MILK ANALYSIS

Identification of Some Nitrogenous Constituents of Cow's Milk by Ion Exchange and Paper Chromatography

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The nonprotein nitrogen fraction of fresh cow's milk has been analyzed qualitatively by ion exchange chromatography. Thirty-one ninhydrin-positive peaks were obtained, 24 of which were characterized or identified. Several new micro methods and techniques were developed and applied in the characterization of some of the components. A number of previously unidentified nitrogenous compounds are reported. The use of identification procedures to supplement positional evidence is illustrated.

IDENTIFICATION of the constituents comprising the nonprotein nitrogen (NPN) fraction of milk has been undertaken from time to time using, for the most part, classical methods of isolation and characterization. On the basis of the quantitative summation of the nitrogen in the compounds identified thus far, a small part of the NPN fraction still remains unidentified.

This paper reports the probable identity of a number of nitrogenous compounds present in fresh cow's milk. A preliminary report of this study has been presented (15). Evidence to substantiate the identity of the compounds is described, together with several new micro techniques and methods which should be useful in work of this type. Since the presentation of this paper, a study of similar nature has been reported by Deutsch and Samuelsson (4) and by Samuelsson (12).

Experimental

Preparation of Sample. The ion exchange technique described by Moore and Stein (11) was employed to resolve the ninhydrin-positive compounds from fresh whole milk. The latter was prepared for analysis as follows: To a 50-ml. sample, 25 ml. of distilled water and 50 ml. of 20% trichloracetic acid were added. This was mixed thoroughly, allowed to stand for a few minutes, and centrifuged 10 minutes at 8000 r.p.m. in the Servall angle head centrifuge. The supernatant was decanted through a plug of glass wool and an 80-ml. aliquot was extracted with four 50-ml. portions of diethyl ether. The ether layer was siphoned off after each extraction. The aqueous layer was evaporated to about 2 ml. on a rotary evaporator, transferred quantitatively to a 10-ml. volumetric flask with the aid of five 1-ml. portions

of 0.4M citrate buffer (pH 2.2), and made to volume with distilled water. A 5-ml. aliquot was chromatographed on Dowex 50 (200 mesh, 4% cross linked).

Desalting. The even-numbered tubes comprising a given peak were pooled and desalted on short columns of Dowex-2 (OH$^-$ form) or on Amberlite IR-120 (H$^+$ form) essentially according to the method of Dreze, Moore, and Bigwood (5). It was necessary, however, to substitute 5N ammonia for the recommended 4N HCl for eluting the desalted fraction following phenylalanine. Elution with 4N HCl caused breakdown of the resin (both Dowex-50 and Amberlite IR-120), yielding a colored material in amounts large enough to distort considerably the paper chromatograms. The desalted eluates were reduced to dryness in vacuo usually in cellulose nitrate tubes. The residues were dissolved in 0.05 ml. of water and analyzed by paper chromatography.

Paper Chromatography. Unless otherwise stated, all chromatograms were run on 8-inch squares of Whatman No. 1 paper. The paper was pretreated prior to use. This indirectly increased the sensitivity of the color reactions by removing reactive impurities. Pretreatment consisted of desalting the paper. One hundred sheets were tied together with string, placed in a borosilicate glass dish containing distilled water, a large magnetic stirring bar, and 25 grams of Amberlite MB-3, a mixed cation-anion resin. The dish was placed on a large magnetic stirrer, covered with a glass plate, and stirred for 72 hours at room temperature. The packet was then drained of excess water and dried in a forced air oven.

Solvents. Solvent systems for identification purposes were selected, whenever possible, so as to give the maximum

separation of the compound in question from the two compounds occurring to the left and to the right of it on the Moore-Stein map. The following solvent systems were utilized: (A) methanol-water-pyridine (80-20-4); (B) phenol–water–ammonium hydroxide (77.5-21.5-1.0); (C) 2-methyl-2-propanal–water–formic acid (69.5-29.5-1.0); (D) 1-propanol–ammonium hydroxide–water (70-30-10); (E) ethanol-1-butanol-water (40-40-20); (F) 2-methyl-2-propanol-2-butanone-water-diethylamine (40-40-20-4); (G) water-saturated 2-methyl-2-butanol; (H) 80% ethanol; (I) 80% l-propanol; (J) ethyl acetate–methanol–water (50-25-25); (K) l-butanol-acetone–water (10-10-5).

Unidimensional, ascending chromatography was employed throughout except with solvent G in which case the descending technique was used on 6 × 21 inch (unwashed) serrated sheets. Authentic samples were always chromatographed on the same sheet with the unknown.

Detection. Spots of the finished chromatogram were detected mainly with the *tert*-butyl hypochlorite–Starch–KI reagent described by Schwartz and Pallansch (14) and with a 0.5% solution of ninhydrin in alcohol. The former reagent was more sensitive than ninhydrin for many of the compounds, and also facilitated the accumulation of more data than would have been possible with ninhydrin alone. Some ninhydrin-negative, nitrogen-containing compounds were detected under some of the peaks. The blue-black color of the iodine-starch complex could be readily removed by steaming the paper in an autoclave (125° C., dry steam, 15 minutes). This permitted the application of ninhydrin or other nonoxidizing reagents to the marked spots or over the entire sheet.
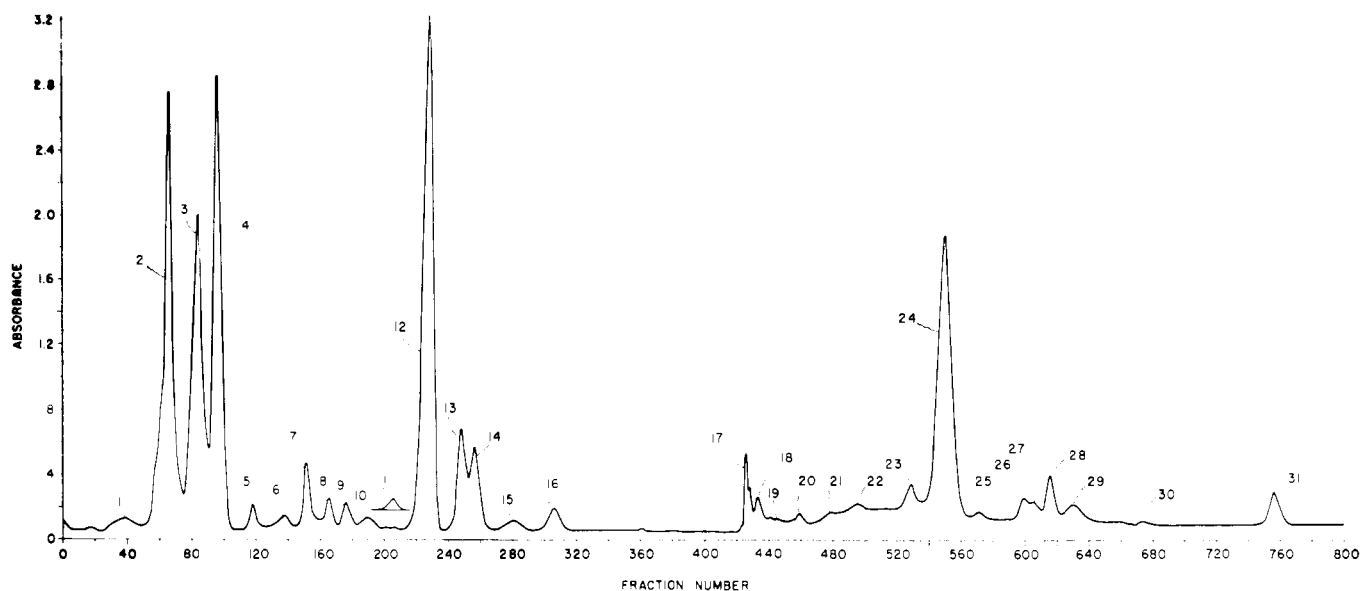
Figure 1. Ion exchange chromatogram of the ninhydrin-positive components of cow's milk serum

Later it was found that hydriodic acid (aqueous, 5% volume in 1% aqueous, soluble starch) could be substituted for potassium iodide without impairing the sensitivity of the reaction. Since hydriodic acid is volatile, steaming the paper removed the acid as well as the iodine from the starch complex, thus allowing oxidizing agents to be applied to the paper as well.

Formation of Dinitrophenyl (DNP) Derivatives. When unusual compounds were suspected, it was desirable to form the 2,4-dinitrophenyl derivative of the compound. Because of the limitation set by the concentration of isolated unknown, the derivative was formed directly at the origin of a papergram by adapting the method of Isherwood and Cruickshank (9) who used 1-fluoro-2,4-dinitrobenzene as a spray reagent for detecting amino acids on finished chromatograms. The procedure used is as follows: The unknown (minimum 4 μg.) was spotted on the starting line with a microliter pipet and 1 μl. of a freshly prepared solution containing 0.02 ml. of 1-fluoro-2,4-dinitrobenzene, 1.7 ml. of 0.2M borate buffer (pH 8.4), and 3.3 ml. of ethanol was spotted on top of the unknown. The paper was heated for 30 minutes at 80° C. to accelerate the reaction. Authentic DNP derivatives were chromatographed with the unknown. Water-saturated 1-butanol was used as developer.

Preliminary experiments with amino acids and authentic crystalline DNP-amino acids showed that the dinitrophenylation procedure was valid and that the small amount of buffer and fluorodinitrobenzene present on the spot did not alter the mobility of the DNP-amino acid synthesized on the paper. Dinitroaniline and dinitrophenol were formed in small amounts.

The former moved near the front and did not interfere. The latter was detected when it faded on exposure of the chromatogram to hydrochloric acid vapors. The dinitrophenylation did not go to completion under the prescribed conditions.

Results and Discussion

A typical map of the ion exchange chromatography of the nonprotein nitrogen fraction of milk is shown in Figure 1. A qualitatively identical map was obtained when milk, dialyzed to equilibrium against water, was chromatographed.

PEAK 1, UNIDENTIFIED. Cysteic acid and phosphoserine emerge from the Dowex chromatogram in this area. The material under this peak was not identifiable with either of these. After this work was completed, the authors were informed (18) that acidic compounds such as phosphoserine and cysteic acid may not be eluted from Dowex 2 under the desalting conditions used in the authors' laboratory. Sparsity of material prevented further analyses.

PEAK 2, PHOSPHOGLYCEROETHANOL-AMINE (PGE). PGE comprised the bulk of the ninhydrin-positive material under this peak. Another ninhydrin-positive component was also noted on the papergrams. This was not identifiable with any of the compounds on the Moore-Stein map that emerge in this general area. Uric acid, which is ninhydrin-negative, was also detected in this fraction. The following evidence is offered to confirm the presence of PGE. The unknown traveled with the same mobility as an authentic sample of L-α-phosphoglyceroethanolamine in solvents A through E and in G and J, but was always accompanied by a fluorescent spot of approximately the same R_f,

which was not present in the authentic sample. Both known and unknown gave more intense spots with tert-butyl hypochlorite than with ninhydrin; both were readily oxidized on paper by periodic acid yielding formaldehyde which was detected by the method of Schwartz (13). The test of Hanes and Isherwood (7) for phosphate esters was positive. Attempts to acid hydrolyze the unknown resulted in severe browning even at room temperature. Separation of chromatographically pure PGE from the impurities in the fraction was achieved by ionophoresis on Whatman 3MM paper in 2N ammonia after ionophoresis in a γ-collidine–acetic acid buffer, pH 7.0, and in acetic acid, pH 3.1, had failed. In all instances, however, the unknown moved at the same rate as authentic PGE.

The desired band from the ammonia ionogram was eluted with water and passed over Dowex-2 (OH$^-$) and eluted with 1N acetic acid. An aliquot of the residue hydrolyzed in 5.7N HCl at 100° C. for 24 hours gave one spot positive to ninhydrin and to tert-butyl hypochlorite, corresponding in position to ethanolamine in solvents, A, D, and F. Hydrolysis in 5.7N HCl at room temperature for 2 hours gave spots corresponding to ethanolamine and unhydrolyzed PGE in solvents A and B.

Additional evidence that the compound is PGE was obtained on chromatograms of its dinitrophenyl derivative.

The evidence presented here strongly indicates that PGE is a normal constituent of cow's milk. Phosphoglyceroethanolamine might arise from the acid hydrolysis of plasmalogens which are normally present in milk. However, the authors' analysis of dialyzed milk also gave phosphoglyceroethanolamine. That the position of the phosphoethanol-

amine residue is probably alpha is suggested by the fact that formaldehyde is liberated from the molecule when oxidation with periodate is carried out. This does not, however, exclude the possibility that both the alpha- and beta-isomers may be present under the peak. Conceivably they would have similar chromatographic properties.

PEAK 3, O-PHOSPHOETHANOLAMINE. The material under peak 3 has been identified as O-phosphoethanolamine based on the following evidence: This fraction and the authentic sample gave a better response to tert-butyl hypochlorite than to ninhydrin on paper chromatograms. The test for phosphate esters was positive. The unknown and authentic O-phosphoethanolamine had the same R_f in solvents A through E. Hydrolysis for 24 hours at 100° C. in 5.7N HCl gave a spot corresponding to authentic ethanolamine and phosphoethanolamine in solvent B, with the former component much stronger.

As an additional check, a micro modification of the macro method of Billman, Parker, and Smith (1) for the conversion of ethanolamine to glycine was undertaken. Five microliters of the unknown containing approximately 8 μg. per μl. was sealed in a capillary with 0.05 ml. of 6N H_2SO_4 and heated for 24 hours at 110° C. A small crystal of $KMnO_4$ was added; the solution was allowed to stand for 1 hour, transferred to a short (1 × 2 cm.) column of Dowex-2 (OH⁻), and washed free of potassium ions. Elution of the column with 1N acetic acid followed by paper chromatography of the residue in solvents A and C gave glycine as the only spot positive to tert-butyl hypochlorite or to ninhydrin.

Supplementary evidence that this fraction was O-phosphoethanolamine was obtained by the DNP technique.

PEAK 4, UREA. This fraction could not be desalted on short columns of cation or anion exchangers. Treatment of a concentrate of the peak with urease, according to Feigl (6), followed by Nessler's reagent gave a positive test for ammonia.

PEAK 5, UNIDENTIFIED. Two components, ninhydrin-negative on paper but positive to tert-butyl hypochlorite, were obtained from the desalted fraction. Both spots remained unchanged after acid hydrolysis. No new spots were formed from this treatment.

PEAK 6. The material under this peak has been tentatively identified as phenylacetylglutamine. Evidence for its occurrence in milk will be reported elsewhere. Phenylacetylglutamine is ninhydrin-negative. The small ninhydrin-peak is probably due to the slight breakdown of the glutamine radical to give ammonia during the heating conditions employed in the ninhydrin test. Methionine sulfoxides and hydroxyproline

emerge in this area, but could not be detected.

PEAK 7, ASPARTIC ACID. The main ninhydrin-positive component under peak 7 was aspartic acid. Traces of threonine were also detected. Solvents C and F were used. The blue color given by aspartic acid on paper when treated with ninhydrin in acetic acid–collidine solution (10) was used as confirmatory evidence.

PEAK 8, THREONINE. This amino acid was identified in solvents B and C. Its presence was confirmed by the specific test described by Schwartz (13).

PEAK 9, SERINE. This amino acid was identified in solvents C and F. The test described by Schwartz (13) was used to substantiate the chromatographic evidence.

PEAK 10, SARCOSINE (N-METHYLGLYCINE). This substituted amino acid constituted the entire ninhydrin-positive material under peak 10. Asparagine and glutamine which emerge from the Dowex chromatogram at the same place were absent. Paper chromatograms in solvents, A, B, C, and E, confirmed the presence of sarcosine. Additional evidence was obtained by the DNP method. The sarcosine content of fresh milk increased approximately seven times when protein- and lactose-free serum was hydrolyzed in acid.

PEAK 11, PROLINE. This amino acid is easily identified by the atypical color it gives with ninhydrin, its absorption maximum being different. Desalted fractions of the peak gave spots corresponding to proline in solvents A and C. The isatin test (3) was positive.

PEAK 12, GLUTAMIC ACID. Characterized in solvents A, B, and C. Identical migration of an unknown with an authentic compound on paper, even in several solvent systems, is not accepted by many investigators as proof of identity. Since the authors were unable to perform specific tests for this compound, and also did not attempt to make derivatives, the presence of this amino acid in milk should be considered probable, but not unequivocal. These reservations should also be made for most of the compounds which follow.

PEAK 13, GLYCINE. Characterized in solvents A, B, and C. Alanine was also detected in this fraction.

PEAK 14, ALANINE. Characterized in solvents A, B, and C. Glycine was present in the alanine peak.

PEAK 15, α-AMINO-n-BUTYRIC ACID. Characterized in solvents A, C, G, and K.

PEAK 16, VALINE. Characterized in solvents C and G.

PEAK 17, ISOLEUCINE. Characterized in solvent G. After chromatography for 2 days at 20° C. leucine and isoleucine are well separated (20). Leucine was also detected in peak 17.

PEAK 18, LEUCINE. Characterized in solvent G as described for isoleucine.

PEAKS 19, 20, AND 21, UNIDENTIFIED.

PEAK 22, PHENYLALANINE. Characterized in solvents A and B.

PEAK 23, HYDROXYLYSINE. The acid is reported to emerge here. One chromatogram (solvent A) gave a spot corresponding to hydroxylysine. Its existence in milk should, therefore, be considered tentative. Difficulty was encountered in obtaining known hydroxylysine from citrate buffer. Desalting of peak 23 also gave poor yields.

PEAK 24, AMMONIA.

PEAK 25, UNIDENTIFIED.

PEAK 26, ORNITHINE. This amino acid was characterized in solvents A and C. It was contaminated with lysine and creatinine.

PEAK 27, LYSINE. Characterized in solvents A, C, and K but contaminated with creatinine and ornithine.

PEAK 28, CREATININE. Identified in solvents A, C, and K and by the Jaffe test (3).

PEAK 29, HISTIDINE. Identified in solvents A, B, C, and K. The spot corresponding to histidine gave a red spot with diazotized sulfanilic acid (3).

PEAK 30, UNIDENTIFIED.

PEAK 31, ARGININE. Identified in solvent C. The specific Sakaguchi test described by Block (3) was positive.

Discussion

The characterization and/or identification of new components in cow's milk is always important from a nutritional and biological standpoint. A knowledge of trace constituents may also be valuable in the processing of milk per se and in the processing of milk into various other edible forms such as evaporated milk, milk powder, cheese, and ice cream. The ease with which milk and milk products undergo nonenzymatic flavor changes, of which little is known, may involve reactions of trace constituents with each other or with major milk constituents under the influence of heat, time, or both.

This study clearly emphasizes the importance of the possible misinterpretation of a standard map or chromatogram as the sole means of identification of a compound. A number of instances have shown that unidentified compounds can emerge in positions normally occupied by standards. In some instances, the so-called standards themselves are completely absent in the material being investigated. This was realized by Stein (16) and by Stein and Moore (17) in their early analysis of biological fluids by the ion exchange technique.

As has been shown, a number of components may be present under an otherwise symmetrical peak. In the quantitative analyses of nonprotein nitrogen fractions of biological material by ion exchange chromatography, the necessity for checking the purity of a fraction

is obviously essential. A comparison of this work with that reported by Deutsch and Samuelsson (4) and by Samuelsson (12) shows a difference in the characterization and/or identification of several of the peaks. The present work failed to detect methionine sulfoxide, citrulline, and asparagine, which were reported by those investigators. However, those investigators were unable to find phenylacetylglutamine and sarcosine, which were identified here.

In contrast to the possible shortcomings of the ion exchange method mentioned above, the superiority of ion exchange chromatography even as a qualitative tool over paper chromatography should be pointed out. Thus, paper chromatography of the ninhydrin-positive components in desalted milk serum has been applied by several investigators (2, 8, 19). That paper chromatography alone is quite limited in its scope is borne out by the fact that a maximum of only 14 spots were detected, compared to 31 peaks in the present study. This limitation is set by the amount of material which can be applied to a sheet of paper, and by the possible disproportionality of the constituents present in the sample.

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MILK PRESERVATION

The Use of Antioxidants in Frozen Whole Milk

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Various antioxidants—ethyl hydrocaffeate, gentisic acid, sodium gentisate, and Ionol—were tested with whole milk at −10° F. for a 6-month storage period with and without the addition of copper. All compounds, with the exception of Ionol, showed good antioxidant properties, the sodium gentisate being superior to the others. Ionol imparted an off-flavor to the milk and precipitated the milk proteins. By use of proper antioxidants and storage conditions, frozen whole milk may be kept in good condition for at least 6 months.

THERE are numerous occasions when it might be advisable to store frozen milk for future use; however, the development of oxidized flavors in stored frozen fluid dairy products usually presents a serious economic problem. This problem has attracted the attention of research workers in the field of dairy and food technology (7–9). Prevention of oxidized flavor development in stored frozen cream has been investigated by using various substances and

procedures, including high temperature heat treatments, homogenization, the use of ascorbic acid (1, 2), and nordihydroguaiaretic acid (N.D.G.A.) (7, 8). Gelpi et al. (5, 6) have reported that butyl hydroxyanisole, Tenox II (butylated hydroxyanisole + citric acid + propyl gallate in propylene glycol), ethyl caffeate, and ethyl hydrocaffeate showed varying degrees of effectiveness in delaying or preventing the development of oxidized flavors in stored frozen cream. The results indicated that ethyl caffeate and the hydrogenated form (ethyl hydrocaffeate) possessed some outstanding properties as antioxidants. Frozen cream containing

0.004% of ethyl hydrocaffeate + 0.5 p.p.m. of copper failed to develop an oxidized flavor after 12 months of storage at −10° F.

The present study includes the use of ethyl hydrocaffeate, gentisic acid, sodium gentisate, and Ionol as antioxidants in frozen milk for a 6-month storage period.

The gentisates (2,5-dihydroxy benzoic acid), as represented by gentisic acid and sodium gentisate, possess good antioxidant properties in certain food fats (4). They are appreciably water-soluble, fairly heat stable, and do not show carry-through properties in the Schaal oven. Gentisic acid has been

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