sions of some duration, may not be unique with nitric oxide. If the reaction proceeds by some mechanism involving such an intermediate as N_2O_3 (or N_2O_4)' the negative temperature coefficient may be understandable due to the thermal instability of these compounds. Certainly little or no N_2O_3 exists in the gas phase at low pressures and high temperatures, but lowering the temperature would at least favor its formation.

If intermediate compound formation is involved in the reaction mechanism, low temperature would favor the formation of intermediates, and a very rapid rate may be expected. Certainly several of the gases (particularly HNO_3, H_2O and NO_2) are easily condensed even at relatively low pressures at 0°. Intermolecular attractions to form intermediates involving these gases are also high at this temperature. A pronounced negative temperature coefficient might then be expected for this reaction, on the assumption that it is a termolecular process. The negative temperature coefficient is incredibly sharp below 20°, however, and it seems likely that other factors are involved.

In view of the complexity of the reaction, particularly the great effect of surface on the rate, the likelihood of alternative explanations of the temperature behavior cannot be ignored. If, as seems likely, the reaction as observed is largely a surface phenomenon, the faster rate at low temperatures may perhaps be attributed simply to increased absorption. The extremely rapid, quite erratic, rate in the neighborhood of 0° indicates this. Certainly surface effects are involved in, and perhaps they are largely responsible for, the unusually large negative temperature coefficient in this region.

Summary

1. A preliminary study of the oxidation of nitric oxide with nitric acid vapor in the gas phase at low pressures is reported. Rate measurements show that the kinetics of the reaction are complex and that surface effects are very prominent.

2. The rate is influenced by the reaction products, and some termolecular characteristics are noted. The rate equation $d[NO_2]/dt = k\,[NO]\cdot[HNO_3]\,[NO_2]$ approximately fits the data. The high order reaction is further complicated by strong catalytic effects due to water vapor and surface.

3. A pronounced negative temperature coefficient in the range from 0 to 30° further suggests termolecular characteristics, although adsorption and surface effects may provide an alternative explanation.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL, AND THE MEDICAL CLINIC, PETER BENT BRIGHAM HOSPITAL]

Preparation and Properties of Serum and Plasma Proteins. XII. The Refractive Properties of the Proteins of Human Plasma and Certain Purified Fractions[1,2]

BY S. H. ARMSTRONG, JR.,[3] M. J. E. BUDKA, K. C. MORRISON AND M. HASSON

The refractive properties of proteins in solution are widely employed both in methods for rapid estimation of protein concentrations[4] and in the optical systems introduced by the Scandinavian workers for analysis of concentration gradients at boundaries formed in solutions both of pure proteins and of mixtures separable by various types of force.[5]

The general assumption of identity of specific refractive increments has been generally recognized as a first approximation,[6] particularly in instances wherein components are associated with appreciable quantities of non-protein materials.[7] The early literature yielded considerable discrepancies of the actual values of specific refractive increments for human plasma proteins (for example, 1.66×10^{-4} to 2.00×10^{-4}, in terms of grams per liter, for albumin).[8]

This study presents serial determinations of refractive index increments on pools of normal

(1) This work has been carried out in part under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

(2) This is Number 59 in the series "Studies on Plasma Proteins" from Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

(3) Welch Fellow in Internal Medicine of the National Research Council.

(4) Earlier literature on rapid protein determinations by refractometric methods, together with the presentation of new measurements on a variety of normal and pathological plasmas of varying protein content, has been recently summarized by F. W. Sunderman (*J. Biol. Chem.*, **153**, 139 (1944)).

(5) O. Lamm, *Nova Acta. Regiae Soc. Sci. Upsaliensis*, IV, **10**, 6 (1937); A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937); T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, London, 1940.

(6) L. G. Longsworth, *Chem. Rev.*, **30**, 323 (1942); V. P. Dole, *J. Clin. Investigation*, **23**, 705 (1944).

(7) L. G. Longsworth, T. Shedlovsky and D. A. MacInnes, *J. Expt. Med.*, **70**, 399 (1939); L. G. Longsworth and D. A. MacInnes, *ibid.*, **71**, 77 (1940); L. G. Longsworth, R. M. Curtis and R. H. Pembroke, Jr., *J. Clin. Investigation*, **24**, 46 (1945).

(8) The values of the earlier literature have been well summarized, together with consideration of the possible bases for the discrepancies, in the papers of Adair and Robinson (G. S. Adair and M. E. Robinson, *Biochem. J.*, **24**, 993 (1930)) and Putzeys (M. P. Putzeys and Mlle. J. Brosteaux, *Bull. Soc. chem. biol.*, **18**, 1681 (1936)). The values of these workers, together with the careful measurements of McFarlane (A. S. McFarlane, *Biochem. J.*, **29**, 407 (1935)), would appear to constitute the most reliable data on animal plasma proteins.

human plasma together with fractions obtained by methods detailed in other communications of this series.[9] Comparison with measurements on certain pathological plasmas, and the range of values considered in terms of the refractive properties of non-protein materials known to be associated with certain of the plasma proteins in their natural state are given.

Strictly, the specific refractive increment cannot be considered a molecular property in that, at a given temperature and wave length, it is a function of the refractive index of the solvent as well as that of the class of molecule. From the data on refractive increments, estimates of the refractive index of dried protein have been made for certain purified materials.

Experimental

Preparation of Solutions for Determination of Refractive Index Increment, Dried Weight and Nitrogen Content.—Plasma and plasma fractions were stored between +8 and −10° without preservative. Those lipoproteins extraordinarily subject to growth of molds and unstable when frozen at low temperatures were stored at 0° following a sterilizing Seitz filtration.

Protein solutions were dialyzed in cellophane sacks for at least five days against several changes of sodium chloride of ionic strength 0.3. This high ionic strength in monovalent ions was chosen to keep inequalities in distribution of diffusible ions across the membranes (as calculated and measured by Scatchard and associates) well under 1%, a value assumed to be less than the combined experimental error of the measurements.[10] That such inequalities did not give rise to systematic errors is seen in the absence of significant differences in nitrogen factors or specific refractive index increments for crystalline albumin measured at various *p*H's in several salts and after exhaustive electrodialysis (*cf.* Table II).

Measurement of Refractive Indices.—Protein solution and dialysate were brought to constant temperature (20–25°) in a water-bath; refractive index measurements were carried out *in situ* by the Bausch and Lomb dipping refractometer. The optical system of this instrument contains a prism monochromator yielding values for the sodium D line, a wave length (5893 Å.) very close to the range of the principal energy of the light source.[11]

Determination of Weight of Dried Proteins.[12]—Determinations of dried weights were made on both protein solutions and the dialysate. The protein concentration was taken as the dried weight of the protein solution minus the dried weight of the dialysate, and is expressed in this report in terms of grams per liter.

Because of variability in results yielded by the drying method standard in the laboratory (air oven at 110°) (particularly in the case of proteins associated in the natural state with lipids), systematic comparison has been carried on with drying processes in which the condition of the protein, the temperature and the pressure have been varied.

By the standard method, proteins relatively poor in lipid (ranging from crystalline albumin, γ-globulin, and fibrinogen to whole plasma, which usually contains less than 0.1 g. of lipid per gram of protein) dried to constant weights which differed very little from the weights obtained under various other conditions. The data for a typical experiment on a pool of normal plasma are presented schematically below. The values of refractive increment and nitrogen factor are calculated from weights determined on a single solution of measured nitrogen content and refractive properties.

The dried weight plotted against time of a protein low in lipid (electrophoretically separated albumin) and a protein high in lipid (Fraction III-O) under various drying conditions is contrasted in Fig. 1. Under standard conditions, the protein low in lipid lost approximately 1 mg. (less than 1 per cent. of its total weight) within the first four to five days; thereafter, weights were apparently constant over a period of a week although during the next three months loss of another milligram occurred; subsequently, the weight appeared constant over several months. The weight at the first plateau has been considered the weight of the dried sample. *Per contra*, the protein high in lipid under standard conditions showed a rapid and continuous loss approximating 15 mg. (over 10% of the total weight) in ten days. *In vacuo* at 100° over phosphorus pentoxide the rate of loss was less rapid. At either 80 or 50° *in vacuo* over phosphorus pentoxide lipoproteins behaved much as did lipid-poor proteins dried by the standard method.

Therefore, values obtained *in vacuo* at 50 to 80°

(9) 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); E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, *ibid.*, **69**, 1753 (1947); P. R. Morrison, J. T. Edsall and S. G. Miller, in preparation; J. L. Oncley, M. Melin, D. A. Richert, J. W. Cameron, and P. M. Gross, in preparation.

(10) G. Scatchard, in E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Chap. III, Reinhold Publishing Company, New York, N. Y., 1943, p. 46; G. Scatchard, THIS JOURNAL, **68**, 2315 (1946); G. Scatchard, A. C. Batchelder and A. Brown, *ibid.*, **68**, 2320 (1946); G. Scatchard, A. C. Batchelder, A. Brown and M. Zosa, *ibid.*, **68**, 2610 (1946).

These studies, together with others subsequently carried out in Professor Scatchard's laboratory, indicate that albumin at the ionic strength here employed may bind about 7 moles of chloride per mole of protein. The effect of this binding on distribution across a membrane is opposite in sign to the Donnan effect. Measurements of greater accuracy, carried out below a critical but presumably low sodium chloride concentration, would reflect this binding in variations of refractive increment as a function of salt concentration.

(11) The light source comprised a mercury arc lamp, General Electric Type AH4 in conjunction with a yellow filter (Wratten #22) whose principal energy by spectroscopic examination lay in the range of the yellow mercury lines (5770, 5791 Å.). This light source is in routine use for electrophoretic measurements.

(12) We wish to express our appreciation to Mrs. M. Y. Donath for her assistance in carrying out a systematic study of conditions affecting dried weight determinations.

TABLE

Drying conditions			$\frac{\Delta n \times 10^4}{\Delta W\left(\frac{\text{g. prot.}}{\text{liter}}\right)}$	$\frac{\Delta W\left(\frac{\text{g. prot.}}{\text{liter}}\right)}{\Delta N\left(\frac{\text{g. N}}{\text{liter}}\right)}$
Air oven, 105 ± 5° to constant weight	No preliminary precipitation	No final vacuum drying	1.831	6.69
		Further dried *in vacuo* over P_2O_5, 100°	1.831	6.69
	Preliminary precipitation at 60% ethanol	No final vacuum drying	1.836	6.68
		Further dried *in vacuo* over P_2O_5, 100°	1.826	6.72
		Limiting values	1.836	6.68
Dried *in vacuo* from frozen state Condenser: CO_2 ice Twenty-four hours		No final drying	1.806	6.79
		Further dried *in vacuo* over P_2O_5 at room temp. to constant weight	1.831	6.69
		Further dried *in vacuo* over P_2O_5, 80°	1.831	6.69
		Further dried *in vacuo* over P_2O_5, 100°	1.821	6.73
		Further dried in air oven, 105 ± 5°	1.836	6.68
		Limiting values	1.836	6.68

have been used in this study. Samples thus kept for a period of months exhibited further rapid weight loss if returned to 100°.

Nitrogen Analyses.—A modification of Pregl's method was employed.[13]

TABLE I

REFRACTIVE INDEX INCREMENTS AND NITROGEN FACTORS OF PROTEINS OF NORMAL POOLED HUMAN PLASMA

Pool number	$\frac{\Delta n \times 10^4}{\Delta W\left(\frac{\text{g. protein}}{\text{liter}}\right)}$	$\frac{\Delta n \times 10^3}{\Delta N\left(\frac{\text{g. N}}{\text{liter}}\right)}$	$\frac{\Delta W\left(\frac{\text{g. protein}}{\text{liter}}\right)}{\Delta N\left(\frac{\text{g. N}}{\text{liter}}\right)}$
97	1.84	1.23	6.67
98	1.84	1.23	6.66
99	1.83	1.24	6.80
100	1.84	1.24	6.70
102	1.82	1.20	6.59
104	1.82	1.27	7.02
105	1.82	1.25	6.88
115	1.84	1.24	6.75
119	1.83	1.23	6.75
121	1.82	1.25	6.88
123	1.83	1.24	6.76
124	1.82	1.23	6.76
125	1.82	1.23	6.78
127	1.86	1.24	6.67
129	1.85	1.24	6.69
130	1.83	1.26	6.86
133	1.83	1.25	6.80
154	1.83	1.24	6.74
162	1.83	1.23	6.69
Average values: plasma pools	1.83	1.23	6.73
Standard deviation	0.012	0.014	0.10
Coefficient of variation	.7	1.1	2.1

(13) F. Pregl, "Quantitative Organic Microanalysis," The Blakiston Co., Philadelphia, Pa., 1930.

Cholesterol Analyses.[14]—The method of Bloor, Pelkan and Allen[15] was used.

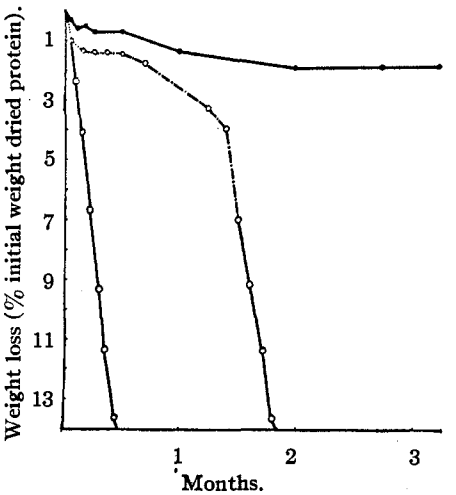

Fig. 1.—●, albumin (lipid <2%); ○, β-globulins (lipid >15%); ———, 110 ± 5° air; —·—·—, 100° *in vacuo*; ....., 80° *in vacuo*.

Results

Pooled Normal Human Plasma Proteins.—Measurements on 19 pools, obtained at different seasons over a period of two years and each representing over 150 individual plasmas, carried out at protein concentrations varying between 30 and 60 g. per liter, are given in Table I. Electrophoretic analyses of many of these pools have been presented in paper Number XI of this series.[16]

(14) We are indebted to Mr. Paul Gross for carrying out the cholesterol measurements.

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

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

TABLE II

REFRACTIVE INDEX INCREMENT AND NITROGEN FACTOR OF CRYSTALLINE HUMAN ALBUMIN C_1 DETERMINED UNDER VARIOUS CONDITIONS

Dialysis conditions				$\frac{\Delta n \times 10^4}{\Delta W\left(\frac{\text{g. protein}}{\text{liter}}\right)}$	$\frac{\Delta n \times 10^4}{\Delta N\left(\frac{\text{g. N}}{\text{liter}}\right)}$	$\frac{\Delta W\left(\frac{\text{g. protein}}{\text{liter}}\right)}{\Delta N\left(\frac{\text{g. N}}{\text{liter}}\right)}$
Gr. protein/liter	Salts	$\Gamma/2$	pH			
79.0	Sodium chloride + Sodium citrate	0.2 + .1	...	1.86	1.16	6.26
74.2	Potassium phosphate	.2	7.7	...	1.15	...
40.6	Sodium chloride	.3	7.3	1.86	1.15	6.13
51.0	Sodium chloride	.3	5.3	1.87	1.16	6.20
95.5	Electrodialyzed 48 hours	...	...	1.86	1.18	6.36
146.8	Electrodialyzed 72 hours	...	4.4	1.87	1.17	6.26
56.8	Electrodialyzed 96 hours	...	...	1.85	1.16	6.25
27.9	Electrodialyzed 96 hours	...	5.2	1.87	1.17	6.27
Average values				1.86	1.16	6.25

The average value of the refractive index increment in terms of weight of dried protein per liter is 1.833×10^{-4} (standard deviation $\pm$ 0.012), and in terms of nitrogen, 1.239×10^{-3} (standard deviation $\pm$ 0.014). The average value for nitrogen factor is 6.73 (standard deviation $\pm$ 0.10). In the range studied, individual values appeared independent of protein concentration.

When corrected to 0°,[17] the resultant value, 1.87×10^{-4}, for the specific increment in terms of weight, is in agreement with the value of 1.86×10^{-4} employed by Longsworth and his colleagues.[7]

The average of the nitrogen factors for these plasma pools, 6.73, is somewhat higher than the conventional factor, 6.25, in general use for the plasma proteins, and is in good agreement with the value of 6.8 given in the studies of Melin from This Laboratory, on four plasma pools reported in paper Number IV of this series.[9]

Pathological Human Plasma Proteins.—The specific refractive increment in terms of weight of dried protein of pathological plasma proteins in the absence of lipemia has in general fallen within the limits given above. With increasing plasma lipid (which in general does not pass through the membrane on dialysis of the plasma proteins) the values observed have been progressively lower, the limit thus far observed being 1.76×10^{-4}. When the refractive index increment is expressed in terms of nitrogen, deviations from the normal value in pathological plasma proteins associated with materials poor in nitrogen are far greater, the observed upper limit being 2.10×10^{-3}.[18]

(17) The specific refractive increments at 0° have been determined on several plasma pools and on many subfractions. The observed difference between values at 25° (0.03×10^{-4}) has been identical within the limits of measurement in all instances. The values at the lower temperature have not been used in calculation of refractive indices because of the arbitrary error introduced by reason of the temperature gradient in the prism. Techniques recently developed by Longsworth (personal communication) do not have this disadvantage.

(18) The calculation of conversion factors based on variations of refractive increments of components present in a mixture is considered in paper Number XI of this series,[16] and their use is extensively illustrated, together with the presentation of a method for estimation of total plasma lipid based on refractive index measurements, in the studies made in this Laboratory in collaboration with Hutchins and Janeway (to be published).

Plasma Fractions.—Measurements on crystalline human albumin made under differing conditions of pH, ionic strength and protein concentration (Table II) illustrate the insensitivity to variations in these ranges (as observed by these techniques) for several plasma fractions.[19]

The specific increment in terms of weight of this crystalline albumin is in good agreement with the figure of 1.88×10^{-4} employed by Grinstein[20] for human albumin, and is slightly higher than 1.83×10^{-4} cited by both Adair and Robinson and McFarlane[8] for horse serum albumin.[21] The nitrogen factor of the crystalline albumin here reported, namely, 6.25, is in agreement with the factor 6.27 cited by Brand and his co-workers[22] for another preparation of crystalline human albumin from This Laboratory.

That the values for albumin preparations of differing purity with respect to the lipid- and carbohydrate-containing α- and β-globulins diverge from those for crystalline albumin in the same directions as values for lipemic plasmas diverge from those for normal is exemplified in the following measurements on electrophoretically separated albumin before and after reprecipitation to remove globulins

	Distribution of electrophoretic components						
	Albumins	α-Globulins	β-Globulins	Cholesterol $\left(\frac{\text{g.}}{100\text{ g. prot.}}\right)$	$\left\lvert\frac{\Delta n \times 10^4}{\Delta W}\right\rvert$	$\left\lvert\frac{\Delta n \times 10^4}{\Delta N}\right\rvert$	$\frac{\Delta W}{\Delta N}$
Unreprecipitated	93	6	1	1.30	1.83	1.19	6.49
Reprecipitated	98	2	...	0.15	1.86	1.15	6.25

(19) Longsworth and Perlmann (personal communication), using methods applicable to very dilute solutions, have noted for certain proteins systematic variations of increment as a function of pH.

(20) M. Grinstein, *Anales Asoc. quimica argentina*, **26**, 106 (1938).

(21) In the former studies, the wave length is not given; in the latter, the wave length was comparable to that here employed.

(22) E. Brand, B. Kassell and L. J. Saidel, *J. Clin. Investigation*, **23**, 437 (1944). As in this study, the value of Brand and co-workers is based on dry weight measurements without either heat precipitation or extraction. It is quite possible that where dry weight measurements carried out on a heat precipitated, alcohol and ether dried protein (with suitable corrections for ash), a lower nitrogen factor would be obtained.

The effects of chemical purifications have in general proved analogous to those of electrophoretic purification of albumin (*cf.* Table VII of paper XI of this series[16]).

The nitrogen factors of the purest human preparations have been close to McMeekin's figure of 6.21 for crystalline, carbohydrate-free horse albumin (paper II of this series); preparations containing globulins in considerable quantity have been in the higher ranges reported for McMeekin's carbohydrate-containing, crystalline horse albumin (which itself was characterized by an electrophoretic mobility in the α-globulin range).

TABLE III

REFRACTIVE PROPERTIES AND NITROGEN FACTORS OF PLASMA FRACTIONS

Fraction[a]	$\frac{\Delta n}{\Delta W}\left(\frac{\text{g. prot.}}{\text{liter}}\right) \times 10^4$	$\frac{\Delta n}{\Delta N}\left(\frac{\text{g. N}}{\text{liter}}\right) \times 10^3$	$\frac{\Delta W}{\Delta N}\left(\frac{\text{g. prot.}}{\text{g. N}}\right)$	Estimated refractive index for water-free protein[b]
Chief Fractions				
I[c]	1.88	1.17	6.2	...
II + III[c]	1.80	1.37	7.6	...
IV[d]	1.79	1.34	7.5	...
IV-1[e]	1.80	1.51	8.4	...
IV-4[e]	1.84	1.23	6.7	...
V[e,d]	1.86	1.17	6.3	...
Subfractions				
I-2 fibrinogen[e]	1.88	1.14	5.9	1.603
II γ-globulin[f]	1.88	1.17	6.2	1.618
III-0 crude β-lipoprotein[f]	1.77	2.03	11.5	...
β_1-lipoprotein[g]	1.71	4.05	23.7	1.514
IV-1,1 α_1-lipoprotein[h]	1.78	2.22	12.5	1.563
IV-6,2 α_2-globulin[h]	1.83	1.28	7.0	...
IV-7 β_1-globulin (lipid-poor)[h]	1.85	1.21	6.6	...
Crystalline albumin	1.86	1.17	6.2	1.598

[a] Values are in general rounded off averages for several fractionations. In the instance of lipid-rich fractions, range of variation proved somewhat greater than the range for pooled plasma (*cf.* Table I), presumably representing magnification in these fractions of differences in lipid content of the pools. For the fractions poor in lipid, range of experimental variation was as small as that for crystalline albumin (Table II). [b] The Lorenz–Lorentz relation for this case is

$$\frac{n_2^2 - 1}{n_2^2 + 2} = c_p\bar{v}_p\left(\frac{n_p^2 - 1}{n_p^2 + 2}\right) + (1 - c_p\bar{v}_p)\left(\frac{n_1^2 - 1}{n_1^2 + 2}\right)$$

where n_p, n_1, n_2 are the refractive indices of the water-free protein, the solvent (0.3 *M* sodium chloride), and the solution, respectively; $\bar{v}_p$ the apparent partial specific volume of the protein, and c_p the protein concentration expressed in *grams per cc*. The partial specific volumes are given by J. L. Oncley, G. Scatchard and A. Brown (*J. Phys. Coll. Chem.*, **51**, 184 (1947)), with the exception of that for fibrinogen, which on the preparation here studied is 0.725 cc./g. Heller (*Phys. Rev.*, **68**, 5 (1945)) has recently treated alternative relations for mixtures. His empirical equation 5 leads to refractive indices very close to those given by the Lorenz–Lorentz rule. [c] Prepared by Method 6.[9] [d] Prepared by Method 5.[9] [e] Over 90 per cent. of protein clottable. For preparation see ref. 9. [f] For preparation see ref. 9. [g] Concentration from III-0 in the ultracentrifuge by Dr. J. L. Oncley. [h] Approximately 90% a single electrophoretic component (sodium diethylbarbiturate, *p*H 8.6).

The specific refractive increments found in the chief fractions of plasma and certain subfractions (Table III) serve to extend the observed range of effects of association of lipid and carbohydrate with protein (estimates of nitrogen, lipid, and carbohydrate components of these fractions have been presented in Table VIII of paper IV of this series). For those fractions notably poor in lipid (fibrinogen, albumin, γ-globulin), the increments are quite alike and slightly higher than the value for plasma.

Refractive indices for the water-free protein, computed by the Lorenz–Lorentz relation, lie in the range computed by Putzeys and Brosteaux for a variety of proteins of non-human origin; the value for crystalline human albumin (1.598) being identical with that based on Adair and Robinson's measurement of horse albumin (1.599).[8]

With increasing associated non-nitrogenous material, lower increments were found. The shift in the increment in terms of weight, as in the instance of progressively lipemic plasmas, is less than differences between increments in terms of nitrogen. The lowest value observed was 1.71×10^{-4} in β_1-lipoprotein, whose lipid content was approximately 75 per cent. by weight.

Unpublished studies by Melin[23] and others in This Laboratory have found that the lipids thus far extracted from lipoprotein fractions studied have approximately the same relative proportions reported for the lipids of normal plasma by Peters and his co-workers.[24] The constancy of these proportions is reflected in the rough linear relation between the cholesterol content (in weight) of these lipoprotein fractions and their per cent. nitrogen (% nitrogen = 16.0 − 0.4(% cholesterol)). The plot at the bottom of Fig. 2, presenting over a wide range of cholesterol concentrations representative data which enter into the average nitrogen factors of Table III, illustrates certain of the largest deviations encountered from this rule. A similar plot at the top of Fig. 2 presents a rough linear correlation of specific refractive increments in terms of weight with the cholesterol content of these same fractions $\left(\frac{\Delta n}{\Delta W} \times 10^4 = 1.85 - 0.043\right.$ (% cholesterol)). Obviously neither of these rules is applicable to pathological plasmas or their fractions in which the ratios of the various protein-bound lipids or carbohydrates to cholesterol are grossly altered.

If the partial specific volume of a lipoprotein of a carbohydrate-containing protein can be considered an additive function over the entire range of combining ratios of the partial specific volumes of the unassociated lipid, carbohydrate, and pro-

(23) M. Melin, personal communication.

(24) The studies of Peters, together with other literature on serum lipids, are summarized in J. P. Peters and D. D. VanSlyke, "Quantitative Clinical Chemistry," 2nd edition, Williams & Wilkins Co., Baltimore, 1946, pp. 467, *et seq.*

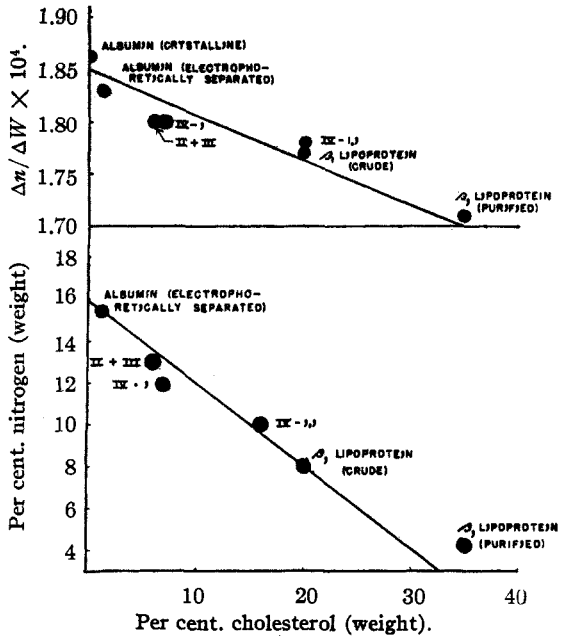

Fig. 2.

tein, respectively, it may be shown by simple algebra that for the protein complex by the Lorenz–Lorentz relation the function $(n^2 - 1)/(n^2 + 2)$ or by the simpler Gladstone–Dale relation, the function $(n - 1)$, is linear in the volume fraction of the non-protein component in the lipoprotein or carbohydrate-containing protein complex. The intercept at zero volume fraction gives the value characteristic of unassociated protein; the intercept at unity volume fraction gives the value characteristic of the pure non-protein component. This linearity reflects the additivity of these functions.[25]

That this, in first approximation, is the case for plasma lipoproteins is indicated by plots of these functions against volume fraction mixed lipids for

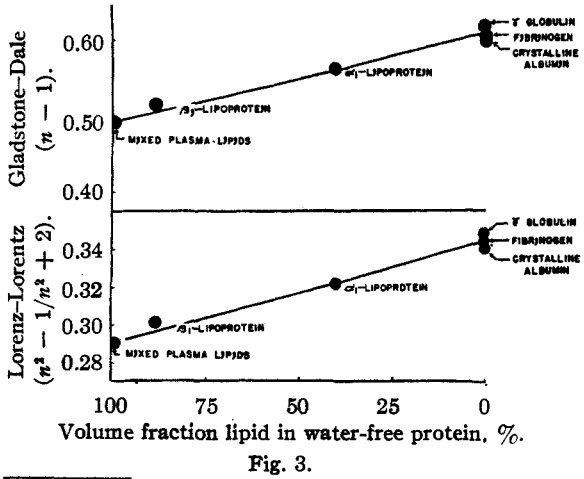

Fig. 3.

(25) The Gladstone–Dale and Lorenz–Lorentz formulas for binary mixtures have been recently presented *in extenso* by Putzeys and Brosteaux.[6]

those plasma protein fractions for which partial specific volume measurements have been done (Fig. 3). The lipoprotein points lie quite close to straight lines connecting the values for mixed plasma lipids at one extreme, with the range for proteins unassociated with lipid at the other. The experimental deviations from each relationship are of about the same magnitude.[26,27]

That carbohydrate association will yield effects of similar sign but smaller size can be predicted from the range of specific refractive increments which is close to that of lipids and significantly lower than those of most proteins. The available data on the composition and partial specific volumes of plasma proteins rich in these materials do not yet permit the quantitative treatment possible for lipid association, although the value for carbohydrate-rich Fraction IV-4 is in qualitative agreement.

Acknowledgment.—The authors wish to express their appreciation to Professor J. L. Oncley for his interest and assistance in planning and carrying out this study.

Summary

Refractive index measurements, in terms of weight of dried protein and of protein nitrogen, are given for a series of pools of normal human plasma proteins, for pathological plasma proteins of varying lipid association, and for the chief fractions and certain of the more highly purified subfractions of normal human plasma.

A narrow range of refractive increments in terms of dried weight (about 1.87×10^{-4} (g./liter)$^{-1}$) has been found characteristic of those proteins poor in associated lipid or carbohydrate. The estimated corresponding refractive index of water-free protein is approximately 1.60.

With increasing lipid or carbohydrate associated, progressively lower values have been found; the lowest observed (β_1-lipoprotein), 1.71×10^{-4} (g./liter)$^{-1}$, corresponds to a refractive index of 1.51.

Whether calculated by the Lorenz–Lorentz or the Gladstone–Dale relationship, the data for lipid-containing proteins of normal plasma are in agreement with predictions for mixtures of ma-

(26) Sufficiently accurate density studies on these lipoproteins and their associated lipids are not available to give evidence on the question of volume changes consequent upon spatial arrangements in the lipid-protein association.

(27) The measured refractive index for mixed plasma lipids (1.4924 at 26°) is somewhat higher than that predicted from the refractive index of cholesterol (based on atomic refractivities) and the refractive indices of fatty acids (A. Dorinson, M. R. McCorkle and A. W. Ralston, THIS JOURNAL, **64**, 2739 (1942)). The measurements were made on mixed lipids extracted by a modification of the method of Hardy and Gardiner (W. B. Hardy and S. Gardiner, *J. Physiol.*, **40**, lxviii (1910)) from a crude β_1-lipoprotein (the method of extraction fails to remove some phospholipids). We are indebted to Mr. M. Melin and Mrs. B. J. Livingstone for the extraction. Because melting points of certain of the components are above room temperature, it was necessary to make refractive index measurements on the supercooled system produced by heating the mixed lipids to 70°, followed by rapid cooling to the desired temperature.

terial of refractive index characteristic of lipid-free protein with mixed plasma lipids in volume fraction corresponding to the analytical figure for the lipoproteins.

Specific refractive increments in terms of nitrogen range from 1.17×10^{-3} (g. N/liter)$^{-1}$, characteristic of lipid- and carbohydrate-poor proteins, to 4.05×10^{-3} (g. N/liter)$^{-1}$ for β_1-lipoprotein. The variation between proteins is roughly inversely proportional to the nitrogen factors.

Corresponding nitrogen factors are given together with empirical rules relating the weight content cholesterol of normal human plasma lipoprotein fractions to weight content nitrogen and refractive index increment in terms of weight of dried protein.

The conditions for dry weight measurements on plasma lipoproteins are re-examined experimentally.

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

Preparation and Properties of Serum and Plasma Proteins. XIII. Crystallization of Serum Albumins from Ethanol–Water Mixtures[1a,b]

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I

Roughly half of the proteins of plasma are albumins. The albumins all appear to be of closely the same molecular weight and of closely the same isoelectric point. They are not only among the most stable, but also among the most soluble of the plasma proteins. In the classical procedure for the separation of proteins by "salting-out" the albumins remain soluble in neutral, two molal, that is to say in half-saturated, ammonium sulfate solution.[2] In the methods which have been developed recently for the separation of proteins in ethanol–water mixtures of controlled pH, ionic strength, and temperature,[3,4] the albumins remained in solution when the γ-globulins and certain of the β-globulins were precipitated from an ethanol–water mixture of mole fraction 0.091 at pH 6.8 and $-5°$ and the remaining β-globulins and α-globulins were precipitated by increasing the ethanol to mole fraction 0.163 at pH 5.8 and $-5°$. The supernatant solution, in the case of the fractionated human plasma proteins, contained 92% of the serum albumins.[4] The albumins were readily precipitated by adjusting the pH to 4.8. The fraction separated by this change in pH in a 0.163 mole fraction ethanol–water mixture at $-5°$ has been called Fraction V in our system of plasma fractionation. It has proved a convenient starting material (1) for purification of the albumins, by precipitating most of the remaining globulins (in Fraction V-1) from a more concentrated solution of the crude fraction at lower ethanol and salt concentrations, and (2) for crystallization of the albumins.

The albumins of different species vary greatly in the ease with which they may be crystallized. Horse serum albumin crystallizes readily from half-saturated ammonium sulfate solution upon acidification[5,6,7]; that is to say, by the method for egg albumin used by Hopkins and Pinkus[8] and later studied in such great detail by Sørensen and Høyrup.[9] Crystallization of human serum albumins from concentrated ammonium sulfate solutions has been carried out by Adair and Taylor[10] and special conditions developed, so that the procedure might consistently yield crystalline preparations, by Kendall.[11] The albumins from bovine plasma have, however, resisted crystallization from ammonium sulfate solution.

The difference in the ease of crystallization of these different species may be related to differences in the polypeptide structure of the albumins, or in the nature and amounts of the carbohydrate or lipid present. Hewitt and Kekwick demonstrated that horse serum albumin could be fractionated into a carbohydrate-free and a carbohydrate-rich fraction,[6,12] and McMeekin succeeded in crystallizing an "albumin" fraction with a carbohydrate content as high as 5.5%.[13] The nitrogen content of his carbohydrate-rich fraction

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(1b) This paper is Number 60 in the series "Studies on Plasma Proteins" from the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

(2) The earlier literature with respect to the "salting-out" of the plasma proteins is referred to in the first paper in this series: E. J. Cohn, T. L. McMeekin, J. L. Oncley, J. M. Newell and W. L. Hughes, Jr., THIS JOURNAL, **62**, 3386 (1940).

(3) E. J. Cohn, J. A. Luetscher, Jr., J. L. Oncley, S. H. Armstrong Jr., and B. D. Davis, *ibid.*, **62**, 3396 (1940).

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

(5) S. P. L. Sørensen, *Compt. rend. trav. lab. Carlsberg*, **18**, No. 5, 1 (1930).

(6) L. F. Hewitt, *Biochem. J.*, **30**, 2229 (1936).

(7) T. L. McMeekin, THIS JOURNAL, **61**, 2884 (1939).

(8) F. G. Hopkins and S. N. Pinkus, *J. Physiol.*, **23**, 130 (1898).

(9) S. P. L. Sørensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg*, **12**, 164 (1917).

(10) M. E. Adair and G. L. Taylor, *Nature*, **135**, 307 (1935).

(11) F. E. Kendall, *J. Biol. Chem.*, **138**, 97 (1941).

(12) R. A. Kekwick, *Biochem. J.*, **32**, 552 (1938).

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