[CONTRIBUTION FROM THE MEDICAL CLINIC, MASSACHUSETTS GENERAL HOSPITAL, AND THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO PUBLIC HEALTH AND MEDICINE, HARVARD MEDICAL SCHOOL]

Isolation of a Group of α_2-Glycoproteins from Human Plasma[1]

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A method is described for the preparation of a group of hitherto little known glycoproteins from normal human plasma. On ultracentrifugal analysis, these plasma constituents appeared as a homogeneous fraction with a sedimentation constant of 2.4 $S_{20,w}$ at a concentration of 1% in 0.15 *M* NaCl. On electrophoretic analysis under standard conditions (*p*H 8.6 barbiturate buffer, ionic strength 0.1), again a homogeneous component, which migrated with a mobility of -4.2×10^{-5} cm.^{2}/volt sec., corresponding to that of the α_2-globulins, was observed. However, in acetate buffer solution at *p*H 3.5 and 4.0, three major components were noted.

I. Introduction

In recent publications[2,3] it has been shown that normal human plasma contains in small concentration certain glycoproteins which are left in the supernatant solution following precipitation of more than 98% of the proteins from normal human plasma.[4,5] These glycoproteins have very low molecular weights and, electrophoretically, belong to the α_1- and α_2-globulins. The α_1-globulin, the acid glycoprotein, has been isolated in pure form, and some of its properties have been reported.[2]

It is the purpose of this paper to describe the isolation of the α_2-glycoprotein fraction.[6]

II. Materials and Methods

Starting Material.—Pooled, normal human plasma has been fractionated according to the low temperature-low salt–ethanol–water method.[4] The solution obtained after removal of the human serum albumin fraction (Fraction V) represented the starting material.[7]

This solution, containing 40% ethanol, had a *p*H value of 4.8 and an ionic strength of 0.11. Its volume was four times that of the original plasma volume. The temperature was $-5°$. The optical density in a 1-cm. cuvette was found to be 0.86 at 278 mμ and 0.69 at 290 mμ. These values are for the most part due to non-proteinaceous dialyzable plasma constituents. After dialysis against water the optical densities were reduced to 0.26 at 278 mμ and 0.16 at 290 mμ.

Methods.—Most of the methods used during these investigations have been described earlier.[2] A Spinco Model E ultracentrifuge[8] and a Perkin–Elmer electrophoresis apparatus were employed.[9] The relative distribution of the different components as well as their sedimentation constants and their electrophoretic mobilities,[10] respectively, were calculated by the conventional methods: resolution of the enlarged tracings in Gaussian curves and planimetric measurements of the relative areas. Refractive indices of all components were assumed equal. In order to minimize a temperature correction, the temperature of the ultracentrifuge rotor was kept between 19.5–20.5°.

III. Fractionation of the Proteins of Fraction VI

Precipitation of Fraction VI.—One hundred and fifty-three l. of the supernatant solution of Fraction V (run 181)[11] obtained from 38 l. of human plasma were mixed, at $-5°$, with 10.3 l. of 1 *M* aqueous zinc acetate solution precooled to 0°. This relatively high concentration of zinc acetate (final concentration 0.06 *M*) was necessary for a complete adsorption of the proteins to the zinc hydroxide. The turbidity formed at *p*H 4.9 indicated the formation of insoluble complexes of certain proteins with zinc ions. To precipitate the zinc hydroxide, the *p*H value of the suspension was raised to 7.9 by the addition of 18 l. of *p*H 10.5 ammonium chloride–ammonium hydroxide buffer precooled to $-5°$.[12] Through the addition of these aqueous reagents the ethanol concentration fell from 40 to 34%. It proved important to cool and stir the suspension well not only during this operation, but for at least 30 minutes afterwards.[13]

The precipitate which was centrifuged off at a rate of 50 l. per hour provided 3.6 kg. of zinc hydroxide paste containing approximately 36 g. of protein. This value was obtained by the refractive index measurements of a dissolved aliquot following dialysis against water. The dry weight averaged 24% of the protein paste. The electrophoretic and ultracentrifugal distribution of the proteins in Fraction VI is found in Table II.

The supernatant solution showed an optical density in a 1-cm. cuvette of 0.15 at 278 mμ and 0.10 at 290 mμ. After dialyzing an acidified aliquot of the supernatant solution of Fraction VI against water, the corresponding values were 0.03 at 278 mμ and 0.02 at 290 mμ. As shown in a previous publication[2] zinc hydroxide adsorbed, not only proteins, but also uncharacterized, dialysable blood constituents which, in neutral solution, have a maximal light absorption at 290 mμ.

Subfractionation of the Proteins Precipitated in Fraction VI.—The fractionation was carried out at $-5°$, and all reagents were precooled to this temperature.

To reduce the concentration of ethanol to 19%, the zinc hydroxide paste was suspended in 2.2 l. of precooled distilled water, then, to raise the final volume to 11.9 l., or 0.31 plasma volume, 2.2 l. of 19% ethanol were added. Decomposition of the zinc hydroxide, providing the release of the proteins, was effected by adding, in small aliquots, *p*H 4.0 acetate buffer of 0.5 ionic strength in 19% ethanol.

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(6) Notes on the preparation of these glycoproteins have been published previously, *cf.* K. Schmid, *ibid.*, **75**, 2532 (1953); K. Schmid, *Federation Proc.*, **13**, 291 (1954).

(7) The author wishes to express his thanks to Dr. J. A. McComb, director of the Division of Biologic Laboratories, Massachusetts Department of Health, for providing the starting material.

(8) The ultracentrifugal analyses were carried out at a speed of 56,100 r.p.m.

(9) A comparative study with a Klett and a Perkin–Elmer electrophoresis apparatus, using simple mixtures of plasma proteins as well as more complex mixtures of egg proteins, revealed qualitative and essentially quantitative agreement of the results obtained.

(10) Both the "closed" and the "open" systems were used. The latter was applied whenever the salt boundaries were detectable. The advantage was that the starting boundary did not need to be shifted into view, because the entire solution in the cell moved at a constant rate toward the anode. This application affords a good separation of the α_1-globulins from albumin even in normal human plasma.

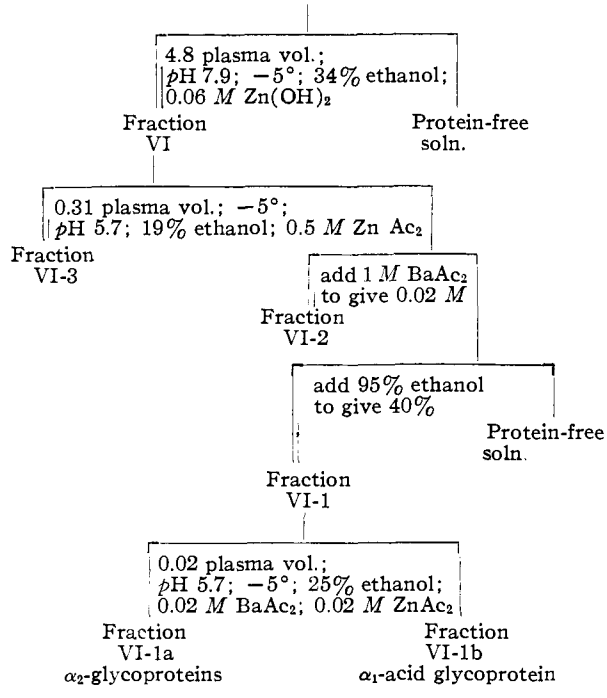
(11) This was one of several runs showing the typical features of the preparation of the α_2-glycoproteins.

(12) This buffer contained 12 g. of NH_4Cl and 100 ml. of 58% NH_4OH per liter.

(13) It is advisable to check the *p*H value of the suspension after this operation and, if necessary, to readjust it.

TABLE I

SEPARATION OF THE PROTEINS PRECIPITATED IN FRACTION VI



diaminetetraacetic acid.[14] Since the binding of the zinc ions and barium ions to this chelating agent was accompanied by a release of hydrogen ions and, consequently, a decrease of the *p*H value, the hydrogen ion concentration of the protein solution was maintained at a neutral value by simultaneously adding precooled *p*H 10 glycinate buffer solution. Following further dialysis the protein solution was dried from the frozen state. The residue dissolved in 476 ml. of ice-water was mixed with 1 *M* zinc acetate and 1 *M* barium acetate to give each salt a concentration of 0.02 *M*. Since, in the presence of 19% ethanol, part of the α_2-glycoproteins was still soluble, as judged from the brown color of the supernatant solution and by electrophoretic analysis, the ethanol concentration of the protein solution had to be increased to 25% by introducing 169 ml. of 95% ethanol precooled to −70°. The proteins thus rendered insoluble, Fraction VI-1a, were centrifuged off, and the precipitate immediately washed twice with 100 ml. of a solution containing 25% ethanol, 0.02 *M* barium acetate and 0.02 *M* zinc acetate.

The protein, Fraction VI-1b, of the supernatant solution, which had been combined with the washings of Fraction VI-1a, consisted essentially of the α_1-acid glycoprotein. After passing through an ion-exchange column (Dowex-50, Na-cycle) the solution was lyophilized.

IV. Results

The results obtained by fractionating these very soluble plasma proteins are given in Table II. Cohn and co-workers[4] reported that the supernatant solution of Fraction V, designated here as Fraction VI, contained approximately 1 g. of protein per liter of plasma, a value which agrees with the one found during this study. Electrophoretic and ultracentrifugal analyses of Fraction VI and its subfractions showed the largest part to be human serum albumin. The albumin was immunochemically identical with that of Fraction V[15] and was concentrated in Fraction VI-3. Without further extraction this subfraction contained approximately 8% acid glycoprotein as shown by electrophoretic analysis at *p*H 4.5.

TABLE II

PROPERTIES OF THE PROTEINS OF FRACTION VI AND ITS SUBFRACTION (RUN 181)

Fraction	Amount of protein Mg./l. of plasma	% of total plasma protein	Electrophoretic distribution[a] Alb.	α_1	α_2	β_1	β_2	Ultracentrifugal analysis[c] "Slow" comp. %	$S_{20,w}$	"Fast" comp. %	$S_{20,w}$	Protein concn., %
VI	942	1.35	50	35	11	4		72	2.7	28	3.4	0.8
VI-3	376	0.54	92	8[b]	..	..	..	12	..	88	3.7	1.1
VI-2	78	.11	6	76	16	2		87	2.5	13	..	1.2
VI-1	488	.70	..	88	12	..	..	100	2.4	0		1.2
VI-1a	98	.14	..	4	91	5		100	2.8	0		1.0
VI-1b	390	.56	..	96	4	..	..	100	2.4	0		1.1

[a] In diethyl barbiturate buffer, *p*H 8.6, ionic strength 0.1. All fractions contained a component which moved with an electrophoretic mobility of -13×10^{-5} cm.^{2}/volt sec. [b] Electrophoresis at *p*H 4.5, ionic strength 0.1 in acetate buffer showed that this fraction contained 8% α_1-acid glycoprotein. [c] In 0.15 *M* NaCl solution.

Five liters of this buffer were required to obtain the desired *p*H value of 5.75. During this operation additional cooling was necessary to remove the heat of neutralization. Complete decomposition was obtained when the *p*H value of the suspension remained constant after further stirring, and the insoluble material became readily soluble in water. The undissolved portion, Fraction VI-3, was subsequently removed by centrifugation (Table II).

Following the addition of 0.24 l. of 1 *M* barium acetate to the supernatant solution of Fraction VI-3, insoluble material separated slowly. This precipitate, Fraction VI-2, was centrifuged off after two days.

The ethanol concentration of the remaining solution was raised to 40% by introducing 4.7 l. of 95% ethanol precooled to −70°, thus rendering the proteins, Fraction VI-1, insoluble. The optical density of the supernatant solution, as measured in a 1-cm. cuvette was 2.13 at 278 mμ and 2.38 at 290 mμ. After dialysis against water the corresponding values were 0.06 and 0.05, respectively.

Subfractionation of the Proteins Precipitated in Fraction VI-1.—After centrifugation, the protein precipitate was immediately suspended in an equal volume of ice-water and dialyzed against precooled water, permitting the removal of the major part of coprecipitated inorganic salts. Thereafter the suspension was dissolved with neutralized ethylene-

The addition of barium acetate to the supernatant solution of Fraction VI-3 rendered insoluble a further small amount of albumin together with some α- and β-globulins, designated as Fraction VI-2.

Fraction VI-1 consisted of α_1- and α_2-globulins as judged by electrophoretic analysis and contained only an extremely small amount of human serum albumin as shown by the corresponding antibody reaction.[15] It is of interest that both the α_1-

(14) It is a pleasure to acknowledge the gift of ethylenediaminetetraacetic acid from Alrose Chemical Co., Providence, Rhode Island.

(15) I am obliged to Dr. David Gitlin, Childrens' Hospital, Boston, for carrying out these determinations.

and the α_2-globulins have very similar sedimentation constants. On the basis of the large difference in solubility (Table I), the α_2-glycoproteins (Fraction VI-1a) were readily separated from α_1-acid glycoprotein (Fraction VI-1b). Rework of Fraction VI-1b under conditions indicated in a previous publication[2] led to the recovery of the α_2-globulins from this fraction.

V. Electrophoretic and Ultracentrifugal Study of Fraction VI-1a

Fraction VI-1a was analyzed electrophoretically over a *p*H range from 1.6 to 12 (Fig. 1). Under standard conditions, *p*H 8.6 barbiturate buffer, ionic strength 0.1, this protein fraction appeared essentially homogeneous, showing only 4% α_1- and 5% β-globulin. In alkaline glycinate buffers, the main component split forming two major components. In glycinate buffer solutions, *p*H 9.3 and 9.8, the electrophoretic mobilities of the two components were almost equal so that only asymmetric schlieren diagrams could be observed. Similar results were obtained in cacodylate buffers inasmuch as the main component of Fraction VI-1a separated into two components at *p*H 5.8. In acetate buffer, especially, at rela ively acid *p*H values, a separation into three components was noted. Following analysis at *p*H 3.5, if the protein solution was reinvestigated in *p*H 8.6 barbituric buffer, homogeneity was again observed, without change of the electrophoretic mobility.

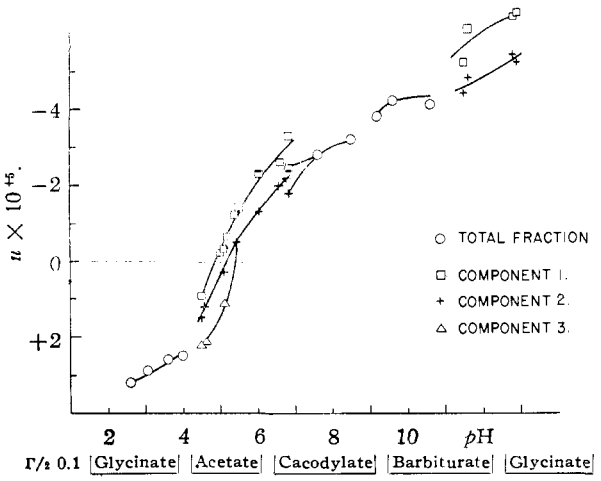


Fig. 1.—Electrophoretic mobility (u) of the α_2-glycoproteins of fraction VI-1a as a function of the *p*H.

The stability of the proteins of Fraction VI-1a was investigated by ultracentrifugal analyses. In both acid and alkaline glycinate buffers as well as in all the other buffer solutions used, symmetric peaks were observed.

As judged by the same criteria, these α_2-glycoproteins did not show any denaturation at *p*H 13, even after 45 days. However, certain changes had taken place after 6 days as noted by the pink color developed in the solution. Some of these changes, most likely hydrolysis, had occurred after two days at *p*H 10.7 and resulted in an increased negative electrophoretic mobility when reinvestigated at *p*H 8.6 in barbiturate buffer, although the apparent homogeneity was maintained. In *p*H 2.0 glycinate buffer the α_2-glycoproteins were less stable. On ultracentrifugal analysis two peaks were apparent after two weeks.

VI. Discussion

The procedure for the separation of the proteins precipitated in Fraction VI, derived from plasma fractionated by Method 6,[4] was similar to that for the separation of the proteins of Fraction VI, derived from plasma which was fractionated by Method 10[5]. However, the changes introduced in the fractionation procedure described here, especially the increase in the volume of the solution from 0.15 to 0.33 plasma volume, offered several important advantages: (1) Fraction VI-3 contained only a small amount of acid glycoprotein; (2) Fraction VI-2 contained the last traces of human serum albumin as judged by electrophoretic analysis; (3) at the increased volume, the α_2-glycoproteins remained essentially in the supernatant solution of Fraction VI-2, and (4) the α_2-glycoproteins together with the α_1-acid glycoprotein were concentrated in Fraction VI-1.

When the volume of the solution to be fractionated was further increased, an appreciable amount of β-globulins appeared in the supernatant solution of Fraction VI-2.

The electrophoretic study of Fraction VI-1a showed that the separation of these α_2-glycoproteins from each other can be followed electrophoretically in acetate buffer and, as mentioned earlier,[6] by extinction coefficient measurements. The curve, expressing the electrophoretic mobility of the components of Fraction VI-1a *vs.* *p*H value of the buffer solution, appeared to have many similarities to the corresponding titration curves, modified by the interaction between the proteins and the ions of the buffers used. Because of the possible interaction of these α_2-glycoproteins at *p*H 4.0, their exact isoelectric points can only be established on the single components.

Figure 1 shows that the α_2-glycoproteins were isoelectric at *p*H values between 3.7 and 4.4. At *p*H 4.5 the α_1[2]- and α_2-glycoproteins were negatively charged in contrast to all the other plasma proteins. Therefore, on electrophoretic analysis at *p*H 4.5, these α-glycoproteins migrated in opposite direction from the remaining plasma proteins. This has been recognized earlier by Mehl and co-workers[16] who showed the changes in concentration of the α_1- and α_2-glycoproteins in various diseases (cancer, pneumonia, rheumatoid arthritis, etc.) using whole plasma.[17]

Increased concentrations of the α_1- and α_2-globulins in blood accompanied by an increased level of protein-bound carbohydrate are observed in almost all acute, infectious states of diseases.[19-23] The α_2-

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globulins together with protein-bound carbohydrates can be specifically elevated in certain conditions of diabetes.[24,25]

The function of these α_2-glycoproteins in the human organism is unknown. However, certain properties of their activities were observed. In contrast to the α_1-acid glycoprotein[26] the α_2-glycoproteins do not seem to have the property of affecting the restoration *in vitro* of the submicroscopic structure of collagen.[27] Further work on the inhibition of the hemagglutinating effect of inactivated influenza virus showed that the α_2-glycoprotein fraction described here strongly exhibits this effect.[22] In their studies with thyroxin, Petermann and co-workers[28,29] found that this α_2-glycoprotein fraction (probably one of its components) may be the carrier of this hormone.

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[CONTRIBUTION FROM THE RESEARCH AND DEVELOPMENT DIVISION, SMITH, KLINE AND FRENCH LABORATORIES]

Reductive Desulfurization of Thiohydantoins and Thiobarbituric Acids with Raney Nickel

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The preparation of a number of substituted 4-imidazolidones, hexahydropyrimidine-4,6-diones and 4,6-dihydroxypyrimidines by the Raney nickel desulfurization of the corresponding 2-thiohydantoins and 2-thiobarbituric acids is described. On the basis of isolated intermediate products, possible mechanisms for the desulfurization reaction are proposed.

Recent reports[2] that the replacement of the 2-carbonyl of 5,5-disubstituted barbituric acids with a methylene group resulted in 5,5-disubstituted hexahydropyrimidine-4,6-diones (VIII) having utility as anticonvulsant agents became of interest to us because a similar transformation in the hydantoin series was reported to yield an inactive compound. Thus 5-ethyl-5-phenylhexahydropyrimidine-4,6-dione (VIIIa) has been used successfully in the treatment of epilepsy[3] and is marketed in England under the trademark Mysoline, while 5,5-diphenyl-4-imidazolidone (Va) has been reported to be ineffective in raising the convulsant threshold of cats.[4] We decided to study the preparation of the latter and to provide an authentic sample for re-evaluation as an anticonvulsant agent.

We chose to prepare 5,5-diphenyl-4-imidazolidone from 5,5-diphenyl-2-thiohydantoin (Ia) by the previously employed[5,6] sodium and amyl alcohol reductive desulfurization procedure and to attempt the desulfurization with Raney nickel catalyst in a manner analogous to the transformation[2a] of 5-ethyl-5-phenyl-2-thiobarbituric acid (VIa) into 5-ethyl-5-phenylhexahydropyrimidine-4,6-dione (VIIIa). The latter transformation also was studied.

During the course of our work the preparation of 5,5-diphenyl-4-imidazolidone by the Raney nickel method was reported.[7] Subsequent to our work the synthesis of a series of imidazolidones[8] and the formation of 2-alkoxy-5,5-disubstituted hexahydropyrimidine-4,6-dione (VII) intermediate reduction products[9] from the Raney nickel reduction of 5,5-disubstituted-2-thiobarbituric acids was published.

While our work duplicates in part and is in substantial agreement with the results obtained by others, we obtained a number of intermediate reduction products and several other compounds not previously reported.

When a solution of 5,5-diphenyl-2-thiohydantoin (Ia) in ethanol was refluxed for 30 minutes with three-week old Raney nickel catalyst, the product was a mixture of several difficultly separable compounds. 5,5-Diphenyl-4-imidazolidone[5-8] (Va) was always produced in low yield, accompanied by the sulfide (IIIa) and 5,5-diphenyl-2-hydroxy-4-imidazolidone[5,6,8] (IIa).[10] Sublimation of IIa readily

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