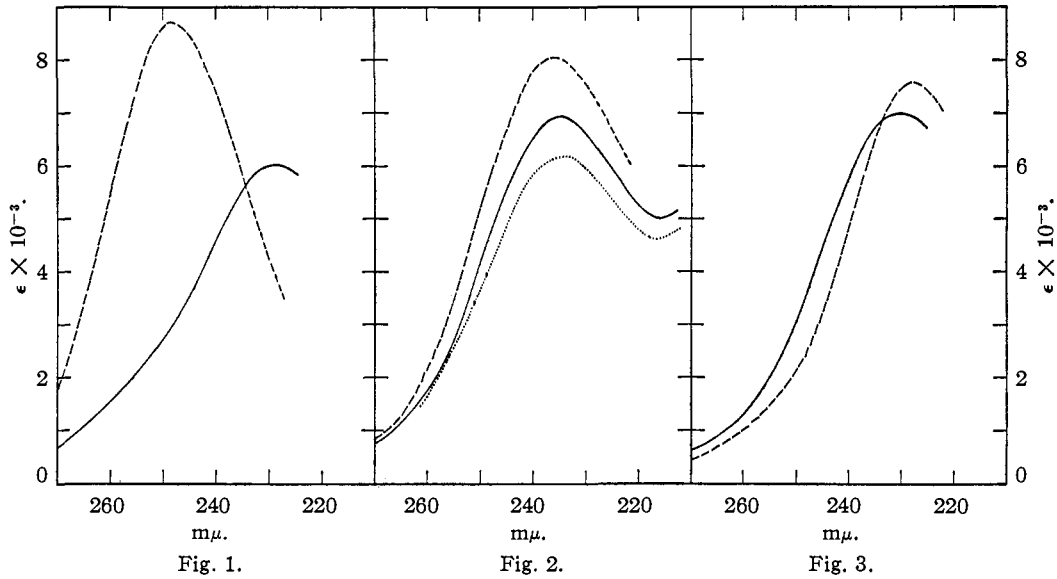

Fig. 1. Fig. 2. Fig. 3.

Fig. 1.—Solid curve: tris-(hydroxymethyl)-methylnitrosohydroxylamine in water; dash curve, same in 0.25 *N* NaOH.

Fig. 2.—Solid curve: tris-(hydroxymethyl)-methylnitramine in water; dash curve, same in *N* NaOH; dotted curve: same in *N* HCl.

Fig. 3.—Solid curve: methylnitramine in *N* HCl; dash curve, same in 0.5 *N* KOH.

10^3 in water to 7.60 × 10^3 in 0.5 *N* potassium hydroxide. No further increase of $\epsilon_{max.}$ was noted in *N* potassium hydroxide. The spectrum of free methylnitramine was obtained by dissolving potassium methylnitramine in a large excess of dilute hydrochloric acid.

Summary

The ultraviolet absorption spectrum of tris-(hydroxymethyl) - methylnitrosohydroxylamine in neutral aqueous solution is similar in location and intensity to that of tris-(hydroxymethyl)-methylnitramine and methylnitramine in neutral, aqueous solution. In aqueous alkaline solution the absorption of the nitrosohydroxylamine is greatly intensified and the maximum shifts 20 mμ toward the visible region. The spectra of the alkylnitramines are intensified by conversion into the salts but the location of the maximum remains almost unchanged. This differing behavior in alkaline medium affords a means of distinguishing nitramines from isonitramines (nitrosohydroxylamines).

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[CONTRIBUTION FROM DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

The Separation of Choline Esterase, Mucoprotein, and Metal-Combining Protein into Subfractions of Human Plasma[1a,b,c]

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A system for the separation of the protein components of human plasma has been described in an earlier paper in this series.[2] The fractions so obtained contain groups of proteins of somewhat similar solubility characteristics, each of which is susceptible of further purification. Many of the components so isolated, either in

(1a) This work was originally supported by grants from the Rockefeller Foundation and from funds of Harvard University. It was aided early in 1941 by grants from the Committee on Medicine of the National Research Council, which included a grant from the American College of Physicians. From August, 1941, to July, 1946 it was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. Since then it has been aided by a grant recommended by the Panel on Hematology of the National Institute of Health.

(1b) This paper is Number XX in the series "Preparation and Properties of Serum and Plasma Proteins" from the Department of Physical Chemistry, Harvard Medical School.

(1c) This paper is Number 75 in the series "Studies on the Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

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(2) 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).

pure or concentrated form, have been found of physiological importance and some of them of clinical use. This was true of Fraction I which contains fibrinogen and the antihemophilic globulin[3]; of Fraction II which contains various antibodies, and of Fraction III which contains the isoagglutinins, prothrombin and the fibrinolytic enzyme precursor, plasminogen.[4]

Fraction IV, originally separated to render the albumin in Fraction V as pure as possible, has since been removed in two steps (2), Fraction IV-1 separated the α_1-lipoprotein under conditions of lower ethanol concentration than were required to precipitate the whole Fraction IV. The remainder of this fraction, IV-4, contained those proteins soluble in 0.062 mole fraction (18 per cent.) ethanol[5] at *p*H 5.2 and $-5°$,—used for the precipitation of Fraction IV-1—and insoluble in 0.163 mole fraction (40%) ethanol at *p*H 5.8 at the same temperature. It comprised slightly more than 7% of the plasma proteins. In addition to a small amount of albumin which separated with this fraction, it was composed mainly of α_2- and β_1-globulins. It has been routinely prepared free of pressor or depressor activity, and has been injected intravenously without untoward reactions.[6]

A number of labile components have also been recognized in Fraction IV-4. These include α_1-lipoprotein, α_2 carbohydrate-containing globulins, and hypertensinogen. It was early recognized that "precipitation of Fraction IV-4 by 0.163 mole fraction ethanol at *p*H 5.8 and $-5°$ led to the destruction of hypertensinogen and presumably of other labile components of the fraction without, however, destroying gross molecular structure."[7] Attempts to isolate certain of these components from Fraction IV-4 in their native states, particularly the various α-globulins, have thus far met with only moderate success. The new method of fractionation now being developed should yield more satisfactory preparations of these and of other labile components of plasma.[8]

This communication is concerned with the separation of Fraction IV-4 of human plasma into subfractions in which have been concentrated certain of the more stable physiologically important components. These include plasma choline esterase and the β_1 metal-combining globulin.[9,10]

These subfractions have been used for certain clinical investigations,[11,12,13] and have been the starting materials for chemical studies, to be reported later, on the purification and crystallization of the metal-combining globulin[14] and on the further concentration and purification of plasma esterase.[15]

II. Materials and Methods

Fraction IV-4 used in these studies came from three sources. The major part was from substandard or outdated dried plasma being fractionated in the interest of recovering albumin, γ-globulin, and other stable protein components of proven therapeutic or prophylactic value for distribution by the American Red Cross.[16] Some was prepared from fresh plasma at the Harvard Pilot Plant during the war. Finally, this fraction was made available from the fresh plasma fractionated at the Division of Biologic Laboratories, Massachusetts Department of Public Health, from blood collected by the American Red Cross.[17]

Carbohydrate analyses were performed by a modification by the method of Sørensen and Haugaard.[18] An equimolar mixture of galactose and mannose was used as the standard.[19] To 1 ml. of solution, containing 0.02 to 0.10 mg. of hexose, in a colorimeter cuvette, was added 5 ml. of 0.1% orcinol in 67 volume per cent. sulfuric acid. After heating for exactly ten minutes in an 80° bath, the tubes were chilled rapidly in an ice-bath. The solutions were kept in the dark and read within an hour against a protein-free blank in a Klett photoelectric colorimeter using Corning glass filters number 5031 and 3389 (420 to 580 mμ). A series of standard tubes, containing varying amounts of the galactose–mannose standard, were always read at the same time as the unknowns.

Hexoseamine determinations were carried out by the method of Elson and Morgan,[20] as adapted by Rimington.[21] Glucoseamine was used as the standard.

Metal-combining capacities were measured by a modification of the method of Schade.[9,10] Determinations were carried out in *p*H 7.4 sodium diethyl barbiturate buffer of ionic strength 0.1, using a standard solution of ferrous ammonium sulfate (200 γ Fe/ml.). The solutions were allowed to stand sixty minutes and their extinctions were read in the Beckman spectrophotometer against an iron-free blank at 460 mμ.[22]

Solubility studies with plasma choline esterase were made with small sized aliquots of a standard solution of the enzyme, adjusted to the desired *p*H, ethanol concentration, and ionic strength; the total amount of protein in the system was the same throughout. After equilibration for twenty-four hours at the desired temperature, the precipitates were removed by centrifugation in a refrigerated angle centrifuge. The supernatant solutions were dried

(3) J. T. Edsall, R. M. Ferry and S. H. Armstrong, Jr., *J. Clin. Invest.*, **23**, 557 (1944).

(4) J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron and P. M. Gross, Jr., THIS JOURNAL, **71**, 541 (1949).

(5) Ethanol concentrations are given in mole fraction and volume per cent. at 25°.

(6) C. A. Janeway and F. L. Plachte, Report to the Subcommittee on Blood Substitutes, Division of Medical Sciences, National Research Council, March 14, 1943; Bull. Blood Subst., pp. 1335 and 1400.

(7) Ref. 2, pp. 469-470.

(8) E. J. Cohn, personal communication.

(9) A. L. Schade and L. Caroline, *Science*, **104**, 340 (1946).

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

(11) R. D. Barnard and J. W. Mentha, *Science*, **107**, 195 (1948).

(12) C. E. Rath and C. A. Finch, *J. Clin. Invest.*, **28**, 79 (1949).

(13) G. E. Cartwright and M. M. Wintrobe, *J. Clin. Invest.*, **28**, 86 (1949).

(14) B. A. Koechlin, in preparation.

(15) D. M. Surgenor and D. Ellis, in preparation.

(16) These preparations were carried out at E. R. Squibb and Son, New Brunswick, New Jersey, and released by the American Red Cross for these investigations.

(17) These preparations were obtained through the courtesy of Drs. Getting, G. Edsall and Mulford.

(18) M. Sørensen and G. Haugaard, *Compt. rend. trav. lab. Carlsberg*, **19**, No. 12, p. 1 (1933).

(19) L. F. Hewitt, *Biochem. J.*, **33**, 1496 (1939).

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

(21) C. Rimington, *Biochem. J.*, **34**, 931 (1940).

(22) We wish to acknowledge the collaboration of Dr. Schade during this work. Periodic microbiological assays of iron-binding capacity, performed under his direction, served as a check on the spectrophotometric determination which, because of its facility, was of great use in guiding the fractionation.

from the frozen state to remove ethanol, and redissolved in water for analysis.[23]

Other methods and the equipment used in this Laboratory for the fractionation of proteins in ethanol–water mixtures of controlled *p*H, ionic strength, temperature, and protein concentration have been described elsewhere.[2]

We are glad to acknowledge the assistance of M. M. Hasson, B. J. Learned and W. O'Donnell during this investigation, as well as that of M. J. E. Budka for the electrophoretic analyses and C. G. Gordon and P. M. Baker, under the direction of J. L. Oncley, for the ultracentrifugal analyses.

III. System of Fractionation

The conditions that have been found effective for the optimum separation of proteins from each other, in the five variable system here employed, have evolved slowly and are always subject to further modification. The lability of some of the components imposed limits on the conditions deemed acceptable. The precipitation of certain components in Fraction IV-4 rather than in Fraction IV-1 or V was considered evidence of protein–protein interactions. When purified, many of the proteins in Fraction IV-4 proved to be isoelectric and of low solubility near or below *p*H 5.0.

Electrophoretic analyses, assays for plasma esterase activity and metal-combining capacity were the principal analytical tools employed. Since a well-planned scheme of fractionation concentrates unknown, as well as known, components, it was hoped that an inclusive subfractionation of Fraction IV-4 would be obtained. This was indeed the case for several components for which no assay was carried out but which were later recognized in one or another subfraction. Because of the possible clinical utilization of some of these components, the number of subfractions was maintained as small as possible and those procedures were adopted which were suitable for large-scale processing.

Fraction IV-5+6.—When a solution of Fraction IV-4 was dialyzed against a buffer of low ionic strength, or diluted to 1% protein concentration (ionic strength 0.005) at 0°, a euglobulin precipitated. The minimum solubility of this precipitate, largely α_1-lipoprotein, was near *p*H 5.0. This precipitate contained 85 to 90% of the lipid, measured as cholesterol, in Fraction IV-4. The supernatant solution contained albumin, α_2- and β_1-globulins; all of the esterase and metal-combining activity.

Although all of the esterase could be precipitated from this solution at *p*H 5.2 by adding ethanol to a concentration of 0.062 mole fraction (18%), a large amount of albumin and β_1-globulin were precipitated as well. An attempt was therefore made to find conditions under which the albumin and β_1-globulin remained soluble while the esterase remained insoluble. On increasing the ionic strength at 0.062 mole fraction ethanol, the solubility of the impurities increased more rapidly than did the esterase. Moreover, on lowering the *p*H to 4.7, the impurities became more soluble. At a *p*H near *p*H 4.4, the esterase as well as the other proteins dissolved. This behavior was reminiscent of the principle[24] that acid to the isoelectric points of the proteins in a fraction, the tendency to interaction diminished and more satisfactory separations were achieved. Thus, at *p*H 4.7, ionic strength 0.02 and 0.062 mole fraction (18%) ethanol at −5°, the esterase was precipitated with a minimum of impurities.

Since the subsequent separation of the esterase involves many additional steps, considerable advantage was gained by proceeding directly to the conditions just described, for the precipitation of the esterase. A large subfraction, Fraction IV-5+6, was obtained, which contained the same components that were found in the earlier two subfractions. This reduced the number of steps and removed the limitation on protein concentration imposed by the low ionic strength necessary to precipitate the α_1-lipoprotein, thus making it possible to use increased protein concentrations throughout.

Fraction IV-5.—In the subfractionation of Fraction IV-5+6 the lipoprotein was separated into Fraction IV-5 by employing the conditions previously found to precipitate this component: *p*H 4.9, and ionic strength 0.002 at 0°. The low ionic strength was attained at a protein concentration of 2.5%, instead of the 1% protein previously employed. The further treatment of Fraction IV-5+6 was intended to separate the plasma esterase, as well as to recover the serum albumins and β_1 metal-combining globulin in this fraction.

Fraction IV-6.—The solution from which Fraction IV-5 was separated was essentially free of lipid, measured as cholesterol. Solubility experiments on the protein remaining in the supernatant showed that the choline esterase had a minimum solubility near *p*H 4.9 (Fig. 1). At this *p*H, in 0.062 mole fraction (18%) ethanol, the solubility of the enzyme increased almost a thousand fold on increasing the ionic strength from 0.02 to 0.10. On lowering the *p*H, the inactive protein dissolved more readily than the esterase. At *p*H 4.4, ionic strength 0.02 and 0.062 mole fraction ethanol, all of the esterase was precipitated into Fraction IV-6 while a considerable fraction of the protein remained soluble.

Fraction IV-7.—The solution from which Fraction IV-5+6 separated consisted mainly of albumin and β_1-globulin. All of the protein in solution could be precipitated, without changing the *p*H or ionic strength, by raising the ethanol concentration to 0.163 mole fraction (40%). However, the β_1 metal-combining globulin could be precipitated in a smaller fraction (IV-7)

(23) We are indebted to Dr. Ralph W. Brauer, and, more recently, to Dr. Avram Goldstein for the plasma esterase assays which they kindly carried out in the Department of Pharmacology, Harvard Medical School.

(24) A. A. Green, THIS JOURNAL, 60, 1108 (1938).

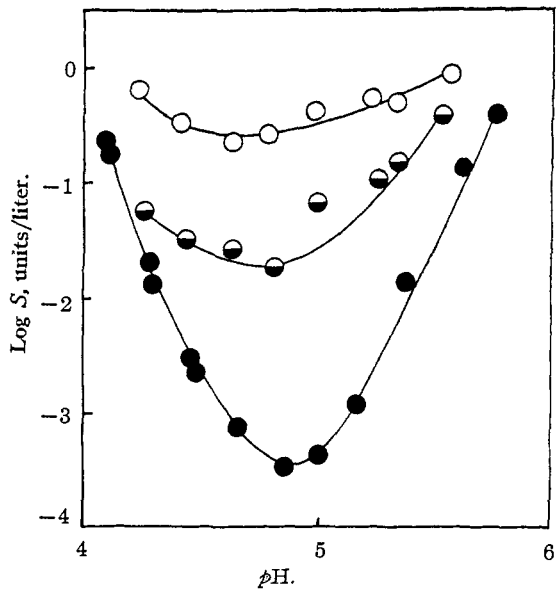

Fig. 1.—Solubility of plasma choline esterase in Fraction IV−5+6 in 0.062 mole fraction ethanol at −5°: ○, Γ/2 = 0.10; ◒, Γ/2 = 0.05; ●, Γ/2 = 0.02 [*cf.* footnote (a) of Table IV for definition of unit of enzyme activity].

formed at *p*H 5.9, ionic strength 0.09 and 0.163 mole fraction ethanol.

Fraction IV-8.—The albumin remaining in solution after removal of Fraction IV-7, could be precipitated, as Fraction IV-8, by lowering the *p*H to 4.9. This fraction contained only small amounts of globulins.

Fraction IV-9.—The solution, after the precipitation of Fraction IV-8, was essentially free of protein. That remaining in solution after precipitation of Fraction IV-6, was mainly albumin and β_1-globulin. These were precipitated together, as Fraction IV-9, by adjusting the *p*H to 5.2 and increasing the ethanol concentration to 0.091 mole fraction (25%) ethanol.

The detailed procedures for carrying out the separation of Fractions IV-5, IV-6, IV-7, IV-8 and IV-9 from Fraction IV-4 are given below. These have been tentatively recommended to the American Red Cross to make these fractions available for clinical studies.

Subfractionation of Fraction IV-4

Fraction IV-4 separated from human plasma by method (6)[25] is the starting material for this preparation.[26] Each kg. of paste is suspended in 8.5 liters of water and ice. The resulting suspension is adjusted to *p*H 4.7, ionic strength 0.02 and 18% ethanol. This requires, for each liter of suspension, 26 millimoles of sodium acetate buffer diluted to 167 ml. with water, and 500 ml. of 53.3% ethanol. The buffer consists of the amount of *p*H 4 acetate buffer necessary to adjust the *p*H, together with sufficient *p*H 4.7 acetate buffer[27] so that the total amount of sodium acetate in the two buffers is 26 millimoles. The diluted buffers and then the ethanol should be added, with vigorous stirring, through capillary jets. The rate of addition should be 80–100 ml. per jet per minute.[28] During the addition of the ethanol the suspension should be kept at the freezing point until a temperature of −5° is reached, and then maintained at −5° throughout the remainder of the addition.

Precipitate IV-5+6 is removed by centrifugation at −5°. The fraction *may* be further separated as described below, or dried from the frozen state. The latter is conveniently done after resuspending the paste in 2 or 3 volumes of water and ice.

The supernatant solution is brought to *p*H 5.9–6.0, ionic strength 0.09 and 40 per cent. ethanol. The *p*H is adjusted with sodium bicarbonate[29] and sodium chloride is added to make a total of 114 millimoles of sodium bicarbonate and sodium chloride for each liter of supernatant. The combined salts are added in 50 ml. of water, after which 437 ml. of 95% ethanol is introduced. The reagents are added through capillary jets, as before, but the rate of addition of the ethanol should be somewhat slower, *i. e.*, 40–50 ml. per jet per minute. The temperature is maintained at −5° throughout the addition.[30]

Precipitate IV-7 is a concentrated source of the metal-combining globulin. The fraction is removed by centrifugation at −5°. It should be dissolved in 3 volumes of water and ice, and dried from the frozen state.

The albumin remaining in solution is precipitated by adjusting the *p*H to 4.9 with *p*H 4 sodium acetate buffer. The buffer should be adjusted with 95% ethanol so that it has an ethanol concentration of 40%. It should be added through capillary jets at the rate of 50 ml. per jet per minute, keeping the temperature constant at −5° and the ethanol concentration at 40%.

Precipitate IV-8 is obtained by centrifuging at −5°. The paste should be redissolved in 3 volumes of ice and water, adjusted to *p*H 6.0–6.5, and dried from the frozen state. The supernatant solution may be discarded.

Subfractionation of Fraction IV-5+6

The IV-5+6 paste[26] is resuspended in 9 volumes of ice and water.[31] This gives a protein concentration of 2.5 per cent., ionic strength 0.002 and 1% ethanol. The *p*H is adjusted with sodium bicarbonate to 4.9 and the suspension should be stirred at least two hours at 0°.

Precipitate IV-5 is removed by centrifugation at 0° at a sufficiently slow rate to give a clear supernatant solution.[32] This fraction is largely α_1-lipoprotein; when derived from dried plasma or dried fractions thereof, it is largely denatured. It may be resuspended in water and dried from the frozen state.

The solution is next brought to 18% ethanol, *p*H 4.4 and ionic strength 0.02. This requires 29 millimoles of ace-

(25) Ref. 2, p. 470.

(26) Storage at −5° of plasma esterase fractions in paste (containing ethanol) will generally result in loss of esterase activity in approximately a month. No appreciable loss of activity has been detected on storage of the dry powder. It is therefore advisable to use the wet paste immediately, or, if this is not possible, to dry the fraction from the frozen state and store the powder in the cold.

(27) W. C. Boyd, THIS JOURNAL, **67**, 1035 (1945).

(28) If dried Fraction IV-4 is used as the starting material, these same conditions will be attained by suspending each 300 g. of powder in 8.7 liters of water. Each liter will require 26 millimoles of the required buffers, diluted to 110 ml. with water, and 556 ml. of 53.3% ethanol.

(29) This is a difficult *p*H adjustment because of the solubility of carbon dioxide in the solution. Thorough equilibration is necessary. The *p*H should be determined with a sample which has been diluted with 3 volumes of 0.02 *M* NaCl without shaking or aerating the solution.

(30) Starting with 2 kg. of IV-4 paste, which is approximately the amount derived from 100 liters of plasma, the volume at this point in the procedure will be 40 liters.

(31) If dry powder is used, each 250 g. should be suspended in 10 liters of water at 0°. It is unnecessary to adjust the ethanol concentration.

(32) In certain cases, for example, in the preparation of the α_2-mucoprotein, it is of advantage to clarify this solution. This can be done by suspending 0.25% of washed standard super-cel and filtering at 0° through Hormann type D-7 pads.

tate buffers, diluted to 50 ml., and 500 ml. of 53.3% ethanol for each liter of solution.[33] The buffers include the amount of *p*H 4 acetate buffer needed to lower the *p*H to 4.4 and enough *p*H 4.4 acetate buffer so that the total amount of sodium acetate in the two buffers is 29 millimoles. The diluted buffers and then the ethanol should be added through capillary jets at the rate of 80–100 ml. per jet per minute. During the addition of the ethanol the temperature should be kept at the freezing point until $-5°$ is reached, and maintained at $-5°$ thereafter. The suspension should be stirred at least two hours, and then centrifuged as soon as possible.[34]

Precipitate IV-6 may be further subfractionated immediately, or resuspended in 2 volumes of ice and water, adjusted to *p*H 5 with sodium bicarbonate, and dried from the frozen state.

Finally, the supernatant solution is adjusted to *p*H 5.2 and 25% ethanol. This requires, for each liter, the necessary amount of sodium bicarbonate, diluted to 25 ml. with water, and 106 ml. of 95% ethanol. The bicarbonate and ethanol should be added through capillary jets as before, maintaining the temperature at $-5°$ throughout.

Precipitate IV-9 may be redissolved in 3 volumes of ice and water, and dried from the frozen state.

IV. Distribution and Properties of Protein Components in Fraction IV-4 and its Subfractions

As in previous studies,[2,4] we have made use of chemical and physical measurements at our disposal to characterize the various components. The distribution of nitrogen and the actual yields of total protein have been determined by direct analysis of the separated subfractions and are given in Table I.

TABLE I

ESTIMATED DISTRIBUTION OF NITROGEN AND PROTEIN INTO SUBFRACTIONS OF FRACTION IV-4

Fraction	Total nitrogen[a]	Per cent. of total protein	$\left(\frac{\text{g. protein}}{\text{g. nitrogen}}\right)$
IV-4	100	100	7.1[b]
IV-5+6	59.8	61.8	7.3
IV-7	14.8	13.8	6.6[c]
IV-8	14.0	12.4	6.3
Supernatant soln.	4.2	3.8	6.4
Total	92.8	91.8	

[a] Based on averages from direct analysis of precipitates in two runs. [b] From ref. 2, Table VII, p. 473 [c] Measurement made by J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. and Coll. Chem.*, **51**, 184(1947).

From these data, and the results of the electrophoretic analyses,[35] the yields of the electrophoretic components in the subfractions have been calculated. The yields are presented in Tables II and III. The limitations in the quantitative interpretation of electrophoretic Schlieren diagrams, discussed in a previous paper in this series,[36] were quite apparent, especially in the determination of α-globulins. Indeed, we have found the electrophoretic technique of but limited use in characterizing fractions containing high percentages of α_2-globulins. The protein components of interest have been most readily followed during subfractionations by means of assays for lipid, carbohydrate, plasma esterase and iron-binding capacity. Results of certain experiments suggesting the distribution of lipoprotein, carbohydrate-rich proteins, plasma esterase and metal-combining globulin are reported in Table IV.

(33) If dry Fraction IV-5+6 were used, each liter of solution requires 535 ml. of 53.3% ethanol.

(34) Although serum esterase has proved to be among the more stable of the plasma proteins at acid *p*H, it is advisible to expose solutions of the enzyme to acid conditions for as short a time as possibe.

(35) Electrophoretic analyses have not been corrected for refractive index increments.

(36) S. H. Armstrong, Jr., M. J. E. Budka and K. C. Morrison, THIS JOURNAL, **69**, 416 (1947).

TABLE II

ESTIMATED DISTRIBUTION OF PROTEINS INTO SUBFRACTIONS OF FRACTION IV-4[a]

Fraction	Albumin	α_1	α_2	β_1	β_2	Total
	Per cent. of subfraction					
IV-4[b]	30	17	24	28	1	100
IV-5+6	15	17	44	23	1	100
IV-7	13	1	7	76	3	100
IV-8	91	3	3	3		100
	Grams per liter of plasma recovered in subfractions					
IV-4[b]	1.5	0.9	1.1	1.6	0.1	5.2
IV-5+6	0.5	0.5	1.4	0.7	0.1	3.2
IV-7	0.1	<0.1	<0.1	0.6	<0.1	0.7
IV-8	0.7	<0.1	<0.1	<0.1	0	0.7
Total	1.3	0.5	1.4	1.3	0.1	4.6

[a] The strong interactions of the α-globulins resulted in low apparent values for α_1-globulins and correspondingly high apparent values for α_2-globulins as determined from the Schlieren diagrams. [b] These values were obtained from three preparations of Fraction IV-4 used in this work, and have been used rather than the previously published values for Fraction IV-4 (ref. 2, Table IX, p. 479) in preparing this table, since the fraction obtained from outdated dried plasma or from fresh plasma in which acid citrate dextrose, "ACD," was used as the diluent, usually contained more albumin, and was therefore correspondingly larger in size, than Fraction IV-4 from fresh citrated plasma.

TABLE III

ESTIMATED DISTRIBUTION PROTEINS INTO SUBFRACTIONS OF FRACTION IV-5+6

Fraction	Albumin	α_1	α_2	β_1	β_2	Total
	Per cent. of subfraction					
IV-5+6	15	17	44	23	1	100
IV-5	8	73	14	4	1	100
IV-6	6	11	77	6		100
IV-9	30	0	28	41	1	100
	Grams per liter of plasma recovered in subfractions					
IV-5+6	0.5	0.5	1.4	0.7	0.1	3.2
IV-5	<0.1	0.5	0.1	<0.1	<0.1	0.6
IV-6	0.1	0.1	0.8	<0.1	0	1.0
IV-9	0.3	0	0.3	0.5	<0.1	1.1
Total	0.4	0.6	1.2	0.5	<0.1	2.7

Fraction IV-5.—The α_1-lipoprotein separated in this fraction was largely denatured. Cholesterol determinations and qualitative observations, however, led to the conclusion that this component was probably identical with the α_1-lipoprotein which separated into Fraction IV-1.

Fraction IV-6.—This complex fraction contained at least two carbohydrate-rich α_2-globulins,

TABLE IV

ESTIMATED DISTRIBUTION OF CARBOHYDRATE, CHOLESTEROL, IRON-BINDING CAPACITY AND PLASMA CHOLINE ESTERASE IN SUBFRACTIONS

Fraction	Carbohydrate, %	Cholesterol, %	Units plasma[a] esterase/g.	Iron-binding capacity, mg. Fe/g.
Plasma	(1.16)[b]	2.3[b]	1.5–2.0	0.04[c]
IV-4	2.9[b,d]	1.7[b]	20	.30
IV-5+6	3.1	2.1	32[e]	.24
IV-5		5.3		
IV-6	3.8		45	
IV-7	2.3	<0.1	<1	.85
IV-8	0.4	<0.1	0	.05
IV-9	1.7		1	.44

[a] Assays for esterase activity were made on appropriate dilutions of the enzyme, by measuring in the Warburg apparatus, at 37°, the release of carbon dioxide from a solution of sodium bicarbonate buffered at *p*H 7.4 with carbon dioxide. Acetylcholine, 0.0805 molar, was used as the substrate. We have used the unit of esterase activity as that amount of enzyme which would split 1 millimole of acetylcholine per hour under the above conditions. [b] Values taken from Table VIII, ref. 2, p. 474. [c] Based on estimates of the amount of metal-combining protein in plasma. This agrees quite well with the value obtained by direct analysis of plasma. [d] Higher values have been obtained using the carbazole reaction, by F. B. Seibert, M. L. Pfaff and M. V. Seibert, *Arch. Biochem.*, **18**, 279 (1948). [e] Plasma esterase represents only a minor part of the protein in this fraction, and its properties cannot therefore be deduced from the properties of this fraction.

in addition to plasma choline esterase. From the results of studies now in progress[15] which indicate that the pure enzyme may have an activity of at least 15,000 times its activity in plasma, it has been estimated that less than 1 per cent. of the protein in the fraction was esterase.

Fraction IV-7.—The main component of this fraction was the metal-combining globulin, so designated because of certain *in vitro* evidence that it is capable of binding other metals as well as iron.[10,14,37] Subsequent purification and crystallization of this component[14] has proved that the β_1-globulin which separated with Fraction IV-4, and was further concentrated in Fraction IV-7, is indeed the component responsible for the iron-binding capacity of the serum.[9]

Of the impurities in Fraction IV-7, the α_2-globulin, although rich in carbohydrate, has been distinguished from the α_2-globulins of Fraction IV-6 by its ability to bind bilirubin. This property was not shared by the α_2-globulins of Fraction IV-6.[38]

Fraction IV-7, rather than the crystallized β_1 metal-combining globulin obtained therefrom, has been recommended to the American Red Cross as the most convenient source of metal-combining globulin for clinical investigation.[10,12,13]

(37) This component has been called "siderophyllin" by Schade [A. L. Schade, R. W. Reinhart and H. Levy, *Arch. Biochem.*, **20**, 170 (1949)] and "transferrin" by Holmberg and Laurell (C. G. Holmberg and C.-B. Laurell, *Acta Chem. Scand.*, **I**, 944 (1947)).

(38) N. H. Martin, THIS JOURNAL, **71**, 1230 (1949).

Fraction IV-8.—Recovery of the albumins in Fraction IV-4 and their addition to those in Fraction V[2] represents an additional yield of 2%. The albumins in Fraction IV-8 were similar in ultracentrifugal behavior and in ability to bind hematin,[39] to the albumins separated into Fraction V. No differences between them have yet been observed.

V. Studies with the α_2-Mucoprotein

Protein-bound carbohydrate is distributed rather generally throughout the globulins in the plasma. Of the various components isolated from plasma, only the crystallized serum albumin was free of carbohydrate.[40] The highest concentration of bound carbohydrate was found in Fraction IV-4.[41]

The carbohydrate-rich proteins fall into two classes, the mucoproteins and the glycoproteins.[42] Examples of these are the mucoprotein fraction from guinea pig serum, associated with one of the components of complement,[43] and the glycoprotein from horse serum, associated with albumin.[44] Mucoproteins have been characterized by euglobulin properties, high viscosity in solution, and a tacky behavior when precipitated. Glycoproteins have often appeared to be pseudoglobulins, resembling the albumins in their solubility properties.

In the course of the studies with Fraction IV-4 we have recognized members of both classes of carbohydrate-rich proteins. These were largely separated, with the α_2-globulins, into Fraction IV-6 by the procedures just described. The results of some studies with an α_2-mucoprotein are outlined here; studies on the glycoproteins will be the subject of a later communication.

The mucoprotein has proved to be extremely labile under conditions of acid *p*H. Preparations derived from Fraction IV-6, which was precipitated at *p*H 4.4, were usually partially denatured, as evidenced by a large amount of fast-sedimenting, inhomogeneous material in the ultracentrifuge.[45] It therefore seemed necessary to prepare mucoprotein by means of separations which did not involve the use of acidities below *p*H 5. In this

(39) M. Rosenfeld, in preparation.

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

(41) Ref. 2, Table VIII.

(42) The nature of the carbohydrate moiety has received considerable attention and the carbohydrate content has been the principal means of characterization. The presence of glucoseamine was early demonstrated by H. W. Bywaters [*Biochem. Z.*, **15**, 322 (1909)]. The evidence indicates a polysaccharide of the composition glucoseamine:mannose:galactose [C. Rimington, *Biochem. J.*, **34**, 931 (1940)]; ref. 19.

(43) L. Pillemer, E. E. Ecker, J. L. Oncley and E. J. Cohn, *J. Exp. Med.*, **74**, 297 (1941).

(44) T. L. McMeekin, THIS JOURNAL, **62**, 3393 (1940).

(45) It is difficult to decide, on the basis of ultracentrifugal behavior, whether a part of this denatured material was already present in the starting material because of the small amount that would have been present in Fraction IV-4.

way, it was hoped to obtain the native protein, although in lower yield and of less purity. Considerable difficulty was encountered in freeing the preparations of the euglobulin lipoprotein, as well as of the last traces of metal-combining globulin, which seemed to interact strongly with the mucoprotein.

Experimental

Purified α_2-mucoprotein was prepared by a series of separations involving varying conditions of *p*H, ionic strength, ethanol concentration, protein concentration and temperature. (1) Fraction IV-4 was resuspended and adjusted to *p*H 5.2, ionic strength 0.02, 18% ethanol, 2% protein and −5°. (2) The precipitate, which contained all the α_1-lipoprotein, α_2-globulins, together with some albumin and β_1 metal-combining globulin, was brought to *p*H 5.0, ionic strength 0.001, 1.5% protein and 0°. The euglobulin precipitate, largely lipoprotein, was discarded. (3) Crude mucoprotein was precipitated by adjusting the solution to *p*H 5.1, ionic strength 0.005, 10% ethanol and −3°. The esterase and more soluble α_2-glycoproteins remained in solution under these conditions. (4) β_1 Metal-combining globulin was removed from the crude mucoprotein precipitate by resuspending and adjusting the conditions to *p*H 6.1, ionic strength 0.02, 19% ethanol, 1.2% protein and −5°. The mucoprotein, which remained soluble under these conditions, was reprecipitated (5) by returning to the first set of conditions: *p*H 5.2, ionic strength 0.02, 17% ethanol and −5°. The precipitate contained 6% of the protein from Fraction IV-4.

Further purification was effected (6) by reprecipitation of the mucoprotein at *p*H 5.1, ionic strength 0.003, 10% ethanol, 0.3% protein and −3°. (7) The precipitate was redissolved in water at a protein concentration of 0.5% and filtered through a Seitz type S-3 pad under pressure to remove the slight turbidity due to some residual lipoprotein in the preparation. The product was 82 per cent. α_2-globulin by electrophoresis, the impurities being albumin (6%) α_1-globulin (8%), and β_1-globulin (4%).

In the ultracentrifuge three poorly resolved components were observed: (a) about 10% sedimenting much like albumin and β_1 metal-combining globulin (s = 4.5 to 5.5), (b) 15 to 25%, quite heterogeneous, sedimenting rapidly (s = 12 to 20), and (c) the third and principal component (65 to 75 per cent.) sedimenting with s = 6 to 9, depending on the protein concentration. The extrapolated value at infinite dilution was near s = 11. This behavior is somewhat like that to be expected of a highly asymmetrical molecule, but more homogeneous preparations must be studied before this can be definitely proved.

The results of dry weight, nitrogen, and carbohydrate determinations are summarized in Table V. The hexose–hexoseamine ratio was close to 3 to 2, instead of 2 to 1.[40] This may be due to the fact that our preparation was not pure.

TABLE V

PROPERTIES OF PURIFIED MUCOPROTEIN

g. protein/g. nitrogen	6.90
g. hexose/g. protein	0.0435
g. hexoseamine/g. protein	0.0284
Hexose/hexoseamine	1.53

The protein could be dried from the frozen state; it was insoluble in water and soluble in dilute sodium chloride, acetate, or bicarbonate. On boiling solutions up to 2% in concentration for four hours, there was no visible change. The protein was precipitated, however, with 7% trichloroacetic acid.

The mucoprotein had a marked accelerating effect on the sedimentation rate of erythrocytes which was accompanied by rouleau formation. Added to whole blood in concentrations of 0.5 g. per 100 ml., the mucoprotein increased the rate 2.5-fold over the control. Fibrinogen, which is the principal component affecting erythrocyte sedimentation rates,[46] produced a 5-fold acceleration in the same concentration. Other fractions tested, including Fraction IV-4, had no effect, or diminished the rate.

Summary

Under controlled conditions of *p*H, temperature, ethanol, salt and protein concentrations, Fraction IV-4 of human plasma has been separated into a series of subfractions into which were concentrated plasma choline esterase, carbohydrate-rich α_2-globulins, both glycoprotein and mucoprotein, the β_1-metal-combining globulin and albumins. These subfraction are of use in chemical physiological and clinical studies and as intermediates in the purification and crystallization of the various protein components.

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(46) R. Fåhraeus, *Acta med. scand.*, **55**, 1 (1921); S. J. Gray and E. B. Mitchell, *Proc. Soc. Expil. Biol. Med.*, **51**, 403 (1942); K. Meyer, E. Hahnel and R. R. Feiner, *ibid.*, **58**, 36 (1945).