Anal. Calcd. for $C_{19}H_{29}ClN_2O_2$: C, 64.66; H, 8.28. Found: C, 64.90; H, 8.24.

1-Benzoyl-2-(β-N-piperidylethyl)-1,2,3,4-tetrahydroquinoline.—From 6.1 g. (0.025 mole) of 2-(β-N-piperidylethyl)-1,2,3,4-tetrahydroquinoline and 14 g. (0.1 mole) of benzoyl chloride by the same method was obtained an oil which solidified after distillation and was recrystallized from Skellysolve B. The yield was 2.9 g. (33%). It distilled at 188–196° at 0.1 mm., and melted at 102–103°.

Anal. Calcd. for $C_{23}H_{28}N_2O$: C, 79.27; H, 8.10. Found: C, 79.22; H, 7.95.

The hydrochloride salt, recrystallized from alcohol and ether, melted at 236°.

Anal. Calcd. for $C_{23}H_{29}ClN_2O$: C, 71.76; H, 7.59. Found: C, 71.54; H, 7.66.

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[CONTRIBUTION FROM THE UNIVERSITY LABORATORY OF PHYSICAL CHEMISTRY RELATED TO MEDICINE AND PUBLIC HEALTH, HARVARD UNIVERSITY]

Preparation and Properties of Serum and Plasma Proteins. XXIX. Separation from Human Plasma of Polysaccharides, Peptides and Proteins of Low Molecular Weight. Crystallization of an Acid Glycoprotein[1a,b,c]

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A method is described for the separation into five fractions of the components of human plasma which remained in solution following precipitation of the major plasma proteins by procedures formerly published. All the proteins not previously precipitated were concentrated with the aid of zinc hydroxide (Fraction VI); among them were hitherto little known glycoproteins of low molecular weight. Unknown polysaccharides with specific blood group activity were selectively rendered insoluble with calcium hydroxide (Fraction VII), and amino acids and peptides were adsorbed on an ion exchange resin (Fraction VIII). After concentration of the final solution, the blood constituents were separated into a lipophilic (Fraction IX) and a hydrophilic fraction (Fraction X). In addition a method is described for subfractionation of the proteins precipitated as Fraction VI. An acid glycoprotein has been separated in a homogeneous state, as judged by electrophoretic and ultracentrifugal analyses, over the *p*H range 1.9 to 9.6. It was isoelectric, in a phosphate buffer solution of ionic strength 0.1, at *p*H 2.7. Its sedimentation constant, $S_{20,w}$, in 0.15 *M* NaCl solution at *p*H 6.5, extrapolated to zero concentration, was 3.5 *S*. The chemical composition and physico-chemical properties of the acid glycoprotein differed widely from those of other plasma proteins. Its concentration in normal plasma was 0.5 g. per liter. The acid glycoprotein has been crystallized as a lead salt.

I. Introduction

A method for the fractionation of human plasma in ethanol–water mixtures at low temperatures[2,3] was introduced in 1941–1944 by which the plasma proteins were separated into six principal fractions: I, II + III, IV-1, IV-4, V and VI. Recently this method has been greatly improved by transferring immediately all proteins to the solid state in order to prevent further chemical, especially enzymatic, changes. In Method 10[4] the albumins, α_1-lipoproteins, α_2-glycoproteins, and the β_1-metal-combining protein were precipitated in an ethanol–water mixture of mole fraction 0.066, and *p*H 5.8 at −5°, by the addition of zinc acetate, following precipitation of the less soluble plasma globulins in Fraction I + II + III.[4,5]

The components which remained in solution included small amounts of certain very soluble blood constituents totalling 1.5 to 2% of the plasma proteins. A method for the concentration and fractionation of these blood components into five fractions denoted as VI, VII, VIII, IX and X is reported in this paper and represents an extension of Method 10.[1c] One of the major components of Fraction VI has been further purified and crystallized as a lead salt.

The separation of these very soluble plasma components was based on selective precipitation by ions and hydroxides of alkaline earths and heavy metals such as calcium, barium, zinc, cadmium and lead. Our experiments indicated that complex formation with low concentration of ions of heavy metals did not denature the investigated plasma proteins.

II. Materials and Methods

The solution remaining after precipitation of over 98% of the protein (Fractions I–V) from pooled normal human plasma[4] was the *starting material* for these investigations. In Method 10 the volume of this supernatant solution was five times that of the original plasma volume. It contained 0.066 mole fraction ethanol (19% ethanol at 25°) and slightly

(1) (a) This work has been supported by funds of Harvard University and the Eugene Higgins Trust, by grants from the Rockefeller Foundation and the National Institutes of Health, and by contributions from industry. (b) This paper is No. 98 in the series "Studies on the Plasma Proteins" from blood collected by the American Red Cross, on products developed by the University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University. (c) A preliminary report of some of this work was presented at the 119th Meeting of the American Chemical Society in Boston, Massachusetts, April 1–5, 1951. See Abstracts of this meeting, p. 28 C. (d) Present address: Research Laboratories of the Medical Clinic, Massachusetts General Hospital, Boston, Massachusetts.

(2) E. J. Cohn, J. L. Oncley, L. E. Strong, W. L. Hughes, Jr., and S. H. Armstrong, Jr., *J. Clin. Invest.*, **23**, 417 (1944).

(3) E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin and H. L. Taylor, THIS JOURNAL, **68**, 459 (1946).

(4) E. J. Cohn, F. R. N. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. Mittelman, R. F. Mouton, K. Schmid and E. Uroma, *ibid.*, **72**, 465 (1950).

(5) In a more recent method the human plasma proteins have been fractionated in an aqueous system. A fraction closely resembling Fraction I+II+III was rendered insoluble by complexing with zinc ions. From the remaining "stable plasma protein solution" (S.P.P.S.) a fraction similar to Fraction IV + V was precipitated by complexing with mercuric ions.[4]

less than 0.02 *M* zinc acetate per liter. The optical density at 278 mμ in a 1-cm. cuvette was 0.49 and the *p*H value 5.8. The nitrogen content was about 60 mg. per liter.

Nitrogen content of the fractions was determined by micro-Kjeldahl analyses using copper sulfate and selenium oxychloride as catalysts for the digestion.

Dry weight of the samples was measured after heating to constant weight in an air oven at 110°.[6]

Biuret reaction described by Mehl[7] was used for measuring the polypeptide content of the protein. The results were referred to human serum albumin as standard. Since the samples very often contained small amounts of barium ions, it proved useful to replace copper sulfate with copper chloride.

Optical densities in the absence of interfering non-protein material were measured by determination of light absorption at 278 mμ in a 1-cm. cuvette in the Beckman quartz spectrophotometer. The method was quantitative only if the specific light absorption of the proteins was known.

Carbohydrate analyses were performed by a modification of the method of Sørensen and Haugaard.[8] The standard for this orcinol reaction was an equimolar mixture of galactose and mannose.[9]

Hexosamine was determined by the method of Elson and Morgan,[10] as modified by Rimington.[11]

Hexuronic acid was measured indirectly. The absorption spectrum obtained from protein samples was measured with a Beckman quartz spectrophotometer under the conditions indicated by the method of Dische,[12] and compared with that produced with pure hexuronic acid under the same conditions.

Fatty acids were extracted with methanol at room temperature as previously described.[13]

Cholesterol was separated from proteins by extraction with a mixture of ethanol and ether at the boiling point of this mixed solvent.[14,15]

Phospholipid determinations were carried out by the method of Gortner[16] using an extraction with hot ethanol–ether.

Free sulfhydryl groups of the proteins were measured by the method recently developed by Hughes,[17] which involved addition of an excess of methyl-mercuric iodide and back-titration of the free reagent with dithizone.

Sulfuric Acid Esters.—The proteins were hydrolyzed with 10 *N* hydrochloric acid. After evaporation of the free hydrochloric acid, barium chloride was added to the hydrolysate to precipitate the liberated sulfuric acid.[18]

Zinc ions were titrated with dithizone in alkaline solutions.[19]

Phosphoric acid was measured colorimetrically by the molybdate method of Gortner,[16] following wet-ashing of the protein samples.

β_1-Metal-combining protein was determined according to a previously described method.[20] The proteins were saturated with ferric ions in a neutral buffer solution and the resulting red color measured in the Beckman spectrophotometer at 460 mμ.

Immunological assay for human serum albumin was carried out with horse serum containing an antibody to human serum albumin.[21]

Electrophoretic and ultracentrifugal analyses were performed by M. J. E. Budka, C. G. Gordon and T. E. Thompson under the direction of Dr. J. L. Oncley.

Refractive index increment analyses were performed by procedures previously described.[6]

Determinations of *p*H values were carried out with a glass electrode (Cambridge research model) at room temperature.[3]

III. Preparation of Fractions VI, VII, VIII, IX and X

Fraction VI.—For the separation of all proteins from the starting solution without increase of the ethanol concentration, zinc hydroxide proved very satisfactory. The concentration of zinc ions remaining in solution after removal of the albumin fraction[4] was found sufficient for this purpose.

The *p*H of the starting solution was adjusted to 7.5 by slowly adding, at −5°, the required amount of 1 *M* NH_4OH–NH_4Cl buffer[22] of *p*H 10.4 in 0.066 mole fraction ethanol and precooled to −5°. The resulting suspension was stirred for 30 minutes after the reagent had been added and kept overnight at −5° to allow the zinc hydroxide to settle. After decanting most of the clear supernatant solution, the remaining suspension was centrifuged at −5° in a Sharples centrifuge, and the supernatant solutions were combined. Following separation of this fraction, the solution had an optical density of 0.10 to 0.12.[23]

In one experiment, 67 l. of solution, corresponding to 13.4 l. of plasma, yielded 820 g. of wet paste which contained 10 g. of protein, as estimated by refractive index measurements following dialysis of acidified aliquots against water. The protein of Fraction VI, therefore, comprised approximately 1.5% of the plasma proteins.

Fraction VII.—Lead hydroxide precipitated a wide variety of compounds, including certain polysaccharides, from the supernatant solution of Fraction VI with reduction of the optical density to less than 0.025. Since these polysaccharides appeared to have interesting physiological properties a more selective precipitation was attempted. Calcium hydroxide proved a convenient reagent for this purpose.

Fraction VIII.—The residual solution, following separation of the calcium hydroxide paste, was a source of peptides and amino acids.[24] Since these compounds were difficult to precipitate, their concentration on a column of ion exchange resin was studied. It was assumed that these blood constituents had a certain net charge, under the conditions chosen for this procedure, and could, therefore, be readily separated from uncharged molecules, such as glucose, which passed through the column. A sulfonated resin on the hydrogen cycle proved satisfactory. The resin removed all compounds which gave a positive reaction with ninhydrin. Thus, the nitrogen content of the effluent solution, derived from one liter of plasma, was reduced to as little as 3 mg. as compared to the total nitrogen content of plasma of 11,500 mg. per liter.[25] The elution of the retained plasma components was performed with a 7% aqueous pyridine solution.[26] Drying from the frozen state yielded about 17 mg. of a brown residue per liter of original plasma. Paper partition

(6) S. H. Armstrong, Jr., M. J. E. Budka, K. C. Morrison and M. Hasson, THIS JOURNAL, **69**, 1747 (1947).

(7) J. W. Mehl, *J. Biol. Chem.*, **157**, 173 (1945).

(8) M. Sørensen and G. Haugaard, *Compt. rend. trav. lab. Carlsberg*, **19**, No. 12, 1 (1933); *Biochem. Z.*, **260**, 247 (1933).

(9) D. M. Surgenor, L. E. Strong, H. L. Taylor, R. S. Gordon, Jr., and D. M. Gibson, THIS JOURNAL, **71**, 1223 (1949).

(10) L. E. Elson and W. T. J. Morgan, *Biochem. J.*, **27**, 1824 (1933).

(11) C. Rimington, *ibid.*, **34**, 931 (1940).

(12) Z. A. Dische, *J. Biol. Chem.*, **167**, 189 (1947).

(13) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(14) W. R. Bloor, K. F. Pelkan and D. W. Allen, *J. Biol. Chem.*, **52**, 191 (1922).

(15) J. L. Oncley, F. R. N. Gurd and M. Melin, THIS JOURNAL, **72** 458 (1950).

(16) W. A. Gortner, *J. Biol. Chem.*, **159**, 97 (1945).

(17) W. L. Hughes, Jr., *Cold Spring Harbor Symposia on Quantitative Biology*, **14**, 79 (1950).

(18) J. W. H. Lugg, *Biochem. J.*, **32**, 2114, 2123 (1938).

(19) W. L. Hughes, Jr., personal communication; F. R. N. Gurd and D. S. Goodman, THIS JOURNAL, **74**, 670 (1952).

(20) D. M. Surgenor, B. A. Koechlin and L. E. Strong, *J. Clin. Invest.*, **28**, 73 (1949).

(21) D. Gitlin, C. A. Davison and L. H. Wetterlow, *J. Immunol.*, **63**, 415 (1949).

(22) One liter of this buffer contained 3.1 g. of NH_4Cl, 57 ml. of concentrated NH_4OH, d = 0.90, and 200 ml. of 95% ethanol. Alternately, ethanolamine could be used.

(23) In an alternate method Fraction VI was rendered insoluble after adding barium acetate to give a concentration of 0.005 *M* and increasing the ethanol concentration to 0.163 mole fraction (40% ethanol at 25°). The *p*H of the solution remained at 5.8. Following removal of this precipitate the optical density was reduced from 0.49 to 0.38. The difference in the optical densities observed in these two procedures was due to the additional adsorption of dialyzable compounds by zinc hydroxide.

(24) R. Fuerst, A. J. Landua and J. Awapara, *Science*, 111, 635 (1950); C. Ågren and T. Nilsson, *Acta Chem. Scand.*, **3**, 525 (1949); H. N. Christensen, P. F. Cooper, R. D. Johnson and E. L. Lynch, *J. Biol. Chem.*, **168**, 191 (1947); H. A. Krebs, *Ann. Rev. Biochem.*, **19**, 409 (1950).

(25) W. F. Lever, F. R. N. Gurd, E. Uroma, R. K. Brown, B. A. Barnes, K. Schmid and E. L. Schultz, *J. Clin. Invest.*, **30**, 99 (1951).

(26) A. Tiselius, *Advances in Protein Chem.*, **3**, 91 (1947).

chromatography, before and after acid hydrolysis, indicated the presence of peptides and amino acids in this fraction.

Fractions IX and X.—The solution which contained the components not retained by the passage over the ion exchange resin was concentrated 40-fold *in vacuo*. The *p*H decreased from 1.9 to 0.85. The blood constituents in this concentrate were extracted with ether, whereby a further separation into lipophilic (Fraction IX) and hydrophilic (Fraction X) substances was achieved. The glucose occurring free in the blood remained in Fraction X.

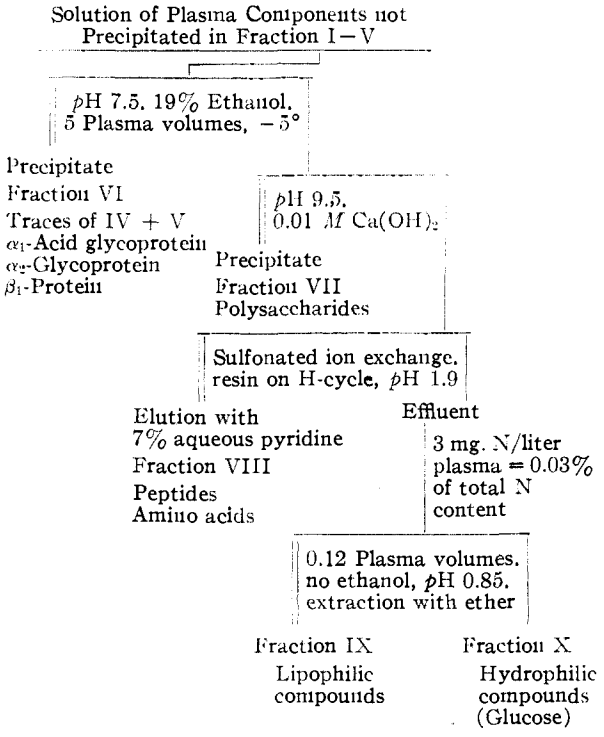

Fig. 1.—Separation of very soluble proteins and other components of normal human plasma in Fractions VI, VII, VIII, IX and X.

IV. Polysaccharides of Human Plasma[27]

Following separation of the zinc hydroxide precipitate, the solution was mixed at −5° with precooled 1 *N* sodium hydroxide, added through a capillary jet, to bring the *p*H to 9.0.[28] After settling for 12 hours, most of the clear supernatant solution could be decanted, only a small volume requiring centrifugation. The precipitate contained traces of protein.

A freshly prepared lead hydroxide suspension[29] was added with stirring to the combined supernatant solutions. The optimal conditions for the precipitation were obtained at *p*H 9 and at a lead concentration of 0.011 *M*. The suspension was allowed to stand overnight; then as much as possible of the supernatant solution was decanted and the remainder centrifuged. Approximately 250 g. of wet paste were obtained from a liter of plasma. The optical density of the supernatant solution was thereby reduced from 0.11 to 0.015.[30]

The lead hydroxide paste was dissolved by adding a neutralized solution of ethylenediaminetetraacetic acid and dialyzed exhaustively against water. The solution was then dried from the frozen state. The residue was taken up in a small volume of water. Non-dialyzable material was found which gave a positive orcinol reaction and contained nitrogen. A concentrated neutral solution of these substances gave no ninhydrin reaction before or after acid hydrolysis. These observations suggested a polysaccharide character. The absorption spectrum of these substances had no peak at 278 mμ, indicating the absence of tyrosine and tryptophan, nor was there a peak at 260 or 350 mμ, such as reported by Holzman and Niemann[31] for blood group A-substance from gastric mucin. This aqueous solution was not stable. Within three days it changed from colorless to brown.

The amount of the polysaccharides was about 10 mg. per liter of plasma. A preparation obtained from mixed pooled plasma, heterogeneous in the ultracentrifuge, had three components with sedimentation constants of 2.9, 4.0 and 4.9 *S*, respectively. These polysaccharides have been shown to inhibit the agglutination of A and B, but not Rh^+ erythrocytes by the corresponding agglutinin[32] suggesting possible specific blood group character of these plasma constituents. It is noteworthy that the polysaccharides were not precipitated in the previous step by zinc hydroxide. A similar observation was made by Rapoport[33] who investigated "blood-group-specific polysaccharides" from human meconium.

V. Fractionation of the Proteins Precipitated with Zinc Hydroxide (Fraction VI)

The zinc hydroxide paste was suspended in an equal volume of 0.066 mole fraction ethanol, precooled to −5°. The *p*H of the suspension was readjusted to 5.8, with *p*H 4.0 acetate buffer of ionic strength 0.5 containing 0.066 mole fraction ethanol. The total zinc ion concentration (approximately 0.5 *M*) was far greater than in the solution (0.02 *M*) from which Fraction IV+V had been precipitated. The proteins insoluble under these conditions, Fraction

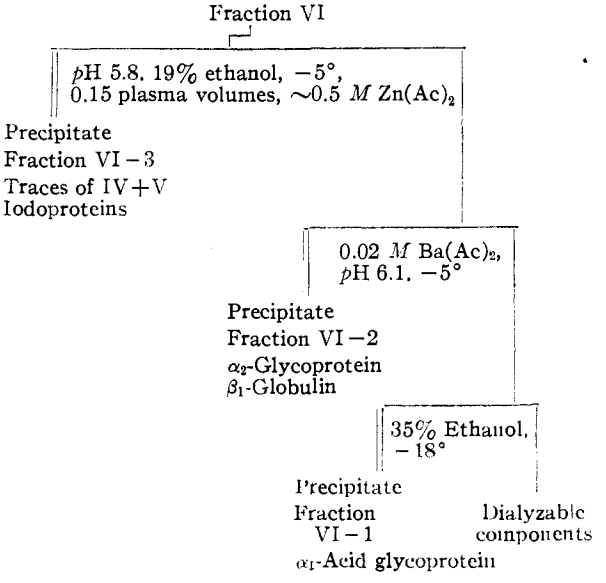

Fig. 2.—Separation of proteins of normal human plasma concentrated in Fraction VI.

(27) The term polysaccharide, referring to human plasma, has often been used in a different sense, meaning the mixture of glycoproteins which includes the "acid glycoprotein" described here. Since real polysaccharides have now been discovered in plasma, it is suggested that the term "serum polysaccharides" be reserved for this class of substances.

(28) The *p*H adjustment could also be made by adding 1 *M* ethanolamine in 19% ethanol.

(29) 7.05 g. of lead subacetate (34.8% PbO) was dissolved in 28 ml. of water. Insoluble material was removed by centrifugation. The solution was then mixed with 10 ml. of 1 *N* NaOH. The reaction of this suspension was close to *p*H 9.0. This amount of suspension was required for one liter of supernatant solution of Fraction VI.

(30) Lead hydroxide precipitated besides these polysaccharides the larger part of Fractions VIII, IX and X. In the method for the separation of human plasma proteins in an aqueous system[5] Fractions VI and VII, together with the bulk of Fractions VIII, IX and X, were rendered insoluble at *p*H 7 with lead subacetate.

(31) O. Holzman and C. Niemann, THIS JOURNAL, **72**, 2044 (1950).

(32) W. E. Wheeler, personal communication made in April, 1950, when working in this Laboratory, as a collaborating guest.

(33) S. Rapoport and D. T. Buchanan, *Science*, **112**, 150 (1950).

VI-3, were removed by centrifugation at −5°. These proteins represented the traces of the albumins and other components of Fractions IV and V which remained in the starting solution and therefore precipitated in Fraction VI.

Sufficient barium acetate was added to the solution to bring its concentration to 0.02 *M* and the *p*H was adjusted to 6.1 with 1 *M* sodium glycinate buffer of *p*H 9.5.[34] After settling overnight, the paste, Fraction VI-2, was centrifuged.

The supernatant solution contained an α_1-glycoprotein averaging 93% of the remaining proteins. For the precipitation of this protein, ethanol precooled to −70° was introduced into the solution to raise the ethanol concentration to 0.136 mole fraction (35% ethanol at 25°). Simultaneously, the temperature of the solution was lowered to −18°. Under these conditions 95 to 98% of the protein was precipitated. Alternately, the protein could be precipitated at −5°, if the ethanol concentration was increased to 0.163 mole fraction (40% ethanol at 25°). After standing overnight at −18°, the precipitate, Fraction VI-1, was separated by centrifugation.

The residual solution contained dialyzable compounds and had an optical density of 1.6 to 2.1 with a maximum at about 290 mμ.

Increase in the ethanol concentration which resulted in precipitation of the protein, Fraction VI-1, decreased the solubility of zinc acetate from 0.5 to 0.2 *M*. Precipitation of this extremely soluble protein from the supernatant solution of Fraction VI-2 was, therefore, attempted without change in ethanol concentration. Copper, cobalt, manganese, nickel, thorium and chromium, added as acetates or sulfates, at a concentration of 0.02 *M* and at −5° did not even produce a turbidity. Uranyl acetate increased turbidity; mercuric chloride produced a slight precipitation. Ions of silver, lead, lanthanum and thallium precipitated about half of the glycoprotein present in the supernatant solution of Fraction VI-2. Lead subacetate and silver nitrate in relatively high concentration (0.076 *M*) rendered insoluble about 99% of the protein in this solution.

VI. Distribution and Characterization of the Proteins in Fraction VI and its Subfractions

a. Electrophoretic Distribution of the Proteins.—The electrophoretic patterns of Fraction VI (Table I) revealed the presence of albumin and α- and β-globulins among which the major component was an α_1-protein. The protein mixture of Fraction VI-3 contained, in addition to α- and β-globulins, practically all albumin present in Fraction VI and had a composition as judged by electrophoretic mobility similar to that of Fraction IV+V, albumins being the major component. Fraction VI-2 contained α- and β-globulins, Fraction VI-1 an α_1-globulin in a state of high purity.

TABLE I

AVERAGE ELECTROPHORETIC PATTERN OF FRACTION VI AND ITS SUBFRACTIONS (EXPRESSED IN %)

Fraction	Albumin	α_1	α_2	β_1	β_2
VI	11	59	20	8	1
VI-1	0	93	6	1	0
VI-2	1	44	34	18	3
VI-3	48	24	21	6	1
IV + V[a]	81	8	5	5	1
$u \times 10^5$ [b]	6.1	5.2	4.3	3.2	2.3

[a] These results are reported by W. F. Lever, F. R. N. Gurd, E. Uroma, R. K. Brown, B. A. Barnes, K. Schmid and E. L. Schultz, *J. Clin. Invest.*, **30**, 99 (1951). [b] Electrophoretic mobility (cm.²/volt sec.) in diethyl barbiturate buffer at *p*H 8.6, ionic strength 0.1.

Electrophoretic analysis of the subfractions of Fraction VI showed that the supernatant solution of Fraction VI-2 contained a "fast-moving" component with a negative mobility of 13 to 14 × 10^{-5} cm.²/volt sec.[35] in diethyl barbiturate buffer of *p*H 8.6 and ionic strength 0.1. This component, which was lost by dialysis against distilled water, amounted to 3–6% of the subfraction.

Detailed analysis was carried out on the protein fractions from a single run (run 59). The results reported in Table II reveal the respective amounts of proteins in subfractions of Fraction VI. The total cholesterol content of Fraction VI was precipitated in Fraction VI-3. Ultracentrifugal analyses indicated that a large part of the proteins of Fractions VI-1 and VI-2 had lower molecular weights than human serum albumin. Fraction VI-2 and its supernatant solution contained a further constituent with a very low sedimentation constant of about 1 *S*. The concentration of this component represented approximately 10% of the fraction. This component was also observed in the same protein fractions of other runs.

TABLE II

PROPERTIES OF THE PROTEINS IN FRACTION VI (Run No. 59)

Fraction	Dry weight mg./liter of plasma	Total plasma protein, %	Cholesterol, %	Sedimentation constant,[a] $S_{20,w}$
VI-1	527	0.81	0	3.1(94); 4.7(3.3); 2.0(2.5)
VI-2	276	.42	0	3.0(76); 6.3(24)
VI-3	280	.44	0.7	4.6(98)

[a] The figures in parentheses represent percentage of the fractions.

The electrophoretic distribution of the proteins in Fraction VI was very similar to that listed in Table I. However, Fraction VI-3 contained more albumin due to the precipitation of the albumin fraction, which in this run left slightly more albumin in the supernatant solution. According to the electrophoretic distribution of the protein in the three subfractions and to the weight of these fractions as reported in Table II, the percentage of the total plasma proteins for the different components of the Fractions VI-1 and VI-2 amounts to 1.07 for the α_1-globulins, 0.16 for α_2-globulins, 0.03 for β_1-globulins and 0.01 for β_2-globulins.

In one experiment 36 l. of plasma yielded 18 g. of α_1-glycoprotein including the extracted α_1-glycoprotein from Fractions VI-3 and VI-2. Therefore, the concentration of this blood constituent amounts to approximately 0.75% of the total plasma protein.

b. Characterization of the Proteins Concentrated in Subfraction VI-3.—It was important to establish whether the proteins of Fraction VI-3 were identical with those occurring in Fraction IV+V. This was investigated by subjecting the proteins of Fraction VI-3 to the same conditions under which Fraction IV+V was obtained, *i.e.*, 0.02 *M* sodium chloride, *p*H 5.8, 0.066 mole fraction ethanol, −5° and 0.02 *M* zinc acetate. Under these conditions only 5 to 7% of the proteins, mostly α_1-*glycoprotein* of Fraction VI-1, was extractable. Moreover, the protein components of Fraction VI-3 were tested for

(34) One liter of this buffer contained 75 g. of glycine, 20 g. of sodium hydroxide and 200 ml. of 95% ethanol; precooled to −5°.

(35) A similar observation was made by J. B. Kirsner, A. L. Sheffner, W. L. Palmer and K. Sterling, *J. Clin. Invest.*, **29**, 867 (1950).

the specific properties of the proteins of Fraction IV + V. *Albumin* was extracted (0.001 *M* zinc acetate, 0.02 *M* sodium chloride, 0.01 *M* barium acetate, 0.051 mole fraction ethanol, −5°, *p*H 5.75, 0.6% protein) from Fraction VI-3 and titrated with an antibody of human albumin from horse serum.[21] In this way it was possible to demonstrate that the extracted protein was albumin, immunochemically identical with the albumin of Fraction V. In addition, the albumin from both fractions (Fraction VI-3 and Fraction V) showed a sedimentation constant of $S_{20,w}$ 4.6 (Table II). Following the removal of the albumin from Fraction VI-3, the protein mixture was tested for the "iron-binding capacity," which is specific for a β_1-globulin of Fraction IV. This test proved the identity of the β_1-globulin of Fraction VI-3 with the *β_1-metal-combining protein* of Fraction IV. The ratio of the *α_1-globulin* to the cholesterol content of Fraction VI-3 and Fraction IV was similar. Therefore, it was assumed that the proteins of Fraction VI-3, at least in large part, were identical with those of Fraction IV+V of Method 10, and comprise only 0.5% of the latter fraction. The *protein-bound iodine* of this protein fraction was far higher than that of any other protein fraction of human plasma.[36]

c. Characterization of the Proteins of Fraction VI-2.—The protein mixture precipitated with the aid of barium ions was essentially free of albumin; it contained α_1-glycoproteins and α_2- and β_1-globulins, the latter two being characteristic of this fraction. These proteins were free of cholesterol, contained hexose and were partly precipitable with trichloroacetic acid. The α_2-protein was found to have a sedimentation constant of about 3.5 *S* and that of the β_1-globulin was about 5 *S*. This α_2-protein represented about 0.1% of the plasma proteins. Like the α_1-globulin of Fraction VI-1, it is a hitherto little known glycoprotein with a low sedimentation constant. The β_1-protein did not seem to bind iron and, therefore, is probably different from β_1-metal-combining protein of Fraction IV. The proteins of Fraction VI-2 interacted very specifically with barium ions as shown by their precipitation. It is interesting to note that barium acetate cannot be replaced by the acetate of calcium or lead.

VII. Purification of the Acid Glycoproteins

Fraction VI-1 (as described in section V) was used for the purification of the acid glycoprotein.[37] Under the conditions of its precipitation, this fraction contained zinc salts in addition to protein. Dialysis against ice-water was first used for the removal of the bulk of inorganic salts. After drying from the frozen state the protein still contained approximately 0.1% zinc. Practically complete removal of the remaining zinc ions, as indicated by a negative dithizone test, could be effected by passing a 10% aqueous solution of such a preparation through a column of a carboxylated resin on the sodium cycle at *p*H 8 and at 0°, provided the flow rate was maintained sufficiently low. Due to a higher affinity, the removal of barium ions with this ion exchange resin was much easier to effect than that of the zinc ions. Since the dialysis required several days, it was more convenient to extract most of the inorganic salts and dialyzable organic compounds from Fraction VI-1, by suspending this protein paste twice, at −5°, in 10 paste volumes of a solution containing 0.02 *M* barium acetate and 0.163 mole fraction ethanol. The residue was again suspended in an equal volume of ice-water, dissolved with a small amount of carboxylated resin on the sodium cycle and, for the removal of the last traces of salt, passed through a column of mixed ion exchange resins. The latter was prepared in such a manner as to prevent denaturation due to locally extreme *p*H values of the solution.[38] The protein solution, freed from ions of barium and zinc, was then dried from the frozen state. The protein obtained by this procedure contained only 0.4% $Zn_2P_2O_7$ as ash.

The resulting protein preparation was dissolved in a small volume of ice-water so that a concentration of about 7% was obtained. Ethanol, precooled to −70°, was introduced into this solution with stirring until a concentration of 0.066 mole fraction was obtained. Simultaneously, the temperature of the solution was lowered to −5°, after which sufficient precooled solutions of zinc acetate and barium acetate were added to bring their concentrations to 0.02 *M*. After standing one day at −8°, the precipitate was removed by centrifugation at the same temperature. The colorless supernatant solution was dried from the frozen state following removal of the ions of zinc and barium with the aid of a carboxylated resin. Such protein preparations were stored at −8° and used for the chemical and physico-chemical analyses which follow.

VIII. Properties of the Acid Glycoprotein[39]

Over a range of *p*H values from 1.9 to 9.6 (Fig. 3), the acid glycoprotein appeared electrophoretically homogeneous in phosphate, formate, acetate, diethyl barbiturate and glycinate buffers. It was isoelectric at *p*H 2.7[40] in phosphate buffer of ionic strength 0.1. Therefore, this protein has been designated as "acid glycoprotein." Asymmetry between ascending and descending boundaries was observed[41,42] from *p*H 5.1 to 9.6. Since the electrophoretic mobilities and the isoelectric point of a protein depend upon both the buffer and the ionic strength,[43,44] the curve in Fig. 3 is drawn only through points representing electrophoretic mobilities in one type of these buffers. Due to strong interaction, the isoelectric point of the acid glycoprotein changed remarkably in the presence of strong acids, such as trichloroacetic acid. In trichloroacetate buffer of ionic strength 0.1, the isoelectric point appeared near *p*H 1. If this buffer solution was 5 times less concentrated, but the ionic strength brought to 0.1 with sodium chloride, the isoelectric point was shifted to near *p*H 1.8.

In the ultracentrifuge the acid glycoprotein appeared again as a single component over the *p*H range of 1.9 to 9.6 if the same buffer solutions as in the electrophoretic study were used. The sedimentation constant at *p*H 6 and in 0.15 *M* NaCl was only slightly dependent upon the protein concentration, pointing to a relatively small asymmetry of the shape of the protein molecule. The average

(36) Thanks are due to Dr. W. T. Salter, Department of Pharmacology, Yale University, and Drs. D. S. Riggs and E. A. Carr, Jr., Department of Pharmacology, Harvard Medical School, for the determinations of protein-bound iodine.

(37) The name "acid glycoprotein" was given to the α_1-glycoprotein of Fraction VI-1 as explained in the following section.

(38) H. M. Dintzis, personal communication. Quantitative removal of zinc, as well as other metals, is now readily achieved by the use of cationic complexing agents.

(39) A short note on the preparation and properties of this protein has been published previously, *cf.* K. Schmid, THIS JOURNAL, **72**, 2816 (1950).

(40) This value is more accurate than that of 2.9 to 3.0 previously reported, *cf.* (39).

(41) Similar effects have been described by L. G. Longsworth, *J. Phys. Colloid Chem.*, **51**, 171 (1947).

(42) J. F. McCrea, *Biochem. J.*, **48**, xlix (1951).

(43) B. D. Davis and E. J. Cohn, THIS JOURNAL, **61**, 2092 (1939).

(44) L. G. Longsworth and C. F. Jacobsen, *J. Phys. Colloid Chem.*, **53**, 126 (1949).

value of the sedimentation constants, $S_{w,20}$, of the acid glycoprotein extrapolated to zero concentration was 3.5 *S*.

The stability of the acid glycoprotein is unusually high as found by the electrophoretic and ultracentrifugal investigations. It is also stable in distilled water at 100°.[45] The sedimentation constant of the protein was unchanged after being subjected to this temperature. The high stability of this protein may be due to the high content of hexose and hexosamine. In trichloroacetate buffer solutions the glycoprotein was denatured. However, the denatured form of the acid glycoprotein was very soluble in water and in buffer solutions of which the *p*H values covered the range of the solubility minimum of this glycoprotein.[46]

TABLE III

ANALYSIS OF ACID GLYCOPROTEIN

Nitrogen content, %		10.7
Polypeptide content, %		66
Non-polypeptide content, %		30
Hexose	17.2[a]	
Hexosamine	11.5[a]	
Phosphoric acid	1.2	

Other possible protein components

Hexuronic acid, Sulfuric acid ester, Fatty acid, Cholesterol, Phospholipid, Free SH-groups	Not present

Physico-chemical constants

$E^{1\%}_{1\ cm.}$ at 278 mμ	8.93
Optical rotation $[\alpha]^{21°}_{5461Å}$	−24°
Refractive index increment, $\Delta n/\Delta w$ (g. protein/liter)	1.80×10^{-4}
Isoelectric point (phosphate buffer, ionic strength 0.1)	2.7
Sedimentation constant, $S_{20,w}$, at infinite dilution	3.5

Precipitants

Effective	Not effective
Ammonium sulfate, saturated	Trichloroacetic acid, 20%
Phosphate, saturated, *p*H 3.0 to 7.0	Perchloric acid, 1.8 *M*
Phosphotungstic acid, 5% in 2 *N* HCl	Sulfosalicylic acid, 0.6 *M*
70% ethanol, *p*H 6.2, ionic strength 0.05, −5°	Boiling in distilled water

[a] These values are not corrected for the additional water taken up during hydrolysis.

As shown by Werner and Odin (*Uppsala Läk. For.*, **1/2**, 69 (1949)) and earlier investigators (see footnote 47) the acid glycoprotein contained galactose, mannose and glucosamine. In addition, Odin and Werner (*Acta Soc. Med. Ups.*, **57**, 227 (1952)) found that part of the glucosamine, mannose and galactose was present in form of sialic acid.

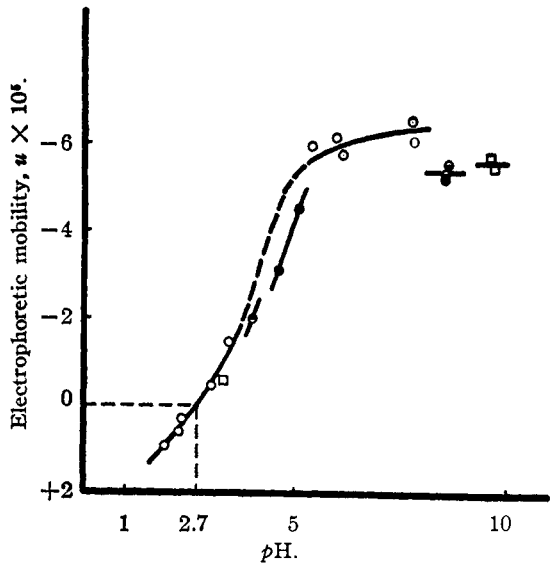

Fig. 3.—Acid glycoprotein; electrophoretic mobility (u) as a function of the *p*H: □, glycinate, ionic strength 0.1; ◒, diethyl-barbiturate, ionic strength 0.1; ○, phosphate, ionic strength 0.1; ●, acetate, ionic strength 0.1; ◓, formate, ionic strength 0.1.

The nitrogen content of the acid glycoprotein was lower than that of any other plasma protein except the β_1-lipoprotein[15] (Table III). Both the hexose and hexosamine contents were very high. According to the classification of Meyer,[47] the acid glycoprotein would fall into the category of the mucoproteins. Taking human serum albumin as standard for the determination of the polypeptide content of the α_1-glycoprotein, the orcinol method for the hexose measurements, and Rimington's method for the hexosamine determinations, the three components total 96%.

The acid glycoprotein is the most soluble protein hitherto encountered in human plasma.[4,48] The high solubility in neutral solutions is in large part due to the low molecular weight[49] and also due to the high net charge of this protein as indicated by the curve in Fig. 3. At *p*H 6.2, ionic strength 0.005 (sodium chloride) and at −5°, the solubility of the acid glycoprotein in 0.415 mole fraction ethanol (70% ethanol at 25°) still amounted to 0.75 g. per liter. At an ethanol concentration of 0.318 mole fraction (60% ethanol at 25°) but under otherwise identical conditions, the acid glycoprotein was very soluble. At *p*H 4.5, ionic strength 0.05 (sodium acetate buffer) and at −18°, an ethanol concentration of 0.270 mole fraction (55% ethanol at 25°) did not precipitate more than a small amount of protein from a 12% solution. In Fig. 4 the solubility of this glycoprotein (S = grams protein per liter) in 0.136 mole fraction ethanol (35%

(45) This is the basis for one of the described methods for the preparation of the protein fraction which contained the acid glycoprotein as the major component. For literature see: R. J. Winzler, A. W. Devor, J. W. Mehl and J. M. Smyth, *J. Clin. Invest.*, **27**, 609 (1948).

(46) After precipitation with 80% methanol at room temperature as used for determination of fatty acids or by an ethanol–ether mixture at the boiling point of this mixture for lipid extraction, which also denatured the protein, the product was still very soluble in water. The amount extracted with ethanol–ether was 0.6% containing only a trace of phosphoric acid and that of the methanol extraction was 0.3% but included no fatty acids.

(47) K. Meyer, *Advances in Protein Chem.*, **2**, 249 (1945).

(48) The acid glycoprotein was very soluble in water even in the absence of salt. On the basis of this property and other characteristics, it appeared that this protein differed from the protein described by McCarty (*J. Exptl. Med.*, **85**, 491 (1947)).

(49) E. L. Smith, D. M. Brown, H. E. Weimer and R. J. Winzler, *J. Biol. Chem.*, **188**, 569 (1950).

ethanol at 25°) at *p*H 6.2 and at low ionic strength is plotted against the concentrations of the acetates of different metals.

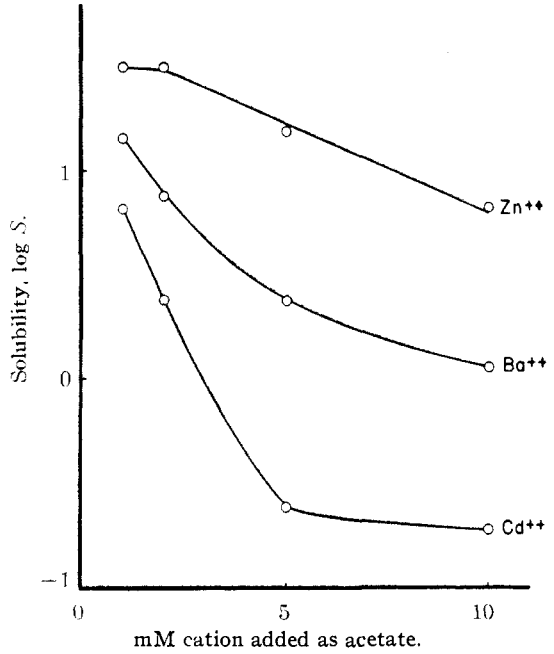

Fig. 4.—Acid glycoprotein; solubility (S) as a function of the concentration of different heavy metal ions at *p*H 6.2. −8°, 0.136 mole fraction ethanol and 0.003 M sodium chloride; S = grams protein per liter.

Under the same conditions the zinc salts of human serum albumins, which are among the most soluble plasma proteins, were insoluble. An ethanol concentration of 0.066 mole fraction gave with human serum albumins a solubility[4] comparable to that of acid glycoprotein obtained at 0.136 mole fraction ethanol.

To render the acid glycoprotein insoluble at 0.066 mole fraction ethanol, ions of heavy metals such as silver, lanthanum, thallium and lead, were required. In Fig. 5 the solubility of the acid glycoprotein is plotted as a function of the concentration of lead acetate. The solubility of this protein under the indicated conditions was similar to that of human serum albumin, when lead ions were replaced by zinc ions. In Fig. 6 the solubility of the acid glycoprotein is plotted as a function of *p*H using phosphate buffer solutions of 0.066 mole fraction ethanol at −5°.[50] In order that solubility values be sufficiently low, these investigations were carried out at an ionic strength of 0.005. The insoluble protein separated as an oil.[51] Ultracentrifugal analysis did not reveal denaturation. The interaction between the acid glycoprotein and the phosphate ions was weak as indicated by the minimal solubility at *p*H 3.0. It was also found that the acid glycoprotein could be rendered insoluble as a sodium salt at its isoelectric point in 0.066 mole fraction ethanol at −5°. The minimal

(50) The *p*H values of these solutions were measured without previous dilution at 25° with a glass electrode.

(51) During the study on whale myoglobin, it was sometimes observed that this protein separated as a dark brown, viscous oil under conditions where it otherwise crystallized. K. Schmid, *Helv. Chim. Acta*, **32**, 109 (1949).

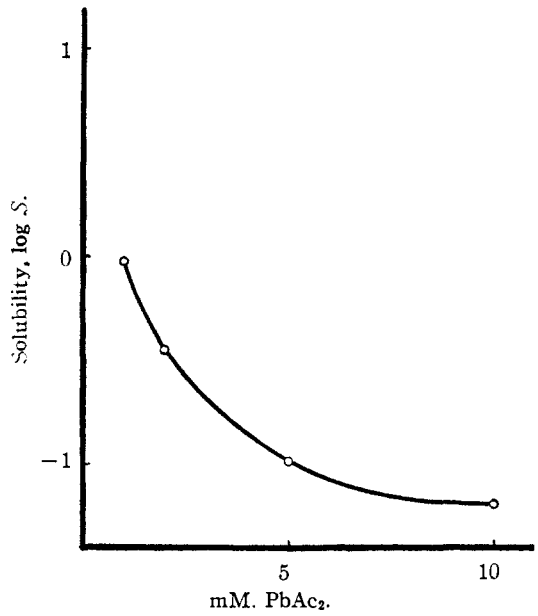

Fig. 5.—Acid glycoprotein; solubility (S) as a function of the concentration of lead acetate at *p*H 6.2. −5°. 0.066 mole fraction ethanol and 0.003 M sodium acetate. S = grams protein per liter.

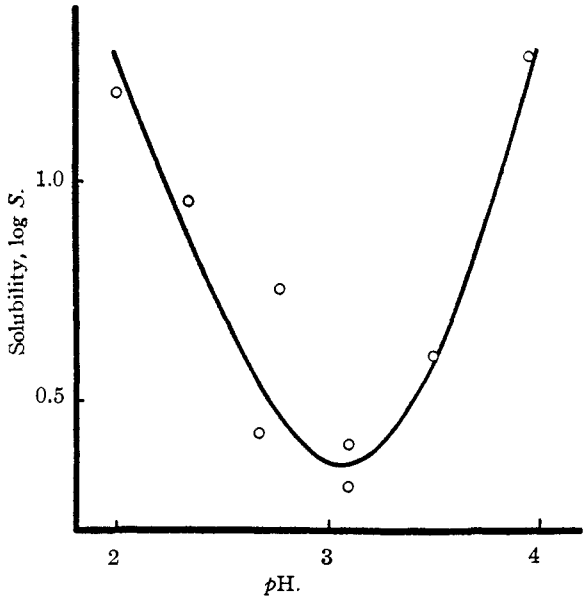

Fig. 6.—Acid glycoprotein; solubility (S) as function of the *p*H values of different sodium phosphate buffers of ionic strength 0.005 in 0.066 mole fraction ethanol and at −5°; S = grams protein per liter.

solubility was much lower than that of human serum albumin at its isoelectric point under otherwise identical conditions, suggesting the pronounced effect of the net charge of the α_1-glycoprotein upon its solubility. The influence of ionic strength upon the solubility of this glycoprotein as shown in Fig. 7 is very high and expresses a strong salting-in effect.

The effect of the different, commonly used, protein precipitating reagents upon a solution of acid glycoprotein may be related to its extremely high solubility in neutral solution as well as to its high

stability (Table III). Addition of solid trichloroacetic acid to a 10% aqueous solution of the acid glycoprotein produced no insoluble material at the boiling point. At room temperature such a solution formed a gel.[52] Perchloric acid,[45] and sulfosalicylic[45] acid were also ineffective in precipitating the glycoprotein. Ammonium sulfate, phosphate and acidified phosphotungstic acid[45] did precipitate the acid glycoprotein.

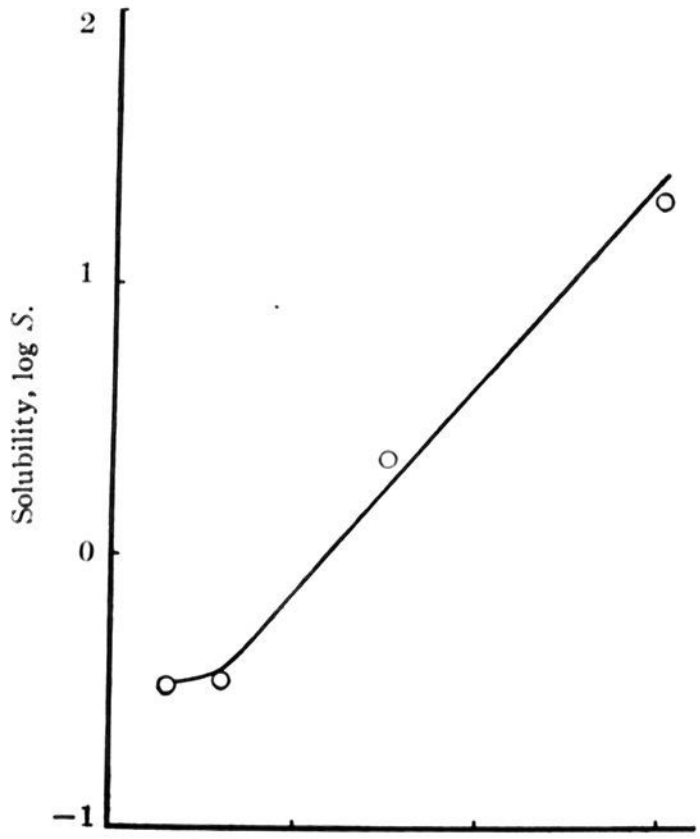

Fig. 7.—Acid glycoprotein; solubility (S) as a function of sodium chloride concentration at pH 6.1, −8°, 0.136 mole fraction ethanol and 0.005 M cadmium acetate. S = grams protein per liter.

The function of the acid glycoprotein in the human organism is not known,[53] although certain properties of its *in vitro* activity have been noted.

IX. Crystallization of the Acid Glycoprotein

Conditions under which the acid glycoprotein crystallized as a lead salt were: 6.3% protein, 0.0072 M lead acetate, pH 5.4, 0 to −8°, 10.6% methanol, 10.6% acetone. These conditions were obtained as follows: 1.0 ml. of 10% aqueous protein solution was mixed with 0.012 ml. of 1 M lead acetate solution and cooled to 0°. With careful stirring, 0.15 ml. of a precooled mixture of methanol–acetone (1:1) was added; the temperature was maintained just above the freezing point of the solution. An additional amount of 0.2 ml. of the mixture of these organic solvents was added using the same precautions. The solution was then carefully warmed until the precipitated protein dissolved. After standing at 0° for at least 24 hours, the acid glycoprotein formed well-defined crystals (Fig. 8). Ultracentrifugal analysis of the crystalline preparation revealed a single component with a sedimentation constant of $S_{20,w}$ 3.16 at a concentration of 0.93% in 0.15 M NaCl solution. This value was identical with that obtained under the same conditions with purified, uncrystallized acid glycoprotein.

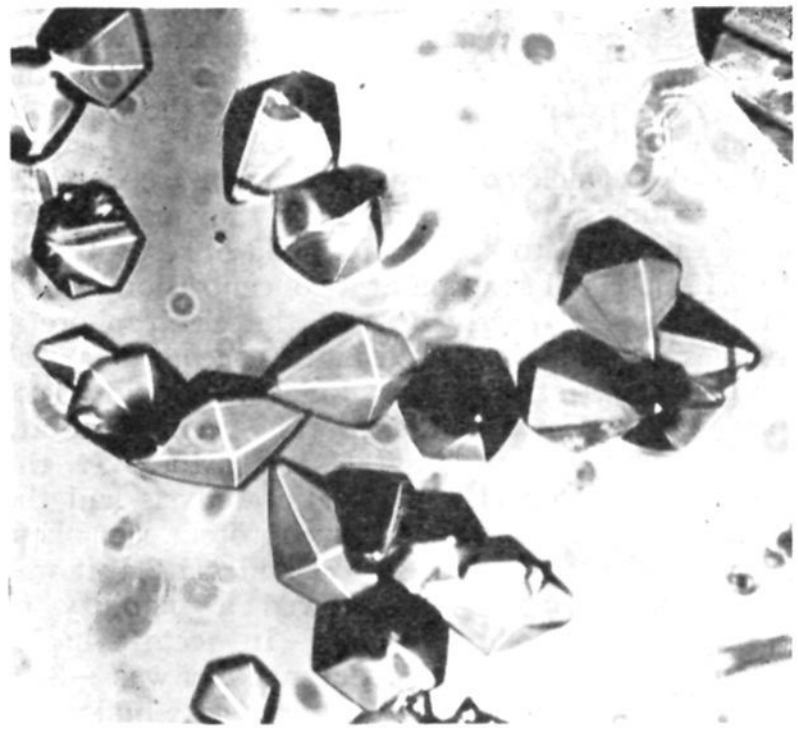

Fig. 8.—Acid glycoprotein; crystallized as Pb salt at pH 5.4, −8°, 10% methanol, 10% acetone and 7% protein.

A glycoprotein of such a high content of hexose and hexosamine has not previously been obtained in a crystalline state.[47,56]

X. Discussion

Comparison of the Acid Glycoprotein with Similar Plasma Proteins Described by Other Authors.

In 1942, Mayer[47,57–59] reported upon two preparations of mucoid-like ("mucoidähnliche") substances prepared from a sulfosalicylic acid filtrate of human serum. The purer fraction contained 8.1% nitrogen, 23.3% hexose and 13.8% glucosamine. The isoelectric point of the preparation was reported to be at pH 3.4. Petermann and Hogness[60] observed an "acid protein" in human plasma with an electrophoretic mobility of -2.7×10^{-5} cm.^{2}/volt sec. at pH 4.0. Winzler and Mehl[45,61,62] investigated similar plasma proteins. Recently they succeeded in preparing an electrophoretically and ultracentrifugally homogeneous mucoprotein containing[63,64,49] 10.3% nitrogen, 16.4% hexose and 11.9% hexos-

(52) H. L. Fevold, *Advances in Protein Chem.*, **6**, 222 (1951).

(53) The acid glycoprotein inhibits the hemagglutinating effect of inactivated influenza virus.[54] It is involved in the restoration *in vitro* of the submicroscopic structure of collagen.[55] These properties are common to similar glycoproteins prepared from animal plasma and tissue.

(54) C. Howe, *J. Immunol*, **66**, 9 (1951).

(55) J. H. Highberger, J. Gross and F. O. Schmitt, *Proc. Natl. Acad. Sci.*, **37**, 286 (1951).

(56) F. Haurowitz, "Chemistry and Biology of Proteins," Academic Press, Inc., New York, N. Y., 1950, p. 198.

(57) K. Mayer, *Z. physiol. Chem.*, **275**, 16 (1942).

(58) M. Stacey, *Advances in Carbohydrate Chem.*, **2**, 194 (1946).

(59) H. O. Bray and M. Stacey, *ibid.*, **4**, 37 (1949).

(60) M. L. Petermann, D. A. Karnofsky and K. R. Hogness, *Cancer* **1**, 104 (1948).

(61) R. J. Winzler, A. W. Devor and D. Burk, *J. Natl. Cancer Inst.*, **4**, 417 (1944).

(62) R. J. Winzler and D. Burk, *Federation Proc.*, **6**, 303 (1947).

(63) H. E. Weimer, J. W. Mehl and R. J. Winzler, *ibid.*, **9**, 244 (1950).

(64) H. W. Weimer, J. W. Mehl and R. J. Winzler, *J. Biol. Chem.*, **185**, 561 (1950).

amine. In sodium chloride–trichloroacetate buffer, the isoelectric point of their preparation was at *p*H 1.8.

The mucoprotein described by Weimer, Mehl and Winzler[64] had properties very similar to those of the acid glycoprotein. The concentration in plasma of both was 0.7% of the total proteins. The content of nitrogen, hexose and hexosamine was essentially the same. The sedimentation constant of the mucoprotein was reported to be 3.1 *S* (Spinco ultracentrifuge) at zero concentration as compared with the value of 3.5 *S* (Pickel's ultracentrifuge) for the acid glycoprotein. The difference in the reported sedimentation constants was resolved by a study by Dr. J. L. Oncley of a preparation of mucoprotein.[65] The sedimentation constants of the mucoprotein were estimated to be 3.80 and 3.55 *S*, respectively, in 0.15 *M* NaCl solution at a concentration of 0.28 and 0.45%, respectively. In the electrophoretic studies, the use of different buffers accounts for the different isoelectric points. Indeed, the acid glycoprotein tested in a sodium chloride–trichloroacetate buffer[64] was isoelectric at *p*H 1.8. Moreover, the mucoprotein[64] on electrophoretic analysis had the same mobilities as the acid glycoprotein. In phosphate buffer, ionic strength 0.1 and *p*H 2.4, it was negatively charged and showed a mobility of $+0.72 \times 10^{-5}$ cm.^{2}/volt sec. In acetate buffer *p*H 4.4, ionic strength 0.1, the mobility was -3.10×10^{-5} cm.^{2}/volt sec., and in phosphate buffer *p*H 7.6, ionic strength 0.1, the mobility was -6.67×10^{-5} cm.^{2}/volt sec. These three values for the mucoprotein were, within the error of the method, identical with those obtained for the acid glycoprotein under the same conditions. The solubilities of both protein preparations, as far as investigated, were also the same.

Other investigators have studied protein mixtures, derived from plasma, with properties similar to those of the acid α_1-glycoprotein. Such preparations were obtained either from sulfosalicylic acid filtrates of plasma,[45,47] from filtrates of plasma after deproteinization by boiling[45] or by fractional precipitation with ammonium sulfate or sodium sulfate.[45,66,67] These mixtures were designated as seromucoids, serum polysaccharides or blood proteoses.

Because the proteins of Fraction I to V of Method 10, representing slightly over 98% of the plasma proteins, would be denatured and rendered insoluble by boiling[68] and since Fraction VII and its supernatant solution were free of protein, the protein mixture previously called "seromucoids, etc. . .," was concentrated in Fraction VI. The acid glycoprotein represents the major component of these very soluble glycoproteins.

Acknowledgment.—The author wishes to express his appreciation of the encouragement and advice of Drs. Edwin J. Cohn, John T. Edsall and John L. Oncley.

(65) These preparations were obtained through the courtesy of Dr. R. J. Winzler, Department of Biological Chemistry, University of Illinois, Chicago 12.

(66) A. B. Gutman, *Advances in Protein Chem.*, **4**, 161 (1948).

(67) M. L. Petermann, N. F. Young and K. R. Hogness, *J. Biol Chem.*, **169**, 379 (1947).

(68) Except the α_2-mucoprotein of Fraction IV which was insoluble in the absence of salt. Its sedimentation constant of 11 *S* at zero concentration (*cf.* (20)) was different from that of the acid glycoprotein.

BOSTON 15, MASS.

[CONTRIBUTION FROM THE WEIZMANN INSTITUTE OF SCIENCE AND THE SCIENTIFIC DEPARTMENT, ISRAELI MINISTRY OF DEFENCE]

Intramolecular Hydrogen Bonds in 2-Aminoalkanols and N-Alkylidene-2-aminoalkanols

BY ERNST D. BERGMANN, E. GIL-AV[1] AND S. PINCHAS

RECEIVED NOVEMBER 23, 1951

Intramolecular hydrogen bonds of the type OH...N involving five-membered rings, have been studied with the help of infrared absorption measurements. 2-Aminoalkanols in carbon tetrachloride solution form such intramolecular hydrogen bonds; in ethanolamine and its N-methyl derivative only intermolecular association has been observed. From the shifts of the hydroxyl absorption, the strength of the intramolecular hydrogen bonds is estimated at about 6 kcal./mole. No analogous effect was observed with certainty in N-alkylidene-2-aminoalkanols, possibly because of the lower basicity of their nitrogen atom.

The strength of internal hydrogen bonding, which depends—among other factors—on the nature of the bonded atoms, the size of the resulting "ring" and possible resonance effects,[2] expresses itself in a shift of the infrared absorption band of the "free" hydroxyl group from about 3600 cm.$^{-1}$ toward longer wave lengths. According to Badger and Bauer,[3] an energy difference of 1 kcal./mole between the two forms causes a shift of 35 cm.$^{-1}$ in the fundamental, of 70 cm.$^{-1}$ in the second, and of 60 cm.$^{-1}$ (estimated) in the first overtone. Six-membered hydrogen bond rings are more favored than five-membered ones.[2-6]

The present paper deals with hydrogen bonds of the type OH···N in amino alcohols and related compounds.

In Table I, the infrared absorption data and (some) molecular weight determinations are listed for a number of 2-aminoalkanols, mostly containing secondary and tertiary nitrogen atoms. Out of 13 substances investigated, all of fairly

(1) Part of a Thesis presented by E. Gil-Av to the Hebrew University, Jerusalem, in partial fulfillment of the requirements for the degree of Ph.D.

(2) L. Pauling, "Nature of the Chemical Bond," 2nd Ed., Cornell University Press, Ithaca, N. Y., 1942, p. 284.

(3) R. M. Badger and S. H. Bauer, *J. Chem. Phys.*, **5**, 839 (1937).

(4) (a) S. B. Hendricks, *et al.*, THIS JOURNAL, **58**, 1991 (1936); (b) O. R. Wulf, *et al.*, *ibid.*, **58**, 2287 (1936).

(5) F. T. Wall and W. F. Claussen, *ibid.*, **61**, 2679 (1939).

(6) L. R. Zumwalt and R. M. Badger, *ibid.*, **62**, 305 (1940).