

BULLETIN No. 215.

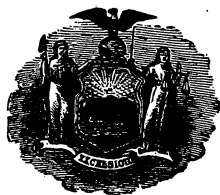
SEPTEMBER, 1902.

New York Agricultural Experiment Station.

GENEVA, N. Y.

METHODS FOR THE ESTIMATION OF THE PROTEO-
LYTIC COMPOUNDS CONTAINED IN
CHEESE AND MILK.

L. L. VAN SLYKE AND E. B. HART.



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METHODS FOR THE ESTIMATION OF THE PRO-
TEOLYTIC COMPOUNDS CONTAINED
IN CHEESE AND MILK.

L. L. VAN SLYKE AND E. B. HART.

INTRODUCTION.

When milk-casein and its salts, or paracasein in cheese and its salts, are acted upon by dilute acids or alkalis under certain conditions or by enzymes or by micro-organisms, the proteid bodies are split up, forming a variety of complex cleavage products. The extent and intensity of such proteolytic changes are measured by the proportions and kinds of the different compounds resulting from the decomposition. In the ripening process that takes place in cheese, we have an instance of extensive proteolysis in the case of the nitrogen compounds of the fresh cheese, due probably to the combined action of dilute acid, enzymes and micro-organisms. In our study of the causes that produce cheese-ripening and of the conditions that influence the process, we were, at the start, brought face to face with the difficulty involved in the lack of satisfactory methods for making quantitative separations and determinations of the products of cleavage formed. While we have well-elaborated methods for estimating the amount of casein and albumin in fresh milk, these are of little aid in studying the products of their decomposition. Little attention has been given to the development of methods that are applicable to the products occurring in this field. Serious difficulties are involved in such a study. First, we know at present only in an extremely meagre and vague way the chemical constitution of milk-casein and its allied compounds. As regards the constitution of the various compounds formed by

the proteolysis of casein, we are largely in the dark, except in the case of some of the simpler ones. In the second place, this field is difficult to study, because the quantities of individual compounds that we have to work with are usually very small. Under these conditions, methods of quantitative separation must, at best, be regarded as largely empirical and more or less tentative. Instead of estimating individual compounds, about which our chemical knowledge is complete, we are compelled, in a large degree, to estimate groups of compounds, the individual members of which we know, for the most part, incompletely, or not at all.

From the peptic digestion of casein, Chittenden has separated the proto- and deuterocaseoses,¹ and, from a tryptic digestion, a deuterocaseose and a peptone.² Alexander³ has separated heterocaseose from a peptic digestion of casein, but only in small quantities. Many investigators have observed paranuclein or pseudonuclein, the insoluble residue remaining from the peptic digestion of casein or paracasein. Emil Fischer and P. A. Lavene⁴ have obtained pyrrolidine- α -carbonic acid from a tryptic digestion of casein.

By hydrolysis of casein with hydrochloric acid, Cohn⁵ obtained l-parahydroxyphenyl- α -amidopropionic acid (tyrosine), l- α -amidoisobutylic acid (leucine), amidosuccinic (aspartic) acid, α -amidoglutaric (glutamic) acid, a pyridine derivative, and ammonia; by the same means, Emil Fischer⁶ has recently isolated, among the monoamido acids, in addition to products previously obtained by others, amidovaleric acid, phenyl- α -amidopropionic acid (phenylalanine), pyrrolidine- α -carbonic acid, and probably amido-acetic acid (glycocoll).

Among the crystallizable end-products which have been found in ripening cheese are the following: Tyrosine, leucine, histidine, α,ϵ -diamidocaproic acid (lysine), tetramethylenediamine (putrescine), pentamethylenediamine (cadaverine), lysatine, guanidine

¹ Studies in Physiol. Chem. Yale Univ., **2**: 156 (1884-5).

² Studies in Physiol. Chem. Yale Univ., **3**: 66 (1887-8).

³ *Zeit. f. Physiol. Chem.*, **25**: 411 (1898).

⁴ *Zeit. f. Physiol. Chem.*, **33**: 170 (1901).

⁵ *Ber. deut. Chem. Ges.*, **29**: 1785 (1896).

⁶ *Zeit. f. Physiol. Chem.*, **33**: 151 (1901).

probably, and ammonia. Most of these products have been recently reported by Winterstein and Thöny⁷ as being found in Emmenthaler cheese. Excepting cadaverine and guanidine, they had also been previously found, though not reported, in American cheddar cheese in this laboratory. It is very probable that other amido compounds will be found sooner or later, among the products formed by the tryptic, if not the peptic, digestion of milk-casein and of cheddar cheese.

In cheese we find, earlier or later, during the ripening process a series of compounds and groups of compounds, which, so far as we know at present, appear in something like the following order of succession: (1) Paracasein, (2) unsaturated paracasein lactate, (3) paranuclein (pseudonuclein), (4) caseoses (albumoses), (5) peptones, (6) amido-acid compounds, and (7) ammonia. After the early stages of ripening, we have present at the same time all these different compounds and groups.

We will consider methods for the separation and estimation of the proteolytic products found, first in cheese, and, second, in milk, following the general order indicated above. It may be well to say at the outset that, in dealing with the separation of nitrogenous bodies so complex in composition as those mentioned above and occurring in very variable quantities, we can, in the study of milk and cheese problems, hope, at present, only to approximate accurate quantitative results. While we have in the Nencki method a very accurate means of estimating ammonia, the methods used in separating peptones from amido compounds can not be relied upon to give us more than approximate results.

⁷ *Zeit. f. Physiol. Chem.*, **36**: 28 (1902).

I. METHODS FOR THE SEPARATION AND ESTIMATION OF THE NITROGEN COMPOUNDS OF CHEESE.

We will present our description and discussion of the methods used for the separation and estimation of the nitrogen compounds of cheese in the following order:

1. Obtaining sample.
2. Determination of total nitrogen in cheese.
3. Extraction of water-soluble products.
4. Determination of total water-soluble nitrogen.
5. Determination of nitrogen in the form of paranuclein.
6. Determination of nitrogen in the form of proteids coagulable by heat in neutral solution.
7. Determination of nitrogen in the form of caseoses (albumoses).
8. Determination of nitrogen in the form of amido-acid compounds.
9. Determination of nitrogen in the form of peptones.
10. Determination of nitrogen in the form of ammonia.
11. Determination of nitrogen in the form of unsaturated paracasein lactate.

I. OBTAINING SAMPLE OF CHEESE.

A sample of cheese is obtained for analysis by means of a cheese-trier, which enables one to secure a round plug of cheese about half an inch in diameter and four to six inches long. Four or five plugs are drawn, one within a short distance of the center of the cheese, one about an inch from the outer circumference, and the others at points equidistant between the two previous ones taken. Samples thus taken represent practically all different conditions existing in the cheese. After each plug of cheese is removed, about an inch of the end having the rind is cut off and the rest placed in a well-stoppered, large-mouthed sample-bottle. The end with the rind is dipped once or twice in melted paraffin and then carefully replaced in the cheese, being pushed in a little below the surface. After all the plugs have been taken and the ends properly replaced in the cheese,

some of the melted paraffin is poured over the surface to fill up and surround the depressions made by replacing the ends of the plugs. This treatment generally insures the exclusion of mould and prevents abnormal loss of moisture in the portions of cheese near the holes left by the removal of the cheese plugs. This is a matter of much importance, when one intends to keep the same cheese for one or two years for systematic examination.

When one has taken all the plugs of cheese needed, the analysis should not be long delayed. The cheese in the bottle is cut into small pieces with a spatula and stirred within the bottle, in order to mix the whole into as homogeneous a mass as possible.

2. DETERMINATION OF TOTAL NITROGEN IN CHEESE.

We weigh out 1 or 2 grams of the cheese, prepared as described above, for the determination of the total nitrogen and treat it according to the Kjeldahl-Gunning method, modified as follows: When the solution has become partially digested, we add a piece of copper sulphate about as large as an ordinary pea. Unless this is done, it will take a long time to convert the organic nitrogen completely into ammonia.

3. EXTRACTION OF WATER-SOLUBLE PRODUCTS.

In a porcelain mortar we thoroughly mix 25 grams of our cheese sample, prepared as indicated above, with about an equal bulk of clean quartz sand. This mixture is transferred to a 450 cc. Erlenmeyer flask, to which we add about 100 cc. of distilled water at a temperature of 50° C. The flask is then placed on a water bath or in some place where it can be kept at a temperature of 122° F. to 131° F. (50° C. to 55° C.), and is allowed to stand for half an hour, being vigorously shaken from time to time. The liquid portion is then decanted through a filter of absorbent cotton into a 500 cc. flask. The residue is again treated with 100 cc. of water, heated, agitated, and the liquid decanted as before. This process is repeated, until the filtrate after being cooled to room temperature, amounts to 500 cc., exclusive of the fat, which usually is present at the top of the liquid.

The cotton filter mentioned is made of two layers of absorbent cotton prepared as follows: In a glass funnel we place some absorbent cotton to the depth of about one inch, moisten this with water, in order to compact it, and then above this place another layer of cotton of the same thickness. Upon this we pour our portions of cheese extract. This kind of filter allows rapid filtration without the aid of a pump, and is as effective in every way as paper, which requires half a day or more for complete filtration of 500 cc. of extract. Several samples of cheese can be extracted at the same time. The upper layer of cotton holds all solid particles and can be returned to the flask for extraction with salt-solution.

The method of making a water-extract of cheese, as described above, insures the complete removal of all water-soluble nitrogen compounds present in the cheese without danger of coagulating any soluble proteids. The use of water at room temperatures is not, in our experience, equally effective in making a complete extraction of the water-soluble products. Under some conditions, as in the early stages of ripening cheese at low temperatures, small amounts of a body are extracted by water which is precipitated by heat in neutral solution. We are unable to say at present whether this body consists of acid salts of paracasein or of hetero-caseose, which are practically insoluble in water, or whether it is some other compound. The temperature 122° F. (50° C.) also has the advantage of arresting further peptic or tryptic action during the extraction. The use of acids in extracting cheese is to be avoided, since a small amount of acid will not only precipitate the soluble nuclein but may form salts with paracasein, which are somewhat soluble in a slightly acid solution; the amount of dissolved paracasein salts under such circumstances depends on the amount of acid used and the time of extraction.

4. DETERMINATION OF TOTAL WATER-SOLUBLE NITROGEN.

For the determination of the amount of total water-soluble nitrogen, we take 50 cc. of the water-extract, prepared as described above, equivalent to 2.5 grams of cheese, and treat it according to the Kjeldahl method for determining nitrogen.

5. DETERMINATION OF NITROGEN IN THE FORM OF PARANUCLEIN (PSEUDONUCLEIN).

To 100 cc. of the water-extract, equivalent to 5 grams of cheese, we add 5 cc. of a 1 per ct. solution of hydrochloric acid, and warm the mixture on the water-bath at 122° F. to 131° F. (50° C. to 55° C.), until complete separation takes place, as shown by a clear supernatant liquid. The precipitate is filtered, washed with water, and then, with the filter paper, treated by the Kjeldahl method to determine the amount of nitrogen. The nitrogen equals nitrogen present in the form of paranuclein (pseudonuclein).

In our early work we used 2 or 3 cc. of a saturated alum solution for this determination, for the reason that, in the separation of casein in milk, we had used this reagent successfully; but at the time we did not know the nature of the body we were precipitating from our water-solution of cheese. Later, when we had studied it and learned its character, we found, on comparing precipitations by use of alum and by hydrochloric acid, that alum gave high results, undoubtedly precipitating some caseoses. In 27 comparative trials with water-extracts of different cheeses, we found in the alum precipitate nitrogen varying from 0.2 to 0.337 per ct. of the cheese and averaging 0.269 per ct., while the nitrogen in the hydrochloric acid precipitate varied from 0.046 to 0.145 per ct. of the cheese and averaged 0.085 per ct. The nitrogen precipitated by alum in these 27 cases was from 2.1 to 5.5 times as much as that precipitated by hydrochloric acid, the average of all being 3.2. Since hydrochloric acid is known to precipitate paranuclein completely, we are justified in assuming that the alum precipitates other compounds, and this is confirmed by other work, showing that when alum is used as the first precipitant, we get smaller quantities of caseoses in the filtrate than we do when we use hydrochloric acid as the precipitant of paranuclein. Alum appears to resemble zinc sulphate as a precipitant of proteids.

Paranuclein (pseudonuclein) results from the breaking down of casein or paracasein and is always found in the water-extracts of ripening cheese, whether salted or unsalted. It may, perhaps,

be regarded more accurately as a residue and probably should not be counted as one of the products to be used in measuring the extent of cheese-ripening. This is undoubtedly the same body as Chittenden's dyspeptone,⁸ which he found as an insoluble residue in a pepsin-hydrochloric-acid digestion of casein.

6. DETERMINATION OF NITROGEN IN THE FORM OF PROTEIDS COAGULABLE BY HEAT IN NEUTRAL SOLUTION.

The filtrate from the preceding determination (5) is made neutral with dilute caustic potash, using phenolphthalein as an indicator. It is then heated at the temperature of boiling water, until any coagulum that forms settles completely, leaving a clear supernatant liquid. The precipitate is washed with water and its nitrogen determined by the Kjeldahl method. In our experience such a precipitate rarely occurs, except in the case of cheese ripened near freezing point. The nature of this body we have not yet investigated.

7. DETERMINATION OF NITROGEN IN THE FORM OF CASEOSES (ALBUMOSES).

The filtrate from the preceding determination (6) is treated with 1 cc. of 50 per ct. sulphuric acid, saturated with c. p. zinc sulphate and then warmed to about 158° F. (70° C.), until the caseoses separate completely and settle. The mixture is allowed to cool and is then filtered. If filtered hot, there will occur a further separation of caseoses in the filtrate on cooling. The precipitate is washed with a saturated solution of zinc sulphate made slightly acid with sulphuric acid. The nitrogen in the precipitate is determined by the Kjeldahl method.

For the determination of caseoses the use of ammonium sulphate was exclusively employed, until Bömer⁹ proposed the use of zinc sulphate, which possesses a distinct advantage in enabling one to determine nitrogen directly in the precipitate or filtrate. This method has been employed in the estimation of caseoses also by the Wisconsin Agricultural Experiment Station. In the present

⁸ Studies in Physiol. Chem. Yale Univ., 3:66 (1887-8).

⁹ *Zeit. f. Analyt. Chem.*, 5: 562 (1895).

state of our knowledge of this class of compounds, zinc sulphate must be regarded as the most available reagent for their quantitative separation.

8. DETERMINATION OF NITROGEN IN THE FORM OF AMIDO-ACID COMPOUNDS.

The amido-acid compounds are determined in the filtrate from the precipitation of peptones (9). For the removal of peptones three reagents have been commonly used—(1) Tannin and sodium chloride, (2) phosphotungstic acid with sulphuric acid, and (3) bromine with hydrochloric acid. After the removal of peptones, the filtrate contains amido-acid and ammonia compounds. After determining the amount of total nitrogen in this filtrate and then the amount of nitrogen present in the form of ammonia, as obtained in 10, p. 99, we subtract the amount of ammonia nitrogen from the combined amount of amido-acid and ammonia nitrogen and thus obtain the amount of amido-acid nitrogen. In the following section (9) we describe the methods involved in removing peptones by the different reagents, and the efficiency of each reagent.

9. DETERMINATION OF NITROGEN IN THE FORM OF PEPTONES.

(1) *By tannin and sodium chloride.*—We place 100 cc. of our water-extract of cheese in a 250 cc. graduated flask, add 1 gram of sodium chloride and a solution containing 12 per ct. of tannin, until one drop added to the clear supernatant liquid gives no further precipitate. We then dilute to the 250 cc. mark, shake, filter through a dry filter and determine the amount of nitrogen in 50 cc. of the filtrate by the Kjeldahl method; this gives us the amount of nitrogen in the form of amido-acid and ammonia compounds. The amount of nitrogen in the form of peptones is determined by difference, that is, by subtracting from the amount of total nitrogen in the water-extract the combined sum of the amounts of nitrogen found in 5, 6, 7, 8 and 10.

The combination of tannin and salt has been settled upon by us as the most satisfactory for the separation of casein-derived peptones from amido-acid compounds in milk and cheese

analysis, when, as is commonly the case, we have large amounts of amido-acid compounds relative to peptones. We have confirmed Schjerning's¹⁰ results showing that this reagent does not precipitate the monoamido-acid compounds, such as leucine, tyrosine, aspartic acid, glutamic acid and amidovaleric acid, nor does it precipitate histidine, arginine, lysine, cadaverine, putrescine, lysatine or ammonia. In our work the tannin-salt solution has nearly as great a precipitating power as phosphotungstic acid, precipitating 93.3 per ct. of the total nitrogen compounds present in a sample of fresh milk; in a study of ripened cheese, it precipitated the uncrystallizable end-products, caseoses and peptones, so completely that no further trouble was experienced in separating the crystallizable end-products.

It is well to record here the fact that, when precipitation of peptones with tannin-salt solution is attempted in a mineral acid solution, no precipitate occurs; it is only in neutral solution that more complete precipitation takes place.

The chief objection to the use of tannin-salt solution as a means of separating caseoses and peptones from amido-acid compounds and ammonia is that it is not a complete precipitant of peptones. Hence, when we use the reagent for this separation, we commonly leave some peptones to be estimated as amido-acid compounds, the amount of peptones thus being made smaller, and the amount of amido-acid compounds larger, than the quantity actually present. Under our discussion of the use of phosphotungstic acid as a reagent for separating these classes of nitrogen compounds, we will give for comparison some results secured by each of the two reagents.

(2) *By phosphotungstic acid with sulphuric acid.*—In a 250 cc. graduated flask we place 100 cc. of the water-extract of cheese, add 100 cc. of water and then 5 cc. of strong sulphuric acid. To this we add phosphotungstic acid of 30 per ct. strength until one drop gives no further precipitation in the clear supernatant liquid. We then dilute to the 250 cc. mark and filter through a dry filter. In 50 cc. or 100 cc. of this filtrate we determine the

¹⁰ *Zeit. f. Analyt. Chem.*, **39**: 545 (1900).

amount of nitrogen by the Kjeldahl method and then the amount of peptones is obtained by difference.

Phosphotungstic acid has come into very general use in this country and in Europe as a means of separating peptones from amido-acid compounds in work with cheese and milk. The work of Stutzer¹¹ and of Bondzynski¹² agrees in showing that phosphotungstic acid is a complete precipitant of casein, caseoses and peptones, while in their experience it does not precipitate the amido-acid compounds or ammonia. Freudenreich and Jensen in all their work, even of recent date, have used this reagent in the cold as a means of separating peptones from amido-acid compounds. Babcock, Russell and Vivian in this country have used it, as well as tannin, designating the different precipitates as "peptones by phosphotungstic acid" and "peptones by tannin."

In the introductory portion of this paper, when mentioning different cleavage products of casein, we included among them the hexon bases, viz: arginine, histidine, and lysine, and, in addition, certain compounds resulting from their cleavage, such as putrescine and cadaverine, and also pyrrolidine- α -carbonic acid, all of which are precipitated by phosphotungstic acid. While all these products have not been separated from ripening cheese, it is probable that they will be sooner or later. We shall soon publish results of work done in this laboratory showing in normal ripening cheese the presence of histidine, lysine, putrescine (derived from arginine)¹³ and Siegfried's lysatine, which is also precipitable by phosphotungstic acid. The quantities of these bases that can be derived from casein are not to be neglected, since, for example, in a hydrochloric-acid cleavage 15.4 per ct. of the nitrogen of the products splits off into the hexon bases. Bondzynski has used phosphotungstic acid in hot solution with arginine and finds the precipitate soluble when hot, separating out on cooling, and this statement we can confirm, the solution, however, being complete only on boiling. In the case of lysine, histidine, and putrescine, the phosphotungstic acid pre-

¹¹ *Zeit. f. Analyt. Chem.*, **35**: 493 (1896).

¹² *Landw. Jahrbuch der Schweiz* (1894).

¹³ A. Ellinger, *Ber. deut. chem. Ges.*, **31**, III: 383 (1898).

cipitate fails to redissolve completely at the temperature of the water-bath or on boiling. This behavior renders worthless the use of phosphotungstic acid as a reagent for the separation of peptones from those amido-acid compounds that are precipitated by it.

We have seen that tannin-salt solution fails to precipitate peptones completely and that phosphotungstic acid precipitates, in addition to peptones, some amido-acid compounds. When, therefore, we use these two reagents in precipitating solutions that contain both peptones and amido-acid compounds, as in the case of normal ripening cheese, we should expect to find the amount of nitrogen compounds left in the filtrate less with phosphotungstic acid than with tannin. This is found invariably to be the case. Vivian¹⁴ has published some results obtained with nine different cheeses which illustrate this point. We give his figures in the table following:

TABLE I.—COMPARISON OF PHOSPHOTUNGSTIC ACID AND TANNIN-SALT SOLUTION IN PRECIPITATING THE NITROGEN COMPOUNDS CONTAINED IN WATER-EXTRACTS OF CHEESE.

No. of sample.....	Percentage of nitrogen in cheese found in filtrates after precipitation by—									Ave.
	1	2	3	4	5	6	7	8	9	
Phosphotungstic acid	1.08	1.00	0.82	0.63	0.67	1.69	0.95	1.10	1.22	1.02
Tannin-salt solution.	1.54	1.26	1.12	0.65	1.16	1.87	1.08	1.36	1.42	1.27

In connection with the above results, nothing was stated in regard to the history of the cheeses used in the work. We have been able to study the action of phosphotungstic acid and tannin-salt solution in connection with samples of cheese that were under known well-controlled conditions, enabling us to know something of the general character of the proteolytic changes taking place. We placed 25 grams of cheese-curd in each of several Erlenmeyer flasks, added 50 cc. of water and sterilized the contents by heat. We then added .5 gram of concentrated lactic acid, in order to convert the paracasein into unsaturated para-

¹⁴ Ann. Rept. Wis. Exp. Sta., 16: 171 (1899).

casein lactate.¹⁵ To some of the flasks thus prepared we added sterilized pepsin, and to others sterilized rennet-extract. For this purpose the enzymes were prepared as follows: We dissolved 600 milligrams of Parke, Davis & Co.'s aseptic pepsin in 25 cc. of water, added 0.5 per ct. of formalin (containing 40 per ct. of formaldehyde), and let the solution stand until bacteriological examinations, made by Mr. H. A. Harding, showed the absence of living organisms. The mixture was then diluted to 100 cc. with water, and to each flask containing cheese we added 10 cc. of this sterilized pepsin solution. One thousand parts of the mixture in each flask thus contained one part of pepsin.

In preparing the sterilized rennet solution, we diluted 5 cc. of Hansen's rennet-extract to 25 cc. with water, added .5 per ct. of formalin, proved the completeness of sterilization by bacteriological examinations, diluted to 100 cc. and added 10 cc. of this preparation to each flask containing cheese.

The flasks thus prepared were kept at 60° F. (15.5° C.). The analytical results given below were obtained at the end of two and four weeks. In this work we had only the enzymes of pepsin and of rennet-pepsin acting upon our proteid. Under these conditions, especially in the given length of time, we should not expect the formation of any considerable amount of amido-acid compounds precipitable by phosphotungstic acid. The results obtained are given in the following table:

TABLE II.—COMPARISON OF PHOSPHOTUNGSTIC ACID AND TANNIN-SALT SOLUTION IN PRECIPITATING THE NITROGEN COMPOUNDS FORMED BY PEPTIC DIGESTION OF CHEESE.

No. of sample	Percentage of nitrogen in cheese found in filtrate after precipitation by—								Ave.
	1	2	3	4	5	6	7	8	
Phosphotungstic acid...	0.10	0.10	0.150	0.175	0.10	0.08	0.16	0.16	0.128
Tannin-salt solution ...	0.45	0.44	0.645	0.685	0.48	0.46	0.73	0.70	0.574

In every case except one the amount of nitrogen not precipitated by tannin-salt solution was more than four times the amount

¹⁵ N. Y. Agr. Exp. Sta. Bul. No. 214:67 (1902).

obtained by phosphotungstic acid. No ammonia was present in any case. In view of these widely differing results in relation to the amounts of amido-acid compounds found, the questions may be asked: Which reagent more nearly represents the amount of amido compounds actually present? Does the tannin-salt reagent fail to precipitate the peptones completely, thus allowing the unprecipitated peptones to be counted among the amido-acid compounds? Or does the amount of nitrogen in the filtrate in case of this reagent really represent amido-acid compounds? Does the phosphotungstic acid precipitate some of the amido-acid compounds simultaneously with the peptones, thus cutting out a part of the amido-acid compounds and counting that part among the peptones? Or is there in the cheese only the small amount of end-products indicated by the action of the phosphotungstic acid?

While we did not by individual isolation determine to what extent amido compounds were present, we are justified in believing that such compounds were not yet present in the cheese in any appreciable degree; and, hence, the figures obtained with phosphotungstic acid are much nearer the truth than those obtained by tannin-salt precipitation. We will state our reasons for this belief. The amido compounds precipitated by phosphotungstic acid are known to be chiefly the diamido compounds, while the monoamido compounds are precipitated little, if at all. Hence, the compounds found in the filtrate of a phosphotungstic acid precipitation are mainly monoamido compounds. Now let us assume temporarily that the amounts of nitrogen in the tannin-salt filtrate, given in the table above, represents the total amido compounds, free from peptones; then, since the monoamido compounds are represented by the amounts of nitrogen obtained in the phosphotungstic acid filtrate, the difference between the two sets of figures, that is, those obtained by tannin-salt solution and those obtained by phosphotungstic acid, represents the amount of diamido compounds. Taking the average of the results given in the table above, the nitrogen of the total amido compounds is 0.574 per ct. of the cheese, while the nitrogen of the monoamido compounds is 0.128 per ct. of the cheese, thus leaving the

difference as the nitrogen of the diamido compounds equivalent to 0.446 per ct. of the cheese. According to these figures, the monoamido compounds constitute about 22 per ct. of the entire amount of amido bodies, while the remainder, 78 per ct., represents largely diamido compounds. In this case, the ratio of monoamido to diamido compounds is as 1 to 3.5. Keeping these data in mind, we will call attention to some work done by Hart¹⁶ in studying the cleavage end-products formed by the action of hydrochloric acid on casein. He found that the diamido compounds formed less than 20 per ct. of the total amido compounds, so that the ratio of monoamido to diamido compounds was as 1 to 0.25 or less; in other words, the monoamido compounds were greatly in excess of the diamido compounds, or just the reverse of what we find to be the case in the results embodied in the table above, based on the assumption that the nitrogen in the tannin-salt filtrate represents the total amido compounds and nothing more. The most obvious and rational explanation of this discrepancy, observed in the ratio of monoamido to diamido compounds, is that it is wrong to assume that the tannin-salt filtrate contains only amido compounds and not any peptones. Withdrawing that assumption, then, and allowing that the nitrogen in the tannin-salt filtrate represents some peptones as well as the amido compounds, how can we tell in this particular case the true amount of amido compounds in the cheese? Unquestionably, the results with phosphotungstic acid more nearly represent the truth in regard to the amido compounds, because, under the conditions of the experiment, we should expect very small amounts of amido compounds, if any; and, in this particular case, the amounts are so small as practically to indicate the absence of amido bodies altogether. From this it may be seen that it is possible for the tannin-salt reagent to give results that are decidedly misleading.

(3) *By bromine with hydrochloric acid.*—To the filtrate from the zinc sulphate precipitate in 7, we add 2 or 3 drops of strong hydrochloric acid and then bromine until the liquid becomes

¹⁶ *Zeit. f. Physiol. Chem.*, **33:247** (1901).

saturated and there remains after vigorous agitation an undissolved globule of bromine amounting to 0.5 cc. to 1 cc. This is allowed to stand over night. The precipitate is then filtered and washed with bromine-saturated water. The nitrogen in the precipitate is then determined by the Kjeldahl method and is called nitrogen in the form of peptones, the filtrate containing the amido-acid compounds and ammonia.

The use of chlorine by Rideal and Stewart¹⁷ in precipitating proteids suggested to Allen and Searle¹⁸ the use of bromine. They reported that bromine quantitatively precipitates the products formed by the peptic digestion of egg-albumin and they developed the method practically as given above. As applied to the separation of peptones from amido-acid compounds in cheese and milk, the method gives varying results, depending upon the age of the cheese or milk used.

In the case of our water-extracts made from cheese, nine months to a year old, crystalline bodies in noticeable quantities are precipitated by bromine along with peptones, due probably to the presence of tyrosine, giving the solution a turbid appearance and rendering filtration difficult. Schjerning¹⁹ has shown that tyrosine behaves in this manner with bromine water. This precipitate is partly retained on the filter paper and is estimated as peptone.

Schjerning has shown also that bromine does not completely precipitate milk proteids and their derived caseoses and peptones. Of the whole proteid, he obtained only 76.7 per ct. by bromine. In the case of milk, we have obtained results varying with the age of the milk. In perfectly fresh milk, when the amount of amido compounds must have been least, we obtained 91.3 per ct. of the entire milk proteids by bromine precipitation. In another case of fresh milk, we compared the precipitation of proteids by bromine and hydrochloric acid with that by tannin and sodium chloride and by solution of phosphotungstic acid and sulphuric acid, with the following results:

¹⁷ *Analyst* 22:228 (1897).

¹⁸ *Analyst* 22:259 (1897).

¹⁹ *Zeit. f. Analyt. Chem.* 39:545 (1900).

Precipitated by—	Percentage of total nitrogen.
Phosphotungstic and sulphuric acids.....	93.8
Tannin and sodium chloride.....	93.3
Bromine and hydrochloric acid.....	91.5

There is also a possible source of error in connection with the use of bromine in precipitating peptones, when the filtrate from the bromine precipitate is used directly for the determination of amido-acid compounds. We have found in the case of water-extracts from cheese over one year old that there is an actual loss of nitrogen when bromine is allowed to stand in contact with the water-extract. In the case of one cheese two years old, the cheese extract, consisting of caseoses, peptones, and amido-acid compounds, contained nitrogen equivalent to 2.74 per ct. of the cheese before adding bromine, while, after standing one hour in contact with bromine in hydrochloric acid solution, there remained only 1.52 per ct. of nitrogen; in other words, there had disappeared 44.6 per ct. of the nitrogen present before the addition of bromine. In cheese one year old we have found the loss varying from nothing in one case to over 5 per ct. in others. To show whether or not this loss came from the action of bromine on the caseoses or peptones, we removed the caseoses with zinc sulphate and, in another sample of cheese extract, we removed the caseoses and peptones with phosphotungstic acid, and the loss still occurred. By passing a current of air through the above extract in contact with bromine and then through potassium hydroxide and through sulphuric acid, these reagents were found free from nitrogen compounds, indicating that the lost nitrogen disappeared in the form of free nitrogen and not in the form of ammonia or nitrogen oxides. We cannot regard the method of determining the amount of peptones in cheese extracts by means of bromine as a reliable method; because, first, bromine precipitates small amounts of tyrosine and perhaps certain other similar compounds; second, it is not a complete precipitant of caseoses and peptones; and, third, its filtrate cannot be used for the determination of amido-acid compounds, especially in old cheeses, owing to the decomposing effect of bromine upon such

compounds, setting nitrogen free. In addition, bromine is a most disagreeable reagent to handle.

(4) *Comparative value of different reagents used in separating peptones and amido-acid compounds.*—We have now considered in some detail each of the three reagents most commonly used in separating peptones from amido-acid compounds, viz: (1) Tannin-salt solution, (2) phosphotungstic acid with sulphuric acid, and (3) bromine with hydrochloric acid. Tannin and salt solution fails as a perfect reagent for the separation, because it does not completely precipitate peptones, which results in making the quantitative results for amido-acid compounds too high and may indicate the presence of considerable quantities of amido compounds even when they are practically absent. Phosphotungstic acid, on the other hand, completely precipitates peptones, but also precipitates some of the amido-acid compounds that are present in cheese and milk, and the consequence is that the amount of amido-acid compounds found is too low. Bromine is open to both objections,—it fails to precipitate peptones completely and at the same time does precipitate some of the amido-acid compounds. While these two sources of error might tend to offset each other under certain conditions, we cannot depend upon such a method for reliable quantitative results.

In our judgment, it is desirable, for best results, to use phosphotungstic acid to separate peptones and amido-acid compounds, when the amount of amido-acid compounds is relatively small as compared with peptones or when they consist mostly of monoamido compounds. This condition occurs in the early stages of cheese ripening and persists longer in cheese cured at low temperatures; it occurs also in milk and cheese acted upon by pepsin enzymes, especially in the presence of chloroform.

Tannin-salt solution can be relied upon to give better results than phosphotungstic acid, when amido-acid compounds are present in proportions that are relatively large compared with peptones or when they consist largely of diamido compounds. The former condition prevails in normal cheese cured under usual conditions, especially after the first few weeks of curing.

10. DETERMINATION OF NITROGEN IN THE FORM OF AMMONIA.

We distil with magnesium oxide 100 cc. of the filtrate from the tannin-salt precipitation, passing the distillate into a standardized acid and titrating in the usual way. In our early work the cheese-mass itself, suspended in water, was used for distillation, giving slightly higher results than the method just described. The small increase is generally accounted for as coming from the proteids themselves present in the solution. Theoretically, it is true that when such bases are present as putrescine and cadaverine, they might distil with the ammonia. In one case where a large quantity of cheese was subjected directly to distillation with magnesium oxide and the distillate examined for these bases, none was found, the distillate consisting entirely of the ammonia salt. The high boiling points of cadaverine and putrescine and the consequent difficulty of distilling them with steam probably accounts for their absence in the distillate.

In our early work on the determination of ammonia in milk and cheese, we subjected to distillation with magnesium oxide and with barium carbonate many different amido compounds, in order to ascertain if any of these bodies, when pure, could split off basic nitrogen. While some of the products used in our work are not at all likely to be found in cheese or milk, we include them with the others in the table given below. The method was carried out as follows: We dissolved 1 gram of each amido body, or, if insoluble, suspended it, in 50 cc. of water, and, for distillation, used 10 cc. of this mixture diluted to 150 cc., adding magnesium oxide or barium carbonate and using ordinary atmospheric pressure.

TABLE III.—EFFECT OF DISTILLING AMIDO BODIES WITH MAGNESIUM OXIDE AND WITH BARIUM CARBONATE.

	Amount distilled over with magnesium oxide.	Amount distilled over with barium carbonate.
Acetamide	0	0
Allantoin	0	0
Arginine ²⁰	0	0
Aspartic acid	0	0
Creatin	0	0
Creatinin	0	0
Diphenylamine	0	0
Glutamic acid	0	0
Glycocoll	0	0
Histidine	0	0
Leucine	0	0
Lysine	0	0
Phenylenediamine	0	0
Trimethylamine	completely distilled.	completely distilled.
Tyrosine	0	0
Uric acid	0	0
Xanthin	0	0

We have also used the excellent Nencki apparatus, distilling under reduced pressure the filtrate from the tannin-salt precipitation. In comparative trials we have obtained no lower results than when we distil under ordinary atmospheric pressure.

II. DETERMINATION OF NITROGEN IN THE FORM OF UNSATURATED PARACASEIN LACTATE.

The residue insoluble in water is treated with several portions of a 5 per ct. solution of sodium chloride, the process being carried out as in preparing the water-extract in 3 above. The nitrogen in an aliquot part of the 500 cc. of this salt-extract is determined by the Kjeldahl method.

²⁰ *Zeit. f. Physiol. Chem.*, **34**:145 (1901).

II. METHODS FOR THE SEPARATION AND ESTIMATION OF THE NITROGEN COMPOUNDS OF MILK AND THEIR PROTEOLYTIC PRODUCTS.

We will briefly describe the methods used for the separation and estimation of the nitrogen compounds of milk and their proteolytic products in the following order:

1. Determination of total nitrogen in milk.
2. " " nitrogen in the form of casein.
3. " " nitrogen in the form of albumin and syntonin.
4. " " nitrogen in the form of caseoses.
5. " " nitrogen in the form of amido-acid compounds.
6. " " nitrogen in the form of peptones.
7. " " nitrogen in the form of ammonia.

I. DETERMINATION OF TOTAL NITROGEN IN MILK.

Weigh about 5 grams of milk and determine the nitrogen by the Kjeldahl method.

2. DETERMINATION OF CASEIN.

To about 10 grams of milk add 90 cc. of water at 104° F. to 108° F. (40° C. to 42° C.) and then 1.5 cc. of 10 per ct. acetic acid. Agitate and warm at the temperature given above until a flocculent precipitate separates, leaving a clear supernatant liquid. Filter, wash and treat by the Kjeldahl method for estimating nitrogen.

In fresh milk, 2 or 3 cc. of a saturated solution of alum may be used in place of acetic acid, usually with little higher results. But when the milk-casein has been proteolyzed to any extent, the use of alum is not permissible, since it precipitates caseoses in addition to casein.

The use of acetic or any other acid in precipitating casein in milk, whose casein has been digested in any degree, precipitates, in addition to casein, any paranuclein that is present. We have not yet succeeded in devising satisfactory methods for the separation of these compounds.

3. DETERMINATION OF NITROGEN IN THE FORM OF ALBUMIN AND SYNTONIN.

The filtrate from 2 is neutralized by caustic alkali, using phenolphthalein as indicator and is then heated at the temperature of boiling water until the precipitate completely separates and settles. The precipitate is then filtered, washed and treated by the Kjeldahl method.

4. DETERMINATION OF NITROGEN IN THE FORM OF CASEOSES.

The filtrate from 3 we heat to 158° F. (70° C.), add 1 cc. of 50 per ct. sulphuric acid and then c. p. zinc sulphate to saturation. Let stand at the temperature indicated until the caseoses completely separate and settle. Then cool the mixture, filter, wash with a saturated solution of zinc sulphate made slightly acid with sulphuric acid, and treat the precipitate by the Kjeldahl method.

5. DETERMINATION OF NITROGEN IN THE FORM OF AMIDO-ACID COMPOUNDS.

Treat about 50 grams of milk with a tannin-salt solution or with phosphotungstic acid as described in case of cheese, under 8, p. 89.

6. DETERMINATION OF NITROGEN IN THE FORM OF PEPTONES.

From the total nitrogen subtract that found in all forms other than that of peptones as indicated in case of cheese, under 9, pp. 89-98.

7. DETERMINATION OF NITROGEN IN THE FORM OF AMMONIA.

See under 10, methods for cheese, p. 99.

III. DETERMINATION OF CHLOROFORM.

When chloroform is used as an antiseptic in milk and cheese, it is very essential to know approximately the amount present in order that we may have a proper control of conditions. We have used the following method successfully: We place 5 grams of milk or cheese in a pressure bottle with about 100 cc. of alcohol and 5 grams of caustic potash. The bottle is then heated in an autoclave for half an hour at 230° F. (110° C.). The resulting chloride is determined by titration as for chlorine in sodium chloride.