Imides could be of two types: cyclic imides involving a single terminal aspartyl residue; and crosslinking imides involving the side-chain carboxyl groups either of terminal or internally located aspartyl residues on separate polypeptide chains. From the standpoint of crosslinking in collagen, interchain imide or ester bonds could serve equally well.

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Studies on the Structure of α_1-Acid Glycoprotein. III. Polymorphism of α_1-Acid Glycoprotein and the Partial Resolution and Characterization of Its Variants*

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α_1-Acid glycoprotein isolated from pooled normal human plasma was resolved on starch gel electrophoresis between *p*H 2 and 4 into seven variants. The best separation was obtained at *p*H 2.9, *i.e.* near the isoelectric point of this protein. It was demonstrated that this resolution is a consequence of the polymorphism of α_1-acid glycoprotein. The sialic acid–free form of pooled α_1-acid glycoprotein was separated into three zones by starch gel electrophoresis near its isoelectric point (*p*H 5.0). A large-scale method is described for the partial resolution of the variants of human α_1-acid glycoprotein by chromatography on diethylaminoethyl cellulose at *p*H 5.0. The obtained variants exhibited chemical and physical-chemical similarities and differences.

α_1-Acid glycoprotein (Schmid, 1953; Weimer *et al.*, 1950), as judged by classical criteria of purity, is one of the most highly purified proteins of normal human plasma. Thus, homogeneity of this protein was observed by free electrophoresis and ultracentrifugation over the *p*H range from 1 to 13, chromatography on Amberlite IRC-50, immunochemical and terminal amino acid analyses (Schmid, 1963). Moreover, monodispersity was also observed on starch gel electrophoresis at *p*H 8.6 (Smithies, 1959).

In the present paper a systematic starch gel electrophoretic investigation over the *p*H range from 1.4 to 8.6 is described which led to the discovery of the polymorphism of α_1-acid glycoprotein (Schmid and Binette, 1961). This observation suggested a study of the differences and similarities of the variants of this plasma protein.

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Material

Pooled normal human α_1-acid glycoprotein was prepared as described earlier (Schmid, 1953). To obtain this protein in immunochemically pure form chromatography on Amberlite IRC-50 (Schmid *et al.*, 1958) was employed subsequent to the fractionation by differential solubility. The size of the plasma pools from which this glycoprotein was isolated varied between 300 and 707 liters, derived from 1350 and 3170 donors, respectively.

Partially hydrolyzed starch was bought from Connaught Medical Research Laboratory, Toronto, Canada.

Vibrio cholera neuraminidase was obtained from Prof. H. E. Schultze, Behringwerke, Marburg/Lahn, Germany, and from Dr. E. H. Eylar, Harvard Medical School, Boston. The enzymatic reaction for the removal of sialic acid was carried out at *p*H 6.0 in $\Gamma/2$ 0.05 phosphate buffer.

Goat serum active against human α_1-acid glycoprotein was received from Dr. S. Cooperband, Harvard Medical School, Boston.

Methods

Starch gel electrophoresis performed according to the technique of Smithies (1959) was carried out in the *p*H region from 1.4 to 8.6. The buffers used to fill the two electrode compartments of the electrophoresis box had an ionic strength of 0.1. These buffers, of which the acidity was determined with a Radiometer *p*H meter, were diluted 5-fold and employed for the preparation of the starch gels. As the best separation was obtained at a very acid *p*H value, it appears important to describe certain details of the technique employed. The $\Gamma/2$ 0.1 phosphate buffer was prepared by adding to a 0.1 M NaH_2PO_4 solution phosphoric acid (85%, d = 1.7) until the desired *p*H of 2.68 to 2.69 was obtained. To adjust the *p*H of 1 liter approximately 1.77 ml of this phosphoric acid was required. Care should be taken that the *p*H be within the indicated range. The *p*H of the diluted buffer varied between 2.78 and 2.80 and, after mixing with the appropriate amount of partially hydrolyzed starch, between 2.88 and 2.89.

The starch gel blocks had the following dimensions: 24.5 × 10.5 × 0.5 cm. Four slots (1.6 cm long, 0.10 – 0.15 cm wide, 0.5 cm deep) were inserted equally spaced on a line 8 cm from one of the shorter edges of the plate. For each analysis 2 mg of protein was dissolved in 0.10 ml of the $\Gamma/2$ 0.02 phosphate buffer mixed with washed starch granules and transferred into one of the slots. Three layers of thick filter paper were used to make the electrical contacts. For the electrophoresis which was carried out in horizontal position in the cold for 6 hours, 150 v was applied on the starch block resulting in a current of 74 to 80 mA. The heat developed in the gel caused the petrolatum cover to soften slightly. After the run the gel was cut, stained with Amidoschwarz 10B (250 ml methanol, 250 ml water, 50 ml glacial acetic acid, and 0.5 g dye), and decolorized with the same solvent mixture without dye. The staining capacity of α_1-acid glycoprotein was found to be weaker than that of albumin, approximately corresponding to the polypeptide moiety of 66% of this α_1-globulin. The individual protein bands could best be observed by transilluminating the gel. The relative percentage of the bound dye of each band was obtained by measuring directly the absorbance of the starch gels with the aid of a Spinco Analytrol. Photographs of the gels were taken with red filters. The relative intensity of each band was also calculated from densitometer measurements of such negatives with a microdensitometer, Model E 12, III (Goyce, Loebel & Co., Ltd.). Both procedures led to essentially the same results.

Elution of the protein from the starch gel was performed by electrophoresis (Moretti *et al.*, 1958). Removal of the soluble starch from the protein was effected by adsorption on Dowex 2-X8, mesh 200–400 (dePailierets *et al.*, 1959).

Immunochemical identification (Poulik, 1957) of the protein variants after starch gel electrophoretic separation was carried out as follows: The *p*H 2.9 starch gel strip containing the separated variants was placed in a Petri dish which was then filled with agar to the level of the starch gel. The agar was previously prepared by dissolving it in *p*H 7.3, $\Gamma/2$ 0.1 phosphate buffer. Parallel to the starch gel block two troughs were cut out into which the antiserum was placed.

To test for the possible cleavage of sialic acid by incubation in *p*H 2.7 phosphate buffer, native α_1-acid glycoprotein was dissolved in $\Gamma/2$ 0.1, *p*H 2.7 phosphate buffer at 25° and 4°, respectively, to obtain a 10% solution which was adjusted to *p*H 2.7 by addition of phosphoric acid. Aliquots containing 300 mg of glycoprotein were withdrawn at 6 and 24 hours of incubation. They were neutralized with Na_2HPO_4 and dialyzed exhaustively against water. The dialysates were concentrated and adjusted to 5 ml. The dialyzed sialic acid was determined according to Werner and Odin (1952), hexose by the orcinol method of Sørensen and Haugaard (1933), and hexosamine (hydrolysis with 4 N HCl at 100° for 4 hours) according to Masamune and Yosizawa (1955).

The isoionic points of the glycoprotein fractions were determined on a 1% aqueous solution which had previously been desalted by passage through an ion exchange resin column (Dintzis, 1952). As reference *p*H 4.0 standard buffer was used.

The extinction coefficients were determined at 278 mμ. The dry weight of the protein fractions was measured after heating at 80° over P_2O_5 at a pressure of 0.01 mm Hg for 4 hours. Further

drying for 17 hours under the same conditions did not lead to a change of the dry weight.

The molar ratio of tyrosine to tryptophan was derived by the spectrophotometric method of Bencze and Schmid (1957) from the slope of the tangent to the absorption curve of the protein, obtained at *p*H 13, where tyrosine is fully dissociated. The content of tyrosine and tryptophan was calculated from the optical density of the higher of the two absorption maxima and the ratio of these two amino acids.

The amino acid analyses were done by Drs. M. Brenner and R. Weber, University of Basel, by the automatic procedure of Moore *et al.* (1958). For each glycoprotein fraction the amino acid composition was determined on a 24-hour and a 72-hour hydrolysate. In Table III the average of these two series of values is reported, except for threonine, serine, and cystine, of which the values were obtained by extrapolation to zero-hour hydrolysis time.

Results

Starch Gel Electrophoresis of α_1-Acid Glycoprotein from pH 1.4 to 8.6.—At *p*H 8.6 in borate (Smithies, 1959) or Tris-EDTA-borate buffer (Poulik, 1957) α_1-acid glycoprotein exhibited a single zone. The apparent electrophoretic mobility was higher than that of albumin and corresponded to that of the slowest moving prealbumin (Poulik, 1957). In *p*H 7.5 cacodylate, *p*H 6.5 phosphate, *p*H 5.5 citrate, and *p*H 4.6 acetate, again no separation was obtained. In *p*H 4.0 acetate or itaconate, *p*H 3.6 and *p*H 2.8 formate or phosphate, and *p*H 2.0 phosphate, resolution into seven zones was observed. The best separation was obtained in *p*H 2.9 phosphate buffer (Fig. 1). Under these conditions no protein remained in or immediately near the slot or migrated toward the anode. The relative percentages of these seven zones arranged according to increasing electrophoretic mobility were 9, 20, 21, 24, 14, 10, and 2, respectively, in terms of bound dye. At *p*H 1.4 in glycerophosphate buffer a broad zone and four minor bands were observed. As expected, the distance and the direction of the movement of the protein variants depended on the *p*H of the buffer used.

Immunochemical Analysis of the α_1-Acid Glycoprotein Variants.—As seen in Figure 2, the seven α_1-acid glycoprotein variants resolved on starch gel electrophoresis at *p*H 2.9 reacted essentially equally with the goat serum active against human pooled α_1-acid glycoprotein. The resulting single precipitin zone which appeared on each side of the starch gel 4 days after the experiment had been started indicated that the protein variants present in the seven zones are immunochemically identical.

Effect of Incubation of α_1-Acid Glycoprotein in Acid Phosphate Buffer.—Incubation of α_1-acid glycoprotein at *p*H 2.7 and 4° for 24 hours did not lead to the release of sialic acid. At 25° only 0.1% of this acid was split off after 6 hours and 1% after 24 hours. In additional control experiments in which α_1-acid glycoprotein was dialyzed under the same conditions without incubation at *p*H 2.7, no sialic acid was hydrolyzed off.

Two per cent solutions of α_1-acid glycoprotein in *p*H 2.9, $\Gamma/2$ 0.1 phosphate buffer preincubated at 24° for 1 and 3 days, respectively, were analyzed at *p*H 2.9 by starch gel electrophoresis concomitantly with untreated glycoprotein. All three specimens exhibited identical patterns. As expected, the same results were obtained from the analysis of the corresponding solutions which had been incubated for the same periods of time at 4°.

Investigation of the α_1-Acid Glycoprotein Variants After Starch Gel Electrophoresis at pH 2.9 and Elution from Starch Gel.—On starch gel electrophoresis in *p*H 8.6 borate buffer the fractions containing the fast (three zones) and slow moving (four zones) variants, respectively, appeared homogeneous and exhibited the same apparent electrophoretic mobility as untreated native α_1-acid glycoprotein. On ultracentrifugal analysis a single peak with a sedimentation coefficient of 3.1 *S* was observed. Repeated starch gel electrophoresis of the "slow" variants at *p*H 2.9 in $\Gamma/2$ 0.02 phosphate buffer revealed four zones of which the apparent mobilities were identical with those of the four bands from which this fraction was recovered.

Effect of Chromatography on Amberlite IRC-50 upon α_1-Acid Glycoprotein.—α_1-Acid glycoprotein prepared by the solubility procedure (Schmid, 1953) was analyzed before and after passage at *p*H 5.2 in citrate buffer through a column of Amberlite IRC-50. No differences between the two preparations were observed as judged by starch gel electrophoresis at *p*H 2.9.

Effect of Ethanol at Low Temperature upon α_1-Acid Glycoprotein.—α_1-Acid glycoprotein was also isolated from the supernatant solution of Cohn's fraction V, Method 10 (19% ethanol, −5°, 0.02 M $ZnAc_2$ and *p*H 5.8 [Lever *et al.*, 1951]) by addition of precooled ethanol and 1 M barium acetate to give 40% ethanol and 0.02 M barium acetate. The resulting fraction VI was chromatographed on Amberlite IRC-50 to isolate the glycoprotein in the pure state. The α_1-acid glycoprotein pattern obtained on starch gel electrophoresis at *p*H 2.9 did not differ from a corresponding pattern of α_1-acid glycoprotein prepared by method 6 (40% ethanol, −5°, *p*H 4.9 [Cohn *et al.*, 1946]).

The same plasma protein isolated from "SPPS" solution (no ethanol, +2°, *p*H 7.4) (method 12 [Surgenor *et al.*, 1960]) and from outdated plasma by chromatography on DEAE column (Peterson and Sober, 1960) afforded patterns on starch gel electrophoresis that were essentially identical with those mentioned above.

Reproducibility.—Four α_1-acid glycoprotein preparations were analyzed simultaneously at *p*H 2.9 on starch gel electrophoresis. They revealed

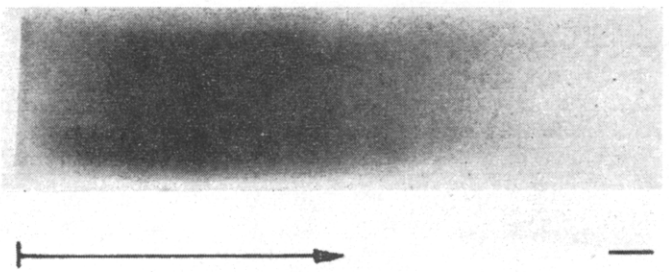

FIG. 1.—Starch gel electrophoresis of pooled normal human α_1-acid glycoprotein at *p*H 2.9 in $\Gamma/2$ 0.02 phosphate buffer. The slot of application of the protein was located at the left end of the starch gel block. The direction of the electrophoretic movement of the glycoprotein variants was toward the cathode, as indicated by the arrow. The fastest moving variant traveled 4.5 cm. The starch gel was stained with Amidoschwarz 10B. The pattern obtained showed seven zones.

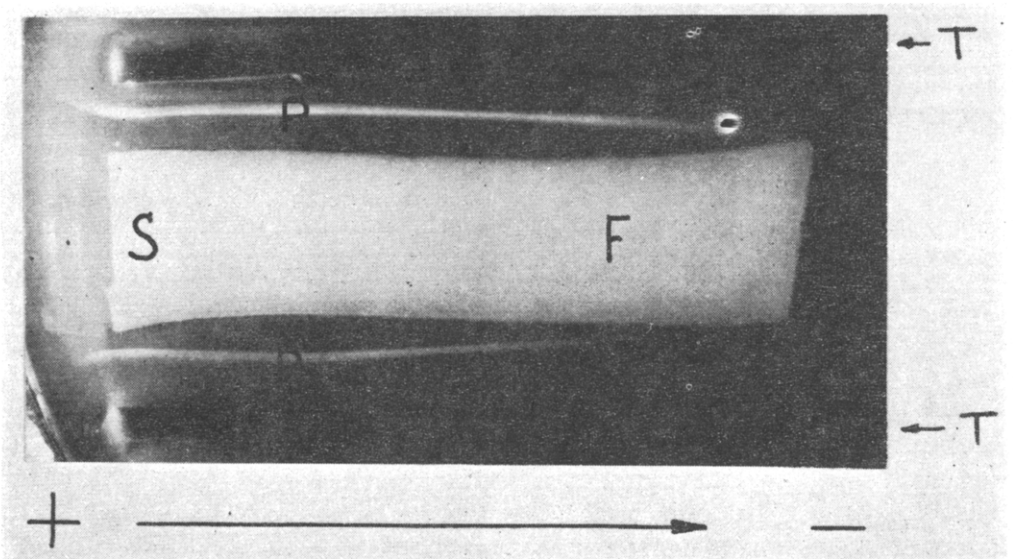

FIG. 2.—Starch gel immunoelectrophoresis of pooled normal α_1-acid glycoprotein. The starch gel electrophoresis was carried out at *p*H 2.9 in $\Gamma/2$ 0.02 phosphate buffer. The direction of the electrophoretic movement of the protein variants indicated by the arrow on the bottom of the figure was toward the cathode. The position of the slowest and fastest moving variant is indicated by "S" and "F," respectively. The actual distance between these two bands was 4.5 cm. In the second step of the analysis the obtained starch gel (white in figure) was immersed in agar (black in figure) into which troughs ("T") were cut for the goat antiserum. The solid agar had previously been dissolved in *p*H 7.3, $\Gamma/2$ 0.1 phosphate buffer. The formed precipitin lines ("P") are represented by the white line on both sides of the starch gel block.

the same pattern, *i.e.* seven zones each. Further, one of these preparations was used as standard over a period of 6 months during which time it was analyzed 30 times. The patterns obtained were essentially identical.

Starch Gel Electrophoresis of Sialic Acid–Free α_1-Acid Glycoprotein.—At *p*H 2.9 and 8.6 the sialic acid–free α_1-acid glycoprotein prepared by treatment with neuraminidase exhibited a single band. The mobility at *p*H 8.6 was approximately half that of the native form. However, at *p*H 5.0 three bands were observed. The apparent electrophoretic mobility at this *p*H was very low so that the bands were located near the slot of application, confirming that the isoelectric point of the enzymatically modified glycoprotein is near *p*H 5.0 (Schmid *et al.*, 1959). The *p*H range in which this resolution took place was found to be relatively narrow. At *p*H 4.5 and 5.5 two main bands and one slowly moving minor zone were noted.

Chromatographic Separation of the α_1-Acid Glycoprotein Variants.—Twenty-three grams of α_1-acid glycoprotein which had not been purified by Amberlite IRC-50 chromatography were dissolved in 750 ml of *p*H 5.0, $\Gamma/2$ 0.012 acetate buffer and applied at 4° to a column of diethylaminoethyl (DEAE)–cellulose (Peterson and Sober, 1960) previously equilibrated against the same buffer. The column was 12.0 cm high and 8.0 cm in diameter. The flow rate was adjusted to 3.0 ml per minute. After washing with 500

TABLE I

PHYSICAL CHEMICAL AND CHEMICAL PROPERTIES OF THE PARTIALLY RESOLVED VARIANTS OF NORMAL HUMAN α_1-ACID GLYCOPROTEIN

Fraction No.	Wt. (g)	Sedimentation Coefficient $s^{1\%}_{20,w}$ (S)	Isoionic Point (pH)[a]	Polypeptide Moiety (Biuret) (%)	Extinction Coefficient $E^{1\%}_{1\,cm.,\,278\,m\mu}$	Tyrosine Tryptophan Molar Ratio	Sialic Acid (%)	Hexose (%)	Glucosamine (%)
1[b]	0.7	—	(3.89)	—	—	—	(9.08)	(10.2)	(11.2)
2	1.2	3.2 ± 0.1[c]	3.54 ± 0.02[c]	66 ± 2[c]	9.2 ± 0.1[c]	2.90 ± 0.05[c]	11.3 ± 0.5[c]	14.0 ± 0.7[c]	14.6 ± 0.7[c]
3	2.7	3.0	3.52	68	9.0	2.84	11.6	14.5	14.9
4	3.6	3.0	3.57	69	9.0	2.85	11.5	15.3	14.7
5	1.4	3.2	3.45	69	9.0	2.81	12.0	15.9	15.6
6	2.6	3.0	3.42	70	9.2	2.74	12.6	15.8	16.8
7	2.9	3.0	3.42	68	9.0	2.80	11.9	16.9	15.8
8	4.6	3.2	3.39	66	8.4	2.85	11.9	14.9	15.1
9	2.2	3.0	3.32	61	7.6	2.66	12.5	14.4	14.8
Unfractionated, pooled normal α_1-acid glycoprotein		3.0[d]	3.53[a]	66[e]	8.93[e]	2.90[f]	11[g]	15[g]	14[g]

[a] Not corrected for excess H-ions (Eylar, 1958). [b] This fraction contained small amounts of albumin and α_2-globulins. [c] The standard error is indicated for the determination of fraction 2 only, but is the same for the corresponding measurements of the subsequent fractions. [d] (Smith *et al.*, 1950). [e] (Schmid, 1953). [f] (Bencze and Schmid, 1957). [g] (Yamashina, 1956).

ml of *p*H 5.0, Γ/2 0.02 acetate buffer, a further 3 liters of a *p*H 5.0 acetate buffer of which the ionic strength had been increased from 0.02 to 0.20 with a gradient were added to the column. Although the absorbance of the effluent protein solution indicated the presence of three main protein fractions (Fig. 3), this solution was separated into nine fractions of varying volume as indicated in Figure 3 and dialyzed against cold distilled water and lyophilized. Thus, the three main glycoprotein fractions included fractions 1 to 3, 4 to 8, and 9, respectively. The recovery of the protein was 95%.

Characterization of the α_1-Acid Glycoprotein Variants.—In spite of the appearance of the three main fractions, each of the nine α_1-acid glycoprotein fractions (Fig. 3) was analyzed in terms of some physical chemical and chemical properties. Paper electrophoresis at *p*H 8.6 in Γ/2 0.1 citrate diethylbarbiturate buffer and ultracentrifugation revealed homogeneity except for the first fraction, which contained small amounts of albumin and α_2-globulins. All fractions exhibited an electrophoretic mobility corresponding to that of the α_1-globulins (Schmid, 1953) and sedimented with coefficients varying in the expected range from 3.0 and 3.2 *S* (Smith *et al.*, 1950) (Table I). The third and the ninth fractions were also analyzed by free moving boundary electrophoresis at *p*H 4.0 in Γ/2 0.1 acetate and at *p*H 2.65 in Γ/2 0.1 phosphate buffer. These two fractions appeared homogeneous in both buffers. At *p*H 4.0 essentially identical electrophoretic mobilities of -2.2×10^{-5} cm²/V.sec were observed which corresponded to that reported earlier for pooled α_1-acid glycoprotein (Schmid, 1953). However, at *p*H 2.6 fraction 3 was essentially immobile ($+0.07 \times 10^{-5}$ cm² V.sec) whereas fraction 9 showed a mobility of -0.36×10^{-5} cm²/V.sec.

Starch gel electrophoresis of the nine chromatographically separated α_1-acid glycoprotein fractions was performed at *p*H 2.9 in Γ/2 0.02 phosphate buffer. The patterns obtained (Fig. 4) demonstrated that the protein eluted first from the column resolved into seven bands. However, the apparent electrophoretic mobility of the fastest-moving bands was higher than that of the corresponding band of the unfractionated glycoprotein, indicated in Figure 4 with REF. The

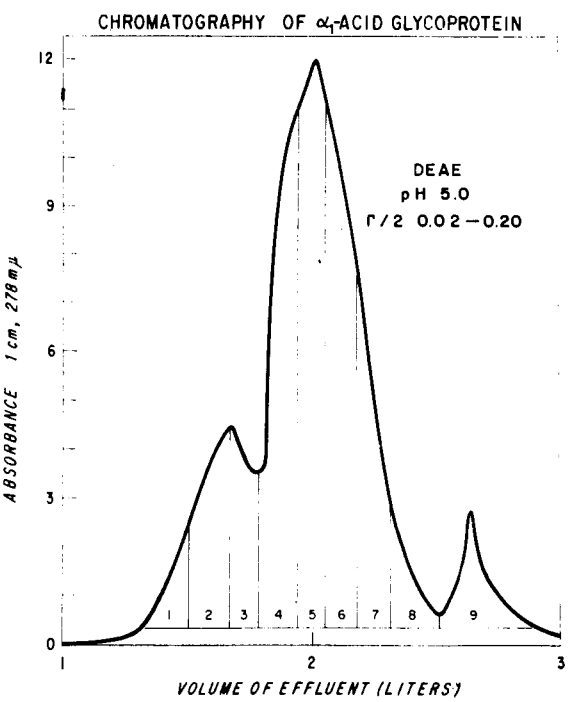

FIG. 3.—Chromatography of pooled normal human α_1-acid glycoprotein on DEAE cellulose at *p*H 5.0 with an ionic strength gradient from 0.02 to 0.20. The absorbance of the effluent was measured at 278 mμ and plotted against the volume of the effluent. The effluent was divided into nine fractions of varying volume as indicated on the abscissa by the numbers 1 to 9.

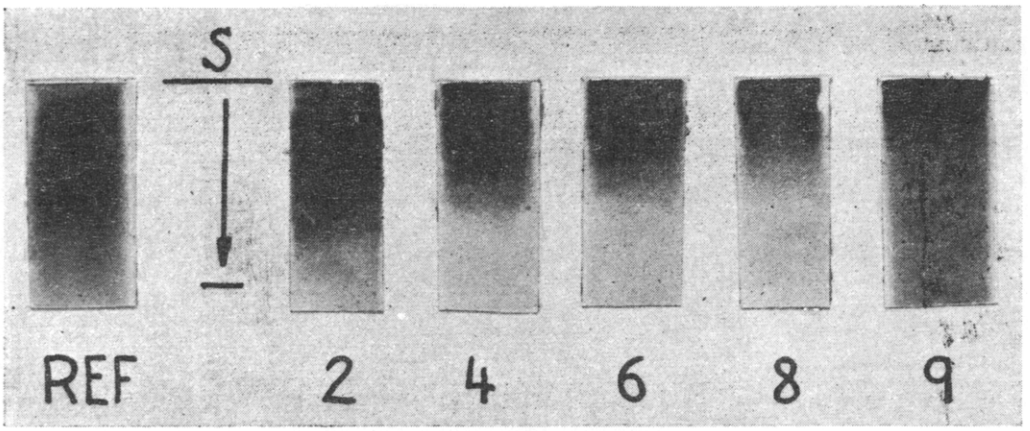

FIG. 4.—Starch gel electrophoretic patterns of fractions 2, 4, 6, 8, and 9 containing the partially separated variants of α_1-acid glycoprotein. These fractions were obtained by chromatography on DEAE cellulose. As reference, unfractionated pooled normal human α_1-acid glycoprotein, designated with REF, was used.

same observation was made with respect to the band that represented the major component. The subsequent fractions separated into a smaller number of components. The mobility of the fastest-moving variant and that of the main component gradually decreased with each successive fraction. The last fraction contained the slowest-moving variants in relatively pure form. In Table II the relative distribution of the α_1-acid glycoprotein variants designated with Greek letters present in fractions 2, 4, 6, 8, and 9 is presented.

TABLE II

THE RELATIVE PERCENTAGE OF THE α_1-ACID GLYCOPROTEIN VARIANTS IN THE FRACTIONS OBTAINED BY CHROMATOGRAPHY OF α_1-ACID GLYCOPROTEIN

α_1-Acid Glycoprotein Fractions, No.	α_1-Acid Glycoprotein Variants (Expressed in Relative Percentage)						
	α[a]	β	γ	δ	ϵ	ζ	η
2	3	10	34	20	20	10	3
4	19	41	19	18	3	—	—
6	55	36	16	3	—	—	—
8	62	32	6	—	—	—	—
9	81	16	3	—	—	—	—
Unfractionated α_1-acid glycoprotein	9	20	21	24	14	10	2

[a] Variant α exhibited the lowest and variant η the highest apparent electrophoretic mobility at *p*H 2.9.

Fractions 2, 5, and 8 exhibited essentially the same amino acid composition (Table III) within the error of the method employed. The variation in the cystine content is probably due to the difficulty in determining this amino acid. Also the difference in the serine content is possibly due to known destruction occurring during hydrolysis of proteins. Further analyses on highly purified variants are needed to explain whether the increased content of arginine of fraction 5 is significant. The lower tyrosine content of fraction 9 was confirmed by independent spectrophotometric determinations. The analysis of fraction 9 differed in that the tyrosine content appeared lower than that of the other fractions, amounting to 9.5 moles of tyrosine per mole of protein as compared with 11 moles per mole of the other variants.

TABLE III

AMINO ACID COMPOSITION OF SOME OF THE PARTIALLY SEPARATED VARIANTS OF α_1-ACID GLYCOPROTEIN (Expressed in Moles per 44,100 g of Protein)

	Glycoprotein Fraction[a]			
	2	5	8	9
Lys	14.07	13.94	13.76	13.14
His	3.31	2.95	2.95	3.22
Arg	7.89	9.00	8.38	7.67
Asp	20.46	21.12	20.51	19.89
Thr	15.88	16.18	16.01	16.93
Ser	8.03	7.72	7.23	9.30
Glu	30.47	30.56	29.64	28.97
Pro	8.03	7.28	7.85	8.47
Gly	7.98	7.85	7.72	8.16
Ala	9.30	9.22	9.22	9.70
$^1/_2$ Cys	3.26	4.10	2.91	4.10
Val	8.78	8.73	8.47	8.95
Met	1.46	1.38	1.32	1.59
iLeu	9.17	9.44	9.17	8.86
Leu	14.99	15.21	14.42	14.33
Tyr	11.29	10.76	10.98	9.57
Phe	9.44	9.57	9.39	9.26
Try	3.90	3.82	3.85	3.60
Total	188	188	184	186

[a] These fractions contain as major component the following variants: Fractions 2, 5, and 8 were enriched in variant δ, β and α, respectively. Fraction 9 was almost pure with regard to variant α, *i.e.* the slowest-moving variant.

The isoionic point (Table I) of fractions 2 to 9,

not corrected for excess of H-ions (Eylar, 1958), decreased gradually from *p*H 3.54 to *p*H 3.32.

The extinction coefficient (Table I) was essentially the same for fractions 2 to 7 ($E^{1\%}_{278m\mu}$ = 9.0). However, fraction 9 exhibited a significantly lower value (7.6) which could be due to either 4.5 moles of tyrosine or 1.0 mole of tryptophan or 1.5 moles of tyrosine plus 0.7 mole of tryptophan less than that of the other fractions. The molar extinction coefficients used for these calculations were taken from Beavan and Holiday (1952). The amino acid composition indicates that the latter assumption is most probable.

The molar ratio of tyrosine to tryptophan (Table I) averaging 2.85 was approximately the same for all fractions except for the significantly lower value of 2.66 of fraction 9. As indicated above, the sum of tyrosine plus tryptophan of the latter fraction was smaller than that of the others. From these data it was calculated that not only the tyrosine content of fraction 9 but also its content of tryptophan is diminished.

The analysis of the carbohydrate moiety (Table I) seems to indicate that all fractions have essentially the same carbohydrate composition. The content of sialic acid averaged 12%, that of the neutral hexoses 15%, and that of the hexosamine 15%. These values agree with those reported for unfractionated α_1-acid glycoprotein.

Discussion

The polymorphism of α_1-acid glycoprotein was observed at a very acid *p*H value. The best resolution was demonstrated at *p*H 2.9, *i.e.* near the isoelectric point of this protein. No specificity of the type of buffer ions with respect to the separation was noted. α_1-Acid glycoprotein appears polymorphic from *p*H 2 to *p*H 4. The significance of this observation has not yet been unraveled. Although this *p*H range roughly coincides with that of the dissociation of sialic acid, it should also be pointed out that the sialic acid content of the partially separated variants of this protein seems to be very nearly the same and that, as reported by Eylar (1958), all sialyl residues appear to dissociate according to the *pK* value of 2.6 of free sialic acid. Polymorphism of other human plasma proteins has been observed at *p*H 8.6 (Wolstenholme and O'Connor, 1959), *i.e.* somewhat alkaline to the isoelectric point of these proteins.

The major question arising from this study is whether the described observation, namely the resolution of α_1-acid glycoprotein into seven zones, is an artifact. The evidence in favor of a separation not due to changes caused by the acidity of *p*H 2.9 or interaction with starch gel is presented in the following seven points: (1) A comparable, probably identical, separation was obtained at *p*H values up to 4.0. (2) Immunochemical analysis of the variants resolved at *p*H 2.9 demonstrated their identity as judged by this criterion. (3) From earlier studies it is known that this glycoprotein is distinguished by an extremely high stability in that it appears homogeneous on free moving boundary electrophoresis and ultracentrifugation over the *p*H range from 1 to 13. (4) Furthermore, investigation of the optical rotation of this protein over the same *p*H range indicated that the changes that are observed at *p*H 2.7 are completely reversible (Schmid and Kamiyama, 1963). (5) The result of the studies on the fast and slow moving variants, separately eluted from the *p*H 2.8 starch gel, were of primary importance in answering the above question concerning the reasons for the resolution of α_1-acid glycoprotein. On ultracentrifugation the "fast" and "slow" fractions revealed a single symmetrical refractive index gradient curve and sedimented with a coefficient of $s^{0}_{20,w}$ = 3.1 *S*, which is identical to the sedimentation constant of pooled α_1-acid glycoprotein (Smith *et al.*, 1950). Analysis of the "slow" fractions at *p*H 2.9 showed that they again resolved into the same number of bands which exhibited the same apparent electrophoretic mobilities as the parent variants. On starch gel electrophoresis at *p*H 8.6 both fractions revealed the mobility of pooled α_1-acid glycoprotein, which corresponds to that of the slowest moving prealbumin, indicating that the sialic acid content had remained unchanged. The latter conclusion was substantiated by the determination of the sialic acid content of the protein after incubation at *p*H 2.9 for a period of time equal to that required for starch gel electrophoresis. Such analysis showed no loss in sialic acid. Further, this glycoprotein, following preincubation at *p*H 2.9, resulted in starch gel electrophoresis at *p*H 2.8 in a pattern which was identical with that of the untreated protein. (6) In additional control experiments it could be demonstrated that chromatography on Amberlite IRC-50 and the presence of 40% ethanol at $-5°$ and *p*H 4.9 did not affect α_1-acid glycoprotein. (7) Moreover, of great significance is the fact that partial separation of the α_1-acid glycoprotein variants could be achieved by chromatography on DEAE cellulose at *p*H 5.0 and physical chemical and chemical differences of the partially separated variants could be demonstrated.

The preparations containing the partially separated variants of this plasma glycoprotein differed in the following properties: The isoionic points and the apparent electrophoretic mobilities of the main zone observed on starch gel electrophoresis decreased with each consecutive fraction. The increasing acidity of these protein fractions and the fact that they were displaced from the DEAE column at increasing ionic strength led to the assumption that the protein eluted last was more tightly bound to the positively charged resin, probably owing to a higher net charge, than that eluted first at low salt concentration. Further evidence in support of this reasoning is that the mobility of

fraction 9 observed on free moving boundary electrophoresis at *p*H 2.65 is significantly more negative than that of fraction 3. The amino acid residue groupings responsible for the differences in the electrostatic net charge of the variants are different from that grouping which is common to all α_1-acid glycoprotein variants and which is responsible for immunologic identity.

Similarity between the α_1-acid glycoprotein variants was found with respect to the following properties: electrophoretic mobility at *p*H 8.6 and 4.0, sedimentation coefficient, extinction coefficient, and chemical composition (amino acids, sialic acid, neutral hexoses, and hexosamine). However, the slowest moving variant, present in relatively pure state in fraction 9, which was separately eluted from the chromatographic column, is an exception. It is distinguished by its lower extinction coefficient, lower molar tyrosine/tryptophan ratio, and lower content of tyrosine. It was estimated that the main variant of this fraction contains probably two moles of tyrosine and one mole of tryptophan less than the second fraction. It should be emphasized, however, that in view of the small differences in the content of the other amino acids further significant differences in the amino acid composition of the other α_1-acid glycoprotein variants might have escaped detection by the method used. Such differences in the amino acid composition might be uncovered after their isolation in pure form. With respect to the three-dimensional structure of the α_1-acid glycoprotein variants it should be noted that, judging from their immunochemical reaction, these variants possess the same immunologic determinant.

The sialic acid–free form of pooled α_1-acid glycoprotein resolved near its isoelectric point into three bands only. Further studies are needed to explain this observation. Nevertheless, it should be added that even on free moving boundary electrophoresis at *p*H 5.0 the modified glycoprotein separated into two peaks (Schmid *et al.*, 1959).

It appears, therefore, that the differences in the electrostatic net charge between the α_1-acid glycoprotein variants leading to the polymorphism of this protein are probably due to differences in the chemical composition and the three-dimensional structure of the variants.

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