[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Specific Refractive Increment of Some Purified Proteins

BY GERTRUDE E. PERLMANN[1] AND L. G. LONGSWORTH

A quantitative interpretation of the electrophoretic patterns of protein mixtures obtained with the aid of the Tiselius method is based, in part, on a knowledge of the specific refraction of the proteins to be analyzed. Since the patterns are recorded at 0.5° and since no precise refractive index measurements of proteins at this temperature are available, an investigation has been made of the specific refractive increment of some purified proteins. A differential prism method, developed in this Laboratory,[2] has been used in conjunction with the optical equipment of the electrophoresis apparatus. This permits data to be obtained under the same conditions as those encountered in the routine electrophoretic analysis of protein mixtures. The results of this investigation are presented in this report.

Experimental

The proteins that have been studied in this research are listed in Table I. The egg albumin and β-lactoglobulin were prepared in this Laboratory; the bovine serum albumin was obtained from the Armour Company, Chicago, and the samples of human serum albumin and γ-globulin were kindly supplied by the Department of Physical Chemistry, Harvard Medical School.[3] In the case of egg albumin the purification procedure, including three recrystallizations, was that used by Sørensen and Høyrup[4] whereas β-lactoglobulin was prepared by a modification of Palmer's method[5] and was recrystallized four times. The modification consists of the removal of casein by the addition of solid ammonium sulfate to 40% saturation. The ammonium sulfate concentration of the filtrate was then increased to 55% saturation, the precipitate discarded and the β-lactoglobulin prepared from the filtrate.

Except for the egg albumin, which was kept as a paste in a concentrated ammonium sulfate solution, all of the protein samples were stored at 2° as dry powders until used. Salt-free solutions of the albumins were prepared by dialysis against distilled water. In the case of the protein solutions containing neutral or buffer salts, a sample was dissolved in the appropriate electrolyte solution and then dialyzed against this solution.

The concentrations of all of the protein solutions, except those dissolved in the sodium diethylbarbiturate buffers, have been determined from nitrogen analysis of weighed portions by the Pregl micro Kjeldahl method with the precautions recommended by Chibnall.[6] The factors for conversion of these results to a dry weight basis have been determined as follows. In the case of the albumins, weighed portions of a salt-free isoelectric solution were used for both the nitrogen and dry weight determinations. The dried residue presumably consisted entirely of protein. In the case of γ-globulin, an isoelectric solution in aqueous sodium chloride was used and correction for the weight of the salt in the residue was made with the aid of the assumption that this was the same as in a mass of the dialysate equal to that desiccated. The factor for the β-lactoglobulin was obtained from a nitrogen determination on a desiccated sample of the solid protein, thus eliminating any uncertainty as to correction for salt in the residue.

TABLE I

NITROGEN CONTENT OF THE PURIFIED PROTEINS

		Egg albumin	Bovine serum albumin	Human serum albumin	β-Lacto-globulin	Human γ-globulin
1	Method of desiccation	100° *in vacuo*	110° in air	110° in air	110° in air	110° in air
2	Percentage of nitrogen	15.72	16.05	15.95	15.53	15.9
3	Nitrogen factor	6.36	6.23[a]	6.27	6.44	6.29
4	References to nitrogen content	4, 6	7	7, 8	6, 9	7, 8

[a] The same value is obtained with water-dialyzed and electrodialyzed samples.

Under the conditions of desiccation given in line 1 of Table I, it was found that the dried residue attained a constant weight in twenty-four hours. The resulting nitrogen factor, line 3, is in good agreement with the best values found in the literature, references to which are indicated in line 4.

The determination of the protein concentration in the presence of the barbiturate buffer will be described later in this paper.

All weight concentrations have been converted to a volume basis, *i. e.*, gram protein per 100 ml. solution, with the aid of the density data in the "International Critical Tables"[10] and in Svedberg and Pedersen's "The Ultracentrifuge."[11] In estimating the density of a given solution the specific volumes of the components have been taken as additive. Moreover, all of the proteins of Table I have been assumed to have the specific volume, 0.741, of the "average protein" as given by Svedberg.[12]

Results

Effect of Protein Concentration in Salt-Free Solutions.—As the results presented in Table II indicate, the specific refractive increment, $k = (n_{solution} - n_{solvent})/p$, of a protein is independent of its concentration, p, over a wide range of this variable, if the concentration is expressed on a volume scale, *e. g.*, g./100 ml. solution. The results in Table II, and also those in Fig. 1 below, indicate that, although the specific refractive increment varies with the protein, this variation is

(1) Commonwealth Fund Fellow, 1945–1947.

(2) Longsworth, *Ind. Eng. Chem., Anal. Ed.*, **18**, 219 (1946).

(3) The products of plasma fractionation employed in this work were developed from blood, collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Harvard University.

(4) Sørensen and Høyrup, *Compt. rend. lab. Carlsberg*, **12**, 1 (1915–1917).

(5) Bull and Currie, THIS JOURNAL, **68**, 742 (1946).

(6) Chibnall, Rees and Williams, *Biochem. J.*, **37**, 354 (1943).

(7) Brand, Kassel and Saidel, *J. Clin. Invest.*, **23**, 437 (1944).

(8) Cohn, Strong, Hughes, Jr., Mulford, Ashworth, Melin and Taylor, THIS JOURNAL, **68**, 459 (1946).

(9) Brand, Saidel, Goldwater, Kassel and Ryan, *ibid.*, **67**, 1524 (1945).

(10) "International Critical Tables," Vol. III, McGraw-Hill Book Co., Inc., New York, N. Y., 1928.

(11) Svedberg and Pedersen, "The Ultracentrifuge," Oxford University Press, 1940, p. 446, app. III.

(12) Svedberg and Pedersen, "The Ultracentrifuge," Oxford University Press, London, 1940, p. 445, app. II.

small. These results confirm and extend those of Adair and Robinson[13] and others.

TABLE II

EFFECT OF PROTEIN CONCENTRATION ON THE SPECIFIC REFRACTIVE INCREMENT IN SALT-FREE SOLUTIONS

Protein	Protein concn., p, g./100 ml.	Specific refractive increment, $k \times 10^6$ at 0.5°
Egg albumin, pH 4.95	1.614	1874
	3.200	1877
	4.026	1878
	6.451	1877
		Av. 1876
Bovine serum albumin, pH 5.05	3.766	1906
	4.740	1902
	5.631	1906
	10.099	1897
		Av. 1901
Human serum albumin, pH 4.85	1.777	1886
	3.456	1887
	5.188	1888
	7.683	1887
		Av. 1887

Effect of Temperature on the Specific Refractive Increment.—Since the differential prism method can readily be adapted for work over a range of temperature, selected solutions have been studied at 0.5, 5, 10, 15, 20 and 25°.

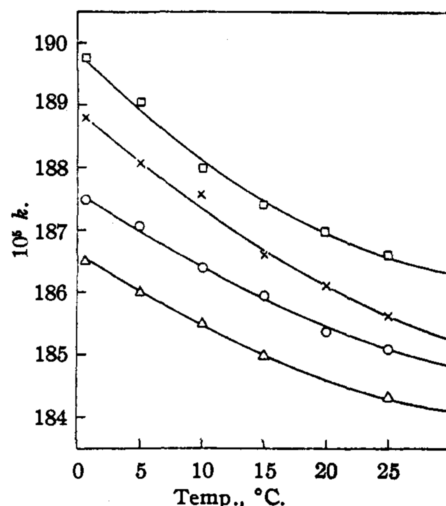

Fig. 1.—Effect of temperature on the specific refractive increment of proteins: □, bovine serum albumin; ×, human serum albumin; O, egg albumin; △, β-lactoglobulin.

These results are presented in Fig. 1 for the three albumins and β-lactoglobulin. Here the specific refractive increment, k, is plotted as ordinate against the temperature as abscissa. Contrary to the general impression, the effect of temperature on the refractive increment is not negligible for most proteins although it is smaller than that observed in the case of salts. This is shown in Table III, which also includes the results that have been obtained on some low molecular weight materials having electrical properties between those of salts and proteins. Of the substances investigated only γ-globulin had a negligible temperature coefficient.

(13) Adair and Robinson, *Biochem. J.*, **24**, 993 (1930).

TABLE III

COMPARISON OF THE EFFECT OF TEMPERATURE ON THE SPECIFIC REFRACTIVE INCREMENT, k, OF PROTEINS WITH ITS EFFECT ON OTHER SUBSTANCES

Substance	$k_5 \times 10^6$	$(k_5 - k_{25}) \times 10^6$
Sodium chloride		+171
Potassium chloride		+100
Glycine, alanine		+ 80
Arginine hydrochloride		+ 79
Glycylglycine		+ 80
Bovine serum albumin	1901	+ 32
Human serum albumin	1887	+ 33
Egg albumin	1876	+ 25
β-Lactoglobulin	1865	+ 23
Human γ-globulin	1875	0

The Specific Refraction at Different Wave Lengths.—Although most of the refractive index measurements have been made at λ = 5780 Å., the mean value of the mercury yellow doublet that is isolated with the Wratten filter number 22, a few solutions have also been studied at other wave lengths. In these measurements a cadmium-mercury lamp of the H4 type is used without a filter, the cell is filled with a fairly concentrated protein solution and, with the aid of the cylindrical lens attachment, the spectral lines are observed directly in the focus of the schlieren camera. With the yellow line as reference the displacements of other lines are measured and the corresponding values of $k_\lambda - k_{5780}$ computed. In Fig. 2, values of $(k_\lambda - k_{5780})/k_{5780}$ are plotted as ordinate against the reciprocal of the square of the wave length as

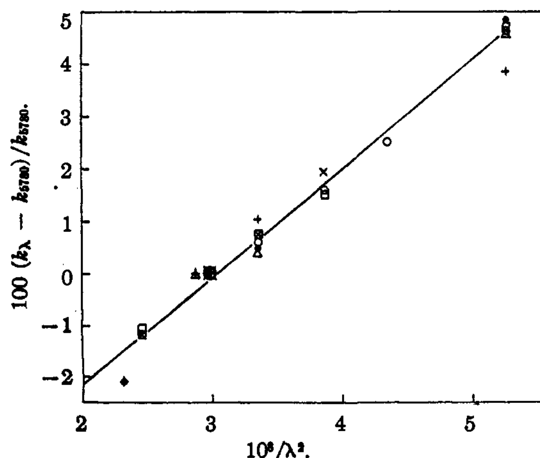

Fig. 2.—Effect of wave length on the specific refraction: □, bovine serum albumin; ×, human serum albumin; O, egg albumin; △, β-lactoglobulin; +, horse serum albumin; ●, horse serum globulin.

abscissa. As will be seen in the figure, all of the points, including those for horse serum albumin and globulin, and β-lactoglobulin taken from the work of Pedersen and Andersson,[14,15] can be adequately represented by a single straight line. Although the specific refraction varies somewhat with the nature of the protein it thus appears that the dispersion is essentially the same for all the proteins that have been studied. Since the slope of the line in Fig. 2 is 0.1946 the relation $k_\lambda = k_{5780}\ (0.940 + 2.00 \times 10^6/\lambda^2)$, where λ is in ångström units, may be used to obtain the specific refraction at any (visible) wave length.

The Specific Refraction of Sodium Proteinates.—The protein solutions employed in an electrophoretic analysis are usually prepared by dialysis against an appropriate buffer solution. The proteins are then present as charged particles in a solution of buffer ions whose composition is given, as a first approximation, by the Donnan equations. If the *p*H of the solution is above the isoelectric *p*H of the protein and if, as is usually the case, sodium buffer salts are employed, the protein is present as a sodium proteinate whose specific refraction differs from that of the isoelectric protein. Moreover, in addition to the acquisition of a net charge the protein may also bind some of the buffer salt. In order to distinguish between the effect of the charge and of bound salt it is essential to determine the refraction of sodium proteinate in the absence of buffer salts for comparison with the values obtained in their presence. Consequently the specific refractions of salt-free isoelectric protein solutions, to which small amounts of sodium hydroxide have been added, were determined with the results given in Table IV.

TABLE IV

EFFECT OF CHARGE ON THE SPECIFIC REFRACTIVE INCREMENT OF CRYSTALLINE EGG ALBUMIN AND BOVINE SERUM ALBUMIN

1 Moles NaOH $\times 10^5$ per 1 g. protein	2 *p*H	3 *p*	4 Δn	5 $k' \times 10^6$	6 a
		Egg albumin			
00.00	4.95	6.451	0.01210_6	1877	..
13.03	5.70	5.951	$.01123_4$	1888	45
19.28	6.20	5.739	$.01086_8$	1894	47
25.38	7.26	5.545	$.01052_3$	1898	44
32.67	8.7	5.334	$.01018_7$	1908	53
42.3	10.1	5.128	.00977	1904	36
44.6	10.5	5.025	$.00959_3$	1909	34
				Mean	44
		Bovine serum albumin			
00.00	5.02	4.740	0.00901_5	1902	..
19.59	7.22	4.340	$.00832_9$	1919	46
34.44	8.32	4.077	$.00788_5$	1934	49
53.68	10.10	3.782	.00735	1944	41
64.75	10.58	3.606	$.00703_6$	1951	40
72.97	10.72	3.525	$.00697_8$	1979	56
				Mean	46

In this table the quantity of alkali added is given in column 1 and the resulting *p*H of the solution in the next column. The protein concentration, column 3, is the value for the isoelectric solution after correction for the dilution due to the added alkali. If this is divided into the observed refractive increment, column 4, a specific refraction, k', column 5, is obtained that differs from the specific refraction k, of the isoelectric protein by an amount that is proportional to the net charge, e, of the protein, *i. e.*

$$k' = k(1 + ae)$$

Here the net charge is given by the values of column 1 since all of the added alkali reacts at the *p*H values studied. The computed values of the proportionality factor, a, column 6, are approximately constant and are essentially the same for the two proteins studied.

Refraction Measurements in Solutions in Sodium Chloride.—In view of the possibility that ions other than the hydrogen ion may be involved in the dissociation equilibria of the proteins, refraction measurements on these materials in solutions of the neutral salt, sodium chloride, have been made as an additional prerequisite to the study of proteins in buffer solutions. The results presented in Table V, where the specific refraction is taken as the difference, per unit concentration of protein, between the refractive indices of the equilibrated solutions, illustrate the two types of behavior that have been encountered. Thus the specific refraction of egg albumin is essentially independent of the concentration of the sodium chloride against which it has been dialyzed, whereas in the case of the bovine and human serum albumin small but significant changes occur. This is consistent with the findings of Scatchard and his associates[16] that these two materials bind some sodium chloride.

An additional feature of the bovine serum albumin used in this research also emerges from the data of Table V. Thus the k-values of 1923 and 1921 $\times 10^{-6}$ for the electrodialyzed solutions Nos. 11 and 13 differ significantly from that of 1902 $\times 10^{-6}$ for the water-dialyzed solution No. 6. If, however, water dialysis is used to remove the salt from solutions that have been equilibrated with aqueous sodium chloride, solutions Nos. 10 and 15, the values obtained, 1919 and 1920 $\times 10^{-6}$, then agree well with those for the solutions prepared by electrodialysis. The origin of these effects is obscure but, together with the observed constancy of the nitrogen factor, suggests the presence in the bovine serum albumin of a volatile, poorly refracting contaminant that is removed by electro- and saline dialysis but not by the or-

(14) Pedersen, *Biochem. J.*, **30**, 961 (1936).

(15) McFarlane, *ibid.*, **29**, 407 (1935).

(16) Scatchard, Batchelder and Brown, THIS JOURNAL, **68**, 2320 (1946).

TABLE V

EFFECT OF SODIUM CHLORIDE ON THE SPECIFIC REFRACTIVE INCREMENT OF CRYSTALLINE EGG ALBUMIN, BOVINE SERUM ALBUMIN AND HUMAN SERUM ALBUMIN

Soln. no.	Protein	Solution prepared by	pH	p	$k \times 10^6$
1	Egg albumin	Dialysis vs. H_2O	5.31	3.154	1869
2		Dialysis vs. 0.1 M NaCl	5.47	3.555	1874
3		Electrodialysis	4.74	3.187	1869
4		Dialysis vs. H_2O	4.95	3.199	1876
5		Dialysis vs. 0.5 M NaCl	5.38	3.604	1869
6	Bovine serum albumin	Dialysis vs. H_2O	5.05	4.740	1902
7		Dialysis vs. 0.1 M NaCl	5.35	5.219	1932
8		Dialysis vs. 0.5 M NaCl	5.31	4.634	1948
9		Dialysis vs. 0.1 M NaCl		3.495	1938
10		Dialysis of soln. No. 9 vs. H_2O		4.539	1919
11		Electrodialysis	5.25	2.223	1923
12		Electrodialysis + dialysis vs. 0.1 M NaCl	5.37	4.103	1943
13		Electrodialysis		4.891	1921
14		Electrodialysis + dialysis vs. 0.1 M NaCl		5.438	1941
15		Dialysis of soln. No. 14 vs. H_2O		6.424	1920
16	Human serum albumin	Dialysis vs. H_2O	4.85	3.424	1887
17		Dialysis vs. 0.5 M NaCl	5.26	2.262	1918

dinary water dialysis. Alcohol or decanol as the contaminant might satisfy these requirements.

Refraction Measurements in Buffer Solutions.—In the following interpretation of the refraction measurements in buffer solutions the assumptions have been made (a) that the refractions due to the various components are additive, (b) that the net charge on the protein may be obtained from the titration curve and (c) that the buffer electrolyte concentrations are given by the first term in the expansion of the Donnan equation. With the aid of these assumptions, together with the measured refractive increment, Δn-obsd., of the protein solution and that, $\Delta n'$-obsd., of the buffer solution against which it has been equilibrated, the specific refraction of the sodium proteinate has been computed as will be described in connection with Table VI. In that table, the concentrations, in equivalents per liter, of the buffer electrolytes in the dialysate, the pH of the protein solution in equilibrium therewith and the protein are given in the first four lines. The protein concentrations in line 5 marked with an asterisk were obtained by mixing weighed amounts of the buffer solution and the protein. Corrections were made not only for moisture content of the protein but also, by rapid weighing of the Cellophane bag and its contents before and after dialysis, for the rather large volume changes that occur during this process. In the case of the concentrations marked with a dagger a weighed sample of the equilibrated protein solution was dialyzed salt-free, transferred quantitatively to a volumetric flask and nitrogen determinations were made on weighed portions of this salt-free solution. It will be noted that the two methods of analysis lead to closely agreeing results for the specific refraction in the one instance where a comparison is possible, *i. e.*, columns III and IV of Table VI. In the phosphate buffers the protein concentration was obtained in the conventional manner since these buffer salts do not interfere with the nitrogen determination.

The sources of the values for the net charge, line 6 of Table VI, are given as footnotes whereas the equivalent concentration of the sodium proteinate, line 7, is simply $C_{NaP} = -10pe$. In the case of the diethylbarbiturate buffers the electrolyte concentrations in the protein solutions, lines 8 and 9, are given by the relations[17]

$$C_{NaR} = C'_{NaR} - {}^1/_2 C_{NaP} \text{ and } C_{HR} = C'_{HR}$$

whereas for the phosphate buffers[18]

$$C_{NaR} = C'_{NaR}\,(1 - 5C_{NaP}) \text{ and } C_{HR} = C'_{HR}\,(1 - 2.5C_{NaP})$$

Owing to small variations of the equivalent refraction with concentration and also to the difficulties of preparing some of the buffer salts for weighing, deviations of as much as 1×10^{-5} in the measured refractive increments of the buffer solutions from those computed by means of the relation

$$\Delta n' = K_{NaR}C'_{NaR} + K_{HR}C'_{HR}$$

have been observed. Here K is the equivalent refraction and, for the computations of Table VI, has been assigned the following values: sodium diethylbarbiturate, 0.04055; diethylbarbituric acid, 0.02905; Na_2HPO_4, 0.01502; and NaH_2PO_4, 0.0172. Correction for the deviations has been made by adding to the observed increment for the protein solution the small difference between the computed and observed increments for the buffer solution. The corrected values of the refractive index increment for the protein solutions are given in line 10 of Table VI.

The contribution of the buffer electrolytes to the

(17) Longsworth, *J. Phys. and Coll. Chem.*, **51**, 171 (1947).

(18) Svensson, *Arkiv. Kemi, Mineral. o. Geol.*, **22A**, No. 10, 27 (1946).

TABLE VI

THE SPECIFIC REFRACTIONS OF PROTEINS IN BUFFER SOLUTIONS

BSA = bovine serum albumin. HSA = human serum albumin. EA = egg albumin. LG = lactoglobulin.

		I	II	III	IV	V	VI	VII	VIII	IX	X
		R = diethylbarbiturate⁻						R = $NaHPO_4^-$			
1	C′(NaR)	0.025	0.050	0.1	0.1	0.1	0.1	0.128	0.134	0.134	0.128
2	C′(HR)	0.005	0.01	0.02	0.02	0.02	0.02	0.008	0.008	0.008	0.008
3	*p*H	8.56	8.59	8.60	8.60	8.60	8.60	7.67	7.71	7.74	7.68
4	Protein	BSA	BSA	BSA	BSA	EA	LG	BSA	HSA	EA	LG
5	*p* − g./100 ml. soln.	2.010*	2.056*	1.9193*	5.024†	1.8115†	2.695†	3.4977	2.9943	2.3565	2.8324
6	−*e*	0.00038[a]	0.00038[a]	0.00038[a]	0.00038[a]	0.00032[a]	0.00051[b]	0.00027[a]	0.00027[c]	0.00027_5[a]	0.00043[b]
7	C(NaP) = −10*pe*	.00764	.00781	.00729	.01909	.00580	.01374	.00944	.00808	.00648	.01218
8	C(NaR)	.02118	.0461	.0963	.0905	.0971	.0931	.1220	.1286	.1297	.1202
9	C(HR)	.005	.01	.02	.02	.02	.02	.0078	.0078	.0079	.0078
10	Δ*n*(obsd. − corr.)	.004986	.006323	.008291	.014179	.008087	.009800	.008665	.007767	.006520	.007297
11	Δ*n*(NaR + HR)	.001004	.002160	.004486	.004251	.004518	.004356	.001966	.002066	.002084	.001939
12	Δ*n*(NaP)	.003982	.004163	.003805	.009928	.003569	.005444	.006699	.005701	.004436	.005358
13	*k*(NaP)	.001981	.002025	.001982	.001976	.001970	.002020	.001915	.001904	.001882	.001892
14	*k*(NaP) $\mu \rightarrow 0$	$.00193_5$	$.00193_5$	$.00193_5$	$.00193_5$	.001902	.001909	$.00192_3$	.00191	$.00189_9$	$.00190_1$

[a] Interpolated from data of Table IV. [b] From Cannan, Palmer and Kibrick, *J. Biol. Chem.*, 142, 803 (1942). [c] Cohn, Strong, Hughes and Blanchard, see Edsall, *Annals N. Y. Acad. Sci.*, 47, 223 (1946).

increment of the protein solution is given by the relation

$$\Delta n(\text{NaR} + \text{HR}) = K_{\text{NaR}}C_{\text{NaR}} + K_{\text{HR}}C_{\text{HR}}$$

line 11 of Table VI, and the difference

$$\Delta n(\text{NaP}) = \Delta n - \Delta n(\text{NaR} + \text{HR})$$

line 12, divided by the protein concentration is the specific refraction of the sodium proteinate, line 13. For comparison, the values of *k* for the sodium proteinates in the absence of salt have been interpolated from the data of Table IV for the *p*H values of Table VI and are given in the last line of that table. It will be noted that the specific increment in the phosphate buffer is essentially the same as in the absence of salt whereas in the presence of the diethylbarbiturate it is significantly greater. Although several explanations for this observation could be advanced an attractive one is that this large organic ion is bound by the protein.

Comparison with Previous Work.—As a test of the validity of the differential prism method used in this research two of the salt solutions studied by Hölemann and his associates[19,20] have been prepared and their refractive increments determined over a sufficient range of temperature and wave length to permit a direct comparison with their results. At 25° and λ = 5876 Å., the increments for 1.6692 molal sodium chloride and 1.2983 molal potassium chloride are 0.01538_5 and 0.01190_6, respectively, and are in agreement with the values of 0.01538 and 0.01190 reported by Hölemann. Additional evidence that the precision of our refractive index measurements is about $\pm 1 \times 10^{-5}$ is afforded by the following fact. At 0.5° and λ = 5780, the refractions of the solutions of potassium chloride recently used in diffusion studies[21] follow, with an average deviation of $\pm 7 \times 10^{-6}$, the simple relation, $\Delta n/C = 0.011405 - 0.00100\sqrt{C}$, over the concentration range studied, *i. e.*, 0.1 to 1.0 normal.

(19) Hölemann and Kohner, *Z. physik. Chem.*, **B13**, 338 (1931).

(20) Shibata and Hölemann, *ibid.*, **13**, 347 (1931).

(21) Longsworth, THIS JOURNAL, **69**, 2510 (1947).

In the case of the specific refractions of proteins, however, the precision of the results is limited by the uncertainty in the protein concentration. As Armstrong, Budka, Morrison and Hasson have shown,[22] the presence of lipid in the protein renders particularly difficult the concentration determination. In the present research this source of error has been reduced by restricting our studies to proteins that contain minimal amounts of lipid and by controlling the nitrogen determinations with dry weight measurements. In spite of these precautions, however, in unfavorable cases the uncertainty in the protein concentration may amount to as much as one per cent. In some instances this is sufficient to mask the variation of the specific refraction from one protein to another or between different preparations of the same protein.

It is of interest that at 20° our values of the specific refraction of human albumin, 1862×10^{-6}, Fig. 1, and γ-globulin, 1875×10^{-6}, agree well with those, 186×10^{-5} and 188×10^{-5}, reported by Armstrong and associates. In the case of β-lactoglobulin, Pedersen reports[14] 1809×10^{-6} and 1812×10^{-6} as the specific refraction, at 20° and λ = 5799 Å., of two different preparations. In these measurements he used nitrogen factors of 6.61 and 6.55, respectively, that were obtained from nitrogen and dry weight determinations on aliquots of a solution of the protein in 0.5 molar sodium chloride. When analyzed similarly, our preparation of this protein gave a factor of 6.62, in essential agreement with Pedersen's values. However, when analyzed, as described earlier in this paper, in such a manner as to eliminate the uncertainty concerning the amount of salt in the dried residue, the nitrogen factor is 6.44. Correction of Pedersen's refraction data to this new factor gives $10^6 k = 1858$ and 1843, in satisfactory agreement with our value of 1846 at 20°.

The authors are glad to acknowledge their indebtedness to D. A. MacInnes of these Laborato-

(22) Armstrong, Budka, Morrison and Hasson, *ibid.*, **69**, 1747 (1947).

ries for his interest in this research and for suggestions in the preparation of this paper.

Summary

With the aid of a hollow, prismatic cell and the optical equipment of the Tiselius electrophoresis apparatus, the refractive index increments of solutions of some purified proteins have been measured as a function of the protein concentration, the temperature, and the wave length of the incident light. The changes in the specific refractive increment that occur on titration of the protein with alkali, in the presence of neutral salts and after equilibration with buffers have also been determined. Such data are necessary for a quantitative interpretation of the electrophoretic patterns of proteins.

NEW YORK, N. Y. RECEIVED APRIL 22, 1948

[CONTRIBUTION FROM THE RESEARCH LABORATORY, UNITED STATES STEEL CORPORATION]

Equilibrium of Iron–Carbon–Silicon and of Iron–Carbon–Manganese Alloys with Mixtures of Methane and Hydrogen at 1000°

BY RODNEY P. SMITH

Measurements of the equilibrium of iron–carbon alloys with mixtures of methane and hydrogen have been reported in an earlier paper.[1] Since most commercial steels contain various amounts of alloying elements, it is of considerable importance to know the effect of these additional elements on this equilibrium. Accordingly measurements were made of the carbon content of iron–silicon alloys containing up to 15% silicon and of iron–manganese alloys containing up to 15% manganese equilibrated at 1000°, with methane–hydrogen atmospheres of known composition. At this temperature the crystal structure of the silicon alloys may be either face-centered cubic (austenite or gamma iron) or body-centered cubic (ferrite or alpha iron) depending on the concentration of carbon and silicon. For these alloys measurements were made in the region in which austenite is stable and in the region in which ferrite is stable and an estimate is given of the phase boundaries in a portion of the isothermal section of the iron–silicon–carbon system. At 1000° the structure of the manganese alloys is austenitic at all compositions investigated. For the iron–manganese–carbon system an estimate is given of the position of the phase boundaries of the austenite–austenite+graphite and a portion of the austenite–austenite+carbide fields.

Experimental

The experimental procedure was similar to that previously described.[1] The dimensions of the samples used were about 1 × 1 × 0.4 cm. For each gas mixture a number of samples of either series of alloys and a sample of electrolytic iron were equilibrated simultaneously. The carbon content of samples containing more than 0.05% carbon were analyzed by the usual combustion method; those with less than 0.05% carbon by the low pressure combustion method described by Gurry and Trigg.[2] The silicon alloys were either milled or broken up in a steel mortar prior to combustion; the manganese alloys were burned without milling, 0.5 to 1.0 g. of Bureau of Standards sample 55b, 0.010% C, being added to start combustion.

The gas composition for each set of samples was derived from the carbon content of the electrolytic iron and the equilibrium data previously published[1] rather than from the analysis of the primary hydrogen–methane mixture and the flowmeter constants. This method has the following advantages: (1) an accurate knowledge of the composition of the primary hydrogen–methane mixture is not required; (2) it is not necessary to make corrections for the small deviations (less than ±1°) from 1000° of the furnace temperature. The minimum equilibration period was one week; that this time was sufficient was established by comparison of the analysis of the electrolytic iron, equilibrated together with a typical set of alloy samples using a primary mixture of known composition, with the results for pure iron–carbon alloys previously published.[1]

The silicon alloys of composition within the range 1.2 to 5.0% Si were commercial quality steels,[3] those between 7.0 and 15.0% Si were prepared from a high-grade ferrosilicon and electrolytic iron. Analysis of a sample of electrolytic iron and of the alloys containing 1.2 to 5.0% Si before and after equilibration with respect to carbon indicated a silicon pick up from the silica furnace tube of 0.02 to 0.06%. The results were not corrected for this small change in silicon content since its effect on the carbon concentration is nearly balanced by that of the manganese present in these alloys.

The manganese alloys were of commercial quality,[4] produced in a 250 lb. arc furnace. Since an alundum furnace tube was used in the equilibration of these alloys with respect to carbon, there was no change in their silicon content. There was, however, a small change in manganese content, that of the electrolytic iron increasing by 0.25%, that of the 14.67% Mn decreasing by 0.41%, the change in the other samples being nearly linear with the manganese content.

Results—Silicon Alloys

Equilibrium with Austenite.—The left portion of Table I gives the carbon content of the austenitic silicon alloys equilibrated with several CH_4–H_2 mixtures containing from 0.14 to 0.86% methane. The marked effect of silicon on this

(1) R. P. Smith, THIS JOURNAL, 68, 1163 (1946).

(2) R. W. Gurry and H. Trigg, *Ind. Eng. Chem., Anal. Ed.*, 16, 248 (1944).

(3) Analysis, C, 0.05–0.07; Mn, 0.2–0.3; P, 0.005–0.01; S, 0.01–0.02.

(4) ANALYSES

Mn	C	P	S	Si
3.92	1.50	0.015	0.014	0.03
6.48	1.64	.023	.011	.01
9.10	0.30	.040	.008	.19
12.87	0.94	.043	.010	.11
14.67	1.33	.040	.007	.09