B were $-0{\cdot}35 \times 10^{-5}$ and $-0{\cdot}60 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$, and indicated that the isoelectric point of the modified protein is close to pH 5 and that the difference in the electrophoretic mobilities of the two main components is very small, perhaps corresponding to 1 charge unit. The relative percentages of the two boundaries appeared to be approx. 1:2. The electrophoretic mobilities of these boundaries of preparation A at this pH were $+0{\cdot}3 \times 10^{-5}$ and $+0{\cdot}7 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$ respectively, and the two boundaries exhibited relative areas like those of preparation B. The free moving-boundary electrophoresis was also extended to pH 2·5 and 10·5. Essential homogeneity was observed at pH 6·1, 8·6 and 10·5. At pH 3·5 in acetate or formate buffer both preparations exhibited a broad descending and a narrow ascending boundary. At pH 2·5 in phosphate buffer both preparations again revealed essentially identical patterns (Fig. 3*b*). However, in glycine buffer at about the same pH, the patterns of preparations A and B differed from one another and each showed two boundaries. On the ascending limb the slower-moving component accounted for the smaller relative area, whereas on the descending limb the faster-moving boundary appeared in relatively smaller concentration (Fig. 3*b*). The electrophoretic mobilities of both preparations were essentially the same in the pH range 2·5–10·5 (Fig. 4). On paper electrophoresis at pH 8·6, 2·9 and even at pH 5 (where a separation occurred on starch gel) no resolution was obtained.

The specific optical rotations, $[\alpha]^{22}_{546}$, of preparation A determined over the pH range 2–12 are

compared with those of preparation B and the native glycoprotein (Fig. 5). Between pH 6 and 10 preparation B and the native protein revealed a plateau with values of 21° and 26° respectively. The optical rotation of preparation B was, over the whole pH range studied, 5–6° less negative than the corresponding values of the native glycoprotein, whereas that of preparation A was about 3° more negative in the same pH range. At more acid or alkaline pH values the rotation increased considerably less steeply than those of preparation B and the native protein. Moreover, the specific optical rotation of preparation B, after heating at pH 1·6 at 80° for 1 hr., was increased by approx. −6° at neutral pH.

Immunoelectrophoretic analysis of the two modified glycoproteins resulted in precipitin lines of the resolved two variants (I and II) that fused completely (Fig. 6).

Cleavage of the sialyl groups from α_1-acid glycoprotein at different pH *values.* In a correlated study the removal of sialyl residues from native α_1-acid glycoprotein was studied as a function of pH when incubated at 80° and for 1 hr. As shown in Fig. 7, at pH 1·0 the sialyl residues were completely cleaved off. At pH 1·6, 5% of the total sialic acid was not hydrolysed off, and at pH 2·1 approx. 10% remained. At higher pH values the percentage of sialic acid released decreased very rapidly. A solution of 'isoionic' protein (pH 3·5) resulted in autodigestion liberating close to 40%. At pH 5 and even 6 small amounts of sialic acid were released, accounting for about 15 and 5% respectively. A drastic change in the rate of release of sialic acid occurred at pH 2·1. Starch-gel electrophoresis at

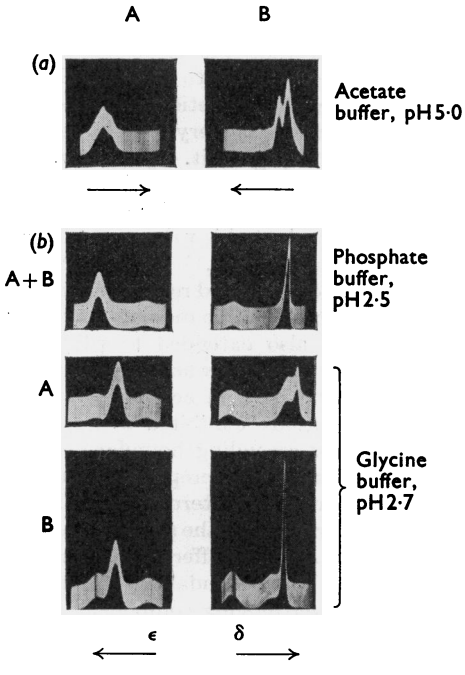

Fig. 3. (*a*) Free moving-boundary electrophoresis of preparations A and B in sodium acetate buffer, pH 5·0 and *I* 0·1. These ascending patterns were taken after 6 hr. of electrophoresis. The direction of migration is indicated by the arrows. (*b*) Free moving-boundary electrophoresis patterns of preparations A and B at pH 2·5 in phosphate buffer and at pH 2·7 in glycine buffer. On the right are the ascending and on the left the descending patterns. The salt boundaries are indicated with δ and ε.

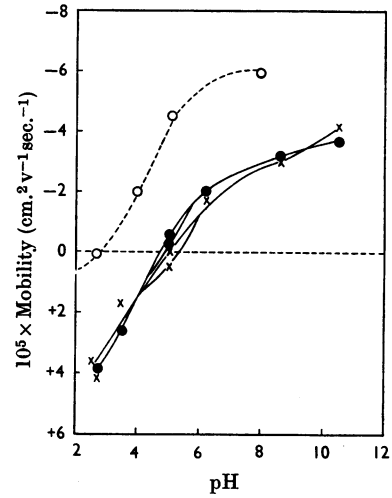

Fig. 4. Electrophoretic mobilities of preparations A (●) and B (×) determined over the pH range 2·5–10·5. For comparison the corresponding values of pooled native α_1-acid glycoprotein (Schmid, 1953) are also included (○).

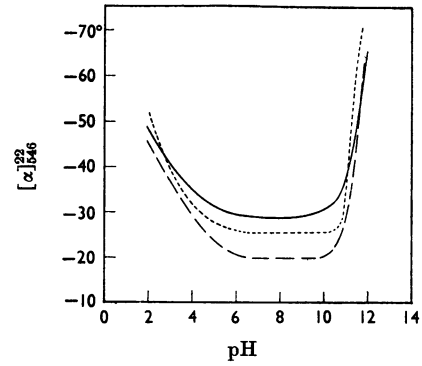

Fig. 5. Specific optical rotation of preparations A (——) and B (— —) and pooled native α_1-acid glycoprotein (------), measured over the pH range 2–12.

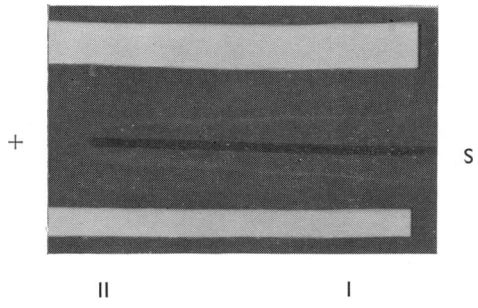

Fig. 6. Immunoelectrophoretic analyses of the variants of α_1-acid glycoprotein. After starch-gel electrophoresis of pooled sialic acid-free α_1-acid glycoprotein at pH 5·0, appropriate gel strips (white) were embedded in agar. A goat antiserum against α_1-acid glycoprotein (S) was placed in the trough between the two gel strips. The precipitin formed a continuous line. Hence the exact location of the two variants (I and II) is not readily discerned.

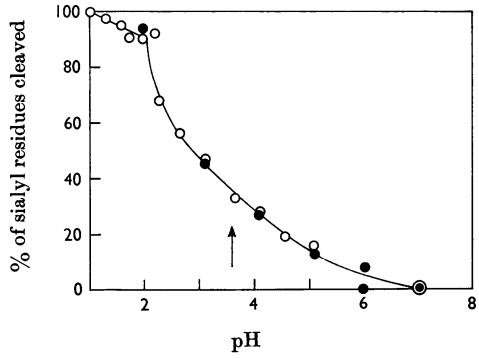

Fig. 7. Content of sialyl residues of pooled α_1-acid glycoprotein after hydrolysis for 1 hr. at 80° at various pH values. The data are based on determination of the cleaved (●) and uncleaved (○) sialic acid. The isoionic point (↑) of this protein is at pH 3·5.

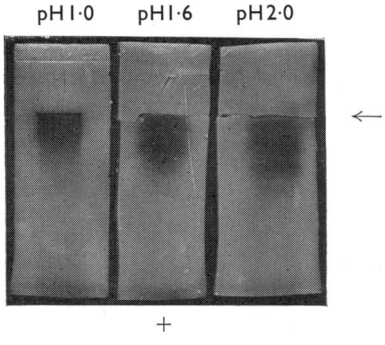

Fig. 8. Starch-gel electrophoresis at pH 5·1 of pooled α_1-acid glycoprotein previously hydrolysed for 1 hr. at 80° and pH 1·0, 1·6 and 2·0 respectively. The position of application of the protein is shown by the arrow.

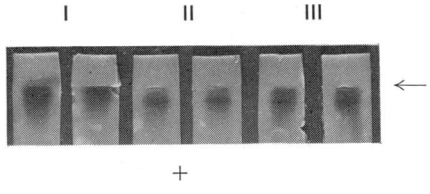

Fig. 9. Starch-gel electrophoresis at pH 5·1 of the two variants (I and II) of α_1-acid glycoprotein and their natural combination (III) prepared by mild acid hydrolysis and by incubation with neuraminidase of the native (individual) α_1-acid glycoprotein preparations. The type of the patterns (I, II or III) is indicated on the top of the gels. The left patterns of each set are derived from the acid-hydrolysed preparations; those on the right are the analysis of the enzymically treated specimens.

pH 5·1 of the resulting protein preparations (Fig. 8) showed that incubation at pH 1·6 led to a pattern that was essentially identical with that of preparation B. Very similar patterns were obtained when the glycoprotein was hydrolysed between pH 1·50 and 1·65. Preparations derived from incubation at 1·0 resulted in an unresolved broad band, whereas those prepared at pH 2·0 yielded patterns similar to those of preparations whose sialyl residues were only partially removed with neuraminidase. These patterns exhibited additional bands with higher migration rates (Schmid *et al.* 1964). The partially separated polymorphic forms of pooled native α_1-acid glycoprotein (Schmid, Binette, Kamiyama, Pfister & Takahashi, 1962), subjected to mild acid hydrolysis as described above, retained the same percentage of the original sialic acid content as pooled α_1-acid glycoprotein included as control in this experiment.

α_1-Acid glycoprotein variants. Preparative starch-gel electrophoretic separation of pooled sialic acid-free α_1-acid glycoprotein yielded the two variants and a faster-moving component (component 3). The recovery accounted for approx. 50% of the starting material. (Control experiments showed that the starch gel itself contributed protein in amounts of approx. 1% of the isolated glycoprotein.) The relative percentage of each component was 40, 50 and 10 respectively. As judged by analytical starch-gel electrophoresis at pH 5·0, variant I and component 3 appeared homogeneous, whereas variant II revealed a purity of about 95%. The apparent electrophoretic mobilities of these preparations were identical with those of the starting material and preparation B. On ultracentrifugal analysis in 0·1 M-sodium chloride each variant revealed a single symmetrical peak sedimenting with a coefficient of approx. 3S, a value identical with that of the starting material and preparations A and B. The schlieren patterns were essentially identical with those shown in Fig. 2.

In view of the higher electrophoretic mobility of the minor component, the sialic acid content was determined on the three fractions. Component 3 contained approx. 1% of sialic acid or about 1 mole/mole of protein. Variant II contained a trace and variant I was devoid of this constituent.

The amide content of the two variants (Table 2) was found to be the same, i.e. 24 ± 1 moles/mole of protein. The same value was obtained for preparations A and B. The native protein and the partially separated polymorphic forms (Schmid *et al.* 1962) contained 25–26 moles/mole of protein. Analysis of appropriate controls showed the following (Table 3): asparagine yielded 1 mole and lysozyme 16 moles of ammonia/mole of compound (Jollès, Jauregui-Adell & Jollés, 1964; Canfield & Anfinsen, 1963), and fetuin (Spiro & Spiro, 1962) 31 moles of ammonia/mole of compound. Glucosamine afforded very little, and sialic acid released a small but significant amount of ammonia. The content of preformed ammonia was very small indeed. However, under the conditions used for cleaving off the sialyl residues (pH 1·6 at 80° for 1 hr.) a distinct amount of ammonia was released from native α_1-acid glycoprotein and a slightly higher amount from the sialic acid-free derivative (preparation A), whereas very little ammonia was released from lysozyme, asparagine and glucosamine.

α_1-Acid glycoprotein variants isolated from blood of normal individuals. Eighteen selected α_1-acid glycoprotein preparations (derived from normal adults), from which sialic acid had been removed either by neuraminidase or by acid (pH 1·6 at 80° for 1 hr.), revealed on starch-gel electrophoresis at

Table 2. *Amide content of the sialic acid-free and native forms of α_1-acid glycoprotein*

The molecular weight of the native glycoprotein was assumed to be 44100 (Weimer *et al.* 1950) and that of the sialic acid-free form and variants 39000, allowing for 11% of sialic acid.

	Amide (moles/mole of protein)
Variant I	24 ± 1
Variant II (neuraminidase-treated)	24 ± 1
Preparations A and B	24 ± 1
Native	26 ± 1

Table 3. *Amide content of native α_1-acid glycoprotein and preparation A and certain control compounds*

	NH_3 or amide (moles of NH_3/mole of compound)		
Compound	Preformed NH_3	NH_3 released by mild acid hydrolysis*	Amide
$(NH_4)_2SO_4$	0·97	—	—
Asparagine	0·0057	0·0067	0·97
Glucosamine	0·0002	0·0014	0·017
Fetuin	—	—	31
Lysozyme	0·0023	0·052	15–16
Sialic acid	0·000	0·000	0·08
Sialic acid-free α_1-acid glycoprotein (prep. A)	0·11	0·82	24
Pooled native α_1-acid glycoprotein	0·19†	0·58	26

* The conditions used for cleavage of the sialyl residues from this glycoprotein were pH 1·6 at 80° for 1 hr.

† Ammonia buffer was initially used for preparation of this protein.

Table 4. *Sedimentation coefficients of the three α_1-acid glycoprotein variant types and of pooled sialic acid-free α_1-acid glycoprotein*

After the first ultracentrifugal run, the protein was carefully redissolved in the buffer of the cell and the cell placed at 25° for subsequent 24hr. For these experiments approx. 1% protein solutions were analysed.

Type	Incubation time at 25° in sodium acetate buffer, pH 5·0 and I 0·1	$S^{1\%}_{20,w}$ (s) Prepared by neuraminidase 1hr.	Prepared by neuraminidase 24hr.	Prepared by mild acid hydrolysis 1hr.	Prepared by mild acid hydrolysis 24hr.
I		2·8	2·9	*	*
II		2·9	2·9	2·8	2·7
III		2·9	2·9	2·8	2·8
Pooled		2·9	2·9	2·7	2·7

* Not enough material available.

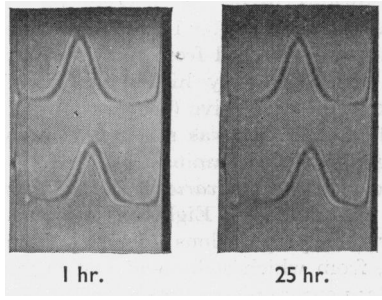

Fig. 10. Ultracentrifugal analysis of variants I and II of α_1-acid glycoprotein incubated at pH 5·0 in sodium acetate buffer, I 0·1, for 1 and 25hr. The normal cell (bottom schlieren curves) contained variant I and the wedged cell (upper curves) variant II. The pictures were taken after 120min. of centrifugation at 56100rev./min. at 25°. The direction of sedimentation is from left to right.

pH 5 six patterns of variant I, six of variant II and six of the natural combination of these two variants (Fig. 9). In a further series of analyses these sialic acid-free specimens were preincubated at pH 5 for 24hr. at 25° before starch-gel electrophoresis was carried out at this pH. The patterns of all three types were independent of the incubation time.

These preparations, prepared by neuraminidase and mild acid hydrolysis, were next studied in the ultracentrifuge at pH 5 in acetate buffer, I 0·1. In an additional series of analyses, samples of these specimens were incubated at room temperature for 25hr. before ultracentrifugation. In all cases homogeneity was observed and the 'peaks' migrated at a rate of 2·8s, independent of the type of variants or the incubation time (Table 4), and yielded patterns (Fig. 10) identical with those of preparations A and B.

DISCUSSION

The cleavage of the sialyl residues from pooled native α_1-acid glycoprotein under optimum acid hydrolysis conditions yielded a protein derivative that revealed on starch-gel electrophoresis at pH 5 a two-band pattern which was essentially identical with that of the corresponding derivatives prepared with neuraminidase. However, these two protein derivatives, although grossly identical, differed from each other with regard to several properties. Chemically, part of the fucose in addition to sialic acid was cleaved off by the mild acid hydrolysis employed, agreeing with earlier reports (Hughes & Jeanloz, 1966; Eylar & Jeanloz, 1962). It should be noted that complete removal of sialic acid from this glycoprotein requires relatively strong hydrolysis, as noticed by Satake, Okuyama, Ishihara & Schmid (1965), leading to further changes readily detectable on starch-gel electrophoresis. It is equally difficult to remove sialic acid completely by neuraminidase (Schmid *et al.* 1965). Physicochemically, the two derivatives of this protein differed from one another as follows. The specific optical rotations at different pH values of the protein prepared by mild acid hydrolyiss were always considerably more negative than those of the enzymically modified protein. These findings probably indicate that mild acid hydrolysis affects native α_1-acid glycoprotein in two ways: (1) removal of the sialyl residues accompanied with a change in the optical rotation from $-26°$ to $-19°$ of preparation B; (2) alteration of the conformation accompanied with a change in the rotation from $-19°$ to $-28°$, as the only minute chemical difference between the two glycoprotein modifications lies in the fucose content. This is in agreement with an earlier report (Schmid & Kamiyama, 1963) on

native α_1-acid glycoprotein, which, when heated at pH 5·8 at 100° for 1 hr., underwent a change in specific optical rotation from −22° to −33°, and with the finding that mild acid hydrolysis of preparation A effected a corresponding increase in the rotation. The optical rotation–pH curve of preparation A differed further in its course from those of the native protein and preparation B. The increase in negative optical rotation below pH 4 and above pH 10 was smaller than that of the other two preparations mentioned. The observation that the specific optical rotations of the enzymically modified glycoprotein were 6° less negative than those of the native protein studied over the whole pH range agrees in principle with previously reported values measured at the *D* line by Schmid & Kamiyama (1963) and is at variance with the report by Bezkorovainy (1965). Further, it should be noted that mild acid hydrolysis affects glycoproteins more than is generally assumed. Nevertheless, these changes are relatively subtle and do not affect the identification of α_1-acid glycoprotein variants on starch-gel electrophoresis.

On ultracentrifugal analysis carried out over the pH range 2·5–10·5 both preparations were monodisperse and sedimented with the same coefficient of 2·8 s, except that preparation A revealed a minor component at pH 3·5 and 2·5. This finding is in agreement with the value observed by Popenoe & Drew (1957) at pH 4·7. As the sedimentation and diffusion coefficients of both preparations were essentially equal to those of native α_1-acid glycoprotein although two components were observed on electrophoresis at pH 5, it was concluded that the molecular weight of preparations A and B is equal to that of the native form except for the difference in the sialic acid content. Smith, Brown, Weimer & Winzler (1950) have shown that the sedimentation coefficient of native glycoprotein is also independent of the pH and that the molecular weight is 44100, confirmed by Bezkorovainy (1965). Sia (1965), employing the field-relaxation technique, determined the molecular weight of native α_1-acid glycoprotein at pH 2·7 and of preparation B at pH 5·4 and reported values of 44000 and 40000 respectively.

Free moving-boundary electrophoresis revealed a resolution into two components within a limited pH range. This pH range appeared to vary to a certain extent depending on the technique used for removal of the sialic acid. At pH 2·5 preparations A and B revealed a single boundary in phosphate buffer, whereas in glycine buffer at about the same pH two boundaries were observed whose shapes were unlike those noted at pH 5 and hence were interpreted to be indicative of protein–buffer ion interactions. Similar observations have been reported for other proteins at other pH values (Cann, 1966; Katz, Mechanic & Glimcher, 1965; Schmid & Polis, 1960).

The cleavage of the sialyl residues from α_1-acid glycoprotein at decreasing pH values revealed the following interesting observation. Two moles of sialic acid of pooled α_1-acid glycoprotein and of the partially separated polymorphic forms thereof appeared to be very strongly bound and required much stronger acidic conditions for their removal.

The variants of α_1-acid glycoprotein were isolated from the pooled sialic acid-free glycoprotein after starch-gel electrophoresis in yields proportional to the ratio observed on analytical starch-gel electrophoresis. They revealed homogeneity on ultracentrifugation, indicating that no gross alteration or even denaturation had taken place during isolation. The observed sedimentation coefficient of 2·8 s of the two variants and of the sialic acid-free and native glycoprotein at pH 5 and 7 also demonstrate that the former are not sub-units of the latter. Moreover, these variants showed the same apparent electrophoretic mobilities on starch-gel electrophoresis at pH 5 as the two components of sialic acid-free α_1-acid glycoprotein prepared either by mild acid hydrolysis or enzymically.

The α_1-acid glycoprotein variants isolated directly from blood of previously typed normal individuals (Schmid *et al.* 1965) without preparative starch-gel electrophoresis, and prepared with neuraminidase, were analysed by starch-gel electrophoresis at pH 5 and by ultracentrifugation. These analyses were carried out in pH 5 buffer after incubation times of 1 and 24 hr. The electrophoretic and ultracentrifugal behaviour of each sample was found to be independent of the incubation time. In other words, variant I or II did not yield a type III pattern. Hence the starch-gel electrophoretic patterns of types I, II and III are not due to an association–dissociation equilibrium [such reactions have been discussed by Schachman (1959) and Gilbert (1959) and summarized by Katz *et al.* (1965)] or isomerization reaction (Cann, 1966; Schmid & Polis, 1960; Petersen & Foster, 1965), and therefore represent true variants characterized with constant sedimentation coefficients and electrophoretic mobilities. Further, the two variants are also not conformational isomeric forms, as described by Craig, King & Crestfield (1963) for ribonuclease. It is important to add that these variants, when prepared by mild acid hydrolysis, revealed the same properties.

Immunoelectrophoretic analysis demonstrated that the two variants possess the same immunochemical determinants, which are shared with the native glycoprotein and hence with its polymorphic forms (Schmid *et al.* 1962). These data show that sialic acid is not a constituent of these determinants.

In view of the suggestion that the microheterogeneity of the 7 s globulins (Feinstein, 1962) may be

a consequence of differences in the amide content, the two variants were therefore analysed for this constituent. Both variants showed the same amide content. Native α_1-acid glycoprotein yielded slightly higher values agreeing essentially with that reported by Weimer *et al.* (1950) and Goa (1961). The difference can probably be explained by the formation of ammonia during the destruction of the sialic acid liberated from the native glycoprotein. Hence the polymorphic forms and variants of α_1-acid glycoprotein are probably not due to a difference in the amide content.

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