THE SOLUBILITY OF CASEIN IN SALTS OF CERTAIN ORGANIC ACIDS, AND ITS FRACTIONATION BY MEANS OF THESE ACIDS

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INTRODUCTION

The study of casein, the principal protein in milk, is being given increasing attention in research laboratories, not only because of the promising new ways in which it may be used in industry, but also because it affords a suitable material for the study of fundamental problems in protein chemistry.

Strong acid and alkaline reagents have generally been used as solvents for casein, but they are not entirely satisfactory. Accordingly, the writers undertook experiments to determine the degree of solubility of casein in solutions of certain salts of organic acids. Nearly neutral aqueous solutions having a pH value of 4.6 (the isoelectric point of casein) were used in these experiments, since very little research work had been done with such solutions as compared to the work done with strong acids and bases.

DEFINITION OF TERMS

The term "casein" as used here means "acid casein" as distinct from rennet casein. Acid casein might be further defined as the phosphoprotein, or aggregation of phosphoproteins, precipitated from skim milk by acid at a pH value of 4.1 to 4.6. The phosphorus content, as determined by different investigators, varies from 0.71 to 0.88 percent.

Rennet casein is evidently a calcium caseinate—calcium phosphate complex—and is not nearly so pure as acid-precipitated casein. Rennet casein is relatively unimportant commercially; approximately 97 percent of all the casein produced is acid casein. Acid casein is the product commonly known in the industry and in the scientific literature as "casein."

By "solubility of casein" is meant the solubility at the isoelectric point rather than that resulting from chemical combination, as, for example, in borax solution where sodium caseinate is formed and dissolved.

The term "solution" in this paper refers both to crystalloidal and to colloidal solutions.

1 Received for publication January 29, 1938; issued August 1938. This paper was presented in part before the meeting of the American Chemical Society, Chapel Hill, N. C., April 1937. It is condensed from the dissertation submitted by Stephen P. Gould in partial fulfillment of the requirements for the degree of doctor of philosophy in the Faculty of Pure Science, Columbia University.

2 The writers acknowledge the generous cooperation and helpful advice of L. A. Rogers, Chief, and E. O. Whittier, senior chemist, Division of Dairy Research Laboratories, Bureau of Dairy Industry.
SOLUBILITY OF ACID-PRECIPITATED CASEIN

EARLIER INVESTIGATIONS DEALING WITH SOLUBILITY OF CASEIN

Much of the earlier work on casein solvents or plasticizers in the organic field was done in connection with casein plastics. Goldsmith \( (12, 13, 14) \) in 1907 found that naphthol and other phenolic compounds and urea would "convert" casein to the "thermoplastic" state. This he believed was due to "the solvent action of the converting agent." Phenolsulphonic acid, tartaric acid, aniline, borax, and naphtholsulphonic acid \( (28) \) are also mentioned in the early patent literature of plastic casein.

Cohn \( (7, 8) \) reported the solubility of casein in water at its isoelectric point as 0.11 g per liter at 25\(^\circ\) C. Euler and Bucht \( (9) \) determined quantitatively the solubility of casein in tartaric acid, sodium potassium tartrate, and chloracetic acid solutions. They determined the amount of casein in the solution by precipitating it with sodium acetate solution or acetic acid, filtering, and drying in a vacuum desiccator. At the Carlsberg Laboratories in 1925 Kondo \( (19) \) examined the effect of hydrochloric acid on casein. By formol titration, he showed that there was no hydrolysis at 18\(^\circ\) C.

Agthe \( (1) \) recorded citric acid, sodium naphthylaminosulphonate, and potassium butyrate as agents for making casein soluble.

In view of the fact that an acid that considerably swells casein will usually dissolve it, the work of Isgarischew and Pomeranzewa \( (16) \) is of great interest. They tested the swelling action of casein in solutions of 23 organic acids. The greatest swelling action was shown by chloracetic, lactic, glycollic, malonic, formic, and citric acids (in the order named). They concluded that a hydroxyl and also a second carboxyl group in the acids greatly increased the swelling of the casein, but that a further increase in carboxyl groups diminished dilatation. With the exception of formic acid, the monobasic fatty acids showed little swelling power.

Sutermeister \( (31, p. 128) \) lists a wide variety of solvents for casein, chiefly inorganic. In addition to the commonly used alkalies, he records less commonly used alkaline solvents such as sodium sulphite, aluminate, arsenate, and citrate, and lithium, magnesium, and cesium hydroxides.

Von Weimarn \( (36, 37) \) formed dispersions of nitrogenous materials in solutions of Ca(CNS)\(_2\), CaBr\(_2\), and LiCNS. The last was by far the most efficient solvent. He observed also that solutions of pyrogallol, resorcinol, thiourea, and guanidine thiocyanate were good dispersing agents—particularly at 108\(^\circ\) C.

In a fundamental study of organic solvents for proteins, Loiseleur \( (22, 23, 24) \) pointed out that they formed true solutions in certain acids, such as formic, while higher acids, such as acetic and propionic, did not bring about solution. However, oxypropionic acids such as lactic or pyruvic readily dissolved casein. Other acids, such as acetic, that did not have the power of dissolving protein acquired this property when amino acids, such as glycocoll or alanine, were added.

In 1932, Soff \( (29) \) at Columbia University worked with new types of solvents developed by the senior author. These are concentrated aqueous solutions of highly soluble salts of organic acids such as

\[3\] Italic numbers in parentheses refer to Literature Cited, p. 141.
sodium cymenesulphonate or sodium benzenesulphonate in which the organic part is a large proportion of the molecule. Such solutions differ from water in their solvent characteristics, as the organic portion of the compounds seems to give them many of the properties of organic solvents. In general, Soff worked with highly concentrated solutions. He found sodium cymenesulphonate solution to be a very powerful solvent for casein and gelatin. He also stated; “There had apparently been no change in properties during storage of these casein solutions over an unusually long period of time.” Nearly neutral solvents such as these salts obviously have a greater advantage in avoiding the factor of hydrolysis over stronger, more commonly used basic or acidic compounds.

Associates of Rogers (2, p. 53) explain that casein is insoluble in the usual neutral organic solvents and in cold dilute acids. Casein at its isoelectric point of 4.6 is considered to be uncombined with acids or bases.

**EXPERIMENTAL PROCEDURE**

**Materials**

Preliminary experiments were made to determine what concentrations would be practical to use for exact solubility work. For these preliminary experiments, sodium cymenesulphonate from the Columbia University chemical engineering laboratory was used. This was fairly pure, but for the more precise work later, the salt was recrystallized from alcohol.

Solubility determinations were run at a pH of 4.6, the isoelectric point of casein, so that the solubility would not be affected by possible alkalinity of the solvent, and also because casein is most stable at its isoelectric point. To adjust the solutions to this point, cymenesulphonic acid was used. This was purified by freeing it from sulphuric acid by the following procedure. The solution was diluted, neutralized with barium hydroxide, and the barium sulphate was filtered off. The cymenesulphonic acid was liberated from its barium salt in the filtrate by the addition of the calculated amount of sulphuric acid. After the barium sulphate was filtered off, the filtrate was evaporated to dryness.

The casein used for the preliminary experiments was a well-washed grain-curd product prepared at the Grove City (Pa.) Creamery. For the later more exact experiments, a purified casein prepared by E. O. Whittier of the Division of Dairy Research Laboratories was employed. This was precipitated from skim milk by adding a 4-percent solution of hydrochloric acid until the pH value was 4.1. The casein was washed first with water slightly acidified with hydrochloric acid, then 8 or 10 times with pure water, next with alcohol, and finally with ether. Thus the redissolving in alkali (which is a concomitant of the Hammarsten process) was avoided. This gave a casein of high purity, containing less than 0.50 percent ash above that due to the P₂O₅ combined in the casein molecule. Hammarsten casein also was used in some of the later experiments.

**Methods**

The general procedure for determining the solubility of casein in the solutions of the salts of organic acids was as follows: The quantity
of salt necessary to give the desired concentration of solution was dissolved in water, enough of the acid or alkali (as the case might be) corresponding to the salt used was added to bring the pH value of the solution to 4.6, and water was added to bring the whole mixture up to 55 cc. At first the casein was added gradually, often in amounts greater than 5 g, but it was found (in agreement with earlier work on solutions in other solvents) that the solubility increased with increasing amounts of the solid phase. This fact in itself was one proof of the nonhomogeneity of casein. Because of this characteristic, it was considered advisable in all cases to use the same gross quantity of casein. Consequently, the solubility values in this paper, unless otherwise noted, have been determined on the empirical basis of 5 g of casein to 50 cc of solution.

In some of the earlier experiments, much difficulty was caused by bacterial decomposition of the solutions. The addition of 0.2 percent of phenol (0.10 g) to 50 cc of solution served effectively to inhibit this action, and it was determined by experiment that phenol at the low concentration employed did not appreciably affect the solubility values.

Five grams of casein and 50 cc of solution at a pH value of 4.6 were put into a 250-cc Erlenmeyer flask. This was well stoppered and placed in an incubator that was held at the necessary temperature by thermostatic control. From two to four determinations were made in establishing the solubility value at each temperature and concentration. The flasks were shaken at frequent intervals.

The progress toward solubility equilibrium was followed by obtaining the refractive index of the solution at suitable intervals. Samples were drawn off by vacuum through a short piece of rubber tubing fitted on the end of a pipette. The tubing contained a wad of cotton to serve as a filter. An Abbé refractometer, with prisms heated to 30° C., was employed, and therefore samples of only 1 or 2 drops of solution were required. The attainment of equilibrium was shown by the constancy of the maximum of the refractive indices over a period of from 2 to 4 days. This was, incidentally, evidence against hydrolytic decomposition of the protein.

After the equilibrium of the solution was established, some of it was filtered as quickly as possible through a wad of cotton placed in the stem of a funnel. From 1 to 2 g of the solution was weighed in a weighing bottle, transferred to a beaker, and diluted with water at a temperature of 35° to 40° C. A few cubic centimeters of 10-percent acetic acid solution were then slowly added to the warm solution to precipitate the casein. At 35° to 40°, the protein separates in a granular, easily filterable condition. The mixture was allowed to stand overnight to promote completeness of precipitation. The casein was filtered off on a Gooch crucible fitted with two pads of filter paper. The filtrate was concentrated nearly to dryness on the steam plate and diluted with water. If any casein remained in solution, it was practically all removed by this method. Sometimes small additional quantities of casein were thus obtained and were added to the main residue. The Millon or biuret test was applied to the filtrate and usually showed the absence of significant amounts of casein. The precipitate was washed free of extraneous matter. Tests for completeness of washing were made with litmus paper. In
addition, lead nitrate solution was used to test for sodium cymenesulphonate; ferric chloride solution was used to test for potassium thiocyanate; and silver nitrate solution to test for sodium benzene-sulphonate.

Acetic acid was chosen as the casein precipitant because this acid, as well as trichloroacetic, has been used for years for this purpose and appears to have been thoroughly tested. Colloid chemists are of the opinion that the action is a salting-out effect. The action is in accordance with the precipitating characteristics of the Hofmeister series—a satisfactory explanation of which is still lacking. To make sure that the precipitates were free from acetic acid, they were always washed until the wash water was neutral to litmus.

In the early experiments, the quantity of precipitated casein was determined by the refractometer. At times, this procedure seemed to give accurate results, but now and then the results were erratic and the source of the error could not be located.

The direct-weight method was finally adopted as the general method for determining the quantity of casein dissolved in the solvents, as it gave more consistent results than the refractometric procedure, was more expeditious than the determination of nitrogen content, and was sufficiently accurate. In obtaining the direct weight of casein, the precipitate on the suction filter was washed free of salts and treated with 60 to 80 cc of acetone and then with the same quantity of ether. The ether was removed by sucking air through the filter for 15 or 20 minutes. The Gooch crucible containing the washed casein was then placed in a desiccator and allowed to remain overnight. From the weight obtained, the number of grams of casein dissolved in 100 g of solvent was calculated.

Unless otherwise indicated, Whittier's purified casein was used for the final solubility determinations.

**SOLUBILITY OF CASEIN IN SOLUTIONS OF RECRYSTALLIZED SODIUM CYMENESULPHONATE**

Solutions containing 5 percent and 7.5 percent of recrystallized sodium cymenesulphonate were used since the high viscosities of the more concentrated solutions prevented effective sampling.

In general, the 5 percent solution was prepared as follows: 2.47 g of the salt was dissolved in water. Two cubic centimeters of phenol solution (containing 0.10 g) was added, followed by 4 drops of cymenesulphonic acid solution (containing 0.03 g) which gave the solution a pH value of 4.6, as shown by bromocresol green paper. The mixture was then made up to 50 cc. The 7.5 percent solution was prepared in the same way except that 3.70 g of sodium cymenesulphonate and 0.05 g of cymenesulphonic acid were used to give the correct concentration and a pH value of 4.6.

The refractive index of the solution was determined, and 5 g of casein was added. The flask was stoppered, placed in an incubator held at the required temperature, and shaken at intervals.

Equilibrium was ascertained by noting or observing the constancy of the maximum of the refractive index over a period of, usually, from 2 to 4 days. Ordinarily, all salts required an average of about 10 or 11 days to attain equilibrium at 15° C., 7 or 8 days at 30°, and 4 or 5 days at 45°.
Percentages of casein were determined by the method of precipitation with 10 percent acetic acid solution, and weighing the casein directly as described above. Some of the early results were also checked by the Van Slyke gasometric micro-Kjeldahl method (34, 35) for determining nitrogen. Results were usually reproducible within narrow limits, although in some cases they were not as close as could be obtained with crystalloidal materials. Proteins, because of their indefiniteness and the huge size of their molecules, never give as exact results as do simpler inorganic and organic compounds.

Final solubility values for all salts (except as otherwise noted) were determined at 15°, 30°, and 45° C. These were considered the most practical temperatures to employ, because of the limiting effect of viscosity. Solubility values are reported as grams of casein dissolved in 100 g of solvent. The solubility values are listed in table 1.

### Table 1.—Solubility of casein in solutions of salts of organic acids having a pH value of 4.6

<table>
<thead>
<tr>
<th>Kind and concentration of the salt solution</th>
<th>Quantity of casein dissolved in 100 g of the solvent at—</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15° C.</td>
</tr>
<tr>
<td>Sodium cymenesulphonate:</td>
<td></td>
</tr>
<tr>
<td>5 percent</td>
<td>3.0480</td>
</tr>
<tr>
<td>7.5 percent</td>
<td>5.2990</td>
</tr>
<tr>
<td>Potassium thiocyanate:</td>
<td></td>
</tr>
<tr>
<td>2.5 percent</td>
<td>1.7850</td>
</tr>
<tr>
<td>5.0 percent</td>
<td>3.5490</td>
</tr>
<tr>
<td>Sodium benzenesulphonate:</td>
<td></td>
</tr>
<tr>
<td>5.0 percent</td>
<td>0.9778</td>
</tr>
<tr>
<td>10.0 percent</td>
<td>2.2070</td>
</tr>
</tbody>
</table>

**SOLUBILITY OF CASEIN IN POTASSIUM THIOCYANATE SOLUTIONS**

The potassium thiocyanate used was a chemically pure analyzed salt. Potassium thiocyanate solution was found to be an excellent solvent for casein. Experimentation established that a 2.5-percent solution of potassium thiocyanate was best adapted to the method of sampling and to the temperatures employed. A 5-percent solution could be used at 15° C., but at 30° and 45° the solution became too dense.

In general, the 2.5-percent solution of potassium thiocyanate was prepared as follows: 1.25 g of KCNS was weighed out, 2 cc of phenol solution was added, and the solution was made up to the mark in a 50-cc volumetric flask. Rather surprisingly, the solution gave at this concentration a pH value close to 4.6. The 5-percent solution was made up somewhat similarly, except that 2.5 g of KCNS was used, and 8 drops of 0.1 N KOH (containing 0.0022 g of alkali) were necessary at this concentration to bring the solution to pH 4.6.

Five grams of casein was added for each determination. Much the same procedure was followed as was used with sodium cymenesulphonate solutions, although in some cases the time required for the solution to reach equilibrium was a day or two longer. The results are shown in table 1.

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4 The authors are indebted to P. D. Watson, of the Division of Dairy Research Laboratories, for making some of the analyses for phosphorus and nitrogen.
SOLUBILITY OF CASEIN IN SODIUM BENZENESULPHONATE SOLUTIONS

The sodium benzenesulphonate used was a commercial product of high purity. It was found that 5- and 10-percent solutions were the most practical to use.

The 5-percent solution was prepared by dissolving 2.5 g of sodium benzenesulphonate in water and adding 2 cc of phenol solution (containing 0.10 g). About 0.25 cc of 0.2 N NaOH solution (containing 0.002 g) was required to give a pH value of 4.6. The volume was made up to 50 cc, and 5 g of casein was added as before. The 10-percent solution was prepared in the same way, except that 5 g of salt was used and about 0.5 cc of 0.2 N NaOH solution (containing 0.004 g) was added to give the required pH value. Equilibrium was attained normally at the customary three temperatures. These results are also given in table 1.

On plotting the solubility values for casein in the 5-percent solution it was noted that the solubility curve was practically a straight line. For the 10-percent solution, there was a slight curve in the line. It was thought advisable to check again very carefully the points on the curve; the values obtained by the recheck showed that the points were correct.

FRACTIONATION OF ACID-PRECIPITATED CASEIN

EARLIER INVESTIGATIONS

Several investigators have believed that acid casein as ordinarily precipitated from milk is not a homogeneous compound but a mixture of closely related proteins or fractions.

According to Linderstrøm-Lang (20), in 1885, Danilewsky and Rodenhausen extracted a substance from casein by boiling it with a 50-percent alcoholic solution and then by means of sulphur determinations they sought to prove a difference between the extract and the residue. In 1918, Osborne and Wakeman (26) treated casein with a 92-percent alcoholic solution and obtained a fraction which differed considerably from casein in phosphorus content as well as in other particulars.

In 1925, Linderstrøm-Lang and Kodama (21) in determining the solubility of casein in hydrochloric acid in the presence of sodium chloride, observed that the solubility increased with the amount of casein added, which suggested that the protein was not a homogeneous material. They apparently proved by the phase rule that there were present more than the four components, i. e., casein, water, sodium chloride, and hydrochloric acid. They succeeded in separating casein into two fractions. The possibility of decomposition by the strong acid at the temperature used was ruled out by formol titrations and by constant solubility values day after day.

Svedberg, Carpenter, and Carpenter (32, 33) found by the Svedberg sedimentation-velocity method and the sedimentation-equilibrium method that Hammarsten casein appeared to contain several proteins of different molecular weight. By extracting casein with alcoholic HCl, they obtained a definite fraction which gave a molecular weight of 375,256 ± 11,000. Carpenter and Hucker (5) also found, by treating casein with acid alcohol and by fractionating it with potassium oxalate, that apparently three proteins having molecular weights of
98,000, 188,000, and 375,000 could be obtained from casein. According to Carpenter, hydrolysis of casein takes place very slowly.

In 1927–29, Linderstrøm-Lang (20) fractionated casein by means of alcoholic HCl at 60° to 70° C. He claimed that phosphorus-nitrogen ratios were excellent bases of differentiation between fractions. For example, in one fraction he found a P/N ratio of 0.0337 and in another fraction a P/N ratio of 0.0490. He postulated that either the casein is very unstable and easily breaks down into its components or else it consists of different molecules which may be slowly separated from each other. He favored the latter view. He found the yields of fractions were not quantitative; the correct proportions were not known. His results were reproducible, although he stated that—naturally such an experiment cannot be reproduced with the same degree of accuracy as solubility determinations with crystalline substances. One cannot expect that a fractionation of this kind will be able to give quite accurately identical results each time it is conducted. There is the possibility that milk from different cows can vary in its composition.

Linderstrøm-Lang concluded that casein is a mixture of colloidal molecules that form a coprecipitation system, and thus to a certain extent seem to act as a unit. He thought a study of fractionation should contribute much to the knowledge of the properties and reactions of casein.

Sørensen (30) made measurements of the solubility of proteins in salt solutions and found that solubility would increase with added amounts of proteins, thus indicating the presence of more than one solid phase. He assumed, however, that the proteins were not primarily mixtures of fractions, but "a complex combination of several components held together by residual valences." He presumed this compound to be easily dissociated into its components by the weak solvents employed, but he did not state whether the dissociation increased with time. If it did, this would be a powerful argument in favor of the dissociation theory.

Cherbuliez and Schneider (6) in 1932 concluded that casein was a mixture of inconstant composition. They used ammonium chloride as the fractionating agent. They stated that casein is very sensitive to the action of water when the pH value of the solution is above 7, but that it is probably perfectly stable in weakly acid solutions as at pH 4.6. These workers found that the components possessed different solubilities in 70-percent pyridine solutions and varied in their rennet-coagulation characteristics.

Berggren (3) separated casein by HCl and lactic acid and a dialysis procedure. She found P/N ratios of 0.0522 and 0.0558 and 0.0444 and 0.0215.

In 1934, Groh (15) fractionally precipitated casein from its 40-percent urea solution and its solution in molten phenol by adding absolute ethanol, and from a 70-percent alcoholic ammonium hydroxide solution by adding dilute HCl. The initial fractions were further divided into subfractions. No evidences of hydrolysis were found.

Giza (11) separated casein by Linderstrøm-Lang's method, and found that fractions differed in phosphorus and in amino acid content as shown by spectrophotometric analysis. He also observed variations in the buffer capacities.
Jirgensons (17, 18) fractionated Hammarsten casein, and precipitated the components by various concentrations of methanol, ethanol, and propanol. Results were plotted. Apparently a uniform protein will give a curve with a single maximum and minimum point, whereas a nonuniform protein ordinarily does not.

In 1935, Carpenter (4) postulated that the reversible dissociation of casein would take place at neutrality by dilution with a large volume of buffer solution.

Suppée (2, p. 52) brought about fractionation of casein by successive sedimentation and washing of fine casein. He found one fraction in particular to be very high in phosphorus.

In 1936, Pedersen (27) concluded that casein would dissociate during sedimentation and produce smaller molecules.

**EXPERIMENTAL PROCEDURE AND EVIDENCES OF FRACTIONATION**

As explained before, the possibility of selective solubility, or fractionation effect, was considered in the course of the solubility determinations. Primary evidence of fractionation was afforded if the refractometer showed an increased index on the addition of further amounts of casein after equilibrium had been attained with the quantity already present. This result, if obtained, was checked by further tests such as phosphorus and nitrogen determinations, formaldehyde absorptions, and solubilities in pyridine solution.

Some hint of fractionation was noticed in an early experiment with sodium cymenesulphonate solution. Seven and five-tenths grams of casein was the starting quantity, and an equilibrium refractive index of 1.3972-3 was first reached. On the addition of a second increment of 5 g of casein, the index jumped to 1.4030 in 1½ hours, indicating the extraction of material from the added casein. As is well known, if the solid phase is homogeneous, the solubility value at equilibrium will not be enhanced by the addition of further amounts of the solid phase. On the other hand, if the solid phase is heterogeneous, the quantities dissolved by the solvent may increase in proportion to the further increments of the material added, provided the solubility of the most soluble fraction has not been exceeded.

The same phenomenon (marked increase of the refractive index on addition of further quantities of casein) was noticed both with a 7.5-percent and a 5-percent sodium cymenesulphonate solution at 45° C., and a 2.5-percent potassium thiocyanate solution at 30°. In the latter case, fractionation was not confirmed by the P/N ratio determinations.

**FRACTIONATION WITH 5-PERCENT SODIUM BENZENESULPHONATE SOLUTION**

Grove City Casein

A 5-percent solution of sodium benzenesulphonate was examined for possible fractionating tendencies. A solution containing 2.5 g of the salt, 0.25 cc of 0.2 N NaOH solution (containing 0.002 g), and 2 cc of phenol solution was made up to a volume of 50 cc. This solution had a pH value of 4.6, and 45° C. was the temperature of the run. Two grams of Grove City casein was added at first. After 4 days the refractive index became stabilized. Two grams of casein was again added, and after 2 days the refractive index showed a second equi-
librium point, thus indicating the possibility of fractionation. One gram more of casein was added at this point, but shortly thereafter the thermostatic control of the incubator failed to function. The solution was then removed from the incubator, as it was thought that enough of the fractions were present to show tests. The solution was filtered through canvas, the residue washed free of sodium benzenesulphonate, as shown by the silver nitrate test, and finally washed with ethanol and ether. The substance dissolved in the filtrate was precipitated with 10-percent acetic acid, filtered, and washed in the same manner. The residue and the soluble material were dried in a vacuum desiccator over calcium chloride. In later experiments, the residue and the soluble material were more effectively dehydrated by washing with acetone and ether, and then drying in the oven at 75° to 80°.

Cherbuliez and Schneider (6) recommended a 70-percent aqueous pyridine solution as a reagent for distinguishing, on a basis of varying solubilities, different casein fractions. They also made use of the rennet-coagulation test to show the same distinction. These tests were applied to the residue and the soluble material obtained in this experiment. One-tenth gram of the residue fraction was added to 20 cc of a 70-percent aqueous pyridine solution. After 24 hours, the residue was only partly dissolved. The fraction obtained from the filtrate was similarly tested and it was found to be completely soluble in only a few hours. The rennet test was also applied to each fraction. To 0.1 g of each fraction was added 0.12 g of calcium hydroxide, 3.2 cc of water, and 0.16 cc of rennet solution. No coagulation was observed in the solution of either fraction.

The results of fractionating different kinds of casein with different agents are shown in Table 2.

Table 2.—Results of fractionating different kinds of casein with different agents 1

<table>
<thead>
<tr>
<th>SODIUM BENZENESULPHONATE (5 PERCENT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kind of casein and fraction</td>
</tr>
<tr>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Grove City casein:</td>
</tr>
<tr>
<td>Insoluble residue</td>
</tr>
<tr>
<td>Fraction from filtrate...</td>
</tr>
<tr>
<td>Whittier casein:</td>
</tr>
<tr>
<td>Insoluble residue</td>
</tr>
<tr>
<td>Fraction from filtrate...</td>
</tr>
<tr>
<td>Hammarsten casein:</td>
</tr>
<tr>
<td>Insoluble residue</td>
</tr>
<tr>
<td>Fraction from filtrate...</td>
</tr>
<tr>
<td>Whittier casein:</td>
</tr>
<tr>
<td>Insoluble residue</td>
</tr>
<tr>
<td>Fraction from filtrate...</td>
</tr>
<tr>
<td>Hammarsten casein:</td>
</tr>
<tr>
<td>Insoluble residue</td>
</tr>
<tr>
<td>Fraction from filtrate...</td>
</tr>
<tr>
<td>SODIUM CYMENESULPHONATE (5 PERCENT)</td>
</tr>
</tbody>
</table>

1 Results listed were calculated on a dry, ashless basis.
Phosphorus and nitrogen analyses were made on the two fractions. The results are shown in table 2.

The phosphorus-nitrogen ratios thus offer strong evidence that the sodium benzenesulphonate solution has fractionated the casein into components of widely differing phosphorus percentages. This evidence was confirmed refractometrically and by pyridine solubility tests.

**Whittier Casein**

The preceding experiment was repeated in the same way except that 5 g of Whittier casein was used. After completion of the run, the residue was washed with water, acetone, and ether. Acetone was substituted for the ethanol used in the preceding experiment because of the possibility that some alcohol-soluble component might be extracted, and also because of the statement by Cherbuliez and Schneider (6) that acetone helps to produce a powdery product that does not stick to the filter. The fraction in the filtrate was precipitated with 10-percent acetic acid at 35° to 40° C. The precipitate at this temperature is granular and easily filtered. At lower temperatures, a fine product is produced which clogs the filter. At higher temperatures a gummy substance is formed which cannot be washed effectively. The fraction from the filtrate was washed in the same way as the residue, and then both fractions were dried overnight in the oven at 75° to 78°.

Phosphorus and nitrogen determinations were made on these fractions. Nitrogen values were obtained by the Kjeldahl-Gunning method. Blanks were run to determine the purity of the reagents. The factor used for converting nitrogen to protein was 6.38. Phosphorus was estimated by a method for analyzing phosphorus in casein previously adapted by the authors from the methods of Meigs, Blatherwick, and Cary (25) and Fales (10, pp. 213–228).

The analytical results of this second fractionation are shown in table 2. Some fractionation is thus indicated, although not as much as when Grove City casein was used, even though this experiment was run for a shorter time.

Different caseins vary in their proportions of fractions, according to the writers’ observations and also those of Linderstrøm-Lang (20).

**Fractionation with 10-percent sodium benzenesulphonate solution**

**Whittier Casein**

Twenty-five grams of sodium benzenesulphonate, 2.5 cc of 0.2 N NaOH solution, and 0.5 g of phenol were made up to 250 cc of solution. This had a pH value of 4.6. Twenty-five grams of Whittier casein was added, the solution was placed in an incubator held at 45° C., and the mixture was shaken at intervals. After 6 days, equilibrium was proved by the constancy of the refractive index at 1.3602. The residue was filtered with suction and through canvas on a Büchner funnel. Because of the larger quantities of casein used, filtration by this method was extremely slow, requiring fully 3 weeks. The fraction in the filtrate was precipitated with 10-percent acetic acid solution. Both fractions were washed with water, acetone, and ether, and dried in the oven at 75° to 80°.
The residue and the fraction precipitated from the filtrate were analyzed for nitrogen by the Kjeldahl-Gunning method, and for phosphorus by the method described on page 135. The values are presented in table 2.

Some fractionation was again obtained. However, it is evident that partial qualitative but not complete or quantitative fractionation may be achieved by the use of the solvents employed. As shown later, fractionation was more definitely evidenced by the formaldehyde absorption and pyridine solubility tests.

Formaldehyde absorption.—Additional tests for fractionation were sought. It was postulated that the two fractions might vary in their affinity or absorbing capacity for formaldehyde. This would imply a difference in the number of free amino groups in each fraction, since formaldehyde is generally considered to react with the casein molecule as follows:

\[ \text{RNH}_2 + \text{HCHO} \rightarrow \text{RN}\text{CH}_2\text{H}_2\text{O} \]

A test of the applicability of this reaction was carried out by exposing the fractions in a desiccator to the vapors of a 37-percent formalin solution. The fractions increased in weight at different rates, but since water vapor was absorbed from the formalin solution along with formaldehyde, a second variable was introduced which affected the reliability of the results.

Later, experiments were run in which the formalin solution was replaced by solid paraformaldehyde. This eliminated the water variable. The paraformaldehyde gradually gave off formaldehyde vapor which was absorbed.

The fractions were screened in order to aid in exposing equal surface areas. Those portions that passed through a No. 40 screen and were retained on a No. 60 screen, were taken for the absorption tests. Two samples of each fraction were weighed into weighing bottles and run simultaneously in the same desiccator. This was kept in the 30° C. incubator. At intervals, the dishes were removed from the desiccator, covered, and the time of covering was taken. The bottles were weighed after being allowed to come to room temperature. After each weighing, the solids in the dishes were mixed by gently shifting them back and forth in order to expose new surfaces to the action of formaldehyde. The absorption proceeded very rapidly for the first day or two, after which it advanced more slowly. Intermediate values did not always check closely, probably because of changes in exposed surfaces; but as the run proceeded, better checks were obtained until final values, after 13 days, were in close agreement. The average of checks for these final values were: For the residue, 2.4 mg; for the fraction from the filtrate, 1.2 mg. Thus the first fraction absorbs twice as much formaldehyde as the second, strongly indicating that there are more free amino groups present in the residue than in the filtrate fraction. This test furnishes an additional proof of fractionation.

Pyridine solubility.—Two-hundredths of a gram each of the residue and the fraction from the filtrate were placed in stoppered test tubes, each tube containing 4 cc of 70-percent aqueous pyridine solution.

\footnote{It was first planned to use the formaldehyde-absorption test as a dynamic one, but intermediate rates of reaction did not check because of differences in particle size. Accordingly, only final weights of formaldehyde absorbed by the fractions were recorded.}
The tubes were warmed to 40° to 45° C. for 1 hour, and then held at room temperature for 24 hours, during which time they were shaken frequently. The residue was only slightly soluble in the pyridine solution. The fraction from the filtrate was entirely soluble.

**Hammarsten Casein**

The preceding experiment was repeated with a different sample of casein—i.e., Hammarsten—to observe how caseins from different sources compared, and to check further the fractionating characteristics of sodium benzenesulphonate. The same kinds and quantities of materials were employed except 25 g of Hammarsten casein was used instead of Whittier casein. The conditions of the determination at 45° C. were practically the same.

Because of the extremely slow filtration resulting from the use of a Büchner funnel and vacuum filtration, a pressure filter was constructed and employed thereafter in this and the subsequent larger-scale fractionations. The pressure filter aided greatly in speeding up the time of filtration, only 9 days being required to complete this fractionation as compared to 3 weeks for the preceding one. The fractions obtained were treated by the usual procedure.

The results of the phosphorus and nitrogen analyses are shown in table 2. Extensive fractionation is shown by the difference in the P/N ratios. Hammarsten casein is obviously fractionated to a greater degree than the Whittier casein by the sodium benzenesulphonate solution.

Not enough material was available from this particular fractionation for additional tests. Accordingly, a second experiment was carried out to obtain additional quantities of the components. Considerably larger quantities were used this time; i.e., 50 g of sodium benzenesulphonate, 5 cc of 0.2 N NaOH, and 1.0 g of phenol were made up to 500 cc, and 50 g of Hammarsten casein was used. The action of the solvent on the casein was continued for 6 days.

The filtration of the larger quantity of residue presented much difficulty, even with a pressure filter. Parts of the mixture were finally removed and filtered by using two Büchner funnels to speed up the process. This involved some loss of material. The filtration required over 3 weeks. The fractions obtained were treated as before. When the yields were weighed, the residue was 29.40 g and the fraction from the filtrate was 1.50 g.

**Formaldehyde absorption.**—Screened 20-mg samples of these fractions were placed in a desiccator, over solid paraformaldehyde, and allowed to absorb formaldehyde vapors. Final values were recorded after 12 days, although absorption was still proceeding at a very slow rate. Ordinarily, 12 or 13 days is sufficient time to attain good checks and establish differences between the fractions. The final values were: For the residue, 4.7 mg; for the fraction from the filtrate, 3.9 mg.

**Pyridine solubility.**—Two-hundredths of a gram of each fraction was taken for the test and the same method was applied as outlined previously. The residue was only slightly soluble in 4 cc of 70-percent pyridine solution, whereas the fraction from the filtrate was wholly soluble.

The pyridine solubility test probably shows differences in the acidic groups of the fractions, whereas the formaldehyde absorption test very likely indicates differences in the free amino groups.
FRACTIONATION WITH 5-PERCENT SOLUTIONS OF SODIUM CYMENESULPHONATE
HAMMARSTEN CASEIN

Recrystallized sodium cymenesulphonate was used for this experiment. Two and forty-seven hundredths grams of salt was weighed out, cymenesulphonic acid solution equivalent to 0.03 g was added (to give the usual pH value), 2 cc of phenol was added and the solution was made up to 50 cc. Small portions of Whittier casein were poured in from time to time and the subsequent establishment of successive constant refractometric readings furnished some indication of fractionation.

The mixture was filtered, and the fractions were prepared in the regular way.

The pyridine and rennet tests were applied to these components as in preceding experiments. The fraction precipitated from the filtrate was largely dissolved in the 70-percent pyridine solution; the residue was not so soluble. The rennet test was negative in both cases. There was not enough material left for phosphorus and nitrogen determinations. Accordingly, another fractionation was attempted on a larger scale.

The solution was prepared in this way: 12.35 g of recrystallized sodium cymenesulphonate was mixed with 0.9 cc of cymenesulphonic acid solution (containing 0.1376 g per cc), 0.5 g of phenol was added, and the solution was diluted to 250 cc. It was now at the usual pH value. A 25-g sample of Hammarsten casein was used for the experiment.

Fractionation was complete in 6 days, and the solution was placed in the pressure filter. Filtration proceeded rapidly, and was finished in 3 days. The fraction from the filtrate was precipitated and both fractions were washed and dried in the customary manner. Lead nitrate solution was used to test for completeness of washing. In this test any residual cymenesulphonate will come down in white flakes as the lead salt.

Nitrogen and phosphorus values are recorded in table 2. These values help to prove that 5-percent sodium cymenesulphonate solution is an effective fractionating agent since the P/N ratios show a wide divergence.

Formaldehyde absorption.—The residue showed a total increase of 4.3 mg, and the fraction from the filtrate, 1.7 mg. Strong confirmatory evidence of fractionation is hereby afforded.

Pyridine solubility.—The difference in solubility was not as marked in these fractions as in the fractions obtained by sodium benzenesulphonate solution. The fraction from the filtrate was evidently more soluble in the pyridine solution than the residue.

WHITTIER CASEIN

The experiment with Whittier casein was on a larger scale than the preceding ones. Forty-nine and four-tenths grams of recrystallized sodium cymenesulphonate solution was weighed out, 3.6 cc of cymenesulphonic acid solution (containing 0.1376 g per cc) was poured in to establish the correct acidity, 2 g of phenol was added, and the total mixture was diluted to 1,000 cc. One hundred grams of Whittier casein was used and the fractionation was carried on for 7 days at 45° C.
Considerable difficulty was experienced with the pressure filter in attempting to handle this large quantity of material, and some was later transferred in successive increments to a Büchner funnel. Some material was lost because of this and other difficulties. Precipitation, washing, and drying were carried out as with the Hammarsten casein. Yields were: Residue, 16.20 g; fraction from the filtrate, 36.90 g.

Phosphorus and nitrogen results are listed in table 2.

Fractionation is again indicated, but in a different manner from that in which it took place when Hammarsten casein was used.

**Formaldehyde absorption.**—Final weights were taken in 12 days, and were: Residue, 5.4 mg; fraction from the filtrate, 2.8 mg.

**Pyridine solubility.**—In this particular case it was very difficult to distinguish between fractions, although the residue appeared to be slightly more soluble than the fraction from the filtrate.

**DISCUSSION AND SUMMARY**

The question of possible hydrolysis of the protein is often raised in connection with fractionation work. However, under the experimental conditions used in this research, the chances of any hydrolytic decomposition are nearly negligible. No evidence of such decomposition (provided a suitable preservative was used) was obtained in the course of the experimentation. The attainment of equilibrium, as shown by constancy of the refractive index, was not dependent on time as would have been the case if hydrolysis were proceeding. The fractions in the various filtrates were always coagulable by acids. Even assuming the presence of any hydrolytic products, most of them would have been leached out by the washing liquids when the fractions were washed, and so would not affect the phosphorus-nitrogen ratios or other tests. Furthermore, the experiments were run at the isoelectric point of casein, a pH value of 4.6, the point at which there is the greatest stability and the least chance of decomposition.

The work of other investigators revealed no evidence of hydrolysis during fractionation even when much stronger reagents were used. (See Linderstrøm-Lang (20) and Kondo (19)). Soff (29) found no indications of hydrolysis even after long storage of casein that was dissolved in the sulphonate solutions.

**SOLUBILITY OF CASEIN**

From the solubility values tabulated in table 1, it will be seen (on the basis of the solubility determined at each of the three temperatures) that in almost all cases solubility increased with the temperature as a straight-line function. With a 10-percent solution of sodium benzenesulphonate, a slight curve was obtained. In the case of a concentrated solution like this, however, some minor deviation from an ideal solubility curve is not unexpected. Increased solvate formation with the sulphonate may be a factor. It will be observed that the determinations generally intercheck, i.e., they rise in a straight line and increase in definite increments between temperatures.

The solubility of casein (on the basis of 5 g to 50 cc of solution at a pH value of 4.6) in a 5-percent aqueous solution of sodium cymene-sulphonate at a constant temperature of 15°, 30°, and 45° C. has been
determined. For 7.50-percent solutions, values at 15° and 30° only could be obtained. The solubilities shown by these determinations were a straight-line function of the temperature.

In the same way, the solubility of casein in a 2.5-percent solution of potassium thiocyanate at 15°, 30°, and 45° C. has been established. For a 5-percent solution of potassium thiocyanate, only the solubility at 15° C. could be determined. For the 2.5-percent solution, the solubility increased with the temperature, and was plotted as a straight line.

The solubility of casein has also been determined in 5-percent and 10-percent sodium benzenesulphonate solutions at 15°, 30°, and 45° C. For the 5-percent solution, the solubility increased in a straight line in the same manner as with the other solvents; but for the 10-percent solution, the solubility showed a slightly curved line, as previously explained.

Potassium thiocyanate solutions were found, for the first time, to be solvents for casein.

The use of the formation of lead cymenesulphonate and silver benzenesulphonate as tests for completeness of washing was developed.

**FRACTIONATION OF CASEIN**

It has been shown for the first time that sodium benzenesulphonate and sodium cymenesulphonate solutions at a pH value of 4.6 will separate casein into at least two components. These components, or fractions, exhibited chemical differences in that their percentages of phosphorus varied; their formaldehyde-absorbing capacities differed (probably indicating unlike numbers of free amino groups); and their solubility values in pyridine solutions varied (evidently showing differences in acidic constituents).

Potassium thiocyanate solution was not a fractionating agent.

Salts of nearly neutral action, such as these sulphonates, should be of great general value for fractionation purposes since the possibility of hydrolyzing proteins is reduced to a minimum. Emphatic criticism of the use of strong acids and alkalies as fractionating reagents has been voiced by some protein chemists because of the likelihood that the molecules may be broken down by such drastic treatment. The use of the nearly neutral sulphonates should obviate any such contingency.

It appears from an inspection of table 2 that qualitative fractionation has been achieved with these agents, but not quantitative fractionation. Strong evidences of fractionation were supplied in the experiments with the Grove City casein and the Hammarsten casein, but when Whittier casein was used the indications of fractionation were not so pronounced. This may have been due to differences in the caseins themselves, or in the methods of preparation. Svedberg, et al. (32, 33) stated that the molecular weight and homogeneity of casein depended on the way in which it had been prepared. There is a possibility that partial pre-fractionation took place during the preparation of the Whittier casein as it was first washed with dilute HCl. (See Linderstrøm-Lang's procedure, p. 132.) The casein was also washed with water in tall cylinders for a considerable period of time, and some of the high-phosphorus fraction might have been separated by sedimentation. (See Supplee's method, p. 133.) Linderstrøm-
Lang (20) commented on the diverse ratios of the fractions in casein from different sources. Possibly, too, Sørensen's theory of the reversible dissociation of casein (30) might explain the lack of fractionation to uniform definite limits in all cases, since the degree of dissociation might vary with many factors involving the casein and reagent used. It will be observed that, when Whittier casein was fractionated with a 5-percent sodium benzenesulphonate solution, the P/N ratios showed an opposite trend from those obtained when fractionation was with a 10-percent solution. This may be due to differing selective solubilities of various concentrations of the salt; or if Sørensen's theory is correct, to the differing dissociating characteristics of the 5-percent and 10-percent solutions.

A new method of testing for differences in fractions has been developed which proves that the amounts of formaldehyde absorbed from paraformaldehyde by the components vary considerably. Results evidently indicate that the fractions are unlike in free amino group contents.

The pyridine solubility test has been employed as a third confirmatory method to further establish the fact of component separation. Differences in the solubility of the fractions are probably due to variations in the acidic groups present.

The refractive indices of the solutions also furnished indications of fractionation; the indices increased beyond previous equilibrium points on the addition of successive increments of casein.

The theory that casein is a mixture or a composite of loosely bound aggregates, rather than a compound, receives emphatic substantiation since a fractionation into constituents has been effected by the nearly neutral, nonhydrolyzing salts of organic acids. The fact that fractionation equilibrium was not dependent on time (as shown by the refractometer) lends evidence to Linderstrøm-Lang's casein-mixture theory rather than to Sørensen's casein-aggregate hypothesis. A further investigation of the fractions obtained should clarify even more the composition and structure of casein.

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