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A Comparison of Plasma Protein Fractions by Kjeldahl and Biuret Analysis and Refractive Index Measurements

By R. William Knouse and J. I. Routh

In electrophoretic studies of serum and plasma the concentration of protein in the various fractions is often expressed in absolute values. These are commonly obtained by calculation from the percentage distribution of the components and the total protein content of the serum or plasma. Although this practice is widespread there is no assurance that the values obtained reflect a true measure of the protein content of the individual components. It is readily apparent from the divergent results obtained by the technique of paper electrophoresis that the major components of serum contain varying concentrations of protein, lipids and carbohydrates and exhibit a varying response to protein dyes.

The total protein of serum or plasma may be determined by a Kjeldahl nitrogen estimation, by the biuret reaction or by refractive index measurements. Although the Kjeldahl method is capable of accurate nitrogen analysis the proper factor for conversion to a particular protein is seldom the commonly used 6.25 times the nitrogen content. In the biuret method the structural unit in the protein molecule that complexes with the copper (1), may not occur at regular intervals and thus produce variable color intensities when applied to equal concentrations of serum fractions. In general, however, the biuret method gives values for protein concentration which bear a linear relationship to those calculated from Kjeldahl nitrogen values (2). The refractive index measurements may also be affected by lipids and carbohydrate complexes in the fractions but exhibit a fairly linear response with change in protein concentration.

To obtain more complete information on the plasma fractions encountered in electrophoretic analysis the separated protein components were subjected to analysis by the Kjeldahl, biuret and refractive index methods.

Experimental

Pooled blood plasma was fractionated by method number six of Cohn, et al., (3) to obtain the fractions commonly encountered in electrophoretic analysis, namely albumin, fibrinogen and alpha, beta and gamma globulins. Cohn's procedure involves the establishment of proper conditions of pH, ionic strength, temperature, and the

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proper mole fraction of ethanol for the precipitation of each fraction. These fractions and the conditions used to precipitate them are presented in Table 1. Whenever the pH was lowered it was done by the addition of the sodium acetate-acetic acid buffer (pH 4.0) through capillary jets. In precipitating fraction IV-4 from the supernatant solution of fraction IV-1, the pH was raised by the addition of a disodium acid phosphate buffer (pH 9.6). A protein solution was constantly stirred whenever an addition was made to it. The ionic strength was decreased by dilution with water or ethanol solutions, and was raised by the addition of acetate buffer. The ethanol and buffer were mixed together before being added to the protein solution. This necessitated titrating an aliquot of the protein solution with part of the alcohol buffer mixture to make sure the final pH would be correct. Readings of pH were made with a glass electrode at ethanol concentrations of 10 per cent (by volume) or less, since ethanol con-

Table 1

Conditions for Plasma Fractionation With Cold Buffered Alcohol Solution (3)

Fraction	pH	Ionic Strength	Temp. °C.	Mole Fraction Ethanol	Protein in System g/1
Plasma	7.4	.16	—	—	60.3
I	7.2	.14	−3.0	.027	51.1
II & III	6.8	.09	−5.0	.091	30.0
IV - 1	5.2	.09	−5.0	.062	15.8
IV - 4	5.8	.09	−5.0	.163	10.1
V	4.8	.11	−5.0	.163	7.5
VI	4.8	.11	−5.0	.163	0.2
III_o	5.7	.005	−5.0	.137	—
II & III_w	5.2	.015	−6.0	.077	—

centrations of 13 per cent or more were found to introduce errors into the pH measurements. Fraction II & III was further treated to give a more nearly complete separation of the beta lipoprotein and gamma globulin by increasing the ethanol concentration to 20 per cent while maintaining the pH at 7.2 and the temperature at 0° C. The precipitated protein fractions were isolated by centrifugation at 2,500 rpm for five minutes at the temperature specified, viz., −3.0° to −6.0° C. As each fraction was obtained in the moist state it was kept cold until transferred to a glass bulb and quickly frozen in a thin layer by rotating the bulb in alcohol and dry ice. The fractions were then lyophilized at a pressure below one mm. Hg. Electrophoretic analyses were then made on aliquots of each fraction to verify their composition. Free electrophoresis was carried out in the Tiselius apparatus at a constant current of 25 milliamperes for 120 minutes. A diethyl barbiturate buffer of pH 8.6 and ionic strength 0.1 was used. The values obtained are compared with those found by Cohn, et al., in Table 2. Complete solution of fractions II & III and IV containing globulins was difficult to attain and

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therefore solutions of known protein concentrations could not be prepared by adding known amounts of the lyophilized product to the buffer solutions. Instead, aliquots of the lyophilized fractions were dried in a vacuum oven and analyzed for nitrogen content. The protein concentration of solutions of these fractions could then be determined by measuring the quantity of dissolved nitrogen. The averaged percentages of nitrogen and those obtained by Cohn, et al., (3) are found in Table 2.

Table 2

Percentage Composition of Fractions Compared with Those Obtained by Cohn (3)

	Fraction						
	I	II & III	II & III$_w$	III$_o$	IV$_1$	IV$_4$	V
Total Nitrogen	15.2		13.9	13.6	13.4	14.0	15.4
Albumin	18		6	22	26	8	98
α	0		16	21	67	53	2
β	6		15	52	7	39	0
γ	0		63	0	0	0	0
Fibrinogen	77		0	5	0	0	0
		Cohn's Fractions					
Total Nitrogen	16.4	13.0			11.9	14.0	15.9
Albumin	7	4			0	16	95
α	8	6			89	46	4
β	15	48			10	38	1
γ	9	37			1	0	0
Fibrinogen	61	5			0	0	0

Similar solutions were prepared for the comparative Kjeldahl, biuret, and refractive index studies. Because these analyses and measurements required several hours to complete, only one fraction was studied at a time, thus avoiding denaturation or bacterial action on standing.

Micro Kjeldahl analyses were carried out on aliquots of the solution by digesting with a mixture of sulfuric and phosphoric acids, and copper and potassium sulfate and heating for one hour after the solutions cleared. Alkali was added and the samples steam distilled in a closed system, the distillate draining directly into a 2 per cent solution of boric acid to which an indicator composed of eight parts of bromcresol green to one part of methyl red had been added. The titration was carried out with dilute hydrochloric acid. The protein concentration in the solution was then calculated using the factor obtained from the nitrogen content of the dried fraction. The biuret analyses were run on aliquots of the same solutions used for the Kjeldahl analyses. Weichselbaum's (4) biuret reagent was employed and the method used was that of Wolfson (5). All specimens were run in duplicate, and read in a Coleman Jr. spectrophotometer. The refractive index measurements were made on aliquots of the same solutions used for the other two procedures, utilizing the Abbe refractometer and a tungsten filament light source. The change in

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Table 3

Comparative Data on Solutions of the Separated Fractions

Fraction	Protein concn. per cent	Kjeldahl mg.N/ml.	Biuret O.D.	Refractive index increments x 10^3
V	4.00	6.27	0.135	110
V	3.00	4.70	0.100	80
V	2.00	3.12	0.067	53
V	1.00	1.56	0.033	23
V	0.20	0.31	0.004	4
I	3.63	5.48	0.165	117
I	2.72	4.09	0.130	88
I	1.82	2.70	0.083	57
I	0.91	1.35	0.022	29
I	0.45	0.67	0.008	15
II & III_w	1.47	2.04	0.070	72
II & III_w	0.98	1.38	0.046	47
II & III_w	0.49	0.69	0.022	23
II & III_w	0.16	0.23	0.007	9
III_o	1.25	1.70	0.076	71
III_o	0.62	0.83	0.037	35
III_o	0.31	0.44	0.018	18
III_o	0.15	0.21	0.009	9
IV - 1	0.87	1.16	0.044	62
IV - 1	0.43	0.53	0.021	31
IV - 1	0.22	0.27	0.010	16
IV - 1	0.11	0.13	0.005	7
IV - 4	5.67	7.95	0.201	160
IV - 4	2.84	3.99	0.100	78
IV - 4	1.42	1.99	0.049	40
IV - 4	0.71	1.00	0.024	19

Table 4

Comparative Data on Solutions of the Fractions Calculated to a One Per Cent Protein Solution

Fraction	Biuret O.D.	Kjeldahl mg.N/ml.	Refractive index increment x 10^3
IV - 1	.086	1.35	71.4
III_o	.060	1.36	56.5
II & III_w	.048	1.37	49.1
I	.046	1.50	32.2
IV - 4	.035	1.38	27.7
V	.032	1.56	26.5

refractive index was calculated by subtracting the readings of the blank (buffer) from that of each aliquot. The data obtained from these studies are shown in Table 3. To facilitate comparison of the fractions, Table 4 presents data calculated for a protein concentration of one per cent.

Discussion

The fractionation procedure was somewhat more efficient in the isolation of fibrinogen than reported by Cohn, et al. (Table 2), whereas they obtained a better isolation of albumin and alpha globulin. As previously discussed, it was not possible to completely dissolve aliquots of fraction III or IV-1 in the barbital buffer. Other solvents were not employed however, since barbital buffer of pH 8.6

and ionic strength of 0.1 is so widely used in electrophoretic analysis of plasma proteins. Since the concentrations of the dissolved fractions are based on their nitrogen content, it is obvious that the variations in optical density with the biuret reaction (Table 3) cannot be assumed to be due solely to changes in the sensitivity of the color reaction to the various types of protein present. They may be due in part to variations in the percentage of total nitrogen bound in peptide linkages, or they may reflect the variations in percentages of nitrogen in the fractions, as given in Table 2. When the data for each fraction in Table 3 were graphed, a linear relationship was observed between the protein concentration and the increases in biuret, Kjeldahl and refractive index values. It was therefore possible to examine the relationships between the fractions by mathematically comparing the values at a given protein concentration. Such a comparison is shown in Table 4 where the fractions are listed in an order that emphasizes the correlations. With the exception of fraction I the biuret and refractive index values decrease as the Kjeldahl values increase. On this basis the proteins having the smaller nitrogen content have a higher percentage of nitrogen in peptide linkages. The two fractions having the highest optical densities and refractive index increments were composed chiefly of lipid-containing alpha and beta globulins. Albumin, which probably has the least lipid, had the lowest value. No relationship between the data of Table 4 and the molecular weight of the chief component of each fraction was apparent.

Summary

Pooled human blood plasma was fractionated and the composition of the fractions determined electrophoretically. Kjeldahl, biuret, and refractive index studies were made on the fractions. The values obtained by each method were linear with respect to concentration of protein. When compared at a given concentration, values obtained by the three methods did not parallel each other, but appeared to differ on the basis of the amounts of lipid and the percentage of nitrogen present in the fractions.

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