ON THE REFRACTIVE INDICES OF SOLUTIONS OF THE CASEINATES AND THE ACID- AND ALKALI-EQUIVALENTS OF CASEIN

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I. Introduction

In carrying out experiments of a physico-chemical character upon proteins, we are continually hampered by our lack of any method of determining quantities and concentrations of proteins with any approach to exactitude. Direct gravimetric methods either involve great loss of material in the process of freeing the protein thoroughly from water, or else are rendered grossly inaccurate through the uncertain hydration of the protein material which is weighed. Volumetric methods which involve titrations are subject, where solutions containing proteins are concerned, to numerous errors of unusual magnitude, arising from a variety of causes. In the first place, the high molecular weights of the proteins lead to a relatively large mass of protein being equivalent to relatively small volumes of the solutions employed in titration, so that the errors arising from the estimation of these volumes are correspondingly high. Then, again, we absolutely lack in most cases that knowledge of the quantitative equivalence between a given protein and some other substance which we must possess before any titration can be successfully employed. Even where we do possess a knowledge of this equivalence, as in the case of casein, we possess no certainty that the equivalent measured represents the formation of a definite chemical compound, so that our estimation of the equivalent usually depends upon the change in color of some arbitrarily chosen indicator, while, owing to the amphoteric character of the proteins it is usually impossible to obtain, in their presence, sharp changes in the color of

indicators. Indirect methods of estimation such as that of determining the nitrogen by the Kjeldahl method and dividing the figure obtained by the percentage of nitrogen in the protein are tedious and subject to numerous inaccuracies.

In the case of casein, thanks to the labors of Söldner,¹ Courant,² de Jager,³ Timpe,⁴ Kobrak,⁵ Laqueur and Sackur,⁶ and Van Slyke and Hart,⁷ we do possess, as I have said, a knowledge of the equivalent between casein and the alkalis and alkaline earths at neutrality to a given indicator, phenolphthalein, and upon this the author based a volumetric method of determining the percentage of casein in solutions which is fairly rapid and, comparatively speaking, accurate,⁸ nevertheless the determinations made by this method are subject to errors of 5% or more.

All these facts strongly suggest that the ordinary chemical methods of estimation cannot be successfully employed where proteins are concerned, and that we must resort to methods depending primarily upon the measurement of *physical* qualities of their solutions. The large size of the protein molecule, which is a drawback when chemical methods of estimation are employed, may, in many cases where physical measurements are in question, be a positive advantage. This is particularly the case where the quantity measured is one, such as the refractive index, which depends upon the *size* of the molecule, being greater the greater the molecular volume. Hence, although the refractive index of their solutions cannot, as a rule, be successfully employed in the quantitative determination of small concentrations of inorganic

¹ Söldner Landw. Versuchsstat., **35**, 351 (1888).

² Courant. Arch. ges. Physiol., **50**, 109 (1891).

³ L. de Jager, Nederl. Tijdschr. v. Geneesk., **2**, 253 (1897), quoted from Jahreshber. f. Thierchem., **27**, 276 (1897).

⁴ H. Timpe, Arch. f. Hyg., **18**, 1 (1893), quoted after Raudnitz, Ergeb. d. Physiol., **2**, 193 (1903).

⁵ E. Kobrak, Arch. f. d. ges. Physiol., **80**, 69 (1900).

⁶ Laqueur and Sackur Beitr. z. Chem. Physiol. u. Path., **3**, 193 (1902).

⁷ Van Slyke and Hart, Am. Chem. Jour., **33**, 461 (1905).

⁸ T. Brailsford Robertson, Jour. Biol. Chem., **2**, 317 (1907).

substances in water, yet there are grounds for hoping that it may be successfully employed for the estimation of the concentration of solutions of substances of high molecular volume such as the proteins.

Accordingly, and with this hope, the following investigations were undertaken.

II. Experimental

The casein employed in these experiments was the C. P. product manufactured by Eimer and Amend and further purified by trituration with large volumes of distilled water, alcohol (absolute) and ether (u. n. d.); it was dried for 24 hours at 36°. The properties of the product thus obtained have been fully described by me in a previous paper;¹ it gives every indication of being a pure product, being insoluble in distilled water (save in traces which adhere to the undissolved particles) and completely precipitated by acetic acid. It neutralizes to phenolphthalein exactly the quantity of base determined by Laqueur and Sackur and by Van Slyke and Hart.² It is free from appreciable water, but is associated with a small quantity of ether, the last traces of which are somewhat difficult to drive off.

Accurately weighed quantities of this product were dissolved each in 100 cc. of solutions of NaOH of various concentrations.³ The refractive indices of the solutions were determined by means of a Pulfrich Refractometer.⁴ In this way it was found that, in the first place, dissolved caseinates change the refractive index of water to a far greater degree than do ordinary inorganic salts in equivalent-molecular concentration and, in the second place, that in a given concentration of alkali *the difference between the refractive indices of two solutions is proportional to the difference between the*

¹ T. Brailsford Robertson, l. c.

² Laqueur and Sackur, l. c. Van Slyke and Hart, l. c.

³ In order to ensure complete solution, the mixtures were stirred rapidly and continuously for an hour at room temperature. In the case of the more concentrated solutions, the stirring was continued for a longer period.

⁴ Very kindly lent to me by the Department of Chemistry.

percentages of casein which they contain. This fact can be expressed algebraically as follows:

$$n - n_1 = a \times c$$

where n is the observed refractive index of the solution, c is the percentage of casein which it contains and n_1 and a are constants.

The following tables summarize the results thus obtained. In the first column of each table is given the amount of casein in grams which was dissolved in 100 cc. of the solution. In the second column is given the refractive index of the solution measured at 20° C. In the third column is given the value of the constant a calculated from the above formula for the given value of the constant n_1 . The constant n_1 is determined in the following manner: the differences between the refractive indices of successive solutions differing equally in casein-content are averaged and this average is subtracted from the refractive index of the solution differing from the solution containing no casein by the same casein-content. Thus, in Table II (solvent N/50 NaOH) differences in refractive index corresponding to successive increments in casein-concentration of 0.5 percent are, respectively, 0.0008, 0.0007, 0.0008, 0.0007, and the average of these is 0.00076; subtracting this from the refractive index of the solution containing 0.5 percent of casein, we obtain $n_1 = 1.33364$. In the fourth column is given the concentration of casein *calculated* from the above formula, for the value of n_1 determined in the manner just described and for the average value of a . At the head of each table is given the concentration of alkali in which the casein was dissolved.

TABLE I
Solvent 0.01 *N* NaOH

Concentration of Casein percent	Refractive index	a for $n_1 = 1.33332$	Concentration of casein (calculated) percent
0.25	1.3337	0.00152	0.253
0.50	1.3341	0.00156	0.520
0.75	1.3344	0.00144	0.720
1.00	1.3348	0.00148	0.987
1.25	1.3352	0.00150	1.253
1.50	1.3356	0.00152	1.520
		average, 0.00150	

TABLE II
Solvent 0.02 *N* NaOH

Concentration of casein percent	Refractive index	a for $n_1 = 1.33364$	Concentration of casein (calculated) percent
0.5	1.3344	0.00152	0.49
1.0	1.3352	0.00156	1.00
1.5	1.3360	0.00157	1.52
2.0	1.3367	0.00153	1.97
2.5	1.3375	0.00154	2.49
3.0	1.3382	0.00152	2.94
		average, 0.00155	

TABLE III
Solvent 0.03 *N* NaOH

Concentration of casein percent	Refractive index	a for $n_1 = 1.33364$	Concentration of casein (calculated) percent
0.5	1.3344	0.00152	0.49
1.0	1.3351	0.00146	0.94
1.5	1.3360	0.00157	1.52
2.0	1.3368	0.00158	2.04
2.5	1.3376	0.00158	2.55
3.0	1.3382	0.00152	2.94
		average, 0.00155	

TABLE IV
Solvent 0.05 *N* NaOH

Concentration of casein percent	Refractive index	a for $n_1 = 1.33396$	Concentration of casein (calculated) percent
0.5	1.3347	0.00148	0.51
1.0	1.3354	0.00144	0.99
1.5	1.3362	0.00149	1.54
2.0	1.3370	0.00152	2.08
2.5	1.3376	0.00146	2.49
3.0	1.3384	0.00148	3.04
		average, 0.00146	

TABLE V
Solvent 0.10 *N* NaOH

Concentration casein percent	Refractive index	a for $n_1 = 1.33444$	Concentration of casein (calculated) percent
0.5	1.3352	0.00152	0.50
1.0	1.3360	0.00156	1.02
1.5	1.3368	0.00157	1.54
2.0	1.3375	0.00153	2.00
2.5	1.3383	0.00154	2.52
3.0	1.3390	0.00152	2.98
4.0	1.3405	0.00152	3.96
5.0	1.3420	0.00151	4.94
6.0	1.3436	0.00153	5.99
		average, 0.00153	

It is at once evident that, in the first place, the formula

$$n - n_1 = a \times c$$

is very accurately obeyed and, in the second place, that these experiments have justified our anticipation that it would be possible to measure the concentration of protein solutions much more accurately by their refractive indices than by chemical methods, for the agreement between the actual and the calculated concentrations of casein is extremely close, far closer than that obtained by methods of estimation

which involve titrations. The Pulfrich Refractometer reads to within $1'$, and $1'$ of the angle of total reflection roughly corresponds to a change of 0.0001 in the refractive index, which is therefore the possible error of the determination; since a change of 0.0015 in the refractive index corresponds to a change of 1 gram in the casein-content of 100 cc., the possible error in the determination of 1 gram of casein in 100 cc. by this method is $1/15$ th, *i. e.*, 7 percent, the possible error for more dilute solutions being proportionately greater, for more concentrated solutions proportionately less. A glance at the Tables I-V will serve to show that the actual error of the determinations is in all cases less than the possible error and usually considerably less, seldom amounting to more than 2 percent.

The value of a is sufficiently constant throughout the series to justify the statement that it is independent of the concentration of the alkali in which the casein is dissolved; its average value for all the determinations is 0.00152. Some of these solutions were acid to phenolphthalein and others were alkaline; now I have pointed out elsewhere¹ that in solutions acid to phenolphthalein the casein is probably polymerized, possessing in these solutions a higher molecular weight than in solutions neutral or alkaline to phenolphthalein. In other words, *no matter in what manner the casein is combined with the base, the change in the refractive index of a given volume of a solution of a base which is brought about by the introduction of a given weight of casein is constant; if the volume be 100 cc and the weight of casein 1 gram, the change in the refractive index is 0.00152.*

The value of the constant n_1 is obviously dependent upon the concentration of the base, being the refractive index of the solution of the base in which no casein is dissolved; the change in hydroxyl concentration consequent upon the addition of casein to these solutions is, apparently,

¹ T. Brailsford Robertson, Jour. Phys. Chem., **10**, 524 (1906); **11**, 453 (1907); **12**, 473 (1908); Jour. Biol. Chem., **4**, 23 (1908). T. Brailsford Robertson and Theo. C. Burnett, Jour. Biol. Chem., **6**, 105 (1909).

in all cases insufficient to appreciably affect their refractive indices although, of course, it is very appreciable to indicators. It was thought better to determine the value of n_1 by interpolation as described above than to determine it directly, since the method of interpolation enables us to determine it with greater precision. The refractive index of N/10 NaOH was, however, determined directly and found to be 1.3344 at 20° C, a value in as good agreement with the value 1.33444 determined by interpolation (cf. Table V.) as the accuracy of the direct determination permits. The equation $n - n_1 = a \times c$ holds good, not only for solutions of casein in solutions of NaOH, but also for solutions of casein in other solvents; this is shown by the following results, in which the solution employed to dissolve the casein was N/20 NH_4OH :

TABLE VI
Solvent 0.05 N NH_4OH

Concentration of casein percent	Refractive index	a for $n_1 = 1.33364$	Concentration of casein (calculated) percent
1.0	1.3351	0.00156	0.99
2.0	1.3368	0.00163	2.06
3.0	1.3383	0.00159	3.01
4.0	1.3399	0.00159	4.04
6.0	1.3428	0.00154	5.86
		average, 0.00158	

In dilute solutions of bases, the differences between the values of n_1 for the different bases are negligible. If 2.5 grams of casein be dissolved in 100 cc each of 0.02 N solutions of NaOH, LiOH, NH_4OH , $\text{Sr}(\text{OH})_2$, $\text{Ba}(\text{OH})_2$ and $\text{Ca}(\text{OH})_2$ the refractive indices of the solutions are in every case identical, namely, 1.3375. Hence the value of a , that is, of the change in refractive index which is brought about by the addition of 1 gram of casein to 100 cc of a solution of a base is independent of the nature of the base. Moreover, the value of a is the same in acid as in alkaline solutions. Thus

the refractive index of a 1.25 percent solution of casein in 0.01 *N* NaOH is 1.3352, and of a 1.25 percent solution of casein in 0.01 *N* NaCl + 0.00513 *N* HCl is also 1.3352. Hence, *whether the casein is acting as an acid or as a base, its effect upon the refractive index of the solution to which it is added is quantitatively the same.*

The Influence of Temperature upon the Refractive Indices of Solutions of the Caseinates

Since the refractive index of fluids varies, as a rule, notably with temperature, it becomes of importance, if the refractive index of casein solutions is to be used as a measure of their concentration, to determine in what manner the refractive indices of solutions of the caseinates is dependent upon the temperature, since it would be inconvenient always to determine the refractive index at a fixed standard temperature. Moreover, the investigation of the change in refractive index, which these solutions undergo between the temperatures of 30° and 40°, is of considerable theoretical interest, since a marked increase in the opalescence, conductivity and alkalinity of casein solutions occurs at a temperature in the neighborhood of 36°. ¹ It has been suggested by W. A. Osborne that this phenomenon is attributable to an increase in the hydrolytic dissociation of the caseinates at this temperature, the insoluble casein thus set free being held in suspension; while I have elsewhere pointed out that the facts are equally readily explained by supposing that the casein undergoes polymerization as the temperature rises, with consequent diminution in its alkali-binding power. ² Two and a half grams of casein were dissolved in 100 cc of NaOH. Some of the solution was placed in the refractometer and surrounded with the jacket supplied with the instrument for maintaining the prism and the solution at a given temperature. The temperature was then slowly raised by running hot water through the jacket; a thermometer was placed

¹ W. A. Osborne, *Jour. Physiol.*, **27**, 398 (1901).

² T. Brailsford Robertson, *Jour. Biol. Chem.*, **5**, 147 (1908).

in the solution and the temperature and the angle of total reflection were read at intervals; the readings were repeated as the solution cooled; both sets of readings are given in Table VII, those which were taken while the solution was *cooling* being distinguished by an asterisk.

Solutions of the caseinates of the alkaline earths and lithium undergo a marked increase in opalescence as the temperature approaches 36° . The caseinates of sodium, potassium and ammonium do not undergo an increase in opalescence at this temperature which is appreciable to the unaided eye, but that they do undergo a slight increase in opalescence is revealed by the fact that the edge of the shadow which marks the angle of total reflection becomes somewhat indistinct and furry as the temperature rises to between 30° and 40° ; hence the experimental error and the difficulty of the determination are somewhat increased at these temperatures.

In the accompanying table, the temperature at which the determination was made is given in the first column, in the second is given the refractive index of the solution at that temperature, and in the third, the difference between the refractive index of the solution and that of water at the same temperature. The refractive index of water at the various temperatures indicated, is estimated by interpolation from the determinations of Rühlmann, quoted in Landolt-Börnstein's *Physikalisch-Chemische Tabellen*, 0.0001 being subtracted from the refractive index of water for every 1° C rise in temperature between 20° and 30° and 0.00013 for every degree rise in temperature between 30° and 40° , the refractive index at 20° being taken as 1.3330.

It will be observed that there is no sudden alteration in the refractive index of this solution between the temperatures 30° and 40° , on the contrary, the difference between the refractive index of the solution and that of water at the same temperature remains appreciably constant. In other words, *the change in refractive index which is brought about by the addition of a given quantity of casein to a given*

TABLE VII

2.5 grams of Casein dissolved in 100 cc of 0.02 N NaOH

Temperature	Refractive index of solution	Difference between refractive index of solution and that of water at the same temperature
20°	1.3375	0.0045
25°	1.3371	0.0046
* 25.5°	1.3370	0.00455
* 26°	1.3369	0.0045
28°	1.3366	0.0044
* 28.5°	1.3365	0.00435
30°	1.3364	0.0044
* 32°	1.3361	0.0044
32.5°	1.3362	0.0045
* 33.5°	1.3360	0.00445
34°	1.3360	0.0045
* 35°	1.3358	0.00445
* 36°	1.3354	0.0042
38.5°	1.3351	0.0042
* 38.5°	1.3353	0.0044
40°	1.3350	0.0043
* 40°	1.3351	0.0044
		average, 0.0044

volume of an alkaline solution is, within the limits of experimental error and between the temperatures 20° C and 40° C, constant. This fact speaks in favor rather of the author's hypothesis regarding the changes in solutions of the caseinates with rising temperature than of Osborne's hypothesis. Were insoluble casein set free and suspended in the solution at a temperature lying between 30° and 40°, we should expect a sudden drop in the refractive index of the solution at this temperature; no such drop is observed. If, on the contrary, the effect of rise of temperature were merely to cause polymerization of the casein molecule, then since, as we have seen, the change in refractive index which is brought about by the addition of casein to an alkaline solution is independent of the manner in which the casein is combined with the base, no sudden change in refractive index would be expected with rise of temperature, on the con-

trary, the observed constancy of the difference between the refractive index of the solution and that of water at the same temperature would rather be expected, since the percentage of casein actually in solution would be the same at all temperatures.

No irreversible change in the refractive index of casein solutions is brought about by heating even to higher temperatures than 40° (unless soluble inorganic salts of the alkaline earths be present, *vide infra*) and there is no appreciable "residual effect" on cooling. Solutions of the caseinates can be rapidly heated to boiling and as rapidly cooled without changing their refractive indices.¹

The Acid-equivalent of Casein

Advantage was taken of the marked change in refractive index which accompanies change in the casein-content of solutions to determine the acid-equivalent of casein, *i. e.*, the maximum amount of casein which a given amount of acid will hold in solution. Here an interesting phenomenon is encountered; if one stirs excess of casein in 0.1 N HCl (or more dilute) rapidly and continuously for over an hour very little or no casein goes into solution, the solution remains perfectly clear on filtration and its refractive index is only very slightly changed. If, however, the casein be previously dissolved in dilute NaOH and then precipitated with HCl, the addition of even slight excess of HCl will then carry it into solution. In other words, dry, granular casein only dissolves with extreme slowness in acid solutions,² while wet, flocculent and freshly precipitated casein dissolves readily

¹ This also shows that the trace of ether associated with the preparation employed in these experiments has no appreciable influence upon the refractive indices of the solutions.

² Van Slyke and Van Slyke, *Am. Chem. Jour.*, **38**, 393 (1907); *Jour. Biol. Chem.*, **4**, 259 (1908), have shown that dry casein, while insoluble in dilute acids, abstracts acid from the solution; they consider that the acid is "adsorbed" or concentrated by capillary forces at the surface of the casein particles. I have elsewhere, *Jour. Biol. Chem.*, **4**, 35 (1908); *Zeit. Chemie und Industrie der Kolloide*, **3**, 18 (1908), dwelt upon the objections to this view.

in excess of acid. This effect is not due to the presence of a trace of salt, such as NaCl, in the latter case, because the addition of 0.01 *N* NaCl to a 0.1 *N* HCl solution does not, to any appreciable extent, enhance the rapidity with which casein dissolves in the solution. The same phenomenon is encountered in the case of serum globulin (the fraction insoluble in distilled water, precipitable by CO₂) and has been repeatedly remarked upon by investigators. Serum globulin which is wet and has never been dried since precipitation, readily and rapidly dissolves in alkaline solutions, while the dried powder dissolves comparatively slowly.

The acid equivalent of casein was determined in the following manner: 2.5 grams of casein were dissolved in 100 cc of 0.02 *N* NaOH and to the solutions thus obtained varying quantities of 0.1 *N* HCl, made up to 100 cc with distilled water, were added, and the resulting mixtures were stirred rapidly for from 1/2 to 1 hour; they were then filtered and the refractive indices of the filtrates were determined. Since the refractive indices of mixtures in the same proportion and concentrations of NaOH and HCl differ only inappreciably from one another, a constant refractive index (= 1.3352, cf. Table I) indicated complete solution of the casein.

In the following tables, the excess of acid (over that required to neutralize the NaOH) is given in the first column, and in the second is given the refractive index of the filtered solution. Since the concentration of Na in all the solutions is 0.01, we may, with sufficient approach to accuracy, write down the casein-content of the solutions from those of solutions of the same refractive index in Table I of this paper; this has been done in column 3. In the fourth column is given the ratio of the excess of acid to the concentration of the dissolved casein.

TABLE VIII

Excess of acid	Refractive index	Concentration of casein	Ratio of excess of HCl to concentration of casein
0.002100 <i>N</i>	1.3341	0.52	0.00404
0.002705 <i>N</i>	1.3344	0.72	0.00376
0.003310 <i>N</i>	1.3348	0.99	0.00334
0.003915 <i>N</i>	1.3351	1.19	0.00330
0.004520 <i>N</i>	1.3352	1.25	—
0.005125 <i>N</i>	1.3352	1.25	—

TABLE IX

Excess of acid	Refractive index	Concentration of casein percent	Ratio of excess of HCl to concentration of casein
0.002100 <i>N</i>	1.3340	0.45	0.0047
0.003610 <i>N</i>	1.3349	1.05	0.0034
0.005125 <i>N</i>	1.3352	1.25	—
0.008150 <i>N</i>	1.3352	1.25	—
0.01420 <i>N</i>	1.3352	1.25	—
0.02025 <i>N</i>	1.3352	1.25	—

The ratios in the fourth column are approximately constant, that is, the increment in casein-concentration is, within the limits of experimental error, very nearly proportional to the excess of HCl in the solution,¹ hence, averaging the increments in casein-concentration for equal increments in HCl-excess we can readily, by interpolation, determine the excess of HCl which was *just* sufficient to completely dissolve one gram of casein; in each of the experiments tabulated above this is found to be 0.00164 *N*. Since 200 cc of 0.00164 *N* HCl = 3.28 cc of *N*/10 HCl, one cc of *N*/10 HCl = 0.305 gram of casein, or, in other words, 1 gram of casein = 32.8×10^{-5} equivalent-gram-molecules of HCl.

The above determinations must be regarded as being

¹ There are, however, indications that the acid-equivalent is higher the more dilute the solution; the same phenomenon is encountered, but to a more marked degree, in acid solutions of Serum Globulin; cf. W. B. Hardy, Jour. Physiol., 33, 266 (1905).

somewhat approximate, since a small amount of NaCl is present in the solution and we do not know whether, or to what extent, salts enhance or depress the solubility of casein in acids. The influence of the salt upon the acid-equivalent cannot, however, be very great, since, if it were so, it might be expected to vary with the nature of the salt, and the equivalent in the presence of NH_4Cl is, within the limits of experimental error, the same as that in the presence of NaCl. This is shown by the following table. The determinations were made in precisely the same manner as those described above, save that N/50 NH_4OH was employed to dissolve the casein instead of N/50 NaOH.

TABLE X

Excess of acid	Refractive index	Concentration of casein percent	Ratio of excess of HCl to concentration of casein
0.002100 N	1.3341	0.52	0.0040
0.003610 N	1.3350	1.12	0.0032
0.005125 N	1.3352	1.25	—

By interpolation we find that the excess of acid which was just sufficient to dissolve 1 gram of casein was 0.00158 N, *i. e.*, 1 gram of casein = 31.6×10^{-5} equivalent-gram-molecules of HCl, which is, within the limits of experimental error, identical with the figure previously obtained. The acid-equivalent of casein is thus much higher than that of serum globulin.¹

The Alkali-equivalent of Casein

In a previous paper I have stated that the maximum amount of casein which an alkaline solution will dissolve is that with which the amount of base present will combine to form "neutral" caseinate, neutral to litmus, containing an amount of the base equivalent to 1.57 percent of CaO .² Using the more sensitive and exact method of determining

¹ Cf. W. B. Hardy, *l. c.*

² T. Brailsford Robertson, *Jour. Biol. Chem.*, **2**, 317 (1907).

the concentration of casein in solution which is described above, however, I was not long in discovering that this statement is erroneous and that casein is far more soluble in alkalies than previous estimates would lead us to suppose.

The source of error lies in the fact that complete "saturation" of an alkaline solution with casein is almost impossible to obtain by shaking the solution with excess of casein. The solutions which I considered "saturated" in my previous publication were, in reality, very far from containing all the casein which the base would dissolve because, after the attainment of neutrality to litmus, additional casein dissolves only with extreme slowness. Even prior to the attainment of neutrality to litmus, however, the process of solution occupies a definite and considerable period of time, and rapidity of solution is less in solutions of weak bases than in solutions of strong bases. This is very clearly shown by the following table. Six grams of casein were stirred rapidly and continuously in 100 cc of the alkali mentioned in the first column, for the time given in the second column. The refractive indices of the filtered solutions are given in the second column, and the estimated casein-contents, calculated from the refractive indices, are given in the fourth column.

TABLE XI

Solvent	Stirred for	Refractive index	Concentration of casein (calculated)
0.02 <i>N</i> NaOH	1 hour	1.3425	5.7
0.02 <i>N</i> LiOH	1 "	1.3426	5.8
0.02 <i>N</i> NH ₄ OH	1 "	1.3411	4.8
0.02 <i>N</i> NH ₄ OH	2½ "	1.3420	5.4
0.02 <i>N</i> NH ₄ OH	4½ "	1.3426	5.8
0.02 <i>N</i> Sr(OH) ₂	2 "	1.3398	4.0
0.02 <i>N</i> Ca(OH) ₂	2 "	1.3382	3.0
0.02 <i>N</i> Ba(OH) ₂	2 "	1.3374	2.4

It is evident that this solution of the casein takes place rapidly at first, very slowly later on, and that the strong bases dissolve casein much more rapidly than weak bases.

Even six hours of stirring with a large excess of casein failed to secure a complete saturation of the alkali. Hence the following procedure was adopted: 4 grams of casein were dissolved in 100 cc of N/40 NaOH and varying amounts of N/10 HCl, made up to 100 cc with distilled water were added, so as to leave varying excesses of NaOH unneutralized by HCl. The excess of alkali which was *just* sufficient to completely dissolve the casein (indicated by a constant refractive index of 1.3367 of the filtered solution) was then determined and found to be 4.55 cc of 0.1 N NaOH. In other words 1 cc of N/10 NaOH = 0.877 gram of casein at "saturation," or 1 gram of casein = 11.4×10^{-5} equivalent-gram-molecules of NaOH, an equivalent of the same order as the alkali equivalent, at "saturation," of serum-globulin.¹ The equivalent for LiOH is identical with that for NaOH.

The alkali-equivalents for $\text{Sr}(\text{OH})_2$, $\text{Ba}(\text{OH})_2$ and $\text{Ca}(\text{OH})_2$ cannot be determined in this manner because the chloride of the alkaline earth completely precipitates the caseinate which is formed.² That this effect is an instance of the well-known phenomenon of "precipitation by a common ion" is shown by the following phenomenon. If to 100 cc of N/50 $\text{Ca}(\text{OH})_2$, containing 2.5 grams of casein, we add 100 cc of N/50 CaCl_2 , no actual precipitation occurs, it is true, but a marked increase in its opalescence is observed. If, however, we add, instead of N/50 CaCl_2 , N/50 SrCl_2 , no change in the opacity of the solution occurs; yet if SrCl_2 be added to a solution of casein in $\text{Sr}(\text{OH})_2$ the caseinate is precipitated just as readily and completely as that in solution in $\text{Ca}(\text{OH})_2$ is precipitated by CaCl_2 . Of course, if sufficient *excess* of a chloride of an alkaline earth be added to a solution of casein in a dilute hydroxide of another alkaline earth, the casein will be precipitated, since, in that case, a sufficient quantity of the caseinate of the first alkaline earth is formed,

¹ W. B. Hardy, l. c.

² Cf. T. Brailsford Robertson, l. c.

by double decomposition, to undergo precipitation by the excess of the chloride.¹

The fully "saturated" solutions of the caseinates obtained by the method outlined above are, of course, acid to litmus, since in solutions neutral to litmus 1 gram of casein = 53×10^{-5} equivalent-gram-molecules of the base,² while in the "saturated" solutions 1 gram of casein = 11.4×10^{-5} equivalent-gram-molecules of the base. The acidity of these solutions is, however, surprisingly disproportionate to the emphatic difference between the alkali-equivalents of the casein in saturated and in neutral solutions. The reactions of the "saturated" solutions to indicators are as follows:

Dimethylaminoazobenzol	Yellow
Congo red	Red
³ Sodium alizarinsulphonate.....	Red
Cochineal	Rose
Paranitrophenol.....	Colorless
Rosolic acid.....	Yellow

indicating a H^+ concentration of between 10^{-5} and 10^{-6} N and a hydroxyl concentration of between 10^{-9} and 10^{-8} N.⁴

¹ It is of interest to note that the casein is precipitated in flocculi from both of these solutions by heating to boiling and that this precipitate does not redissolve on cooling. The same phenomenon, save that it occurs at a lower temperature, was observed by Sidney Ringer, in solutions of paracasein (cf. S. Ringer, Jour. Physiol., **12**, 164 (1891), also Ringer and Sainsbury, Ibid., **11**, 369 (1890); **12**, 170 (1891)). In the absence of soluble inorganic salts of the alkaline earths, of course, no precipitation occurs on heating, and the increase in opalescence which occurs at 36° disappears on cooling.

² Van Slyke and Hart: Am. Chem. Jour., **33**, 470 (1905).

³ It is possible by means of this indicator, to demonstrate in a very striking manner, the amphoteric character of casein. If to a solution of casein in dilute NaOH, and containing sufficient alizarinsulphonate of sodium to render it bright red, one adds sufficient HCl to precipitate and *partially* redissolve the casein, on stirring and allowing the undissolved casein to settle, it can be seen that the supernatant, acid fluid is dark brown, while the precipitate is stained bright pink, or, in other words, that, when suspended in an *acid* solution, casein is acting as a base. All the indicators used were Gruebler's preparations, being the same as those employed by Salm, *vide infra*.

⁴ E. Salm: Zeit. phys. Chem., **57**, 471 (1907).

In other words, although in passing from the "saturated" to the "neutral" caseinate the amount of alkali bound by 1 gram of casein changes by 40×10^{-5} gram-equivalents, yet the hydroxyl-content of a solution containing 1 gram of casein only changes by 10^{-7} or 1/4000th of the change in the concentration of the base. Even in passing from the "saturated" to the "basic" caseinate (neutral to phenolphthalein, 1 gram of casein = 80×10^{-5} equivalent-gram-molecules of alkali, cf. Van Slyke and Hart, l. c.), the change in hydroxyl-concentration of a solution containing 1 gram of casein is still only about 1/350th of the change in the concentration of the base. Hence the caseinates possess in a very high degree the power of maintaining the neutrality of solutions in which they occur. I have elsewhere suggested¹ that this phenomenon is to be attributed to the amphoteric-acid structure of the protein molecule and the power of almost undergoing indefinite polymerization² which this structure confers upon them.

III. Conclusions

(1) The difference between the refractive indices of two solutions of a caseinate which differ only in their casein-content, is proportional to the difference between the percentages of casein which they contain. This relation can be expressed algebraically as follows:

$$n - n_1 = a \times c$$

where n is the observed refractive index of the solution, c is the percentage of casein which it contains, n_1 is a constant the value of which depends upon the concentration and nature of the solvent employed to dissolve the casein, and a is a constant numerically equal to the change in the refractive index of the solvent which is brought about by the addition to 100 cc of 1 gram of casein.

¹ T. Brailsford Robertson: Jour. Phys. Chem., **10**, 524 (1906); **11**, 453 (1907); **12**, 473 (1908); Jour. Biol. Chem., **4**, 23 (1908); **5**, 147 (1908). T. Brailsford Robertson and Theo. C. Burnett: Jour. Biol. Chem., **6**, 105 (1909).

² Or, more accurately, *condensation* with the elimination of the elements of water.

(2) By means of the above formula the concentration of casein in a solution can be very accurately determined, the deviations from accuracy rarely exceeding 2 percent of the quantity of casein contained in 100 cc of the solution.

(3) The change in the refractive index of a given volume of a solution of a base which is brought about by the introduction of a given weight of casein is independent of the concentration of the base and of the nature of the base; if the volume be 100 cc and the weight of casein 1 gram, the change in the refractive index is 0.00152.

(4) The change in the refractive index of a given volume of a solution which is brought about by the introduction of a given weight of casein is the same in solutions of HCl as in solutions of the bases; hence, whether the casein is acting as an acid or as a base, its effect upon the refractive index of the solution to which it is added is quantitatively the same.

(5) The difference between the refractive index of a solution of sodium caseinate and that of water at the same temperature is independent of the temperature between 20° and 40°; hence the change in refractive index which is brought about by the addition of casein to a given volume of an alkaline solution is, between 20° and 40°, constant.

(6) The hydrochloric-acid-equivalent of casein (*i. e.*, the quantity of acid required to just dissolve 1 gram of casein) has been determined, by methods described in this paper, and, for solutions containing 1.25 percent of casein, it was found that 1 gram of casein = approx. 32×10^{-5} equivalent-gram-molecules of HCl.

(7) Casein dissolves in alkaline solutions, at first rapidly and later more slowly. After the attainment of neutrality to litmus, additional casein dissolves only with extreme slowness. For this reason the author's previous statement that the maximum amount of casein which an alkaline solution will dissolve is that with which the amount of base present will combine to form the "neutral" caseinate (neutral to litmus) is erroneous.

(8) The NaOH-equivalent of casein has been determined, by methods described in this paper, for solutions containing 2 percent of casein, and it was found that 1 gram of casein, at "saturation" of the base = 11.4×10^{-5} equivalent-gram-molecules of NaOH. The LiOH-equivalent was found to be identical with the NaOH-equivalent.

(9) The acidity of solutions of bases "saturated" with casein was determined by means of indicators and was found to lie between 10^{-5} and 10^{-6} N H^+ ; the hydroxyl-concentration in these solutions, therefore, lies between 10^{-9} and 10^{-8} N.