# SIALIC ACID AS AN INDEX OF THE κ-CASEIN CONTENT OF BOVINE SKIMMILK [1]

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SUMMARY

The sialic acid content of skimmilk proteins has been examined, using a modification of Warren's thiobarbituric acid method. Lactose interferes with this method and was removed by thorough washing of the proteins. κ-casein and proteose-peptone appear to be the only two skimmilk proteins containing significant amounts of sialic acid. Treatments with DEAE cellulose almost completely removed the low concentrations of sialic acid present in $\alpha_s$- and β-caseins, and examination of these caseins by starch gel electrophoresis indicates that such removal involved traces of κ-casein.

Since the stability of $\alpha_s$-κ-casein mixtures in the presence of calcium appeared to be directly related to the sialic acid content of the mixture at all levels below the 0.37% (uncorrected for moisture) needed to give complete stability, it is concluded that sialic acid measurements can be used as an index of the intact κ-casein content of such mixtures.

Proteose-peptone is precipitated by 12% TCA, but remains in solution at pH 4.5, so analysis of sialic acid can also be used as an index of the κ-casein concentration of whole acid casein. Values of 0.26-0.59% sialic acid were obtained in washed whole acid caseins prepared from individual lots of milk, indicating that the proportion of κ-casein is variable. The sialic acid content of κ-casein appears to be close to 2.3%; on this basis, the proportion of κ-casein in whole acid casein varies between 11-26%.

The κ-casein fraction of bovine milk protein contains a glycomacropeptide which can be released by the action of rennin (1, 7, 12). The peptide and glucide portions of this glycopeptide have been characterized (1, 7), and it has been shown that the glucide portion contains a sialic acid (1) present solely as N-acetyl-neuraminic acid (5).

Since κ-casein appears to be the fraction which stabilizes the casein micelle (22, 24), a measure of its concentration in normal milks would be of considerable interest. Sialic acid determinations have been used as a measure of κ-casein in sedimented fractions of whole casein (17), but various other casein fractions appear to contain some sialic acid (1, 2, 13) and, if so, sialic acid cannot be used as a measure of κ-casein in milk.

Several authors have compared methods for the determination of sialic acids, and have reported that Warren's thiobarbituric acid method (21) is sensitive, specific, and convenient (1, 4, 14). Some workers (1, 3) have reported that it gives slightly lower values than Svennerholm's resin-resorcinal method (18), whereas others have noted excellent agreement between the two methods (4, 14). Other methods for the determination of sialic acid can give erroneously high results, caused by decomposition products formed during hydrolysis (4). The work reported herewith represents an attempt to estimate the extent to which sialic acid determinations indicate the κ-casein content of bovine milk.

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## MATERIALS AND METHODS

Casein from individual lots of raw skimmilk was obtained by adjusting 5.0-ml samples to pH 4.5 (ca. 0.25 ml 1.0 N HCl) in conical centrifuge tubes. Total milk protein was similarly obtained by adding trichloracetic acid to a final concentration of 12% (1.3 ml 60% TCA/5 ml milk). Casein precipitates were washed four times by suspension in 5-ml aliquots of acetate buffer, pH 4.5; TCA-precipitated proteins required five washings with acetate buffer. This extensive washing was necessary to remove lactose which interferes with the determination of sialic acid by giving rise to an orange color (max. absorption at 490 mμ).

Alpha-casein complex (i.e., the α-κ-mixture usually designated as α-casein) and β-casein

TABLE 1

Recovery of sialic acid added to whole acid casein

| Whole acid casein obtained from: | μg Sialic acid: Added to casein prior to hydrolysis | μg Sialic acid: Determined in hydrolysis-wash solution | Total expected | Per cent recovery |
|---|---|---|---|---|
| 5.0 ml skimmilk | 0 | 435 | ..... | ..... |
| | 50 | 487 | 485 | 100.4 |
| | 100 | 520 | 535 | 97.2 |
| | 150 | 591 | 585 | 101.0 |
| 2.5 ml skimmilk | 0 | 226 | ..... | ..... |
| | 50 | 282 | 276 | 102.2 |
| | 100 | 345 | 326 | 105.8 |
| | 150 | 380 | 376 | 101.1 |
| | | | Avg | 101.3 |

were prepared by urea fractionation (6). Calcium-sensitive casein ($\alpha_s$-casein) was prepared as described by Morr (10), and κ-casein by various procedures (9, 10, 19, 25). All casein preparations were freeze-dried and stored at 4 C. All casein fractions, including those purified by chromatography, were characterized by starch gel electrophoresis (11). Starch gel electrophoretic patterns of Samples 1, 2, and 4 of Table 2 (also Figure 3) have been published (11).

TABLE 2

Sialic acid content of casein fractions

| | % Sialic acid in casein: Present study | % Sialic acid in casein: Cayen et al. (2) |
|---|---|---|
| κ-caseins | | |
| 1. Swaisgood-Brunner (19) | 2.14 | 2.22* |
| 2. Morr (10) | 1.81 | |
| 3. Zittle (25) | 1.81 | |
| 4. McKenzie-Wake (9) Preparation 2 (11) | 1.25 | |
| 5. Preparation 1 (11) | 0.65 | |
| 6. S-B (19) + rennin | 0.24 | |
| Other fractions | | |
| α-casein complex (6) | 0.42 | 0.41 |
| $\alpha_s$-casein (10) | 0.06<br>0.08 | 0.06** |
| β (6) | 0.18<br>0.23 | 0.14 |

* Fraction A (2).
** α-Casein—Fraction A (2).

Chromatographic purification of casein fractions on DEAE cellulose was done according to the procedure outlined by Ribadeau-Dumas (13), except that the sodium chloride gradient was 0 to 0.4 M. One-half gram of casein was

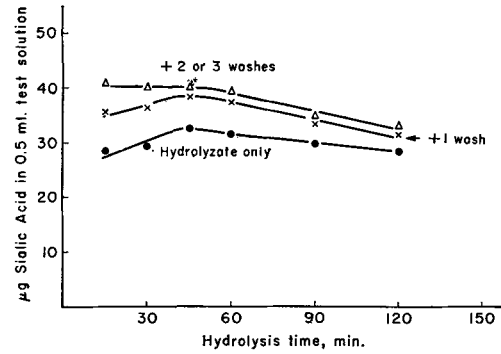


FIG. 1. Effect of time of hydrolysis (80 C, 0.1 N $H_2SO_4$) and number of washings in 0.1 N sodium acetate buffer (pH 4.5) on the apparent sialic acid content of whole casein.

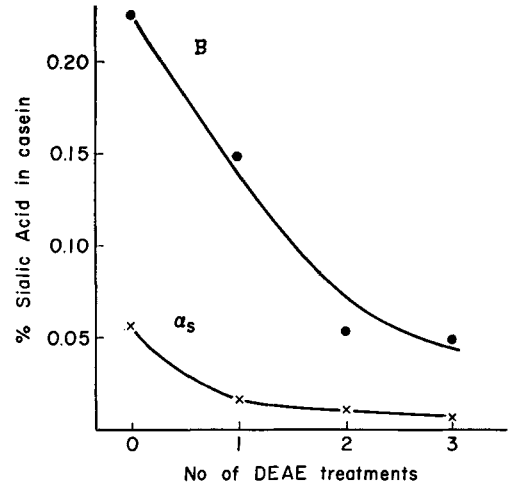


FIG. 2. Removal of sialic acid–containing contaminants from $\alpha_s$- and β-caseins by chromatography on DEAE cellulose.

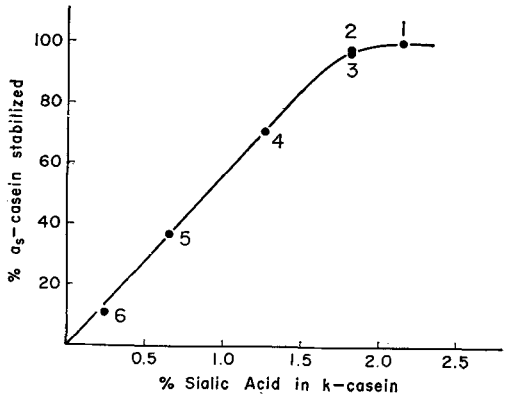


FIG. 3. Relation between the sialic acid content and the stability of suspensions of $\alpha_s$- and κ-casein in the presence of 0.020 M $Ca^{++}$. Composition of the mixtures was as follows: Samples 1-6: four parts $\alpha_s$-casein containing 0.07% sialic acid plus one part of: 1) κ-casein, Swaisgood and Brunner (19); 2) κ-casein, Morr (10); 3) κ-casein, Zittle (25); 4) κ-casein, McKenzie-Wake (9), Preparation 2; 5) same as 4) but Preparation 1; 6) κ-casein, Swaisgood-Brunner (19) treated with rennin.

placed on the column for each treatment; 10-ml fractions were collected, combined according to their light absorption at 280 mμ (Gilson Medical Electronic meter), dialyzed against running tap water for 24 hr, then against distilled water for 24 hr, and finally freeze-dried. Moisture content of all freeze-dried casein preparations was determined by vacuum drying at 85 C.

Warren's thiobarbituric acid method (21) was selected for use in this study. This colorimetric method measures free sialic acid only, and consists of a preliminary mild acid hydrolysis of the sample, followed by periodic acid oxidation, arsenite addition, color development with 2-thiobarbituric acid, and extraction of the red pigment with cyclohexanone. After extensive preliminary testing this method was slightly modified, (a) by using a 0.5-ml aliquot of test sample instead of 0.2 ml, and (b) by using exactly 4.0 ml of cyclohexane per test. Aqueous aliquots below 0.2 ml or above 0.7 ml decreased the sensitivity of the method.

A standard curve, i.e., 5-40 μg sialic acid, was prepared from a mucoid compound containing 18.3% N-acetyl-neuraminic acid (Nutritional Biochemicals Corp.), hydrolyzed in 0.1 N $H_2SO_4$ for 60 min at 80 C. After adjustment to pH 4.5, and addition of chloroform as a preservative, the hydrolyzate (100 μg N-acetyl-neuraminic acid/ml) could be stored for approximately 1 wk at 5 C. Absorption readings were made with a Coleman Jr. spectrophotometer at 545 mμ, with a 1-cm light path.

For analysis of isolated casein fractions, the samples (100-800 μg sialic acid) were weighed directly into 15-ml capacity conical tubes, then hydrolyzed. To hydrolyze, the material was wetted with 4.5 ml of water and 0.5 ml of 1.0 N sulfuric acid was added, and the suspension held at 80 C for exactly 45 min. The samples were then cooled in water for 3 min, and 0.45 ml of 0.1 N sodium hydroxide added (final pH ca. 4.5) with thorough mixing. The precipitated protein was sedimented (2 min, 1,000 × *g*), the supernatant decanted into a graduated cylinder, and the precipitate washed once with 5.0 ml of acetate buffer. The washing was added to the first supernatant, the final volume was recorded (i.e., 10.3 ± 0.5 ml), and the solution was clarified by shaking with 0.1 ml chloroform (16). A 0.5-ml aliquot of this solution was used for the sialic acid analysis. Sialic acid is reported on a dry weight basis.

## RESULTS

*Liberation of sialic acid from casein.* The liberation of sialic acid from acid casein during hydrolysis at 80 C in 0.1 N sulfuric acid appeared to be complete in 15 min (Figure 1), although more than one washing of the precipitate was necessary to remove the liberated sialic acid. Increased hydrolysis times up to 45 min increased recovery of sialic acid in the original supernatant, and in the combined solution after one washing, presumably by altering the properties of the hydrolyzed protein. A 45-min hydrolysis and one washing step appeared to be optimal for the determination of sialic acid. Further washings diluted the solution and made accurate analysis of low levels of sialic acid difficult. Hydrolysis for more than 60 min reduced the apparent sialic acid content of whole casein by 10 to 16%/hr (cf. 2, 4).

Repeated hydrolysis in fresh acid did not release further sialic acid from casein previously hydrolyzed 15, 30, or 45 min and washed three times.

Recovery of added sialic acid from whole casein under these conditions averaged 101.3, with a standard deviation of ± 2.5% (Table 1).

*Sialic acid content of casein fractions.* The sialic acid content of the casein fractions prepared by accepted methods varied from a low of 0.06% in $\alpha_s$-casein to a high of 2.14% in κ-casein (Table 2). The values obtained are

in good agreement with those of Cayen et al. (2). Repeated chromatographic purification of $\alpha_s$-casein on DEAE cellulose reduced the sialic content to very low values (ca. 0.01%; Figure 2). (The sensitivity of the sialic acid method became limiting under these conditions, and it is probable that the sialic acid content of this $\alpha_s$-casein was approaching zero.) Repeated chromatographic purification of $\beta$-casein reduced its sialic acid content by 75 to 80%, and it appeared possible that repeated chromatography would remove additional sialic acid (Figure 2).

The removal of sialic acid from these casein fractions by chromatographic purification was accompanied by a removal of contaminating proteins. With $\alpha_s$-casein, absorption data (280 m$\mu$) on the effluent from the DEAE column indicated that the proportion of material in the major component (based on areas under the absorption curve) increased from 77% on the first treatment to 86% on the third. With $\beta$-casein, the corresponding figures were 72 and 85%. The amount of components present as minor peaks was insufficient to permit further study. Two treatments with the DEAE column reduced the minor protein components of $\alpha_s$-casein below the level that could be detected by starch gel electrophoresis, using 4 mg protein (this amount overloaded the gel with the major component, but increased the probability of detecting minor fractions). Three treatments with the DEAE column were required to reduce the minor components of $\beta$-casein below the level detectable by starch gel electrophoresis.

The sialic acid content of $\kappa$-caseins shown in Table 2 was determined by direct hydrolysis of the freeze-dried preparations. Preliminary studies had revealed that many $\kappa$-casein preparations contained some sialic acid soluble in acetate buffer at pH 4.5, and a few contained some sialic acid soluble in 12% trichloroacetic acid. This soluble sialic acid was not present as the free compound. Solubility at pH 4.5 was especially noticeable in $\kappa$-casein preparations of high moisture content, whereas solubility in 12% TCA may have been favored by storage and/or drastic treatment. However, a thorough study of these factors was not undertaken at this time. $\kappa$-Casein was the only casein fraction that lost any sialic acid to acetate buffer or trichloracetic acid solution.

The stabilizing power of the $\kappa$-casein preparations which did not give essentially 100% stabilization of an $\alpha_s$-casein in the presence of calcium [when tested at a 4:1 ratio (11)] was directly related to their sialic acid content (Figure 3), and a preparation in which the sialic acid content had been reduced by treatment with rennet (Point 6 of Figure 3) also showed this relation. Presumably, these samples were impure, so that their sialic acid content and stabilizing powers were reduced by the presence of components which did not contain sialic acid. This contaminating material may have been denatured protein in our McKenzie and Wake preparations (9). When several preparations of $\kappa$- and of $\alpha_s$-casein, differing in their sialic acid content, were tested at various $\alpha_s$:$\kappa$-casein ratios, the total sialic acid of the mixture appeared to control its stability (data not shown). The observed relation was linear from 10 to 100% stability, and extrapolated to zero stability at 0.06% sialic acid and to 100% stability at 0.37% sialic acid in the test mixture (uncorrected for moisture). The latter result (ca. 0.40% when corrected for moisture) is close to the values reported for $\alpha$-casein complex ($\alpha_s$ + $\kappa$-casein) [(1, 2) and Table 2].

*Recombined casein fractions.* As a check on the accuracy of the sialic acid determination, mixtures of various casein fractions were prepared and analyzed. To secure some recombination of the fractions into complex micelles, they were dissolved in sodium hydroxide at pH 12, to form the monomers; then, within 25 min, they were acidified to pH 4.5 in the presence of calcium, magnesium, phosphate, and citrate (Table 3 headnote), so as to form and coagulate micelles. A sample of whole acid casein was similarly treated. After the alkali treatment, some of the sialic acid originally present in the $\alpha_s$-$\kappa$-casein mixture was soluble at pH 4.5. Therefore, all supernatant solutions were hydrolyzed and analyzed.

The total amount of sialic acid recovered agreed closely with the amount calculated from the analyses of the individual fractions (Table 3). Factors affecting the amount of sialic acid present in the supernatant after alkali treatment of certain casein mixtures are being investigated further.

*Sialic acid content of skimmilk proteins.* Preliminary tests with skimmilk indicated that some 17 to 28% more sialic acid was present in the proteins precipitated with trichloracetic acid than in casein precipitated at pH 4.5. Analysis of the heat-coagulable whey proteins showed that they contained little or no sialic acid. Precipitation of the proteose-peptone fraction from the serum of heated skimmilk (95 C, 15 min) with trichloracetic acid yielded a fraction (sedimented in 1 min at 10,000 rpm

TABLE 3

Recovery of sialic acid from recombined casein fractions (0.020 M Ca, 0.005 M Mg, 0.020 M P, 0.010 M Cit; 25 C)

| Type of casein | Total μg sialic acid found in: Casein hydrolyzate-wash solution | Supernatant hydrolyzate [a] | Sum | Expected [b] | Per cent recovery |
|---|---|---|---|---|---|
| 45 mg $\alpha_s$ + 40 mg $\beta$ | 100 | 0 | 100 | 100 | 100.0 |
| 60 mg $\beta$ + 10 mg $\kappa$ | 306 | 0 | 306 | 310 | 98.7 |
| 60 mg $\alpha_s$ + 15 mg $\kappa$ | 358 | 94 | 452 | 450 | 100.4 |
| 150 mg whole casein | 549 | 0 | 549 | 553 [c] | 99.3 |

[a] 0.61 ml of 1.0 N $H_2SO_4$ was added to the supernatant solution (5.5 ml) prior to hydrolysis.
[b] Based on values of Table 2, using the Swaisgood and Brunner (19) κ-casein.
[c] Based on prior analysis of this whole casein.

in a Spinco L centrifuge, No. 30 head) containing about 1.8% sialic acid $\left(\frac{\text{sialic}}{\text{N} \times 6.38} \times 100\right)$. The sialic acid content of this fraction accounted for the entire difference between the sialic acid content of acid casein and of milk proteins precipitated with trichloracetic acid (Table 4).

TABLE 4

Sialic acid content of casein, proteose-peptone, and TCA-precipitated protein

| | μg Sialic acid per milliliter of original milk or serum | | | |
|---|---|---|---|---|
| | Milk 1 | | Milk 2 | |
| Whole acid casein | 78.4 | 100.4 | 72.4 | 89.4 |
| Proteose-peptone | 22.0 | | 17.0 | |
| Total TCA-precipitated protein | | 100.7 | | 91.6 |

The sialic acid content of caseins from different milks varied from 0.26 to 0.59% (Table 5). Individual values in the literature, i.e., 0.21-0.43% (1, 2, 8, 14, 17), show a similar variability. The sialic acid content of the proteose-peptone fraction varied to about the same extent, but independently of casein-bound sialic acid (Table 5).

TABLE 5

Routine analysis of sialic acid in whole acid casein of bulk and individual raw skimmilks

| Type of milk | Per cent casein [a] | Per cent sialic acid in whole acid casein | Sialic acid in proteose-peptone: μg per milliliter of original milk | Sialic acid in proteose-peptone: Per cent of casein sialic acid |
|---|---|---|---|---|
| Bulk | 2.81 | .29 | | |
| | 2.31 | .33 | | |
| | 2.64 | .41 | | |
| | 2.23 | .53 | 20.8 | 17.6 |
| Individual | 2.78 | .26 | 19.2 | 26.7 |
| | 2.25 | .31 | | |
| | 2.39 | .31 | | |
| | 2.15 | .34 | | |
| | 2.30 | .34 | 22.3 | 28.3 |
| | 3.07 | .37 | 30.7 | 27.0 |
| | 2.10 | .38 | | |
| | 2.63 | .39 | | |
| | 2.47 | .40 | | |
| | 2.95 | .46 | | |
| | 1.98 | .50 | | |
| | 3.35 | .59 | | |

[a] Casein N × 6.38.

## DISCUSSION

Estimation of the κ-casein content of milk by determination of the sialic acid content is possible if the following conditions are fulfilled:

a) Only casein sialic acid is measured and it is measured completely;
b) If other casein fractions contain little or no sialic acid, and
c) If the sialic acid content of κ-casein is known and is constant.

With regard to condition (a), the data (Table 4) show that noncasein sialic acid is present in the proteose-peptone fraction of skimmilk protein. However, this fraction is not precipitated with casein at pH 4.5 (15). The complete recovery of sialic acid added to casein before hydrolysis (Table 1) indicates the reliability of the thiobarbituric acid method for the determination of sialic acid. Also, the agreement between determined and calculated

(based on analyses of the individual fractions) sialic acid contents of reconstituted casein mixtures (Table 3) would appear to indicate that this method determines all of the sialic acid present in mixtures of various casein fractions. It is, therefore, reasonable to assume that all of the sialic acid present in whole acid casein will be determined by this procedure.

With reference to condition (b), the results obtained on $\alpha_s$- and $\beta$-casein purified by repeated chromatography on DEAE cellulose indicate that most, and probably all, of the sialic acid found in normal preparations of these fractions is present as an impurity. Starch gel electrophoresis of the usual preparations of $\alpha_s$- and $\beta$-casein has indicated the presence of a small proportion of protein having a mobility similar to that of $\kappa$-casein (11), and the present work shows that this is removed by chromatographic purification on DEAE cellulose. Therefore, it can be concluded that the sialic acid found in preparations of $\alpha_s$- and $\beta$-casein indicates contamination of these fractions with $\kappa$-casein. Since $\alpha_s$-, $\beta$-, and $\kappa$-casein appear to be the three major fractions of whole casein, a reasonable corollary is that $\kappa$-casein is the only major fraction containing a significant amount of sialic acid.

With reference to condition (c), the variable sialic acid content of different preparations of $\kappa$-casein does not necessarily indicate that the composition of $\kappa$-casein is variable. These preparations are impure (11), and the preparation preferred on the basis of calcium stabilization of $\alpha_s$-casein (11) is also the one with the highest sialic acid content. If, as suggested by Waugh (22, 23), $\alpha_s$- and $\kappa$-casein associate in constant proportions, then the true $\kappa$-casein content of these impure $\kappa$-casein preparations should be related to the amount of $\alpha_s$-casein it can stabilize in the presence of calcium. The observed linear relation between the stability and the sialic acid content of the mixture (Figure 3) is, thus, a strong indication that the measured sialic acid content reflects the amount of intact $\kappa$-casein (i.e., including the glycomacropeptide) present in these preparations. Specific removal of sialic acid, presumably without other change in the molecule, did not reduce its stabilizing power proportionately (20).

The highest sialic acid value found in $\kappa$-casein during the present study was 2.14%. Swaisgood and Brunner (19) have stated that the $\kappa$-casein prepared by their method is approximately 92% pure, so the true sialic acid content of this $\kappa$-casein is probably 2.3%. Values of 2.4 (1) and 2.5% [resorcinol-HCl method (20)] have been reported by others for $\kappa$-casein, and Cayen et al. (2) have found 2.22% in their Fraction A. Sialic acid contents of 0.42 (present work), 0.41 (2), and 0.38% (1) have been reported for $\alpha$-casein complex. If, as seems probable, the urea method (6) isolates a specific complex in which $\alpha_s$- and $\kappa$-caseins are associated in a weight ratio of 4:1 (22, 23), these values would indicate sialic acid contents of 2.10, 2.05, and 1.90%, respectively, in the $\kappa$-casein portion of the complex. However, this calculation does not correct for $\beta$-casein, which may be present as an impurity. Further work is required to establish the sialic acid content of $\kappa$-casein.

These results indicate that the procedure used in the present work fulfills the conditions that must be met if sialic acid determinations are to be used as an index of the $\kappa$-casein content of skimmilk. The only exception is that the exact sialic acid content of $\kappa$-casein has not been established, but a value of 2.3% is suggested as an approximation pending further studies. The 0.26-0.59% sialic acid observed [a] in whole acid casein thus indicates the proportion of $\kappa$-casein varies between 11-26%. This range is similar to that observed by Sullivan et al. (17) in sedimented caseins of varying particle size.

[a] We have since found values as low as 0.21% in whole acid casein.

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