NITROGEN DISTRIBUTION IN MILK

by

Benjamin Berck

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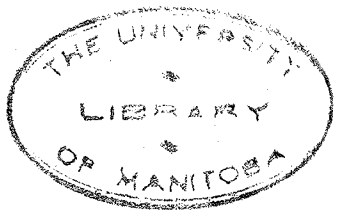

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INTRODUCTION

The high nutritional value of milk in the animal diet is abundantly established. In this regard, proteins have a predominant role. Proteins affect not only the nutritional and physiological relations of milk, but also the quality of milk and milk products resulting from pasteurization, homogenization and other processing treatments. Furthermore, as a result of disease and even mild inflammatory conditions in the animal, proteins will vary in amount and apparently, to some extent, also in kind. To get more meaningful information and to unravel, even in a limited fashion, the multi-sided role of proteins in given cases, we must inquire into the distribution trends of the proteins which are involved. Obviously, a measurement expressed simply as "% protein" would be inadequate for this purpose.

Protein distribution studies are required to clarify the interrelations between proteins and the other constituents (lipids, carbohydrates, minerals, etc.,) with which they are associated in living cells and in our foods. Thus, to promote a better understanding of the physicochemical properties of milk, quantitative knowledge of the distribution of milk proteins in relation to the other

constituents of milk is obviously a fundamental prerequisite. In this regard, available data are far from adequate, and, in recognition of this, the Department of Dairy Science, University of Manitoba, has undertaken a continuing investigation oriented around physico-chemical properties of milk, of which the study entitled "Nitrogen Distribution in Milk" is an integral part, and is reported here in some detail. The investigation, sponsored by the Division of Chemistry, Science Service, Canada Department of Agriculture, is outlined in the section that follows.

SCOPE OF THE INVESTIGATION

1. Definitions.
2. Abbreviations Adopted.
3. Comments on Estimation of Protein Content.

SCOPE OF THE INVESTIGATION

The thesis assignment entailed chemical measurement of the nitrogen (hereinafter called N,) content of milk derived from pure-bred dairy cattle in Manitoba, with specific reference to the following: Total N, casein N, proteose-peptone N, globulin N, albumin N, and non-protein N. N was measured by semi-micro Kjeldahl methods, after Shahani and Sommer (55), with some minor modifications by the writer, as shown later. Pooled samples of milk were obtained at regular intervals (four weeks apart) from twelve herds of pure-bred cows representing the following six breeds: Holstein-Friesian (3), Ayrshire (3), Jersey (3), Guernsey (1), Brown Swiss (1), and Red Poll (1). The fact that Holstein, Ayrshire and Jersey are currently the most popular breeds in Manitoba is reflected in their greater representation here. Concurrently, advantage was taken of the opportunity to do some exploratory fact-finding on the less popular Guernsey, Brown Swiss and Red Poll breeds which, under present circumstances, are each represented by only one herd. The sources of the milk samples analysed may be found in the Appendix, under A.N.1.

In addition to the N determinations mentioned above, the milk samples were examined by others in the Department of Dairy Science for a variety of other physico-chemical

properties, details of which will be published elsewhere. For purposes required in this thesis, data on specific gravity, total solids and butterfat content were made available to the writer. Both the N distribution phase and the other phases of the general problem were, and since it is a continuing investigation are, under the direction of Mr. August Reinart, Chemist-in-charge, Department of Dairy Science.

1. Definitions.

Nitrogen distribution in milk, as used in this study, refers to the N content in milk of (a) certain proteins (simple, conjugated and derived), and of (b) the aggregate of the non-protein fractions, comprising in part the following types of N-containing substances: protein degradation products, such as amino acids, ammonia, urea, creatine, creatinine, uric acid, choline, trimethylamine, and others; milk enzymes, vitamins, phospholipids, cerebrosides; ultramicro quantities of hormones, antibodies and possibly of toxins of certain bacteria. Elemental or atmospheric N, present in milk in minute traces (45), constitutes a third N category, but since it is excluded by the methods of measurement used herein, atmospheric N is not considered in this report.

N is reported here as mg. N / 100 ml. of milk at 60°F., except where comparisons with data of other inves-

tigators are presented in terms of % protein. In the latter regard, the conversion from "mg. N" to "% protein" is readily executed through use of the volume-specific gravity relationships of the particular sample, and of the equation, % Protein = % N x 6.38. The factor 6.38 seems to be the most prevalent in the literature on milk proteins, and accordingly is used here also. However, although "% protein" (i.e., % N x 6.38) is used herein, and for comparative purposes only, it is with the tacit understanding that a given protein conversion factor is not applicable to all milk samples. Two supporting facts may be cited. First, as revealed by electrochemical and ultracentrifuge studies, (19), casein, lactalbumin or lactoglobulin, consists of a group of allied but different caseins, albumins or globulins, each with slightly different physical, and presumably structural, properties. It is highly improbable that these interrelated proteins are present in all milk samples in a fixed and constant proportion, or that all have identical N contents. Secondly, different N factors may stem from the different analytical methods used, as pointed out by (23). The foregoing remarks emphasize that a protein factor is a convenient but also somewhat variable protein: N ratio, and that 'mg. N / 100 ml.' (which is readily convertible to 'mg. N / 100 gm. milk') is, at least, less complicated.

2. Abbreviations Adopted.

Reference to certain N-containing fractions will be frequent and recurring. Accordingly, the following abbreviations will be substituted for the longer terms indicated.

	Abbreviation	Longer Term
1.	TN	Total N
2.	CN	Casein N
3.	NcN	Noncasein N
4.	GN	Globulin N
5.	PPN	Proteose-peptone N
6.	AN	Albumin N
7.	NpN	Nonprotein N
8.	mg. %	mg. / 100 ml. milk

*This abbreviation is reserved for the tabular presentation of results, and is according to current practice, especially in clinical studies, where the weight:volume relation of mg. / 100 ml. of solution is reduced to "mg. %".

3. Comments on Estimation of Protein Content.

A simple method for estimating the protein content of milk is to determine the TN, and multiply by the factor 6.38. This estimation of "total protein" was used in nearly all the earlier and also some recent work, and

apparently is still used in analysis of meat, cereal, vegetable products, and so forth, (each with appropriate factors, of course).

In milk analysis, this calculation has at least two weaknesses:

(a) TN, as determined by Kjeldahl methods, includes all forms of N present in milk, with the exception of atmospheric N, and the possible exception of N in refractory ring compounds, nitro groups, and the like. TN, being nearly all-inclusive, consequently cannot and does not show the amount contributed by NpN. As will be seen from the data reported here, NpN is present in significant quantities in milk, and accounts for 4% to 6% of the TN. Neglecting the representation of NpN in TN will, therefore, make the protein calculation correspondingly too high by 4-6%.

(b) TN, because of its 'totality' basis, cannot show the constant shifts in proportionality of the N components that comprise TN. As will be outlined later, such shifts are normal, but may also be due to abnormal causes (e.g., subclinical mastitis).

In either case, the yields of casein, albumin, etc. may vary considerably from time to time, but the variations will not be reflected in the TN determination. In passing, we might note that points (a) and (b) have some economic

bearing, inasmuch as protein content is used as a basis of payment, in cheese factories for instance, and in calculation of yields.

More precise information is provided by the separate or distribution analysis of the major protein components. The amounts of the protein fractions are ascertained by a sequence of steps involving controlled precipitation from whole milk, followed by filtration to remove the precipitate, and determination of the N content of the precipitate and/or filtrate, (also called serum or whey,) by micro Kjeldahl methods. The scheme of operation outlined in the section entitled "Materials and Methods" shows how the seven N groups are treated. To provide some background for these groups, and to clarify some of the physical differences between them, a review of their position in the conventional protein classification scheme is presented in the Appendix under A.N.2.

REVIEW OF THE LITERATURE

I. Some Notes on Present Knowledge of Milk Protein Fractions.

II. Investigations Concerning N Distribution in Milk.

III. Factors that Influence the Composition of Milk.

IV. Methods of Determining Protein N, with Emphasis on Kjeldahl Methodology.

1. Principle and Sequence of the Kjeldahl Method.
2. Temperature-Time Relations.
3. Choice of Catalyst.
4. Boric Acid Modification.
5. Some Additional Methods of Measuring Protein and Ammonia N.

REVIEW OF THE LITERATURE

The literature providing the background for this study falls naturally into four divisions, namely:

I. Newer knowledge of the milk protein fractions and its possible bearing on this particular N distribution study.

II. The work of other investigators concerned with N distribution in milk.

III. Factors that influence the composition of milk.

IV. Methods of measuring protein N, with particular reference to factors affecting Kjeldahl methodology, and with some consideration of alternative methods of measurement.

These aspects will be treated in the order named.

I. Some Notes on Present Knowledge of Milk Protein Fractions.

The milk protein fractions of interest to us are casein, lactalbumin, lactoglobulin, proteoses-peptones, and nonprotein nitrogen. Our present knowledge of them may be reviewed.

The casein content of cow's milk ranges from 2.0-3.5% and contains approximately 80% of its TN. (TN and total protein are not the same, as pointed out previously.) From extensive ultracentrifugal and electrophoretic investigations, casein is known to be heterogeneous, consisting

of at least three proteins, viz., α, β and γ caseins, although as many as five fractions have been claimed by some workers (17). The fraction in greatest abundance is α casein; at 2°C., its isoelectric point, determined indirectly, is at pH 4.2, in contrast to that of 4.9 exhibited by β casein. β casein was found to have a higher relative viscosity in the pH range 5.5-9.0. The various caseins differ significantly in their respective contents of phosphorus, sulfur, dicarboxylic amino acids (aspartic and glutamic acids), tryptophane, tyrosine, and in coagulability with rennet, in stability to heating, in different rates of alkaline hydrolysis, etc. (17).

Linderstrom-Lang and Kodama (34) consider the parent casein to be a "reversible-dissociable" component system consisting of a loose association of various casein complexes which respond differently to changes in composition of the fractionating solution as, for instance, salt content, pH, alcohol or acetone content, temperature, phosphatases, proteolytic enzymes, etc. The net effect on the parent casein can therefore vary appreciably, and helps to explain why its isoelectric point has been variously reported as pH 4.6 or pH 4.7, and furthermore, that lack of more exact agreement with regard to solubility relations may be due to actual differences in the casein used by various investigators.

When casein is precipitated from milk, and then removed by filtration, the NcN fraction, i.e. the filtrate (also called whey or serum) contains (a) lactalbumin, (b) lactoglobulin, (c) proteoses and peptones, and (d) diverse nonprotein nitrogenous constituents. Lactalbumin is here determined indirectly by deducting the N values of lactoglobulin, of proteoses-peptones, and of the NpN constituents from the NcN fraction.

Both albumins and globulins are simple, non-phosphorylated proteins (see Appendix under A.N.2) and are heat-denaturable. Serological tests have demonstrated that the albumins of milk and blood serum are different, but that the globulins of milk and blood serum are the same (58). As with casein, so with lactalbumin and lactoglobulin do we find heterogeneity of composition, which may be resolved by fractionating techniques and solubility studies. Thus, there are at least 3 albumins, and even more globulins, since β globulin is itself evidently made up of three components (19). The recently discovered therapeutic value of γ globulin is referred to in the Appendix under A.N.3, along with other points. A recent review of the effect of heat on albumins and globulins, as well as the other milk proteins, is given by Rose (46). Pasteurization at 143°F. for 1/2 hour produced about 9% coagulation of albumin and 5% coagulation of globulin (57).

Proteoses and peptones are derived proteins, as indicated in the abridged classification scheme in the Appendix (section A.N.2.). They are produced mainly by prolonged heating of milk, evidently by partial hydrolysis of the denatured albumin and globulin (58) and of partially denatured casein (7). Rowland (47) showed that the soluble protein fraction of normal fresh milk is approximately 76% albumin and globulin, and 24% proteoses-peptones. In earlier work, the total soluble protein N contents of raw and heated milk samples were recorded as the "albumin fraction", i.e., as albumin plus globulin N, and proteoses-peptones N was not recognized as a constituent. Apparently, as pointed out by Davies (7), there are trace quantities of proteoses-peptones in the NpN of the filtrate from the precipitation of milk proteins by trichloracetic acid, inasmuch as a positive biuret test is obtained. (In repeating this, the writer obtained biuret tests that ranged from negative to faintly positive. The quantitative use of the biuret and other tests will be dealt with later.)

The NpN fraction of normal milk contains approximately 6% of the TN, and in this respect resembles GN (58). Variation in this amount is to be expected. Thus, when milk is low in s.n.f. the NpN increases significantly, but the increase in GN is not proportionate. Davies (5)

suggests that when milk is high in chloride and the expected level of lactose is not reached, the casein level is likewise reduced. This produces an upward shift in the relative amounts of soluble (serum) proteins and also of NpN.

Not much information is available concerning the significance of NpN constituents (mentioned previously) with respect to the physiology or pathology of milk secretion. According to recent data of Shahani and Sommer (56), urea is the constituent present in greatest amount, followed by creatine, uric acid, α-amino N, creatinine and ammonia. Their infiltration from the blood to the milk is probable, in view of the parallelism in content between blood and milk NpN (7). Perkins, quoted in (56), indicated a greater effect of dietary and other factors on NpN fractions than on gross composition. Thus, milk from cows maintained on high protein diet contained high NpN.

Pasteurization at 155°F. for 30 min. and homogenization at 2000 lb. pressure lowered GN and AN and raised the NpN, particularly NH_3 and α-amino N (57). Increase of NH_3 probably stems from cleavage of amide linkages of the milk proteins, and amino N from similar protein-splitting. (The effects of various processing treatments on N distribution are admittedly not part of this thesis, but mention is nevertheless made of at least two such studies (39,57), because they suggest much work of both theoretical

and practical interest that remains to be done, such as comparative nutritive values or effects on specific enzymes. As past research has shown with regard to the role of trace substances in other fields, future research on NpN constituents may reveal their influence on quality and physico-chemical properties of milk, which is probably greater than their amounts would indicate.)

As indicated previously, the kind and amount of salts influence the precipitation and denaturability of milk proteins (12,44). It was mentioned as an important detail which could affect the results of N distribution studies. There is another detail which might be added at this point, and which has apparently not received the attention it deserves. Thus, Eagles (11) refers to the finding of Linderstrom-Lang and Ottesen (35) that bacterial enzymes were able to convert ovalbumin to an electrophoretically different albumin without any degradative side-effects. Urea accelerates the transformation. Proteolytic enzymes can affect casein similarly. The foregoing demonstrations of the possible effect of indigenous microorganisms on ultimate isolation of given proteins clearly indicates that this aspect must be considered in the separation of milk proteins by the conventional procedures.

In closing this section, we may conclude that we are confronted with heterogeneous protein molecules that vary

in their amounts in milk. These variations are influenced not only by 'external' factors, which we shall mention in due course, but also by a complex of factors affecting the internal environment the exact interrelationships of which are not too well known as yet. Additionally, as pointed out by Gortner (15) (see also Appendix, A.N.3) our scheme of protein separations is founded on differential solubilities, but since these are a function of various artificial conditions, we may be dealing with artifacts, or with partially peptized products.

II. Investigations Concerning N Distribution in Milk.

Investigations that are reasonably complete with regard to N distribution in milk are relatively few in number. Earlier studies (5, 6, 13) neglected the presence of the PPN, which is approximately 24% of its soluble protein fraction. Thus, as was subsequently shown by Rowland (48), all earlier studies in reporting AN or GN, either separately or combined, are too high by at least 24%. A point not previously discussed here is that different results for casein, albumin and globulin obtained by different workers may be due to differences in analytical techniques or precipitation methods. In the following are summarized some of the more important facts presented by various investigators.

The variation in protein distribution with s.n.f.

content was determined by Davies (5), who found that in normal milk the N distribution (mean values) is as follows: Protein N, 94% (consisting of CN, 76%; AN, 12%; and GN, 6%); and NpN, 6%. As the s.n.f. decrease, less N occurs as casein and more as GN and AN, and protein values fluctuate. With very low s.n.f., the AN and GN are high and variable in consecutive samplings. With regard to milk of low s.n.f., Davies concludes that it takes on the composition of a true milk fraction diluted with a liquid rich in blood constituents. In a later study (6), Davies modified the general N distribution (%) in milk to: protein 94, non-protein 6; casein 77, albumin 13, globulin 4. The variations in AN and GN were higher than for any of the other N fractions, being respectively 14% and 23% on either side of the mean (6). TN varied directly with the s.n.f., and CN and total protein N varied directly with the TN content.

With respect to the NpN fractions of milk considered to be of commercial quality, the most comprehensive data are those of Shahani and Sommer (55, 56, 57). In their reports, data are given on the N distribution of both the protein and NpN fractions, with reference to their content in fresh raw milk and in milk after pasteurization and homogenization. The individual NpN fraction showed large variations. Pasteurization at 143°F. for 1/2 hour produced

coagulation of 9% of the albumin and 5% of the globulin (cf. data of Kieferle and Gloetzi, quoted in Davies (7), showing an increase in the NpN of 10.4% and 18.6% effected by pasteurization and boiling respectively.) Souring also increases the NpN appreciably.

Changes in composition in milk in England in recent years were noted by Rowland (52), particularly a trend to lower s.n.f. The deficiency is largely shown in lowered protein. Thus, as s.n.f. ranged from 8.1-8.5%, protein content ranged from 2.7-3.0%, lactose from 4.60-4.65%, and ash from 0.70-0.73%. Inadequate feeding in the late winter months was apparently largely responsible for extremely low s.n.f. This could be reproduced experimentally by decreasing the starch equivalent and protein equivalent of the feed.

Mean values reported for seven samples of Argentine milk (4) are of interest. Thus, in %, TN, 2.98; CN, 2.30; AN, 0.33; GN, 0.11; NpN, 0.24. TN was determined by the method of Orla-Jensen, GN by that of Moir and Andrade, and CN, AN and NpN were determined by A.O.A.C. methods. The foregoing values, recalculated as % of TN, are: CN, 77.1, AN, 11.1, GN, 3.7, and NpN, 8.1. Although breed of cow, season of the year, etc. are not given in the literature source, it is interesting to note the unusually low TN and unusually high NpN values, in comparison to results

obtained here to date. The AN:GN ratio is high, as is the sum of AN + GN (14.8%). The presence of PPN was clearly neglected, but on correcting for this omission, using the approximate factor of 24%, AN + GN is accordingly reduced from 14.8 to 11.25%, which is in reasonable proximity to our own data.

A note on the composition of colostrum may be of interest. Colostrum is a thick, viscid secretion which can be drawn from the udder immediately after parturition, and differs considerably from normal milk. The most striking difference is in the high GN of colostrum, which may run as high as 13% (45). Colostrum also contains considerably more GN, AN, chlorides, iron, riboflavin, carotene and vitamin A than milk. All of these constituents, present in extra abundance in colostrum, assist the offspring in attaining rapid development by making available to it substances in which the newborn is deficient. In addition, colostrum assists in protection of the calf from disease, and in this respect the high globulin content, which has been shown to be largely γ globulin, apparently transfers maternal antibodies to the calf (45). The importance of human colostrum to the infant is strongly emphasized in present pediatric science. It will be interesting to follow future developments with regard to the contemplated community-scale use of γ globulins as a

preventative and also therapeutic treatment for infants and children against poliomyelitis and other virus infections.

Before closing this particular section, attention will be directed to the influence of bovine diseases, such as mastitis, on N distribution in milk.

Mastitis is an inflammatory disease of the udder. In chronic cases, it is characterized by discharge of pus (high leucocyte count) and streptococci in the milk. The milk is very heat-coagulable, and may have abnormal color. Mastitis is apparently widespread in many parts of the world, certainly in Europe and in the Western hemisphere, and constitutes one of the most serious problems confronting the dairy farmer. It is, moreover, of some public health importance, since occasionally streptococci of a type pathogenic to humans (e.g., S. pyogenes) and also staphylococci may be excreted in the milk and give rise to scarlet fever or septic sore throat in those consuming it in a raw state (45). (It should be noted in passing that staphylococcal toxins as a class are heat-stable and will not be destroyed under commercial pasteurizing conditions.) According to a number of veterinarians, the estimated incidence of mastitis in the cow population in Manitoba is 20-30%, but this does not include the many subclinical or marginal cases which undoubtedly exist.

In addition to decrease in yield and titratable acidity of milk, mastitis causes a decrease in those constituents that involve secretory activity - fat, casein, lactose, citric acid, calcium, phosphorus and potassium, and an increase in those components that are normally low in milk - chlorine, sodium, globulin, catalase, pH (7, 58). The net result is a significantly lower s.n.f. of altered composition.

Two of a number of biochemical indices that have been advanced to make more accurate the diagnosis of mastitis are:

(1) The chlorine-lactose, or "Koestler number" $\frac{\text{Cl \% x 100}}{\text{lactose \%}}$. The basis for this index is that in response to the chlorine-lactose shifts that occur during udder disease, the chlorine:lactose ratio changes proportionately more than either chlorine or lactose alone. The normal Cl content in milk is below 0.14% and the lactose content is at least 4%, but in mastitis, the Koestler number rises well above 3.0 (7). Chlorine in milk may rise appreciably, however, from causes other than mastitis (60), e.g., for healthy animals approaching the end of lactation (53).

(2) The "casein number", $\frac{\text{CN \% x 100}}{\text{TN \%}}$ (see 51, 53, 54).

Based on the comparative N distributions in normal and abnormal milk, Rowland (51) found that the casein number of (a) normal, uninfected milk was surprisingly constant despite fluctuations in TN, and averaged 78.5, with variations of 77.3 to 80.7, whereas that of (b) milk drawn from infected quarters ranged from 65.8 to 73.5. Rowland suggested a casein number of 77.0 as a minimum for normal milk, and showed that the casein number could distinguish between milk samples with pathological and with physiological low s.n.f. The pathological samples had the low CN and high soluble protein content (AN, GN, and PPN) of mastitis milk, on account of the "isotonic diluent" containing only soluble proteins, whereas the physiological cases had a normal protein distribution. Of the pathological samples, 88% had s.n.f. values below 8.80, the average being 7.93%. Physiological cases were comparatively few, and the s.n.f. averaged 8.56% (54). The advantages of the casein number, and comparisons with bacteriological data to show its reliability, are given in (53). It is concluded that subclinical mastitis accounts for a high percentage of milk low in s.n.f. (54).

Vanlandingham et al (60) investigated the relationship of the development of chronic mastitis to changes in the chlorine, lactose, chlorine-lactose number and casein number of the milk. Other chemical and also bacteriological tests were used. It was concluded that both the chlorine-lactose number and the casein number, and to a lesser extent the chlorine or lactose alone, could be recommended for diagnosis of chronic mastitis which had developed to a stage in which the composition of milk is altered. Changes in composition based on differences between quarters of the same udder were preferred to a preset "normal" standard, since they obviated changes associated with age, advanced stage of lactation, or possible singularity of the individual animal.

In this section we have attempted to provide some indication of investigations that have been made on N distribution with reference to various facets of milk composition. A relatively large amount of space has been devoted herein to the possible effect of mastitis on N distribution, because the possibility of further increase in incidence of bovine mastitis would tend to alter our standards for the norms of milk composition. In any event, the writer considered the mastitis question of interest, and found an opportunity during the course of his work to determine the N distribution, the casein number and the chlorine value of milk obtained from two cows afflicted

with mastitis. Details are presented in the Appendix under A.N.4.

III. Factors That Influence the Composition of Milk.

The chemical composition and physical properties of milk reflect the genetic-environmental complex. Since the latter is a dynamic relationship, the protein composition of milk will vary accordingly. Discussions of the influencing factors are contained in texts on dairy chemistry, such as (7, 45, 58). Some of the major factors are:

1. Breed of cow. Differences due to breed are highly significant. The breed characteristic shows up most prominently in the fat content of the milk (45). Milk high in fat is also high in s.n.f., and consequently in protein and in lactose, which are the main components of s.n.f. Lactose appears to show least change (7). In general, the richness of the milk is inversely proportional to the size of the cow (58), e.g., the Jersey and Guernsey produce the richest milk.
2. Individuality of the cow. Within a herd there will be differences among individuals, due presumably to genetic factors, or possibly to some factors not yet identified. Some cows yield persistently milk of low s.n.f. Milk low in s.n.f.

shows marked diurnal fluctuations in non-casein proteins (albumin and particularly globulin) (7).

3. Intervals between milking. The milk of some cows is more affected by the length of interval than others. Morning and evening milk generally differ in composition. It must be recognized that economic and practical conditions often compel the producer to milk the herd at unequal intervals.

4. Completeness of milking. Omission or incompleteness of stripping, or overlong milking periods can account for change in milk composition.

5. Nature and amount of food. As is the case with other animals, the food ingested by the cow influences composition of the milk. Allen (quoted in 58) fed vegetable fats to cows and obtained increases of 0.1-0.3% fat on intake of 1/4 lb. fats daily, and 0.7-1.0% fat on intake of 1 1/4 lbs. daily. Conflicting results by other investigators are reported. A lower plane of nutrition changes both the yield and composition of milk. For farm animals, in Western Canada at least, the nature of the food ingested is closely related to the season of the year. Breirem (2) quotes investigations showing that long-continued deficiency of protein in the diet may lead both to reduced

fat content and lower protein content in the milk. Both fat and protein rise during pasture feeding, only to fall again during indoor feeding. This is particularly the case when the feed is low in protein. In Finnish milk, the casein content fell from 2.55% in 1939 to 2.29% in 1945-46, presumably due to prolonged deficiency of protein. Interestingly, the total protein content of the milk remained essentially unchanged. Other investigators, it should be mentioned, found somewhat different effects on the casein content of milk. Also, as a note of interest regarding the palatability of the diet, especially when milk is to be used for cheese-making, selection of the feed, and also feeding in a manner to avoid digestive disturbances, are matters of importance, as shown in interesting experiments (2).

6. Age of the animal. A decrease in all constituents occurs with increasing age of the animal, except in AN (7).

7. Period of lactation. Milk composition during lactation is influenced by (a) breed, (b) condition before calving, and (c) management and feeding after calving. Commonly the fat content is about 0.5% higher in the last stages of lactation as compared

with the middle period (58). For the first few days of lactation, the milk, called "colostrum", has a strong odor and bitter taste, and is unusually high in GN, the significance of which has been previously mentioned. The milk during the month following the colostral stage is higher in AN and fat than occurs later (45).

8. Climate and weather conditions. Some subdivisions of this item are: (a) season of the year, (b) temperature, and (c) sudden changes in weather. Each and all can influence composition of the milk.

9. Effect of disease. Digestive ailments, fever and even mild inflammations markedly influence N distribution in milk. Similar shifts in N distribution result after injury and during the subsequent healing process, as shown by clinical studies on animal blood. The effects of mastitis on milk composition were mentioned previously.

10. Miscellaneous. (a) Moderate exercise has been shown to increase fat content in milk (58). (b) Hormonal control of lactation, e.g., feeding or administration of thyroxine, has increased the fat content, the s.n.f. and the yield of milk, notably with cows in declining lactation (7). (c) Irritation by insects and other pests, or by inexperienced

milkers, upset normal composition. (d) Excitement or fright will also upset normal equilibria in milk, causing incomplete "let down" (58).

Some of these factors are controllable, other are not. Most of the published literature is concerned with the effects of such factors on butterfat content, inasmuch as commercial emphasis has been, and by and large still is, directed on the butterfat content. It is reasonable to suppose that influence on N distribution will result from the commensurate changes in s.n.f. as butterfat fluctuates. However, direct evidence of the exact magnitude of such effects, under controlled conditions, on N distribution is still lacking for the most part.

IV. Methods of Determining Protein N, with Emphasis on Kjeldahl Methodology.

Since results depend so much on the methods employed, a clear understanding of the possibilities and limitations of the latter is essential. In this section, a preliminary report prepared earlier by the writer (1) is presented in a somewhat abridged form, and additional information available since the time of its writing is included as well.

1. Principle and Sequence of the Kjeldahl Method.

A Kjeldahl determination is essentially a "wet combustion" and involves oxidation-reduction. The procedure is

simple:- the substance is boiled with conc. H_2SO_4 in a Kjeldahl flask for some time (1/2 - 6 hrs.). K_2SO_4 is added to raise the b.p. of the reaction and thus to accelerate the "digestion", during which process the N of the sample is converted to $(NH_4)_2SO_4$. The hot H_2SO_4 acts as an oxidizing agent: $H_2SO_4 \rightarrow H_2O + SO_2 + O$. Oxidation may be accelerated by catalysts, such as compounds of Hg, Se or Cu, and also by oxidants such as H_2O_2 and perchlorates. After clearing, when oxidation is assumed to be complete, the acid digest is cooled, diluted with water, and made alkaline with excess NaOH, whereupon NH_3 is liberated. The liquid is boiled or steam-distilled to drive off the NH_3, which is absorbed in an excess of standard acid. The N content of the original sample is calculated from the titration value (back-titration with standard base, or, using the boric acid modification (61), direct titration with standard acid). Protein is calculated by multiplying N by the appropriate factor, the use of which has been discussed previously.

To meet special requirements of particular problems, or to promote greater accuracy, precision, speed or convenience, many modifications of the Kjeldahl method (30) have been made since its publication 80 years ago. It is only within recent years, however, that intensive studies have been made of the variables that affect the performance

and reliability of some of the commonly used Kjeldahl procedures. The following presents briefly some of the main features in the writer's earlier report (1) of such studies.

2. Temperature-Time Relations.

That the temperature of digestion is critical is indicated in papers by Grunbaum et al (16), Hiller et al (23), Lake et al (32), Ogg and Willits (40), White and Long (62) and Lake with 19 collaborators (33).

Using thermocouples to measure digestion temperatures, Lake et al (32) found that the range 370-410°C. was optimum for their samples. Temperature was controlled by the amount of K_2SO_4 added. Digestion proceeded for 1 hour after clearing. Uniform timing was necessary because temperature tends to increase with digestion time, due to evaporation of H_2SO_4. Consumption of acid depends on the material.

In the conventional open flask digestion, too much K_2SO_4 promotes loss of N (62). Grunbaum et al (16) attribute such loss to overly high temperatures, with resultant oxidation of NH_4^+ ion, and consider that long digestions, even at optimum temperature, may have a similar but slower effect.

Ogg and Willits (40) showed that low temperatures or a short digestion time contributed to low values. About

0.6-0.8 gms. K_2SO_4 / ml. H_2SO_4 are considered optimal. Boiling points of various mixtures of K_2SO_4 and H_2SO_4 are given. (This importance of the correct K_2SO_4:H_2SO_4 proportion interested the writer. Some experiments were done on it, the results of which are reported in the Appendix, under A.N.5.)

Lake et al (33) report less frothing and smoother digestion when $K_2S_2O_7$ -(potassium pyrosulfate)- was substituted for K_2SO_4. The substitution was based on the assumption that the following reaction occurs when the usual K_2SO_4 is used:

$$K_2SO_4 + H_2SO_4 \longrightarrow 2KHSO_4 \longrightarrow K_2S_2O_7 + H_2O$$

Thus, in addition to evolving H_2O which increases frothing, K_2SO_4 combines with an equivalent amount of H_2SO_4. This, added to the acid consumed by the sample, lowers the amount of liquid in the flask so that chances of oxidation of the NH_4^+ ion are increased, thus contributing to lower results. Apparently the use of $K_2S_2O_7$ obviates such tendencies to some extent. This is a modification which seems to warrant further investigation in view of the excessive frothing of some samples.

3. Choice of Catalyst.

Besides the addition of K_2SO_4 to increase the b.p. and thereby accelerate the digestion process, oxidizing agents such as $KMnO_4$, $K_2Cr_2O_7$, H_2O_2, $HClO_4$, and potassium

persulfate may be used. More widely used, however, are Hg, Cu, and Se and compounds of these elements. A large number of papers deal with the advantages and disadvantages of each of these catalysts, some of which have been reviewed previously (1).

Selenium has a synergistic effect on the rate of Kjeldahl oxidation, showing marked accelerative power. Accordingly, as Patel and Sreenivasan (41) point out, use of Se in more than small quantities results in loss of N. The "afterboil" time (i.e., the time after clearing of the digest) is critical, and must be determined and controlled for each type of protein or other nitrogenous material. A mixture of Se and HgO gave better recoveries.

Hiller et al (23) concluded that Hg is the only catalyst capable of yielding results comparable to those of the Dumas combustion method, which was used as a standard of comparison. In the report by Lake et al (33), a number of laboratories stated that Se was not a necessary or even desirable ingredient of the digestion mixture. Kirk (27) found that Hg was the most efficient catalyst, and could not be replaced satisfactorily by Se.

It might be mentioned at this point that Rowland (50) used Se in the digestion mixture for milk protein analysis, and pointed out that N recovery was thereby increased. However, it should be noted that his comparison was made

between Se and $CuSO_4$ as against $CuSO_4$ alone, and that $CuSO_4$ alone is a comparatively slow catalyst, giving low results unless the digestion period is prolonged. The methods of Shahani and Sommer (55), used in this investigation, employ for each sample 1 gm. Na_2SO_4-HgO powder, consisting of Na_2SO_4:HgO as 14:1 by weight.

Digestion conditions evidently become less elastic as the scale of operation is reduced. The effect of the nature of the material is, of course, a major determinant, since amino acids like tryptophane, histidine and proline, because of their type of ring structure, require more drastic treatment than glycine and the like, and these conditions might induce greater losses of N. The conditions for reducing the N to NH_3 and for oxidizing the remainder of the substance obviously must coexist, and according to the newer studies, there is evidently a narrow oxidation-reduction range in which the decomposition can be carried out with quantitatively satisfactory results. This underlines the need for careful adjustment of kind and amount of the various additives available (catalysts, oxidizing agents, reducing agents) with optimum time-temperatures.

4. Boric Acid Modification

The usual method in Kjeldahl distillation is to absorb the NH_3 distillate in a solution of excess standard acid and then to back-titrate with standard alkali. A

useful alternative is to employ boric acid solution (2 or 4%) as the NH_3-absorbing agent. A suitable indicator is added at the same time. The solution is then titrated with standard acid until the pH is lowered to that of a control or blank run. The end point is signalled by the indicator, which may be methyl red, or methyl red + bromcresol green (36), or methyl red + methylene blue (38), or methyl purple (32). (The latter is claimed to be superior in sharpness.)

The use of boric acid as an absorbing agent for NH_3 has a number of advantages, such as:

(1) It eliminates boiling of the distillate to remove CO_2 before titration (cf. Rowland (50)).

(2) It eliminates the use of dil. NaOH, which requires frequent restandardization and is sensitive to CO_2.

(3) The direct titration with standard acid simplifies calculations.

5. Some Additional Methods of Measuring Protein and Ammonia N.

Kjeldahl methods may collectively be considered to consist of three principal stages, namely: (a) reduction of organic N to NH_3, (b) separation of the NH_3, and (c) measurement of the NH_3. By suitable selections, it is possible to modify any or all of the three Kjeldahl stages,

and, in special cases, even to obviate them entirely. Conditional, of course, on the nature of the protein analysis problem, one might use gravimetric (24), titrimetric (32, 33, 36, 37, 50, 55, 61, 62, et al), colorimetric (10, 22, 25, 26), gasometric (23), turbidimetric (31), microdiffusion (16, 27), nesslerization (21, 59), falling drop methods (42), and others. Albumin in clear solutions (sera) can be estimated directly by ultraviolet absorption measurements at 280 mμ (24); δ globulin in dilute sera can be measured turbidimetrically (31). There are as well electrophoresis, already mentioned, and serological methods developed in immunology, such as those employed by Chow et al (3). Some of the features of some titrimetric, colorimetric, gasometric and microdiffusion methods for protein N are given in the Appendix under A.N.6.

In this investigation the methods of Shahani and Sommer (55) were used to study the N distribution in milk. Of a number of procedures which were suggested by the Division of Chemistry, Science Service, Ottawa, for consideration of suitability for this particular study, the Shahani-Sommer procedures were selected because: (a) they appeared to be well-suited for the nature of the problem, and (b) they were based, for the most part, on the methods of Rowland (50) and of Menefee, Overman and Tracy (39),

and these are widely used both in Europe and the Western hemisphere. Accordingly, comparisons of our work with that of a number of other investigators would have a common denominator based, at least, on similar analytical procedures.

EXPERIMENTAL

I. Apparatus.

II. Reagents.

III. Sampling and Analytical Procedures.

1. Sampling.
2. Precautionary Measures.
3. Outline of Fractionation Procedure.
4. Method in Digestion, Distillation and Titration.
5. Conversion and Correction Factors.
6. Modifications in Procedure.

IV. A Simple Adapter for Micro Kjeldahl N Distillation.

1. Details of Construction and Procedure.
2. Advantages.

EXPERIMENTAL

For convenience, this section will be divided into four parts, namely:

I. Apparatus

II. Reagents

III. Sampling and analytical procedures

IV. Development of a simple adapter for micro-Kjeldahl distillations.

I. Apparatus

Two 6-unit digestion racks (Precision Scientific Co.,) with Pyrex manifolds and glass water-aspirators for control of fumes. The racks were mounted on a floor of firebricks laid on the laboratory workbench. A photograph of the digestion setup is shown as Plate 1.

Steam distillation apparatus, modified by the writer as described below in subsection IV.

Microburette, automatic, (Scientific Glass Apparatus Co.,) 5 ml. capacity, graduated in 0.02 ml. divisions.

Glass electrode, Coleman Model 3 D.

Erlenmeyer flasks, 125 ml. size, marked at a 60 ml. level, and used as receivers.

Pipettes, "Normax" precision, (Kimble Glass Co.,) in 1, 5, 10 and 20 ml. sizes.

Semimicro Kjeldahl flasks, 100 ml. bulb capacity.

Volumetric flasks, 50 ml. and 100 ml. sizes.

II. Reagents

Concentrated sulphuric acid, reagent grade, sp. gr. 1.84, tested for freedom from N.

Catalyst mixture made of mercuric oxide and sodium sulphate anhydrous powders, reagent grade, mixed 1:14 by weight, placed in a glass container and shaken in a laboratory shaker for several hours.

Acetic acid, 10% aqueous solution.

Sodium acetate, 1.0 N. solution.

Boric acid-indicator solution. To 4 l. of 2% boric acid solution were added 20 ml. of indicator solution made as follows: 100 mg. methyl red and 30 mg. methylene blue, dissolved in 60 ml. of 95% ethanol, and made up to 100 ml. with distilled H_2O.

Alkali-thiosulfate solution, consisting of 40% sodium hydroxide pellets, C.P., and 5% sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$), C.P., in aqueous solution.

Standard hydrochloric acid, seventieth normal (i.e., 14.28 mM.). The solution was prepared by adding 142.8 ml. of 0.1 N. hydrochloric acid (diluted from 1.0000 N. HCl standard solution, sealed ampoule, British Drug Houses, Ltd.) to a 1-l. volumetric flask and diluting to the mark. With a standard acid of this normality, 1 ml. $\simeq$ 0.2 mg. N, so that calculations of N content from titration values are accordingly simplified.

Standard ammonium chloride, 14.28 mM. This reagent is used for checking the micro-Kjeldahl procedure. Ammonium

hydroxide, C.P., diluted to this molarity, can be used similarly. One ml. of standard ammonium chloride or ammonium hydroxide contains 0.2 mg. N.

III. Sampling and Analytical Procedures.

1. Sampling

By arrangement with the owners of certain pure-bred dairy herds in Manitoba, pooled samples of milk were obtained at regular intervals (four weeks apart) from twelve herds representing the following six breeds: Holstein-Friesian (3), Ayrshire (3), Jersey (3), Guernsey (1), Brown Swiss (1), and Red Poll (1). The sources of the milk samples are listed in the Appendix, under A.N.1.

For analysis, the samples were tempered to 60° F., mixed thoroughly, and treated according to the procedures shown below. Each determination was done in duplicate, and as rapidly as possible after the arrival of the samples. Those not being analysed were stored at 38° F. until needed. No preservatives were added to the samples, and none were stored long enough for acidity to develop as a result of microbiological activity.

2. Precautionary Measures

Each shipper was requested not to include milk from animals suffering from mastitis, since this disease alters the composition of milk. Each of these persons was able to recognize the disease and had his herd serviced by veteri-

narians when necessary, so it is believed that chances of error from this source are slight.

3. Outline of Fractionation Procedure.

The amounts of the protein fractions were ascertained by a sequence of steps involving controlled precipitation, followed by filtration to remove the precipitate, and determination of the N content of the filtrate by micro Kjeldahl methods (55). The scheme of operation outlined below shows how the seven groups mentioned previously were treated. It should be noted that TN, NcN, (PPN + NpN), GN and NpN are determined directly, whereas CN, AN and PPN are determined indirectly by calculations based on results of the former.

TN — Five ml. of whole milk were diluted to 100 ml. A 5 ml. aliquot was transferred to a micro Kjeldahl flask, and analysed as detailed later. (In other words, the equivalent of 0.25 ml. whole milk was analysed for TN).

NcN — Ten ml. of milk were diluted to 70-80 ml. in a 100 ml. volumetric flask, held for 10 mins. at 40°C., and acidified with 1 ml. 10% acetic acid. After 15 mins., 1 ml. N/1 sodium acetate was added, the mixture was cooled rapidly to room temperature, diluted to 100 ml., the pH

adjusted to 4.65 if necessary, and filtered, using a dry pleated Whatman No. 42, 11 cm. paper. A 10 ml. aliquot was transferred to a flask for the rest of the analysis.

GN — A 20 ml. aliquot of the serum obtained in the NcN procedure was chilled for a half hour. Seven ml. absolute methanol (cold) was added to precipitate the globulins. After holding for 2 hours at 0-4° C., it was filtered. Finally, the precipitate and paper were transferred to a flask for the balance of the test.

PPN + NpN — (This fraction is composed of two groups. Later we determine NpN, and by deducting its value from this fraction, obtain PPN.) The procedure is the same as for NcN above, but the 10 ml. milk are first heated in a boiling water bath for 40 mins. to precipitate the proteins denaturable by heating (lactalbumin and lactoglobulin fractions).

NpN — Ten ml. milk were placed in a 50 ml. volumetric flask, diluted to the mark with 15% trichloracetic acid, and shaken to mix. After standing one half hour to allow the precipitated proteins to settle, it was filtered

through a dry pleated Whatman 42 paper. A 20 ml. aliquot of the filtrate was transferred to a Kjeldahl flask, a quartz chip added, and analysed in the usual manner.

The values for CN, AN and PPN are obtained indirectly as follows:

CN	Casein N = TN-NcN.
AN	Albumin N = NcN - (PPN + NpN + GN).
PPN	Proteose-peptone N = (PPN + NpN) - NpN.

4. Method in Digestion, Distillation and Titration.

(Note: The procedures given below are discussed in some detail, inasmuch as observance of small details are often essential to replicability of results. Very few, and in most cases hardly any, minutiae are available in published papers.)

In a clean, dry Kjeldahl flask were placed 1.00 ± .03 gm. catalyst, the sample, and 3 ml. conc. sulfuric acid. The flask was swirled gently to mix, and placed on the digestion rack. It was heated gently with a low flame (about one inch high), until the contents were well charred and the liquid volume was reduced appreciably. The flasks were turned occasionally. If a tendency to "foam up" was evident, a quartz chip was added. If foaming still persisted, the flask was cooled by removing it from

Plate 1. Micro Kjeldahl Digestion Apparatus.

the rack, replacing, and again heating slowly, with turning, repeating the cooling and heating, if necessary. Now the heat was increased until the flask contents were clear. The flask was removed for cooling, the sides washed down with a minimum of distilled water (a polyethylene "squeeze bottle" equipped with a fine delivery jet was found convenient for this purpose). If necessary, a rubber policeman was used to work down any charred substance adhering to the flask that did not wash down readily. The flasks were replaced on the rack and heating continued for 1 1/2 hours. (The latter stage is also known as the "after-boil" time, which ranges from 20 minutes upwards.)

Ten to fifteen ml. water was added to the cool flask. It was then attached to the adapter of the distillation apparatus shown schematically in Figure 1, 18 ml. of alkali-thiosulfate solution was added, and the contents were steam distilled. The liberated NH_3 was absorbed in 20 ml. of boric acid-indicator solution contained in a 125-ml. Erlenmeyer flask, placed under the condenser at an angle such that the delivery tip was submerged as far as possible in the solution. As soon as the bubbles ceased to form, the color of the indicator changed to green if NH_3 was present, whereupon distillation was continued until the 60 ml. level was reached. This took about 3 to 4 minutes. The receiver was lowered, the condenser tip washed, and

The steam vent opened and the flame removed. The receiver and flask were removed and replaced with a fresh receiver and flask, and the distillation was repeated.

While another sample was being distilled, the solution just removed was titrated with standard hydrochloric acid solution. The color changed from green to grey-blue as the end point was approached. The most reproducible end point occurred just before the final disappearance of the grey-blue color. A blank determination was made, both as a check on the reagents and as a color standard. Before distillation of a group of samples was undertaken, a blank run was made with distilled water, in order to moisten the walls of the condenser (cf. ref. 38).

5. Conversion and Correction Factors

(a) Because the normality of the standard acid, viz., 0.01428 N., was selected to facilitate calculations, titration values can be readily converted to mg. N / 100 ml. milk by use of simple factors. These factors are derived from the dilution and aliquot relations of a given determination and are as follows:

Determination	Factor to convert ml. std. acid to mg. N %
TN	80
NcN	20
PPN	20
GN	10
NpN	5

(b) In addition to the foregoing conversion factors, two of the determinations were corrected as follows:

1. NcN calculations were reduced by the factor 0.995 (45) to correct for the volume occupied in the 100 ml. flask by the ppte. of casein and fat (0.5 ml.). This correction was not used by Shahani and Sommer (55), but was adopted here in the interest of greater accuracy. Small variations in fat and casein contents in different samples were not considered, and the factor was used without variation.
2. GN, when determined exactly as prescribed by Shahani and Sommer (55) resulted in values that appeared to be too high, and consequently in AN values that seemed to be comparatively low. Nevertheless, results obtained in these respects by the writer were in reasonable agreement with those of Shahani and Sommer. In either case, however, the GN:AN ratios seemed to be at variance with those of Davies (6), and especially of Rowland (51).

   The reason for this variance was revealed in a recent investigation by the writer, in which it was shown that the filter paper used

in the GN determination had an appreciable blank value. All filter papers tested had a significant N content, and this was reduced only partially by various pre-washing methods (alcohol, ether, hot water, acidulated water). In any event, by deducting the blank value of the paper used in the GN determination, the variance in GN and AN values obtained by the writer, as compared to those obtained by Davies (6) and Rowland (51), was reduced appreciably. It was tentatively surmised that omitting to deduct the blank value of the paper accounted for disparity in GN-AN relations in both the published work of Shahani and Sommer (55) and the earlier determinations of the writer. (On bringing the subject to the attention of a number of other chemists, it was found that: (a) not all include blank determinations in their particular routine, and, (b) some were surprised, as was the writer at first, that purified filter paper (11 cm. size) contained significant amounts (equivalent to 0.1-0.3 mg. N, depending on the paper) of some unidentified nitrogenous compounds.)

6. Modifications in Procedure

Two major and a number of minor changes in the Shahani-Sommer methods were made by the writer, namely:

(a) GN determination. Before placing the folded paper and precipitate into the Kjeldahl flask, the paper was partially dried to remove residual alcohol by placing the funnel and paper in a warm air oven for a few minutes. The semi-drying assisted considerably in eliminating almost completely the excessive frothing that otherwise occurred from the action of the hot sulphuric acid. After placing 1 gm. of catalyst and the paper and precipitate in the flask, 3 ml. sulphuric acid were added, and the flask was heated with a low flame until a spongy char was obtained. The flask was then removed, and an extra 2 ml. of sulphuric acid were added. The additional acid shortened the digestion time, and also compensated for a certain amount of acid consumed by the filter paper. Correction was made for the titration value of the blank paper, as indicated previously.

(b) The time specified for holding the globulins at 0-4°C. after addition of the precipitant

(absolute methanol) was extended from 40 minutes (55) to at least 2 hours. Standing overnight was preferred, since settling of the suspension was thereby promoted. Water was not used to wash the ppte. since it seemed to have a peptizing action, giving low GN values.

(c) Absorption of distillate by the receiving flask proceeded to a standard volume of 60 ± 5 ml. of solution. This promoted better precision within replicate titrations. Combining the indicator solution with the boric acid in one stock solution simplified somewhat a repetitive step, and also made unlikely any variation in concentration of indicator per titration.

(d) Instead of 0.03-0.04 N. hydrochloric acid (55), that of 0.01428 N. was used, as indicated previously. Not only were calculations simplified, but the comparatively greater dilution (approximately 2.5 x) made possible attainment of end-points within closer limits. This was especially useful when the titration value of a sample was low, e.g., 2 ml.

(e) The "after-boil" period was lengthened from one half hour (55) to one and one half hours, in view of the possibility of incomplete digestion,

as pointed out in our previous discussion. The slightly increased recovery, shown in the Appendix under A.N.5, effected by increasing the acid:sulfate ratio of the specified procedure (55), shows the empirical nature of Kjeldahl methods in general. However, in order that comparisons of data might be on a generally similar basis, insofar as procedure is concerned, the 3:1 ratio of acid:sulfate was used throughout.

(f) Steam distillation of the digest was conducted directly from the 100 ml. flask, instead of transferring the digest to a 300 ml. flask as specified by Shahani and Sommer (55), after Menefee and Overman (38). Details are presented in the section following. Excellent replicability was obtained, due presumably to avoidance of loss by transferring. (Distilling from the same flask as used for digestion is, of course, the case in macro procedures.)

IV. A Simple Adapter for Micro Kjeldahl N Distillation.

In the Kjeldahl (30) N procedure, quantitative separation of the NH_3 from the neutralized digest can be accomplished more rapidly by steam distillation than by direct heating of the flask with a gas burner. For micro and semimicro modifications of the Kjeldahl method, steam distillation

apparatus, such as the Parnas-Wagner type, is commercially available from a number of laboratory supply firms. Such apparatus permits automatic evacuation of the contents when distillation is terminated. However, in addition to extra cost and fragility, the need to transfer the digest from the digestion flask to the distillation apparatus uses as much time as that saved by the evacuation feature.

With the aim of avoiding transfer by distilling the digest directly, attempts were made to fit glass tubing of suitable diameter into a no. 1 rubber stopper to fit the neck of a 100 ml. Kjeldahl flask. Results, however, were unsatisfactory in several respects. Attention was accordingly directed to development of suitable modification. The glass-blowing work for a number of these was done by Mr. R. J. Cheale of the Grain Research Laboratory. All test models proved to be impractical because of fragility or were inadequate otherwise, with the exception of the simple adapter shown as part of Figure 1.

Details of Construction and Procedure.

The adapter is made by fusing about 2 inches of the lower section of a test tube, 35 mm. outside diameter (O.D.) (a 50 ml. centrifuge tube may be used), to about 1 1/2 inches of tubing, 20 mm. O.D. (a section of the neck of a 100 ml. Kjeldahl flask is ideal for the latter requirement). The adapter, fitted with a rubber stopper, allows ample

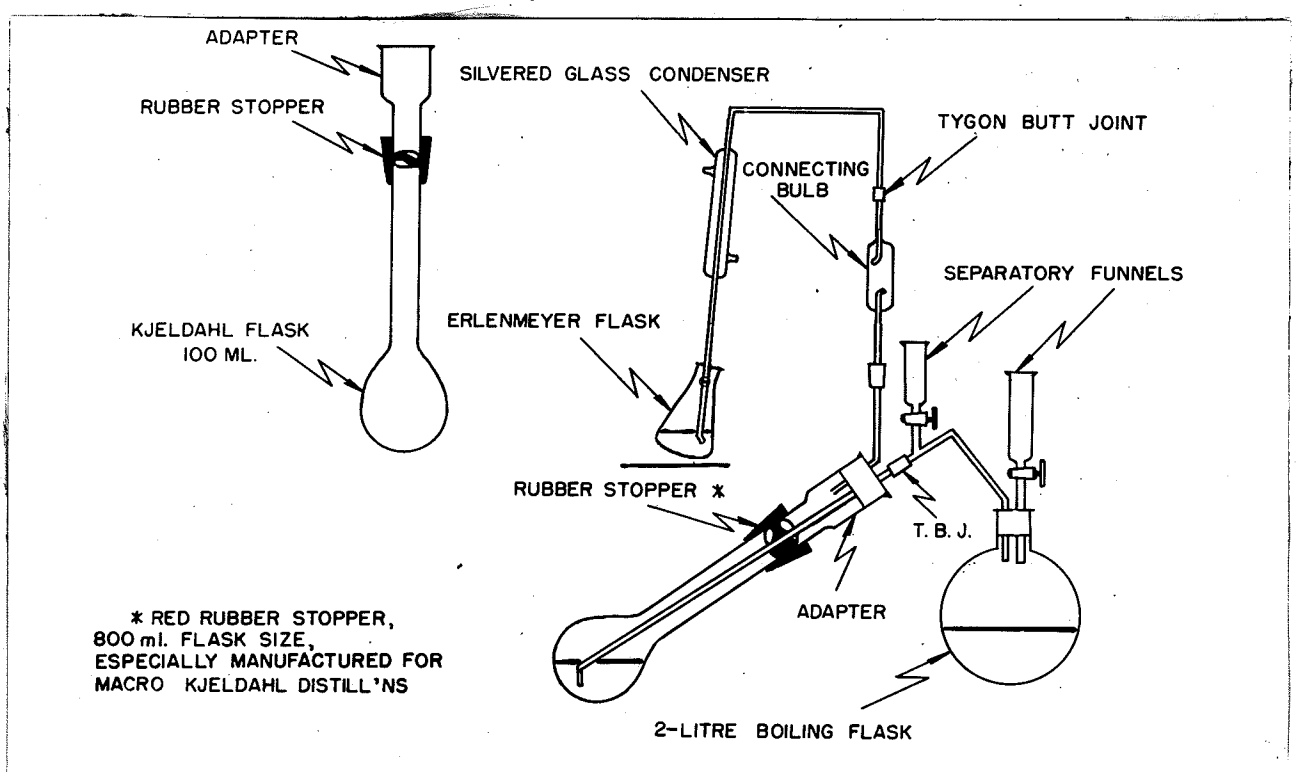

FIGURE I. SCHEMATIC DRAWING OF APPARATUS USED IN MICRO KJELDAHL DISTILLATIONS.

room for the steam inlet tube (6 mm. O.D.) and the outlet tube (10 mm. O.D.), and also serves as an expansion chamber. Mechanical carryover of traces of alkali has not been experienced with the setup as shown.

A special rubber stopper serves as a tight and thick-walled sleeve to connect a digestion flask to the adapter assembly. The stopper, obtainable from Wilkens-Anderson Ltd., Chicago, is of red heat-resistant silicone rubber and has been advocated by that firm because of its proven durability in Kjeldahl distillations. A stopper (800 ml. flask size) is bored through the center with a no. 11 borer. The upper half of the stopper is fitted permanently to the adapter, whereas the lower half accommodates a 100 ml. flask, which is pushed into position with a twisting motion and clamped around the mid-portion of the neck for stability. Flasks of the older type have an appreciable flange or rim extending outwards at the mouth, and with such flasks it is necessary to grind the rim on a carborundum stone until it becomes approximately flush with the neck. With a power-driven stone, such an operation requires less than 1 minute. Ground flasks, and also the newer (narrow rim) bottles, are easily fitted into and removed from position. The stopper of the original model is still in use, and has withstood to-date of writing over 600 distillations with no demonstrable deterioration.

The steam inlet tube extends close to the bottom of the flask. A thistle-top separatory funnel is fused into the upper portion of the inlet tube to permit addition of excess sodium hydroxide-thiosulfate when the flask is in position. The added base always forms a layer below the digest, but after the stopcocks are closed, active intermixing results immediately from entry into the system of steam and air from the boiling flask. Distilled water is used in the boiling flask, and some H_2SO_4 is added to the water to trap any NH_3 that might be present.

The connecting bulb is of a type used in macro Kjeldahl distillation.

Heavy-wall (3/32 inch wall) Tygon tubing proved to be durable, and satisfactory for making butt joints in joining glass to glass.

A silvered glass tube was used as the condenser, after Pregl's methods for micro Kjeldahl N determination.

Advantages.

1. Distillation directly from a micro Kjeldahl flask (the adapter could be scaled down for a 30 ml. flask, if necessary) obviates the necessity for prior transfer to larger flasks. Time is accordingly saved, and, of greater importance, the determination is not subject to possible loss resulting from transfer.

2. Utilizing the speed of steam distillation, distillation with the assembly described can be terminated in 3 or 4 minutes, as against 12-15 minutes for semimicro procedures which employ heating of the flask by direct gas flame (8, 20, 62). U-tubes after Henwood and Garey (8), and the Hengar type, which is hardly any different, are commercially available. The comparatively thin rubber sleeves supplied with the Hengar U-tube did not prove satisfactory in the writer's preliminary trials. Based on the success of the silicone rubber stopper aforementioned, it would seem that the greater thickness as well as the heat-resistant quality of the rubber would, for the Hengar and similar tubes, be sufficient recommendation for a change-over, providing that one used either ground or new-type Kjeldahl flasks.

RESULTS

I. Accuracy and Replicability of the Methods.

II. The N Content of Protein Fractions in the Experimental Samples.

III. The Relative Amounts of the Protein Fractions in the TN of the Experimental Samples.

IV. Analysis of Variance of TN.

1. Synopsis of Calculations.

2. Calculation of Standard Errors and Significant Differences.

RESULTS

For convenience in presentation, this section is divided into four parts:

I. The accuracy and the precision (or replicability) of the methods used herein.

II. The N content of various protein fractions in the experimental samples.

III. The relative amounts of the protein fractions (CN, AN, GN, PPN and NpN) in the TN of the experimental samples.

IV. The analysis of variance of TN.

I. Accuracy and Replicability of the Methods.

To increase reliability of a result, analytical determinations are often done in duplicate. This practise was adopted for this study. With the exception of comparatively few which were done singly, all determinations were made in duplicate, the results of which agreed closely for the most part. Determinations that exceeded a deviation from the arithmetic mean of 0.15 ml. of standard acid (14.28mM) were repeated.

To test the reliability of the Shahani and Sommer (55) micro Kjeldahl methods, modified as described in the previous section, the TN, NcN, PPN, GN and NpN fractions of a random sample of milk (Holstein) were determined in quintuplicate. From the arithmetic mean of each set of determinations, the root mean square deviation, represented by the Greek letter sigma (σ), and commonly known as the standard deviation (S.D.)

was calculated. The S.D. is a measure of the variability of each replicate from the mean, and was calculated according to the formula (Snedecor, "Statistical Methods". 1946):

$$\sigma = \sqrt{\frac{\Sigma (x - M)^2}{n - 1}}$$

where x is the observed determination, M is the arithmetic mean, and n is the total number of determinations. The coefficient of variation, which is $\frac{S.D.}{M} \times 100$, was also calculated. The results are shown in Table 1.

To test the efficiency of performance of the modified steam distillation apparatus shown in Figure 1, and thus to provide an estimate of the accuracy of the method by the degree of recovery, determinations were made on a dilute ammonia solution. The titration equivalent of 5 ml. of this solution was 4.90 ± 0.01 ml. of standard HCl (14.28mM). A 5-ml. aliquot of the ammonia solution was pipetted (submerged) into acidulated distilled water contained in a 100 ml. Kjeldahl flask, which was then fitted to the adapter, made alkaline, and steam distilled in the usual manner. The titration values of six consecutive determinations were: 4.91, 4.92, 4.92, 4.93, 4.92 and 4.93 ml. of the standard HCl.

II. <u>The N Content of Protein Fractions in the Experimental Samples</u>.

The results, expressed as mg. N %, of the TN, CN, AN,

Table 1. The replicability of N determinations of five aliquots of five different milk protein fractions.

Det'n	Replicates, calc'd as mg. N %	Mean	S.D.	Coeff. of Variation (%)
TN	502.4, 504.0, 502.4 504.0, 500.8	502.72	1.329	0.264
NcN	110.8, 111.2, 111.2, 110.2, 111.4	110.96	0.860	0.778
PPN	46.5, 46.0, 46.2, 46.4, 46.0	46.22	0.456	0.99
GN	23.4, 22.4, 21.8, 21.0, 22.0	22.12	0.879	3.97
NpN	24.7, 24.5, 24.8, 24.7, 24.4	24.62	0.164	0.665

GN, PPN and NpN contents determined in the experimental samples of milk are tabulated according to individual herds and months for each of the six N categories in Tables 2, 3, 4, 5, 6 and 7. The means of the data presented in these tables provide the bases for two series of Figures, namely: (a) those which show as histograms a comparison of the 6-month means for each of the six breeds for each of the six categories (arranged together in Figure 2), and (b) those which show the monthly fluctuations of the six categories for each of the six breeds (Figures 3, 4, 5, 6, 7 and 8). In each of the two series, the values for the Red Poll breed (herd no. 12) represent only the period Dec. - Mar. inclusive, inasmuch as samples were not available for October and November. Details relevant to the herds numbered 1-12 are listed in the Appendix under A.N.1. In Table 5, the globulin values were obtained by correcting the analytical values by 12 mg. N (equal to a titration blank of 1.20 ml. standard acid for the filter paper used in the determinations).

Table 2. Total Nitrogen, as mg. N %, for the period Oct. 1952 - Mar. 1953.

Breeds	Herd Numbers	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Mean
Holstein	1	498.3	490.4	507.3	539.2	518.4	469.6	503.9
	2	540.4	518.2	502.6	525.6	553.6	503.2	523.9
	7	548.2	497.4	492.5	494.8	462.0	464.0	493.2
	Mean	529.0	502.0	500.8	519.9	511.3	478.9	507.0
Jersey	3	661.4	600.7	609.1	587.4	539.2	513.6	585.2
	6	681.3	634.7	647.9	661.6	609.6	618.4	642.2
	8	649.5	658.2	645.5	624.8	552.8	517.6	608.1
	Mean	664.1	631.2	634.2	624.6	567.2	549.9	611.8
Ayrshire	4	569.2	552.0	540.2	571.2	547.6	532.0	552.0
	5	588.8	590.8	615.8	609.8	544.8	533.6	580.6
	10	547.1	510.3	513.6	547.6	528.0	557.6	534.0
	Mean	568.4	551.0	556.5	576.2	540.1	541.1	555.5
Guernsey	9	628.4	629.3	624.4	620.8	612.8	559.2	612.5
Br. Swiss	11	589.2	584.9	552.0	533.6	476.0	544.0	546.6
Red Poll	12	-	-	552.4	572.4	576.4	556.8	564.5
Mean for 6 breeds		595.8	579.7	570.0	574.6	547.3	538.3	562.2

Table 3. Casein Nitrogen, as mg. N %, for the period Oct. 1952 - Mar. 1953.

Breeds	Herd Numbers	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Mean
Holstein	1	393.0	372.2	388.3	420.4	414.8	361.9	391.8
	2	417.8	400.9	387.7	410.9	447.6	392.6	409.6
	7	421.1	380.8	388.2	396.3	363.8	374.4	387.4
	Mean	410.6	384.6	388.1	409.2	408.7	376.3	396.2
Jersey	3	528.1	476.5	478.1	464.6	428.9	398.2	462.4
	6	532.8	489.0	510.1	529.1	499.6	492.8	508.9
	8	518.3	527.8	519.5	500.4	437.4	407.6	485.2
	Mean	526.4	497.8	502.6	498.0	455.3	432.9	485.5
Ayrshire	4	423.5	421.6	397.5	456.0	428.5	409.2	422.7
	5	438.5	438.7	476.6	466.9	421.6	403.3	440.9
	10	431.1	399.9	405.6	433.7	418.5	447.0	422.6
	Mean	431.0	420.1	426.6	452.2	422.9	419.8	428.7
Guernsey	9	488.3	482.7	499.0	482.4	479.6	446.0	479.7
Br. Swiss	11	454.0	458.0	442.9	419.4	367.9	448.5	431.8
Red Poll	12	-	-	442.5	462.6	465.3	459.7	457.5
Mean for 6 breeds		458.9	440.7	444.7	453.5	431.1	420.2	441.5

Table 4. Albumin Nitrogen, as mg. N %, for the period Oct. 1952 - Mar. 1953.

Breeds	Herd Numbers	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Mean
Holstein	1	47.2	50.9	48.8	45.8	52.7	48.7	49.0
	2	60.8	44.1	36.3	42.3	54.5	42.4	46.7
	7	40.0	41.1	50.4	41.7	44.7	34.4	42.0
	Mean	49.3	45.4	45.2	43.3	50.6	41.8	45.7
Jersey	3	47.8	62.8	60.8	57.6	35.2	55.2	53.2
	6	54.6	69.2	57.7	53.3	60.8	51.1	57.8
	8	47.1	56.1	66.2	65.0	61.6	42.8	56.5
	Mean	49.8	62.7	61.6	58.6	52.5	49.7	55.8
Ayrshire	4	49.2	48.1	45.3	33.8	41.1	54.6	45.4
	5	52.4	49.0	50.9	56.2	68.2	42.7	53.2
	10	32.1	37.9	44.3	49.5	56.3	47.5	44.6
	Mean	44.6	43.0	46.8	46.5	55.2	48.3	47.7
Guernsey	9	39.9	47.0	50.7	59.0	69.7	45.3	51.9
Br. Swiss	11	63.2	52.2	50.9	58.6	47.3	33.9	51.0
Red Poll	12	-	-	47.4	55.2	58.7	44.3	51.4
Mean for 6 breeds		49.4	50.1	50.4	53.5	55.7	43.9	50.2

Table 5. Globulin Nitrogen, as mg. N %, for the period Oct. 1952 - Mar. 1953.

Breeds	Herd Numbers	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Mean
Holstein	1	14.4	17.4	18.9	19.8	12.9	14.0	16.2
	2	15.4	19.8	21.6	20.8	15.5	22.0	19.2
	7	23.7	23.3	9.0	12.0	17.0	16.2	16.9
	Mean	17.8	20.2	16.5	17.5	15.1	17.4	17.4
Jersey	3	29.0	17.4	16.1	18.5	21.3	18.2	20.1
	6	30.2	21.3	24.0	18.6	13.0	23.7	21.8
	8	27.5	21.9	13.8	18.0	18.6	22.0	20.3
	Mean	28.9	20.2	18.0	18.4	17.6	21.3	20.7
Ayrshire	4	23.7	20.8	27.9	22.4	20.0	15.0	21.6
	5	21.0	29.4	29.7	23.3	11.3	28.0	23.8
	10	24.4	22.8	14.9	11.5	9.3	20.1	17.2
	Mean	23.0	24.3	24.2	19.1	13.5	21.0	20.9
Guernsey	9	31.9	31.8	16.1	21.3	13.3	18.5	22.1
Br. Swiss	11	23.2	20.7	11.1	13.2	15.0	18.0	16.9
Red Poll	12	-	-	14.5	11.9	10.4	14.8	12.9
Mean for 6 breeds		24.9	23.4	16.7	16.9	14.2	18.5	19.1

Table 6. Proteose-peptone Nitrogen, as mg. N %, for the period Oct. 1952 - Mar. 1953.

Breeds	Herd Numbers	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Mean
Holstein	1	16.9	28.4	24.5	24.6	11.3	22.5	21.4
	2	19.7	20.4	30.5	24.7	10.0	21.6	21.2
	7	29.2	26.4	23.5	21.1	13.2	16.9	21.7
	Mean	21.9	25.1	26.2	23.5	11.5	20.3	21.4
Jersey	3	41.6	19.0	23.0	18.9	24.5	16.0	23.8
	6	34.9	31.8	27.9	22.5	13.7	20.6	25.2
	8	28.6	27.9	26.4	17.9	14.4	23.0	23.0
	Mean	35.0	26.2	25.8	19.8	17.5	19.9	24.0
Ayrshire	4	43.1	33.6	33.9	27.9	26.5	22.2	31.2
	5	42.7	45.2	31.1	31.1	21.5	34.6	34.4
	10	30.4	25.2	22.2	22.5	18.9	20.5	23.3
	Mean	38.7	34.7	29.1	27.2	22.3	25.8	30.3
Guernsey	9	41.0	33.5	26.5	30.9	14.8	18.1	24.2
Br. Swiss	11	18.9	23.4	15.7	12.0	15.7	13.5	16.5
Red Poll	12	-	-	25.8	19.5	18.0	17.0	20.1
Mean for 6 breeds		31.1	28.6	24.4	22.2	16.6	19.1	23.8

Table 7. Nonprotein Nitrogen, as mg. N %, for the period Oct. 1952 - Mar. 1953.

Breeds	Herd Numbers	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Mean
Holstein	1	26.8	21.5	26.8	28.6	26.7	23.5	25.6
	2	26.8	23.0	26.5	26.9	26.0	24.6	25.6
	7	34.2	25.8	21.4	23.7	23.3	22.1	25.1
	Mean	29.3	23.4	24.9	26.4	25.3	23.4	25.4
Jersey	3	26.0	25.0	31.1	27.8	29.3	26.0	27.5
	6	28.8	23.4	28.2	28.1	22.5	30.2	26.9
	8	28.0	24.5	19.6	23.5	20.8	22.2	23.1
	Mean	27.6	24.3	26.3	26.5	24.2	26.1	25.8
Ayrshire	4	29.8	27.9	35.6	31.1	31.5	31.0	31.1
	5	34.2	28.5	26.9	31.1	22.2	25.0	28.0
	10	29.1	24.5	26.6	30.4	25.0	22.5	26.4
	Mean	31.0	27.0	29.4	30.9	26.2	26.2	28.5
Guernsey	9	27.3	34.3	32.1	27.2	35.4	31.3	31.3
Br. Swiss	11	25.9	30.6	31.4	30.4	30.1	30.1	29.7
Red Poll	12	-	-	22.2	23.2	24.0	21.0	22.6
Mean for 6 breeds		28.2	27.9	27.7	27.4	27.5	26.4	27.7

H – HOLSTEIN
J – JERSEY
A – AYRSHIRE
G – GUERNSEY
B – BROWN SWISS
R – RED POLL

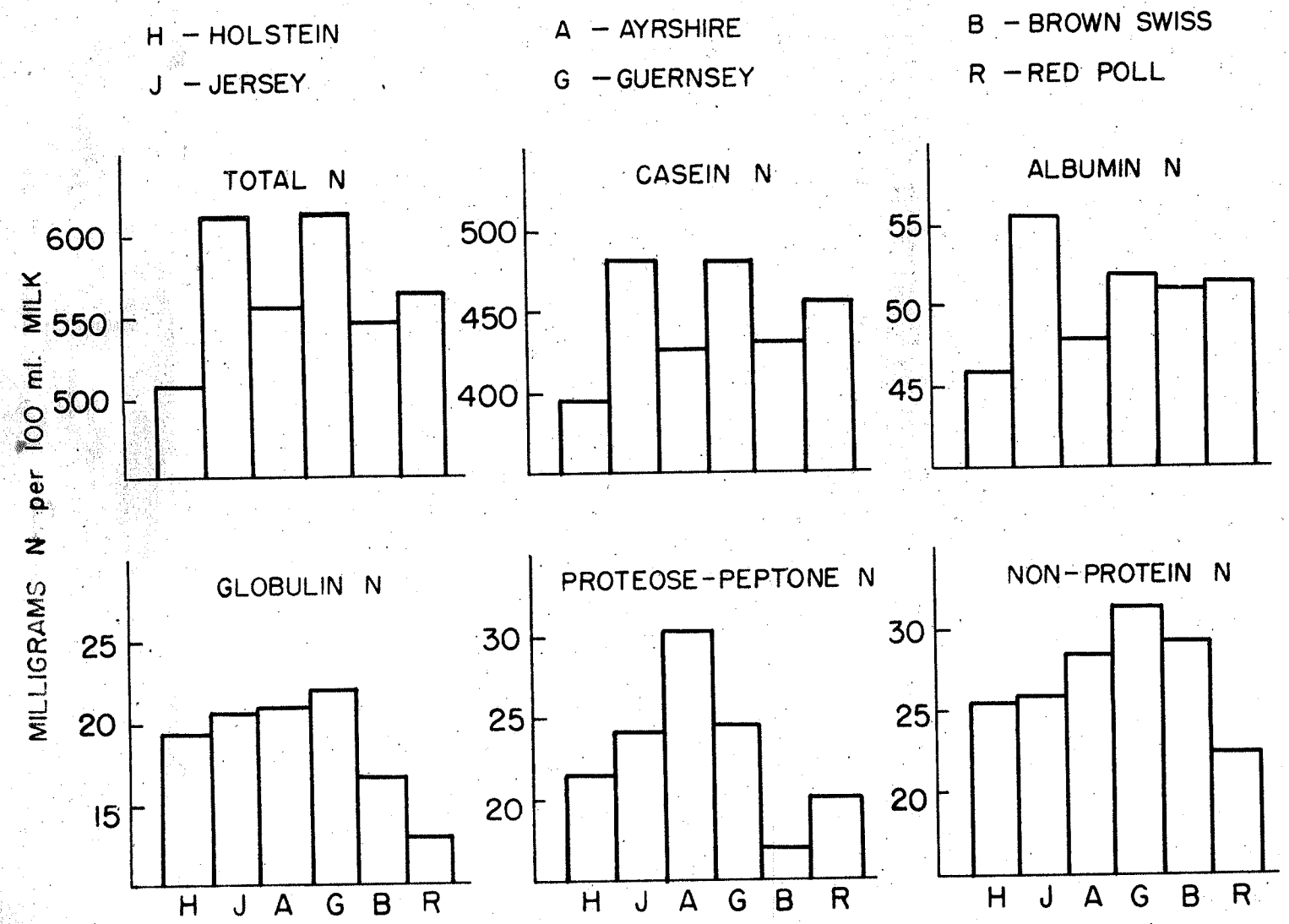

FIGURE 2. MEAN N DISTRIBUTION (OCT.–MAR.) FOR SIX DAIRY BREEDS

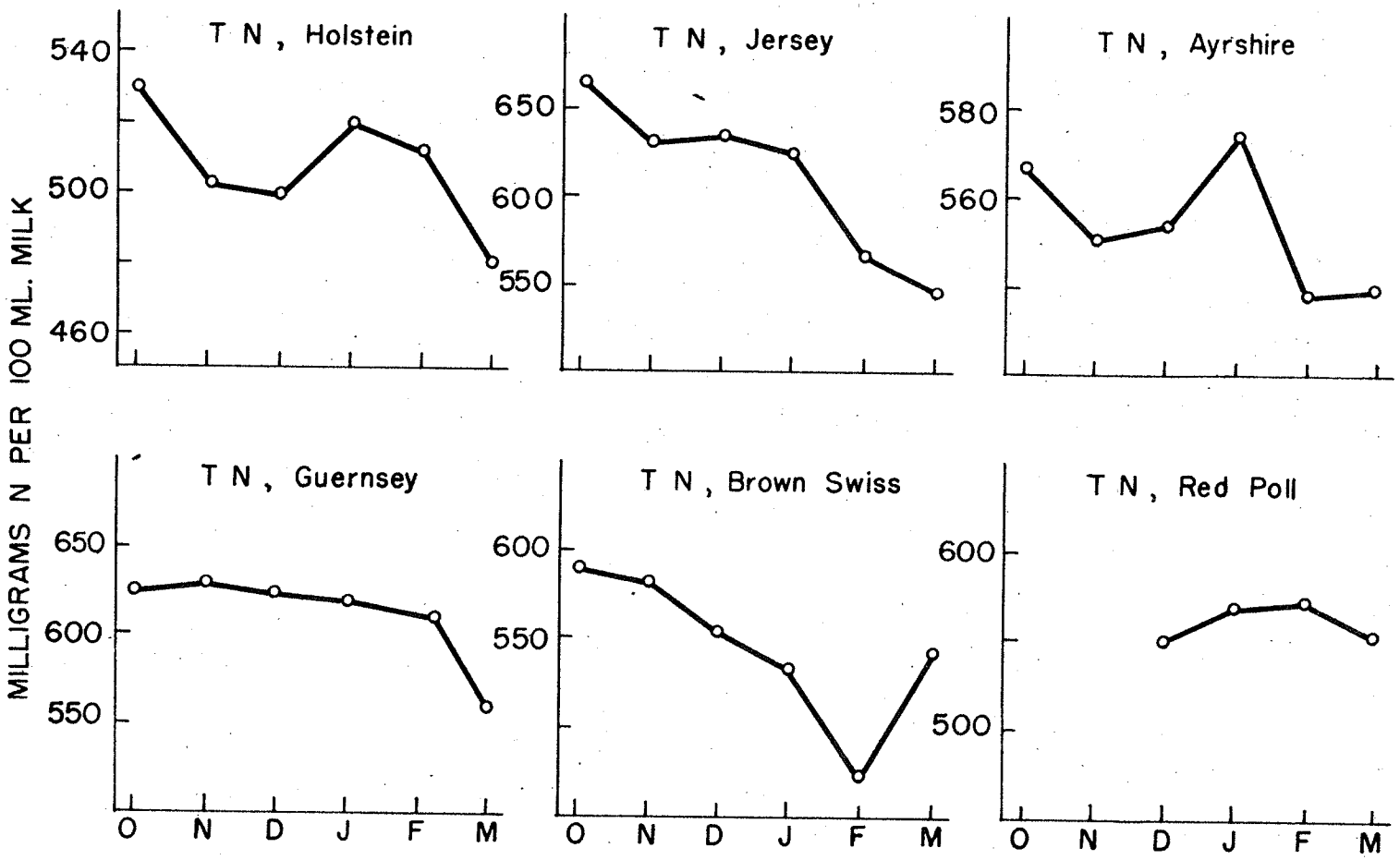

FIGURE 3. MONTHLY FLUCTUATIONS (OCT.–MAR.) IN TOTAL N

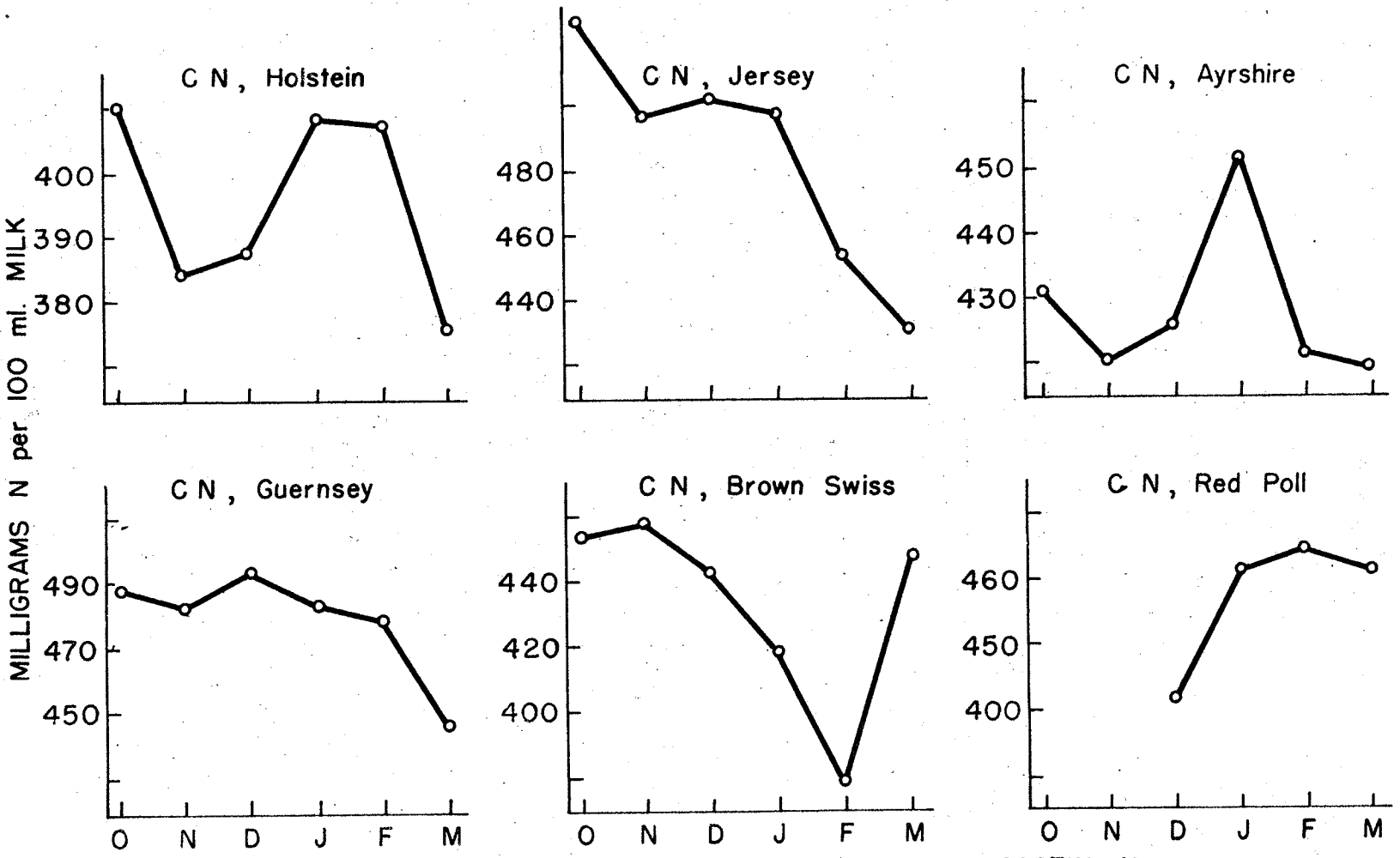

FIGURE 4. MONTHLY FLUCTUATIONS (OCT.—MAR.) IN CASEIN N

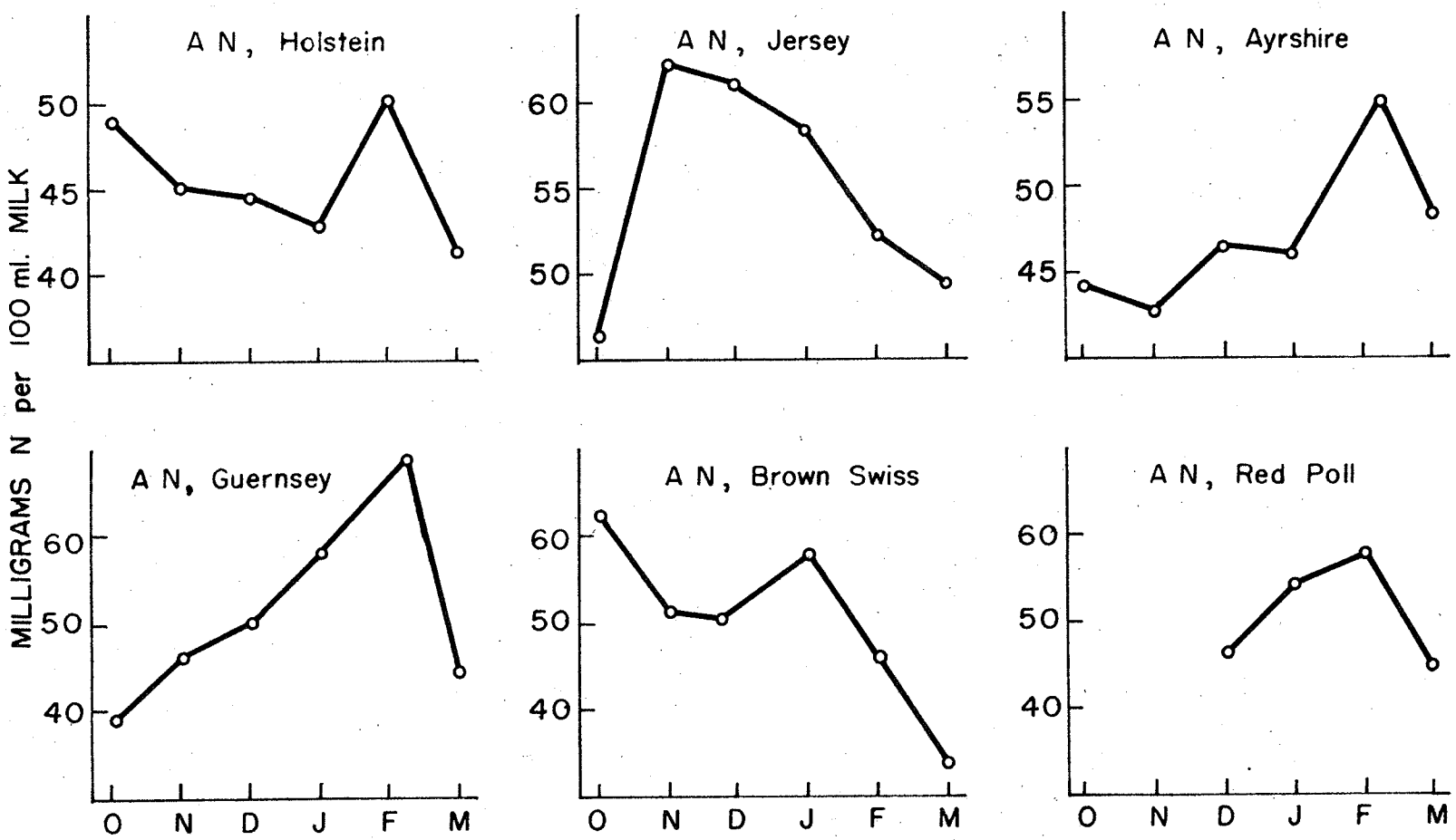

FIGURE 5. MONTHLY FLUCTUATIONS (OCT.–MAR.) IN ALBUMIN N

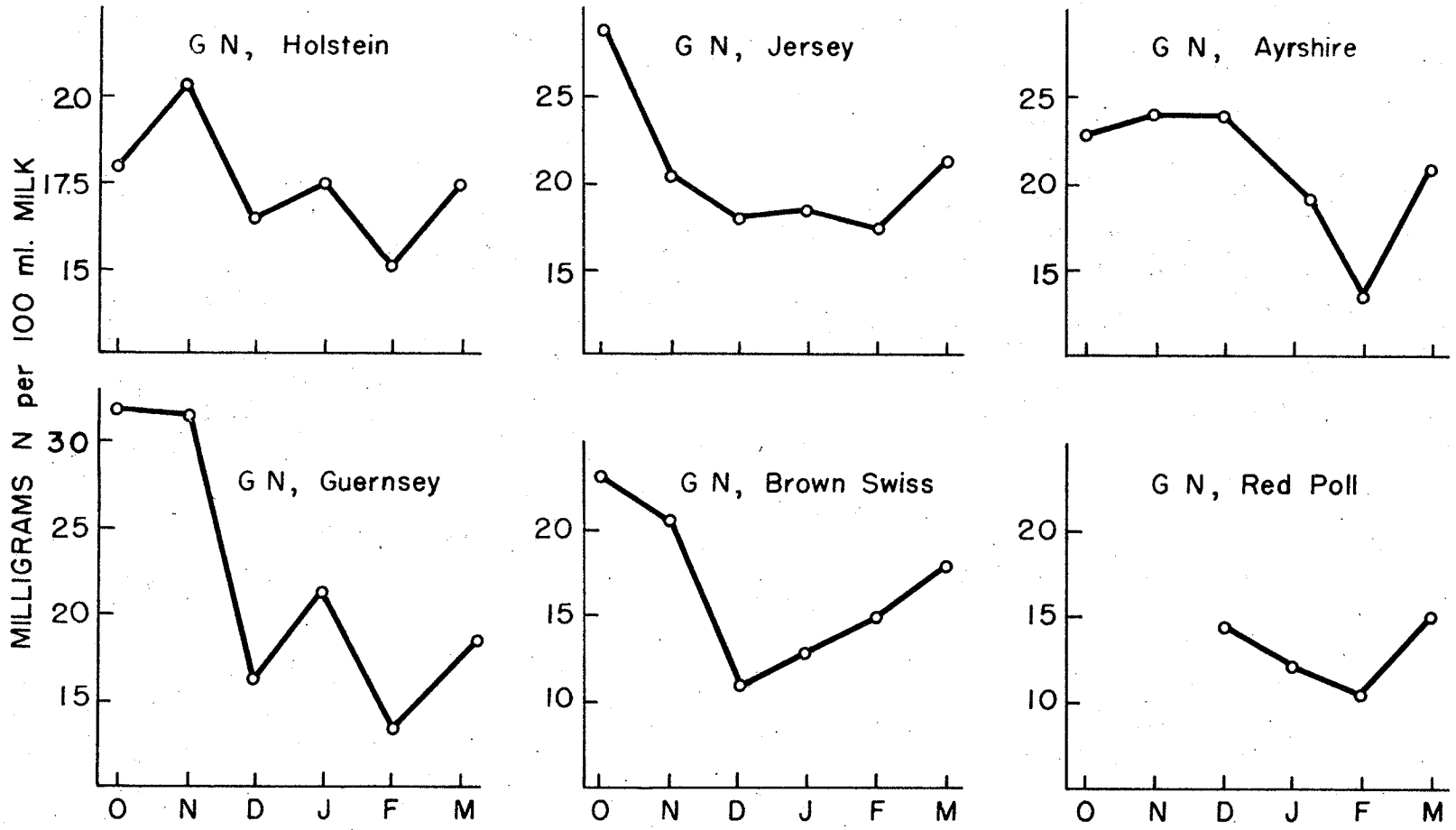

FIGURE 6. MONTHLY FLUCTUATIONS (OCT.–MAR.) IN GLOBULIN N

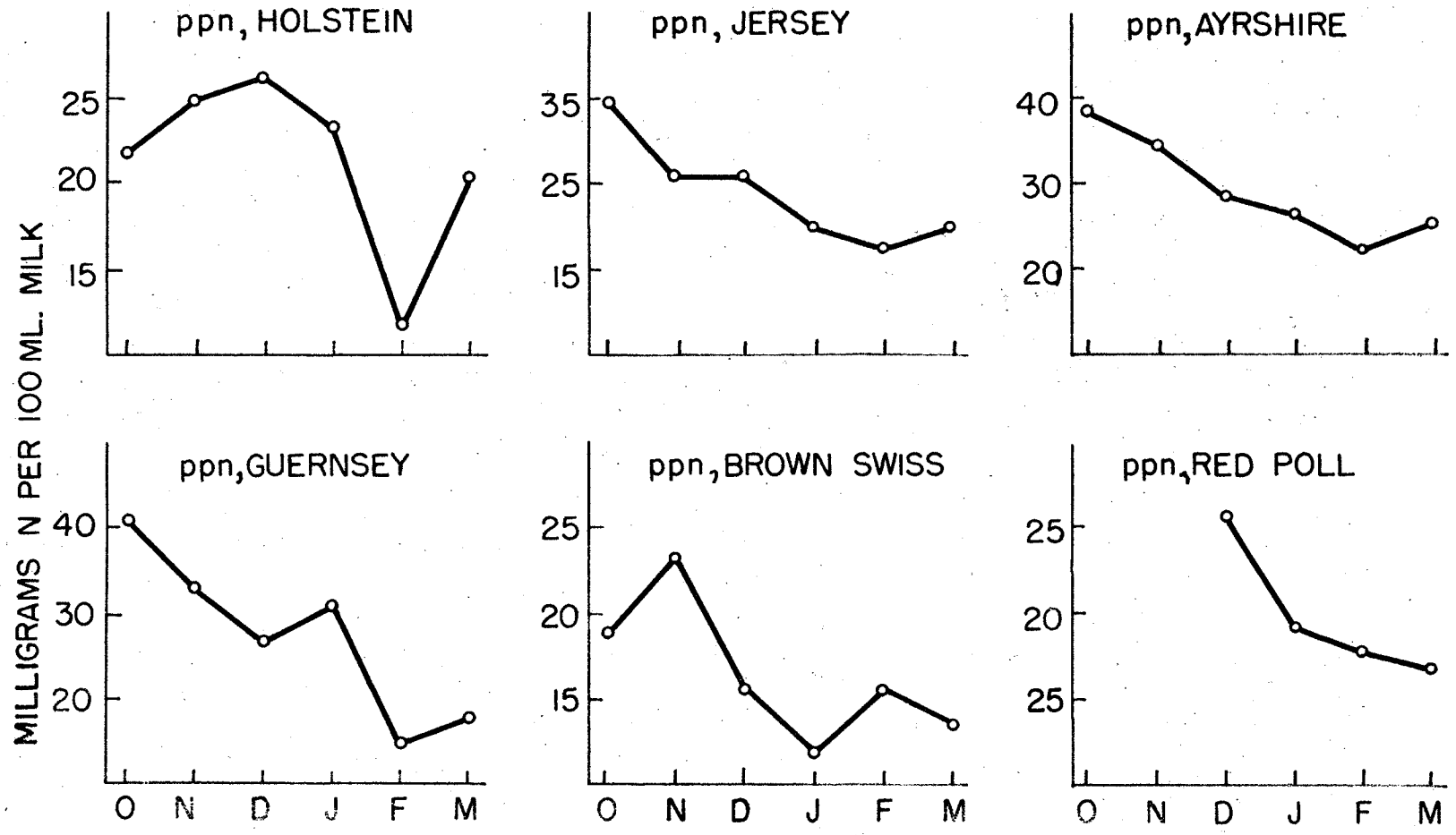

FIGURE 7. MONTHLY FLUCTATIONS (OCT.– MAR.) IN PROTEOSE–PEPTONE N.

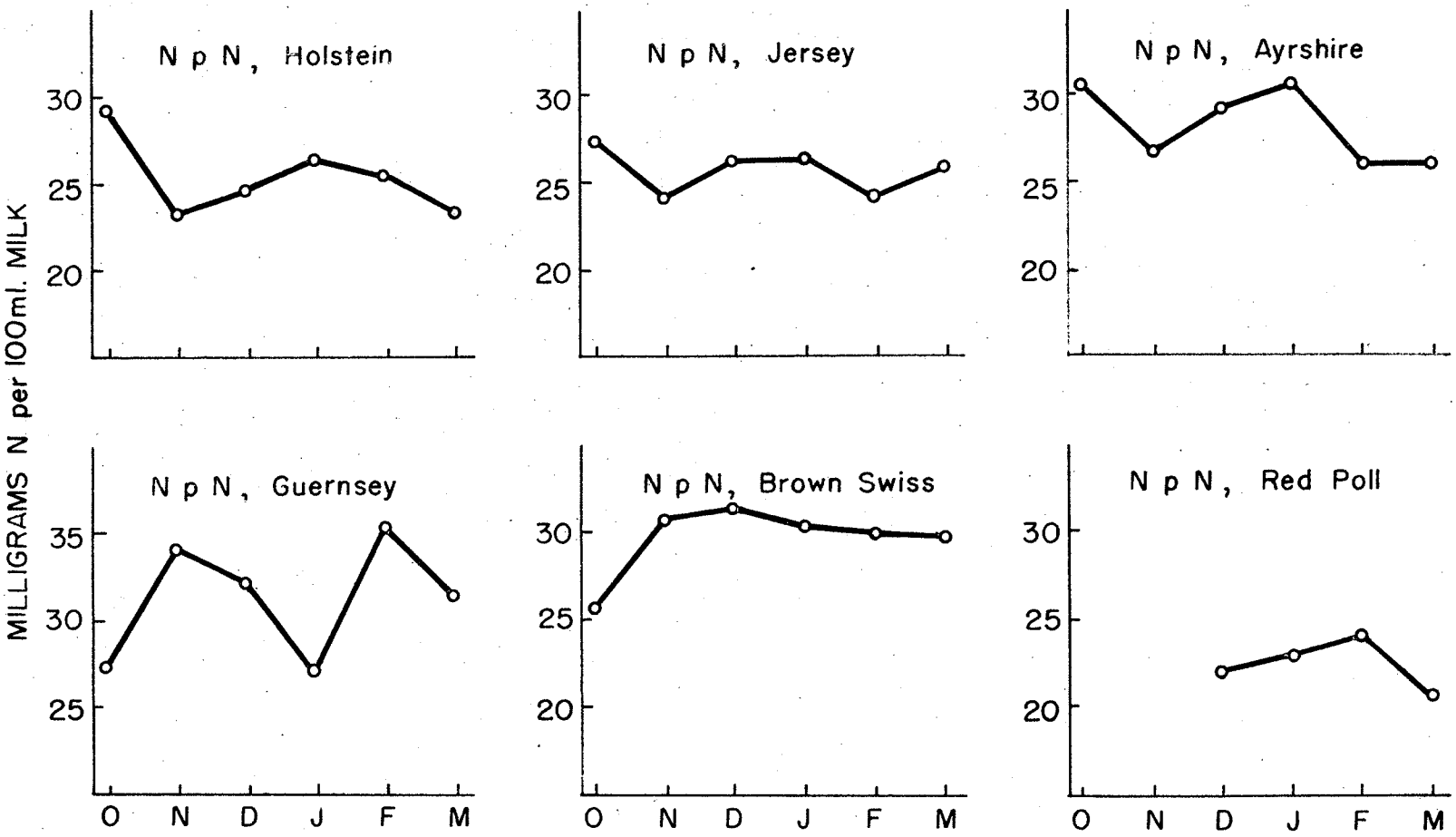

FIGURE 8. MONTHLY FLUCTUATIONS (OCT.–MAR.) IN NON-PROTEIN N.

III. The Relative Amounts of CN, AN, GN, PPN and NpN in the TN of the Experimental Samples.

The data in the previous section (Tables 2-7) concerning comparative N distribution in the milk of six dairy breeds for the first six months of this study were presented in absolute terms, namely, mg. N / 100 ml. milk at 60° F. This section considers the means of the data given in the previous section and presents them in relative terms, as percentage composition of TN, as shown in Table 8 that follows. In the column entitled "TN as protein", TN was equated to total protein, in line with conventional practise, using the conversion factor, % TN (by weight) x 6.38. Using specific gravity values determined at 60° F., volume relations at 60° F. are readily converted to a weight basis, and TN percent by weight is thus obtained.

Table 9 shows a comparison of data on N distribution in milk. The data are derived from four sources, namely, Shahani and Sommer (56), Rowland (51), Davies (6), and the present work. In Table 9, the TN means listed by each source are used as denominators, so that differences due to breed are minimal, as may be inferred from consideration of the data of Table 8. The values abstracted from the three reports in question (56, 51, 6) represent means for pooled (mixed milk) samples, whereas those from the present work are means for six months for all six breeds (see bottom line of Table 8.).

Table 8. Relative amounts of CN, AN, GN, PPN and NpN in the Total N.

Breed	Herd No.'s	TN as Protein	$\frac{CN}{TN}$	$\frac{AN}{TN}$	$\frac{GN}{TN}$	$\frac{PPN}{TN}$	$\frac{NpN}{TN}$
		%	%	%	%	%	%
Holstein	1	3.17	77.8	9.72	3.21	4.25	5.08
	2	3.27	78.2	8.91	3.66	4.05	4.88
	7	3.09	78.5	8.51	3.43	4.40	5.09
	Mean	3.17	78.2	9.05	3.43	4.23	5.02
Jersey	3	3.70	79.0	9.09	3.43	4.07	4.70
	6	3.99	79.4	9.00	3.39	3.92	4.19
	8	3.86	79.9	9.29	3.34	3.78	3.80
	Mean	3.85	79.4	9.13	3.39	3.92	4.23
Ayrshire	4	3.44	76.6	8.22	3.91	5.65	5.63
	5	3.64	76.0	9.16	4.10	5.92	4.82
	10	3.27	79.2	8.35	3.22	4.36	4.94
	Mean	3.45	77.3	8.58	3.74	5.31	5.13
Guernsey	9	3.84	78.3	8.47	3.61	3.95	5.11
Br. Swiss	11	3.38	79.0	9.33	3.09	3.02	5.43
Red Poll	12	3.50	81.0	9.11	2.29	3.56	4.00
Mean for six breeds		3.53	78.6	8.93	3.39	4.24	4.80

Table 9. Comparative data (means) on N distribution in milk.

Data from:	$\frac{CN}{TN}$	$\frac{AN}{TN}$	$\frac{GN}{TN}$	$\frac{PPN}{TN}$	$\frac{NpN}{TN}$
	%	%	%	%	%
1. Rowland (51)	78.3	9.1	3.5	4.1	5.0
2. Davies (6)	76.4*	13.0**	4.6***	-	6.0
3. Shahani - Sommer (56)	78.9	7.7	4.7	3.6	5.0
4. Present work	78.7	8.9	3.4	4.2	4.8

* As pointed out by Rowland (51), Davies' CN values are relatively low because of incomplete recovery of CN by Moir's method which was used (6). (For comparative purposes only, and specifically for the table above, adding 1% to Davies' 76.4 figure would not be out of place as a "correction" due to incomplete recovery.)

** Davies' AN figure of 13.0 includes PPN, which was not separately determined. Deducting 4% for PPN would reduce the AN value to 9.0.

*** Since Davies' CN figure is too low by at least 1% due to inadequacy of method, the GN value is accordingly 1% high, other things being equal, since GN is derived from the NcN.

According to the foregoing rationale, Davies' CN, AN and GN figures, "corrected", would be 77.4, 9.0 and 3.6 respectively. On this basis, the sums of AN + GN for the four sources, in descending order, are 12.6, 12.6, 12.4 and 12.3, whereas the AN:GN ratios, in the same order, are 2.6, 2.5, 1.6 and 2.6.

IV. Analysis of Variance of TN.

It is obvious from the preliminary review of factors that influence composition of milk that a considerable degree of heterogeneity is intrinsic to this experiment. Because of this, arithmetic means, such as those given in Tables 2 - 7, cannot be used per se as absolutely reliable indices of differences in composition due to breed and month. To estimate the degree of certainty or probability attachable to inferences that might be made from Tables 2 - 7, an analysis of variance was attempted. In the latter regard, the writer wishes to acknowledge the active interest of Dr. R. F. Peterson, Officer-in-charge, Dominion Laboratory of Cereal Breeding, Winnipeg, who set up the analysis and checked the procedure that was followed.

The analysis of variance was conducted for the three major breeds, namely, Holstein, Jersey and Ayrshire, and was determined for TN only, inasmuch as TN is widely used as a measure of the total protein content. Table 10 shows the analysis of variance of TN. The appropriate variance to use for testing the significance of differences between breeds for data obtained over the six-month period is the interaction variance, and this procedure was followed in calculating the F values shown in this analysis (cf. Goulden - p. 130, Statistical Methods of Analysis. 1939.)

Table 10. Analysis of Variance of Total N.

Source of Variation	Degrees of Freedom (D.F.)	Sum of Squares (S.S.)	Mean Square (Variance)	F	F, 5% point	F, 1% point
Breeds	2	99,152.0582	49,576.0291	40.01	4.10	7.56
Months	5	24,132.1304	4,826.4261	3.89	3.33	5.64
Interaction	10	12,391.9440	1,239.1944	1.236		
Error	36	36,248.7600	1,006.7600			
Total	53	171,924.8926				

Synopsis of Calculations

1. Total Sum of Squares (T.S.S.)= $498.3^2 + 540.4^2 + \ldots$

$- \frac{T^2}{N}$ (correction factor) = $16,993,393.80 - \frac{30,139.0^2}{54}$

= 171,924.8926.

2. S.S. for months = $\frac{5,284.2^2 + 5,052.7^2 + \ldots}{9} - \frac{T^2}{N}$

= 24,132.1304.

3. S.S. for breeds = $\frac{9,125.7^2 + 11,013.3^2 + \ldots}{18} - \frac{T^2}{N}$

= 99,152.0582.

4. S.S. for interaction = $\frac{1,586.9^2 + 1,992.2^2 + \ldots}{3} - \frac{T^2}{N}$

- S.S. months - S.S. breeds = 12,391.9440.

5. S.S. for error = T.S.S. - S.S. months - S.S. breeds

- S.S. interaction = 36,248.7600.

Calculation of Standard Errors and Significant Differences

The term 'standard error', which is coming into general use in place of the S.D. of a sample mean (Goulden - Methods of Statistical Analysis) is symbolized by $S_{\bar{x}}$, and is also abbreviated by others as S.E. The probability of obtaining a significant result depends in part on the S.E.. Since the exact calculation of probability is quite complicated, use of S.E. by itself can give us approximations that are good enough for practical purposes.

1. The S.E. of a single value = $\sqrt{1,239.1944}$ = 35.2022

2. The S.E. of a breed mean = $\sqrt{\frac{1,239.1944}{18}}$ = 8.2972

3. The S.E. of a monthly mean = $\sqrt{\frac{1,239.1944}{9}}$ = 11.7340

4. The S.E. of a difference between the means of any two breeds is 8.2772 x $\sqrt{2}$ = 11.7322

The difference between the means of the Holstein and Jersey breeds is 611.8 - 507.3 = 104.5.

Applying Student's t-distribution test, the calculated t value = $\frac{104.5}{11.7322}$ = 8.907.

Using the "t" tables, the t values corresponding to the interaction variance at the 5% and 1% points for 10 degrees of freedom are 2.23 and 3.17 respectively. As the calculated t value of 8.907 is considerably larger, this provides statistically valid proof that the difference between the means of the Holstein and Jersey breeds is highly significant. Similarly the means for the Holstein and Ayrshire breeds differ significantly, as do also the means for Jersey and Ayrshire.

DISCUSSION

1. The Accuracy and Precision of the Methods.
2. The Absolute Amounts of N in the Protein Fractions.
3. The Relative Amounts of the Protein Fractions in the TN.
4. The Analysis of Variance of TN.
5. Miscellaneous Aspects.

DISCUSSION

As stated in the Introduction, the general aim of this investigation was to obtain data over a period of time on the N distribution (TN, CN, AN, GN, PPN and NpN) in the milk from twelve herds representing six dairy breeds. We shall attempt to assess the results obtained in this study to date, and to consider some tentative inferences that may be derived therefrom. The term "tentative" is used advisedly, since it is recognized that a half-year is not long enough for a complete study of this kind.

The discussion will deal with the results in the order already given, namely (1) the accuracy and precision of the methods, (2) the absolute amounts of N in the various protein fractions, (3) the relative amounts of the protein fractions in the TN, and (4) the connotations of the analysis of variance of the TN. We shall also consider a fifth section, namely, (5) miscellaneous aspects. The latter is reserved for discussion of various aspects not treated under sub-sections 1 - 4.

1. The Accuracy and Precision of the Methods

The Shahani-Sommer methods (55), with minor modifications as indicated herein, show good precision in the tests made on replicability (Table 1). Except for the GN tests, where a coefficient of variation of 4% was obtained, that for TN, NcN, PPN and NpN were all under 1%. Possibly

a systematic, albeit not necessarily exhaustive, study of factors that influence variability in the GN determinations would indicate modifications that could reduce the GN variation to less than 1%. The accuracy and precision of the distillation step were good. Determination of the accuracy of the digestion step, using purified protein fractions, was omitted, but this might be a fruitful field for study.

The writer's schedule during his term of service allowed little time for research into methodology. Nevertheless, at least three points were indicated in this regard:

(a) GN values should be corrected for the blank value of the filter paper. All filter papers tested, even those taken from the center of a newly opened cellophane-wrapped box, had an apparent GN value which ranged from 5.0 - 15.0 mg. GN, depending on the grade of paper used. The reagents used gave check tests which were invariably N-free. The reciprocal relation between AN and GN has been noted, and the effect of uncorrected GN is to make the AN:GN ratio less than 2.0, such as that obtained by Shahani and Sommer (56), instead of over 2.5, as obtained by Rowland (51), Davies (6), and in the present work. With regard to bulk samples of mixed milk, and in comparison with the all-breed means shown here in Table 8, it is of

interest that the sums of GN + AN are all in reasonably close agreement in the four cases quoted, allowing for experimental differences. The point at issue, accordingly, is one of relative proportionation of AN and GN, and not of absolute levels (see Table 9).

(b) A small but nevertheless significant increase in N recovery could be effected by lowering the sulfuric acid:sodium sulfate ratio from 3:1 (55) to 3:2. The matter was not pursued exhaustively inasmuch as the writer merely wished to ascertain a point raised in recent investigations of others regarding the empirical nature of Kjeldahl methods, and the importance of correct time-temperature relations. Inasmuch as the Shahani-Sommer methods gave consistent and reproducible results, and were based on methods employed by other workers investigating N distribution, no departure from the 3:1 acid:sulfate ratio was made, so that comparisons of the present work with that of others might be made more readily.

(c) A number of ways of saving time in the various stages of the procedures were tried. Changes in the steam-distillation set-up proved convenient, increased the speed of the tests, and also contributed to accuracy and precision by avoiding transfer of the digest. Small changes, such as using 0.01428 N. instead of 0.03-0.04 N. HCl (55) not only expedited calculations but made possible more

precise determination of the end points in the titrations, particularly for samples low in N.

2. The Absolute Amounts of N in the Protein Fractions.

Tables 2 - 7 show the actual amounts of TN, CN, AN, GN, PPN and NpN respectively for six months for the six breeds. In Figure 2, the 6-month means are plotted as histograms for each N fraction. In this Figure, the pattern shown by the six breeds for TN is generally replicated for CN. This is not surprising, in view of the reasonably constant CN:TN ratios, and the fact that CN constitutes almost four-fifths of the TN. The histograms for the other N fractions are not of the same pattern as those for TN and CN, but this is to be expected also, since slight differences in actual amounts are large in relation to the total amounts of these minor constituents. The least variation in composition between the six breeds is for NpN.

Figures 3 - 8 show the fluctuations by month. There is no repetitive pattern here, other than that the levels, with only one exception, are higher in October than in the late winter months. The differences in levels may be manifestations of the effect of season, and of outdoor as against indoor feeding. It would be interesting to observe the trends shown by samples taken during the summer months. Figures 3 - 8 should be taken in apposition with Figure 2.

3. The Relative Amounts of the Protein Fractions in the TN.

With two exceptions, the data of Table 8 (p. 73) indicate that within and between herds for the 6-month interval the CN, AN, GN, PPN and NpN bear a relatively constant ratio to the TN, with deviations not exceeding ∓ 1%. It would seem from this that the factors which influenced the composition of the milk samples affected the absolute amounts of the fractions, but the ratio of the fractions changed comparatively little. Differences in these absolute amounts are, or should be, of prime concern in the processing and use of milk and milk products. Consequently, differences due to breed are of economic significance.

The two exceptions noted above are Ayrshire herds 4 and 5. The CN:TN ratios in the samples of herds 4 and 5 varied from 73.5 to 80%, but were generally below 76%. The milk from the third Ayrshire herd, herd 10, appeared constant in composition, showing little relative change over the months. As Table 8 shows, the low average % CN values of herds 4 and 5 lowered the Ayrshire mean value appreciably. It may be possible to account for the low CN values of the milk from herds 4 and 5 in three ways, namely, (a) physiological reasons -- individual cows may give consistently milk of low casein content (51, 54), (b) pathological reasons -- individual cows may be infected with non-recognizable latent or mild mastitis, and (c) a combination of

physiological and pathological causes. It should be noted (see Table 5) that the GN values for milk from herds 4 and 5 are not much higher, and indeed are sometimes lower, than those for milk from herd 10. Furthermore, the s.n.f. contents for herds 4 and 5 are of the same magnitude as that for herd 10. It is reasonable to suppose, therefore, that the abnormal CN values for herds 4 and 5 are due to physiological reasons. It would be of interest to investigate these herds more closely.

Note should be made of the Red Poll herd 12. All the % CN values for herd 12 are considerably and consistently higher than the 6-breed means. However, only four samples have been analysed to date.

4. The Analysis of Variance of TN.

The value of statistical methods in testing the significance or in assessing the magnitude of the effects indicated by experimental results needs no elaboration here.

It is clear from the data of Table 10 that the differences between breeds are highly significant. The differences in TN values for the various months are less marked, but they are nevertheless significant.

It is of interest that the interaction variance is not much greater than the error variance, the F value in this case being 1.236. This means that the relative TN for the three breeds did not differ significantly from month to month.

Thus, the statistical approach substantiates what Table 8 implies.

5. Miscellaneous Aspects.

(a) Change of TN values to percent total protein (TN % x 6.38) is practised widely. However, there are at least two errors in such a practise, namely:

1. NpN, which is included in the TN, accounts for approximately 5% of the TN, and thus the protein values would be too high by 5%.
2. PPN, which is also included in the TN, accounts for approximately 4.25% of the TN. It should be recalled that proteoses and peptones consist of derived and not well-characterized (i.e., heterogeneous) proteins and protein degradative products. The application of a conversion factor, such as 6.38, presupposes a N distribution that is constant and similar in the protein fractions concerned. The likelihood of this being the case for PPN on the one hand, as with CN, AN, and GN on the other, is open to question.

Although TN is converted to total protein in the simple manner aforementioned, as was done for part of Table 8 (p. 73), the writer feels that the errors and assumptions intrinsic to the conversion should be more widely recognized.

(b) Since the ratios of the protein fractions with respect to TN is similar for the six breeds examined, as shown in Table 8, it is possible to compare the breeds on the basis of the 6-month means shown in Tables 2 and 3. This we do for TN and CN only, inasmuch as the other fractions show greater variations due to their smaller amounts. The Holstein 6-month means for TN and CN are the lowest, and if we assign to them indices of 100, we get comparative figures as follows:

	Jersey	Guernsey	Red Poll	Ayrshire	Br.Swiss	Holstein
TN	120	120	111	109	108	100
CN	122	121	115*	108	109	100

*This "CN number" reflects the relatively high amount of CN obtained in the milk of the Red Poll breed (81%, as Table 8 shows).

On the basis of the ratings assigned above, one may deduce that the milks of the Jersey and the Guernsey breeds contain at least 20% more TN and CN per unit volume than that of the Holstein breed. It must be recognized, however, that while cows of the Holstein breed give lower amounts of protein per gallon in their milk than do those of the other five breeds examined, it does not necessarily follow that the <u>total yield</u> of protein is also lower, since the yield of milk also varies with breed. Thus, while the ratings

above give the Jersey breed a 20% advantage over the Holstein breed, this advantage would be nullified if it were to be shown that Holsteins produce 20% more milk.

Comparative yield data for this experiment are not available at present, since it is first necessary to correct the actual yield data for the ages of the animals, the average period of lactation, and other factors that influence yield. However, information was obtained from Record of Production (Canada Department of Agriculture) data of recent years for registered Jersey and Holstein cows in Manitoba. The Record of Production data are given in terms of annual production per cow of milk and of butterfat derived from the milk. According to these data, the average Holstein cow surpasses the production of milk of the average Jersey cow by an amount ranging from 15% to 40%. This indicates that the total yield of protein could be greater for a Holstein herd. However, the information quoted represents gross production figures which were not partitioned for variance. Interestingly, the same Record of Production data show, that in terms of total yield of butterfat, that the two breeds are rather similar, and in some cases the Jersey surpasses the Holstein breed.

The foregoing discussion has some relation to a feature that may assume a major role, namely, the probability that as milk becomes less important for its butterfat and more

important for its s.n.f. content, that the quantity of proteins present in the milk, and perhaps ultimately the quality also, will become the basis of a rational system of payment.

SUMMARY

1. A study was made of the N distribution in the milks obtained from twelve pure-bred dairy herds in Manitoba. The twelve herds included three each from Holstein, Jersey and Ayrshire, and one each from Guernsey, Brown Swiss, and Red Poll breeds.

2. Data were obtained over a six-month period for the amounts of total N, casein N, albumin N, globulin N, proteose-peptone N and non-protein N, expressed as mg. N per 100 ml. milk at 60°F.

3. The N distribution values varied according to breed and month. It was shown by an analysis of variance that the differences between breeds, and to a lesser extent between months, were significant. Although actual values varied widely, the various fractions occurred in reasonably constant ratios. This was most noticeable with the casein N and total N respectively, the casein N: total N ratio for the six breeds being 0.787. A tentative rating based on the total N and casein N data, assigning an index of 100 to the Holstein breed, placed the six breeds in the following order according to protein content per unit volume of milk: Jersey 120, Guernsey 120, Red Poll 111, Ayrshire 109, Brown Swiss 108, and Holstein 100. For a complete interpretation of the protein content, it is pointed out that information on total yield of protein for each breed is required.

4. Certain modifications were made in the micro Kjeldahl methods of Shahani and Sommer which were used in the investigation. An adapter was designed for and modifications were made in a steam distillation apparatus which eliminated the need to transfer the digest from the Kjeldahl flasks, thereby eliminating a possible source of analytical error and also increasing the speed of analysis. In replicated experiments conducted to assess the precision of the analytical technique, all the determinations had a coefficient of variation of less than 1%, except the determination of globulin N, which showed a variation of 4%. The accuracy and precision of the distillation step were good.

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APPENDIX

APPENDIX

This section contains facts that are considered essential but of secondary importance to the thesis. Each item is accorded an Appendix Note number (abbreviated as A.N.) to which appropriate reference is made in the text of the thesis.

A.N.1. Sources of Milk Samples.

Dairy Breed	Sample No.	Shipper	Location (Man.)
Holstein	1	Univ. of Manitoba	Ft. Garry
"	2	T. Townsend Rockwood Holsteins	St. Norbert
"	7	T. A. Croy	Balmoral
Ayrshire	4	Victor Nordin	Box 137, Teulon
"	5	Dom. Exptl. Station	Morden
"	10	G. Sheach	Forrest
Jersey	3	Camille Carriere	Otterburne
"	6	H. H. Smith	Stonewall
"	8	Alex Airth	Stonewall
Guernsey	9	Art Rampton	Dauphin
Brown Swiss	11	Dr. Louis Delaquis	Notre Dame de Lourdes
Red Poll	12	Geo. Wilkinson	Portage la Prairie.

A.N.2. Classification of Proteins.

According to the conventional protein classification scheme (15), proteins are (a) simple (b) conjugated or (c) derived. For purposes of this report, we are interested mainly in the basis for differentiation, according to this scheme, of the major milk proteins.

(a) Simple proteins on hydrolysis yield only α amino acids or their derivatives, and in this class we place the albumins, which are heat-denaturable, but are H_2O-soluble, and also the globulins, which are also heat-denaturable but are H_2O-insoluble. The globulins are soluble in dilute acids and bases, and in dilute NaCl solution.

(b) Conjugated proteins are simple proteins linked with various nonprotein groups. Here we place casein, which, because it is linked with phosphoric acid, is a phosphoprotein. Casein is precipitated when the pH is lowered to 4.6-4.7, its isoelectric point. (Other conjugated proteins are: chromoproteins (hemoglobin), glycoproteins (mucin), nucleoproteins, and others.

(c) Derived Proteins. This is an artificial group, and consists of derivatives of proteins resulting from the action of heat, enzymes or chemical agents. This group is divided in two classes:

primary - there has been no significant change in size of protein molecule.

<u>secondary</u> - molecules are smaller, because of hydrolysis.

A. <u>proteoses</u>	B. <u>peptones</u>	C. <u>polypeptides</u>
H_2O-soluble, not coagulated by heat; precipitated with saturated $(NH_4)_2SO_4$.	H_2O-soluble, not coagulated by heat, and not precipitated with saturated $(NH_4)_2SO_4$.	definitely characterized combinations of 2 or more amino acids.

Since each of these three subclasses represents a different stage in hydrolysis, A>B>C, as far as molecular size is concerned.

A.N.3. <u>Some Additional Points Concerning Protein Fractionation</u>.

In a criticism of the bases of conventional protein classifications, Gortner (15) points out that differences in <u>solubility</u> and <u>precipitability</u> are emphasized in interdifferentiation, and that so-called proteins may be artifacts, or derived from native proteins. Thus, the amount of globulin which may be extracted from wheat flours, for instance, is a function of the nature of the salts present and their relative concentrations. Since solubility is synonymous with peptization, it is pointed out that isolation of a protein by a prescribed technique does not necessarily involve a chemical entity, but instead may be only a peptized fraction.

The influence of mineral salts, mentioned above in

connection with coagulation of proteins (11, 12, 15), affects at the same time the pH found after the addition of a certain quantity of acid or base to a protein solution, even though the salts have no buffer action themselves (44). These aspects are just a few of the considerations which affect conventional isolation techniques.

Mention should be made of the increasing use of electrophoretic methods for protein separation. By electrophoresis, blood serum globulins have been separated into at least three fractions, viz., α, β, γ. Recently, γ globulin, presently in the news in regard to poliomyelitis therapy, has been resolved into a number of electrophoretically distinct fractions, as well as into at least two closely related antibodies (19). Thus there are available better methods (electrophoresis, ultracentrifugal, serological) for separating proteins which cannot be fractionated by conventional methods.

Finally, as emphasized by Gortner (15), we must bear in mind that the <u>in vitro</u> laboratory study of proteins may have no direct bearing on <u>in vivo</u> properties, inasmuch as proteins are not merely complex chemicals but are also colloidal micelles, and subject to the multitude of interactions characteristic of colloidal systems and certainly of living cells.

A.N.4. Exploratory Tests in the Chemical Diagnosis of Mastitis.

As mentioned in the text (p. 22), milk from cows afflicted with mastitis has a CN:TN ratio that is below normal, the normal being not less than 0.77 according to Rowland (51, 53). In view of the reported prevalence of mastitis in bovine populations, and the possibility that this could conceivably lead to generally lower CN values, the writer was interested in conducting a test of Rowland's (53) "Casein number" $\left(\frac{CN\%}{TN\%} \times 100\right)$.

For purposes of the test, milk was obtained from three cows belonging to the herd of Mr. Joseph Dufily, dairy farmer on Pembina Highway. One of the three cows served as a control, and was selected by Mr. Dufily as being normal in milk production, and in good health, with no past record of mastitis. The other two cows had mastitis in different degrees of severity, and were being treated with aureomycin. However, unlike other cows in the herd who had been treated successfully for mastitis, the two cows were not responding to the aureomycin therapy, according to Dr. M. T. Lewis, the veterinarian in attendance.

Within 2 hours after sampling, analyses for all of the nitrogen fractions were begun to lessen the possibility of change due to microbiological activity. Using an A.O.A.C. (Volhard) method, the chlorides present in the

samples were also determined. The analytical data are listed below in terms of mg. N %. The mastitic cows are designated as M_1 and M_2 respectively.

	Normal	M_1	M_2
TN	507.2	449.6	417.6
CN	403.8	341.6	275.6
AN	45.0	43.8	41.8
GN	18.0	21.0	47.8
PPN	18.6	44.8	31.5
NpN	21.8	22.0	26.9
Chlorides (mg. / 100 ml. milk)	135.0	128.0	231.0

The casein numbers for the normal, M_1 and M_2 samples are 79.6, 76.0 and 66.0 respectively, and the AN:GN ratios are 2.5, 2.1 and 0.88.

It will be evident from the data that the differences between M_1 and M_2 with respect to casein number, AN and GN values, AN:GN ratio, and chloride content are considerable, and that M_1 approaches the control sample in some respects. While the possibility of unknown factors that could vitiate the diagnostic accuracy of the casein number was considered, the correct solution to the anomaly was provided in a subsequent conversation with Mr. Dufily. It was then ascertained that cow M_1 had recovered completely two days after sampling, whereas

cow M_2 was still suffering with mastitis.

From the foregoing tests, it was tentatively concluded that a limited N distribution study (CN:TN ratio) could be applied as a sensitive and reliable indication of mastitis, inasmuch as it could differentiate mastitis that did not respond to therapy (M_2) from that which was in the process of responding (M_1). At the same time, it was recognized that quantitative determination of chlorides was also useful.

A.N.5. Exploratory Tests Involving Alteration of the Sulfate:Acid Ratio.

With reference to accuracy in Kjeldahl digestions, it is pointed out in the text (pp. 29-30) that Ogg and Willits (40) considered that 0.6-0.8 gms. of K_2SO_4 / ml. H_2SO_4 is optimal. By way of contrast, attention is drawn to the fact that the methods of Shahani and Sommer (55) call for approximately 0.3 gms. of K_2SO_4 / ml. H_2SO_4. (The actual quantity is 0.31 gms. K_2SO_4, since 1 gm. of catalyst / 3 ml. H_2SO_4 is prescribed, the catalyst being K_2SO_4:HgO, 14:1.) It was accordingly a matter of interest to the writer to ascertain the effect of raising the sulfate:acid ratio on recovery of N from a protein determination. To do so, the following tests were made:

Determinations were made in triplicate on the TN and NpN of a sample of Jersey milk. For each test the

volume of H_2SO_4 was fixed at 3 ml., but the amounts of catalyst-sulfate mixture were 1.0, 2.0 or 2.4 gms. respectively. During the digestions, flame heights were kept as uniform as possible; the after-boil time was 1 1/2 hours. Replicate determinations agreed within 1% for TN and within 1.3% for NpN, and the means are given as mg. N % below:

Determination	K_2SO_4:H_2SO_4 Ratio		
	0.31	0.62	0.74
TN	608.8	624.0	624.8
NpN	24.2	25.5	25.5

The data show that doubling the amount of catalyst can raise the TN values by about 2.5% and NpN values by about 5%. It was also observed, as was to be expected, that the time necessary for a test to arrive at the "clear" stage was shorter for those having increased amounts of sulfate.

The foregoing determinations, although limited to TN and NpN, confirm the importance (40) of optimal amounts of K_2SO_4 and their role in correct time-temperature relations. They also point to the empirical nature of Kjeldahl procedures. However, no departure from the 1:3 sulfate:acid ratio (55) was made in the procedures used by the writer, so that comparisons of the present work with that of others might be made more readily.

A.N.6. Features of Some Alternative Methods for Determining Protein N.

For determining protein and ammonia N, Kjeldahl methods are certainly the most widely used. The salient features and influencing factors of Kjeldahl methods have already been discussed in some detail (pp. 27-33). At the same time, a considerable variety of other methods are available, some of which have been indicated in the text (p. 34). Each method has particular advantages for as well as limitations in given situations, and should be weighed on its respective merits as an adjunctive or alternative approach. With the latter in mind, we will consider briefly some of the features of sealed tube methods (16, 62) and only cursorily some colorimetric methods, since both have already been discussed in the writer's preliminary report (1).

Colorimetric methods of measuring ammonia, particularly those using spectrophotometry, offer greatly increased sensitivity. When small amounts of ammonia are present in the sample, or when micro samples are used, the Nessler reaction has been applied directly to the digest (21, 59). In this way, one obviates the conventional distillation and titration steps. Other colorimetric methods are of interest, such as the sodium phenate-hypochlorite reaction (28) and the biuret reaction (10, 22, 25, 26). Use of long-path capillary absorption cells

in the spectrophotometer promotes considerable increase in sensitivity (27).

Sealed Tube Methods. To avoid use of high concentrations of K_2SO_4 (too much was shown to be conducive to destruction of the ammonium bisulfate formed in the Kjeldahl digestion (62)), and also to shorten the long digestions often required with refractory materials, White and Long (62) carried out microdetermination of heterocyclic N in heavy-walled, sealed glass (Carius) tubes at 470°C., using conc. H_2SO_4 and HgO catalyst. Using 7-inch tubes sealed with a gas-oxygen torch, and a steel box enclosing the tubes and heated in a conventional muffle furnace, the total time required was about 45 minutes, consisting of 15 minutes to reach 470°C., 15 minutes of required reaction time, and 15 minutes cooling. Accuracy and precision were good, and the pressure developed within the digestion tubes was nominal (estimated to be only a few atmospheres). The sealed tubes were opened, diluted with water, and the solution transferred to a Kjeldahl still containing NaOH-$Na_2S_2O_3$ solution. Titration by conventional methods was employed.

Grunbaum et al (16) have improved the White and Long method and adapted it for micro operation. Tubes are 7 mm. O.D. and 4.5 cm. long, and are readily made from borosilicate glass tubing. Catalyst was found unnecessary, and only conc. H_2SO_4 was added to the sample containing 1 to 15 micrograms

N. Boric acid was used as the trapping liquid. Instead of distillation, the diffusion principle was applied after Conway. It should be noted that phosphorus may also be analysed from the digest in the sealed tubes. From the digest an aliquot can be taken for N analysis, and a second aliquot for phosphorus analysis.

The main purpose in putting down a few of the foregoing details in this report is based on the impression that sealed tube technique offers important advantages over the conventional open-tube semi-micro Kjeldahl methods, and is worth testing for confirmation of the notable convenience, rapidity, accuracy and precision claimed for it. Multiple analyses can be carried out, so that two to three dozen tests may be made per man-day.