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KAPPA-CASEIN IN MILK

AN IMMUNOCHEMICAL INVESTIGATION OF THE κ-CASEIN CONTENT IN MILK FROM DANISH CATTLE BREEDS

By

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THYMANN, MARIANN: *Kappa-casein in milk. An immunochemical investigation of the κ-casein content in milk from Danish cattle breeds.* Acta vet. scand. 1972, *13*, 539—553. — The κ-casein content in milk from the Danish cattle breeds SDM, RDM and Jersey was investigated by means of immunoquantitation in antibody containing agarose gels. The method, which determines the total κ-casein complex, is suitable for routine work. The reproducibility of the method was found to be ± 4.1 %.

The κ-casein concentration, expressed as percentage of the total protein content in milk, was significantly lower in milk from SDM (21.5 %) and RDM (21.3 %) than in Jersey (23.9 %). The investigation showed that the κ-casein constitutes a greater part of the protein in milk than previously assumed.

The amount of κ-casein was positively correlated with the protein content in milk, but there was no relation between the relative κ-casein content and the percentage of fat, milk yield and the time after calving.

k a p p a - c a s e i n; m i l k; c a t t l e; i m m u n o q u a n t i t a t i o n.

Approx. 80 % of the protein in bovine milk is casein, present as a stable suspension of aggregates of calcium-proteinate-phosphate, the micelles. The casein itself is a complex mixture of several components, which were separated into α-, β- and γ-casein by *Mellander* (1939). Later it was shown by *Waugh & von Hippel* (1956) that α-casein is composed of a calcium-sensitive part, α_s-casein and a soluble part, κ-casein. A complete separation of the 20 components, which constitute whole casein, was first ob-

tained by *Wake & Baldwin* (1961) using electrophoresis in starch gel containing urea. Decomposition of casein to the monomeric form with urea has moreover demonstrated genetically determined variants in the fractions α_s-, β-, γ- and κ-casein. In κ-casein intermolecular S-S-bindings have to be broken, for instance with mercaptoethanol, before the different genetic types can be demonstrated (*Neelin* 1964, *Schmidt* 1964). The occurrence of polymorphic milk protein components in different cattle breeds is described by *Aschaffenburg* (1968). Data on the occurrence of genetically determined milk protein variants in Danish cattle and the interaction of the controlling genes have been published by *Larsen & Thymann* (1966).

In contrast to the ability to identify qualitative variations there are, so far, no methods available to determine, in a simple way, the quantitative distribution of the different proteins in whole casein. Investigations of the content of α_s-, β- and κ-casein have formerly been performed by means of sialic acid analysis, which has been used as a κ-casein index (*Marier et al.* 1963), and turbidimetric measurement of the sum of β- and κ-casein (*Tessier et al.* 1963). The κ-casein is, however, a heterogeneous complex, composed of at least seven fractions, only some of which contain sialic acid. The content of sialic acid in different preparations of purified κ-casein has, moreover, been found very variable (*Mackinlay & Wake* 1971). At present it must be assumed that the amount of sialic acid does not reflect the κ-casein concentration in whole casein.

Very laborious methods for quantitating the different casein components have been described by *Ribadeau Dumas* (1968), who used carboxypeptidase A for determination of α_{s_1}-, β- and κ-casein in whole casein, and by *Rose et al.* (1969), who estimated the same fractions by DEAE-cellulose chromatography. Recently *Manning et al.* (1971) quantitated the κ-casein by determinations of the sulfhydryl group content of reduced whole casein. All these methods are very complicated and not suitable for routine work.

κ-casein is the fraction which stabilizes the micelles. Furthermore, it is the substrate for the specific reaction with rennin. Therefore, it seems likely that κ-casein is of importance to the manufacturing properties of milk and should be taken into account on a par with the milk production characters used at present. Consequently it will be of great interest to investigate,

if the content of κ-casein is genetically determined. The possibility of a relation between the known genetically determined κ-casein types and the κ-casein content ought to be examined as well. Then it can be considered, whether the κ-casein content in milk is of significance for the appraisal of the breeding value of an animal. Investigations on a great number of individual milk samples require a rapid and reliable method for the quantitative determinations of κ-casein.

Immunological methods for quantitations of proteins are very suitable for routine work. Therefore, the possibility for an estimation of the κ-casein concentration in milk by means of electrophoresis in antibody containing agarose gel (Laurell-electrophoresis) has been attempted in this work.

MATERIALS AND METHODS

Estimation of proteins by means of quantitative immunoprecipitation implies production of a specific antiserum. The principle in Laurell-electrophoresis is that antigen and antibodies react with one another to form precipitation zones like ascending rockets. The growing of the rockets is due to unbound antigen which, during its electrophoretic migration, redissolves the antigen-antibody-complex in the front. At a given antibody concentration, the final position of the precipitation frontier of each antigen (the peak height) varies with the amount of antigen applied.

Preparation of κ-casein

Whole casein was precipitated from skim milk at 22°C by adjusting the pH to 4.6 with 1 N-HCl. The precipitate was washed four times with water to remove whey proteins. κ-casein was then prepared by the urea-sulfuric acid method according to *Zittle & Custer* (1963), except the final ethanol treatment. The κ-casein was further purified by means of gel filtration on Sephadex G-150 according to the procedure of *Yaguchi et al.* (1968), except that 8 M urea was used in the elution liquid, and gel filtration was performed twice.

Production of antibody

Rabbits were used for the production of antisera. The κ-casein used for immunization consisted of equal amounts of the two

known genetic variants κ-AA and κ-BB isolated and purified from cow milk. During a period of 18 days the animals were initially given two intravenous and then a series of intramuscular injections of 5 mg antigen, with intervals of two days. The antigen solution used for the first intramuscular injection was emulsified with an equal volume of complete Freund's adjuvant. Five days after the last injection blood was drawn from each rabbit for serum production. Reimmunization of the animals was performed approx. four months later by one intravenous and three intramuscular injections of 5 mg antigen.

Electrophoretic methods

Laurell-electrophoresis. The immunoquantitative method according to *Laurell* (1966) was modified for determination of κ-casein in milk. The gels consisted of 1.1 % agarose A 37 (L'industrie Biologique Francaise) and 10 % anti-κ-casein serum. Gel buffer: Tris(hydroxymethyl)aminomethane 4.44 g, citric acid 1.84 g, sodium veronal 3.61 g, distilled water ad 1 l, pH 8.2. Vessel buffer: Tris(hydroxymethyl)aminomethane 6.06 g, citric acid 2.21 g, sodium veronal 4.33 g, distilled water ad 1 l, pH 8.4. A commercial water-cooled equipment (Dansk Laboratorieudstyr A/S) was used. The electrophoresis was run with 3 v/cm for 16 hrs. Samples: skim milk diluted 1+20 with buffer. Ten µl was applied by means of a double constriction-pipette. Standard solution: purified κ-casein dissolved in 0.9 % NaCl. The protein concentration was estimated by averaging three micro-Kjeldahl analyses, employing the factor 6.38 for conversion of nitrogen to protein. The standard solution, containing 33.8 mg κ-casein per 100 ml, was divided into small aliquots, equal to the amount used in one day, and stored at —20°C. On each plate 4—5 different dilutions of the standard solution were applied. A standard curve was constructed plotting peak height against corresponding concentrations of κ-casein.

Starch gel electrophoresis. The previously described method (*Larsen & Thymann* 1966) was modified by use of smaller gels (22 cm × 15 cm × 0.2 cm) and 320 v for 5—6 hrs. at 5°C. A discontinuous Tris-buffer (*Aschaffenburg & Michalak* 1968) was used. Rennin treatment: A 2 % solution of κ-casein in Tris-citric acid buffer was incubated with rennin for 10 min. at 35°C, approx. 0.5 µg crystalline enzyme (Chr. Hansen's Laboratorium

A/S) per mg protein was used. The enzyme was inactivated by the addition of equal volumes of buffer containing 8 M urea and about 0.04 M 2-mercaptoethanol.

Antigen-antibody crossed electrophoresis. The method is described by *Clarke & Freeman* (1966). In the 1st dimension a voltage gradient of 20 v/cm was used for 50 min., and in the 2nd dimension the gradient was 3 v/cm for 16 hrs. The 1.1 % agarose gel contained 10 % anti-κ-casein serum. Samples: The κ-casein solution was 0.2 %, and skim milk was diluted 1+2 with buffer.

Immunological examination of κ-casein

The κ-casein complex was first separated by means of starch gel electrophoresis, where the addition of mercaptoethanol was omitted. Diffusion against anti-κ-casein was then performed in 1.1 % agarose gel (*Brummerstedt-Hansen* 1962). A 4 % solution of κ-casein (κ-AA and κ-BB) treated for 24 hrs. at 5°C with 10 µl 2-mercaptoethanol per 0.5 ml was used.

Protein determination in skim milk

The content of total protein was estimated by the Orange-G method described by *Moustgaard & Neimann-Sørensen* 1959. The linear regression between the Orange-G values and the nitrogen content, estimated by means of micro-Kjeldahl analysis, was calculated on the basis of 49 determinations. The correlation factor ($r=0.78$) was significantly different from zero ($P<0.001$). The agreement between the Orange-G values and the nitrogen content was ± 3.9 %.

Animal material

The milk samples were collected from three breeds (22 Black and White Danish Dairy Cattle (SDM), 21 Red Danish Dairy Cattle (RDM) and 16 Jersey). The time after calving was from 1 to 11 months. An average sample was taken from one milking, and merthiolate 1/10,000 was added as a preservative. The samples were stored at 5°C after the fat had been removed. For two of the breeds (SDM and RDM) new samples were collected from the same animals after 14 and 35 days respectively. At the last collection of samples from SDM, two samples were taken from each cow, as the evening and morning milk samples were collected separately.

RESULTS

Methodological investigations

Immunoquantitation of κ-casein by means of Laurell-electrophoresis (Fig. 1) implies that the produced antiserum solely reacts with κ-casein, failing this, other precipitates originating from antibody impurities must be differentiated from the main immunoprecipitate in such a way that they do not interfere in the evaluation of the results.

During preparation, purity of the κ-casein was examined by means of starch gel electrophoresis. Only those fractions from the gel filtration on Sephadex G-150, which were shown exclusively to contain κ-casein, were used. Incubation of the purified κ-casein with rennin showed that the whole product could be converted to para-κ-casein, thus indicating that no impurities of non rennin-sensitive caseins were present (Fig. 2). It was, therefore, considered that the κ-casein used for immunization did not contain essential impurities.

The rabbit anti-κ-casein sera produced after the first immunization were rather weak. After reimmunization four months later, the anti-κ-casein was usable provided that the agarose gels contained 8—10 % antiserum.

Antigen-antibody crossed electrophoresis in a gel containing anti-κ-casein showed that skim milk (Fig. 3A) only produces one strong precipitate with maximum at the point, where κ-casein

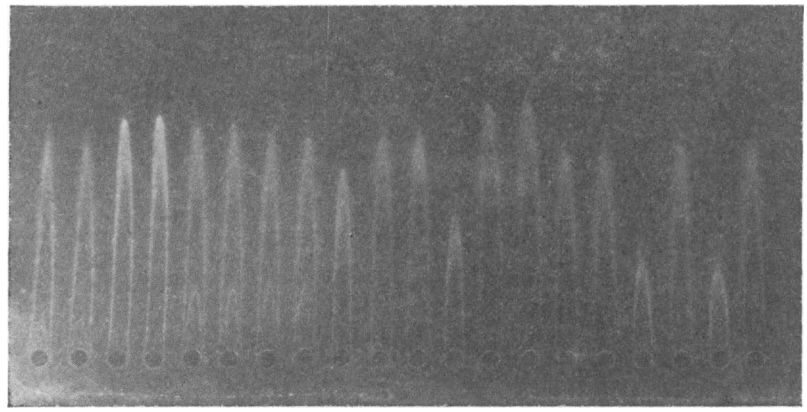

Figure 1. Quantitative determination of κ-casein by electrophoresis in agarose gel containing 10 % rabbit anti-κ-casein serum. Individual samples of skim milk were diluted 1 + 20. Dilutions of a standard solution were applied in holes nos. 3, 4, 9, 12, 17, and 19.

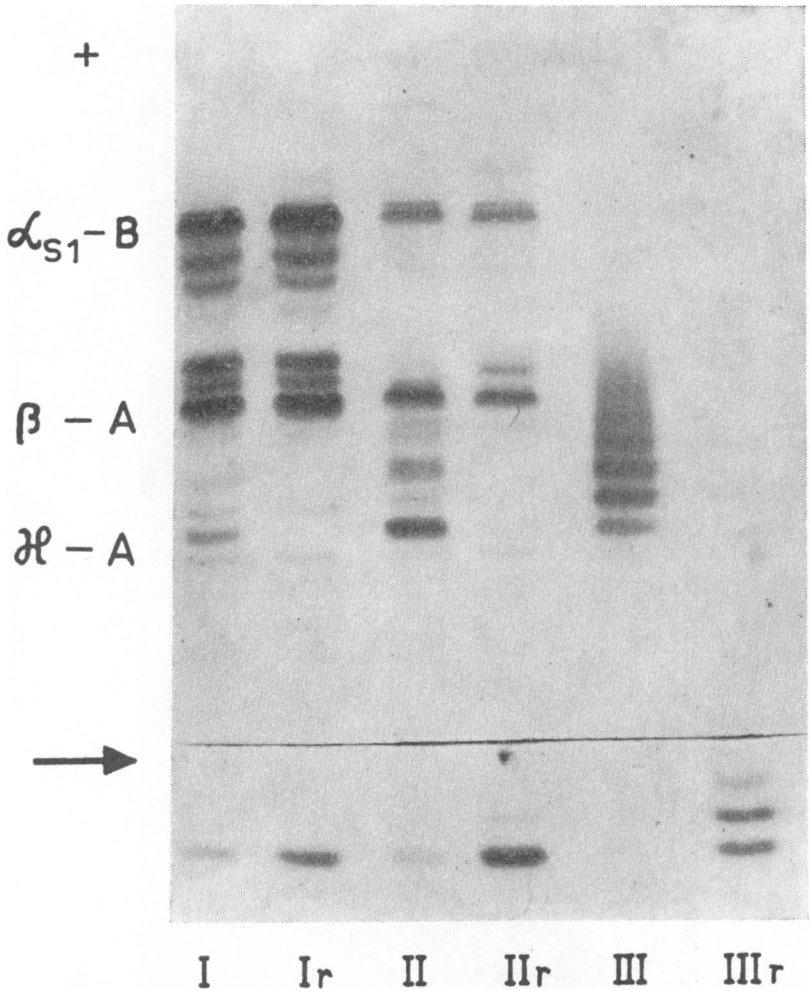

Figure 2. Starch gel electrophoresis of total milk (I), ϰ-casein prepared by the urea-sulfuric acid method according to *Zittle & Custer* (II), ϰ-casein further purified by two gel filtrations (III) before and after rennin treatment. Samples treated with rennin are designated r. Arrow indicates origin.

is found in the electrophoretic picture in 1st dimension (Fig. 3C). Both ϰ-casein and skim milk were investigated in the same anti-ϰ-casein containing gel. The samples were applied in separate grooves at intervals of 1.2 cm, in such a way that the migration routes were the same (Fig. 3B). This analysis showed that the precipitates produced from milk and ϰ-casein, respectively, formed one continuous curve with two maxima, and not two

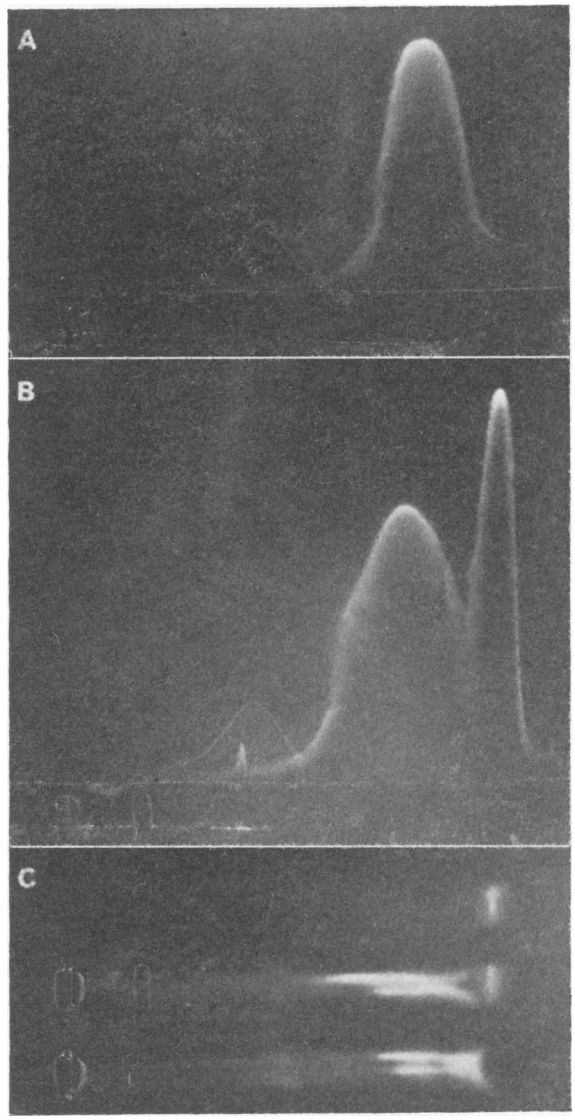

Figure 3. Antigen-antibody crossed electrophoresis. Antigen: A) skim milk, B) skim milk and κ-casein. 1st dimension electrophoresis (C) shows uppermost pure κ-casein, in the middle skim milk and κ-casein, and at the bottom skim milk.

crossing curves. From this result, it can be concluded that the precipitate seen by antigen-antibody crossed electrophoresis of skim milk originates from the reaction between the κ-casein in skim milk and the anti-κ-casein in the produced antiserum. Fig. 3

also shows that the antiserum used is not strictly specific, as a weak precipitation curve can be seen at a position equal to a protein with lower electrophoretic mobility than κ-casein. This protein is, however, not immunologically related to κ-casein. It is assumed to be identical to the protein giving rise to the weak precipitate which can be seen inside the κ-casein/anti-κ-casein rockets in Fig. 1. Investigation on the isolated whole casein, by means of Laurell-electrophoresis, suggests that this weak precipitate originates from a reaction with a whey protein, as no precipitation curve occurs inside the κ-casein rocket, when the whey has been removed before application.

Electrophoresis of the κ-casein complex in starch gel, combined with subsequent diffusion against anti-κ-casein in agarose gel, showed one even curve, not a row of small intersecting curves. Consequently all the κ-casein bands contain proteins which are immunologically related. This implies that the described method quantitates the total κ-casein complex.

The reproducibility of the method was determined by analysing two samples every day for 10 days. The experimental error was ± 4.1 %. After addition of pure κ-casein to skim milk 99.4 % was recovered.

Estimation of the κ-casein content in skim milk

Table 1 shows the results from the determinations of the κ-casein content in 116 milk samples from the Danish cattle breeds SDM, RDM and Jersey. The results are expressed as κ-casein as percentage of the total protein content in skim milk.

The first investigation (August 17) of 22 milk samples from SDM showed an average κ-casein content of 21.5 %. Fourteen days later new samples from the same animals were analysed and the samples from the evening milking (August 30) and the subsequent morning milking (August 31) were analysed separately. The average κ-casein content, 22.3 % and 22.8 % respectively, were not significantly different (t-test). The mean value of these investigations did not significantly differ from the result found two weeks previously.

Milk samples from 19 cows of the breed RDM were analysed. The average κ-casein content was 21.3 %. By analysing the milk samples from 13 of the same animals 35 days later, a mean value of 21.6 %, not significantly different from the previous average, was found.

Table 1. The κ-casein content, as percentage of total protein, in skim milk from Danish cattle breeds.

SDM				RDM			Jersey	
	date				date			date
cow no.	August 17	August 30	August 31	cow no.	August 17	Sept. 21	cow no.	June 10
10	22.4	24.0	24.8	13	17.0	19.3	1	25.9
37	20.4	24.9	22.5	19	17.6	19.0	36	21.8
39	17.5	18.5	21.4	31	18.4	20.8	72	22.1
70	22.9	21.8	22.4	45	17.6	23.8	77	21.5
83	20.5	23.7	22.2	50	20.5	—	94	22.4
91	19.7	23.6	20.6	67	20.7	—	105	19.7
93	22.4	24.2	23.1	72	25.9	—	114	27.9
94	19.3	22.7	20.0	78	—	18.0	120	25.2
97	26.2	19.5	20.4	81	—	21.5	121	25.4
99	20.4	20.9	21.7	100	18.4	21.6	123	25.8
100	18.1	20.3	24.7	470	22.4	22.3	130	23.0
101	18.7	21.1	22.4	476	19.8	22.0	134	24.1
108	18.1	21.1	23.1	478	22.8	—	136	23.4
123	17.9	23.1	24.3	482	20.4	19.9	138	26.5
199	23.3	22.8	22.8	490	21.4	—	141	21.1
215	24.5	22.7	22.0	638	23.9	22.7	143	26.3
256	24.3	19.9	22.5	756	26.2	—		
368	24.4	22.1	23.4	759	22.2	25.3		
375	25.8	24.5	25.5	763	21.8	21.8		
382	24.0	23.2	24.0	771	23.1	19.6		
394	20.5	20.8	22.2	775	25.5	26.0		
397	21.4	24.6	24.9					
mean	21.5	22.3	22.8		21.3	21.6		23.9
±s.e.m.	±0.6	±0.4	±0.3		±0.6	±0.6		±0.6

The κ-casein content in skim milk from Jersey cows was determined in samples from 16 animals. The mean value was 23.9 %.

The results of the three breeds were compared statistically (t-test). The difference in the κ-casein content in milk from SDM and RDM was not significant. But in comparison with the κ-casein concentration in milk from Jersey cows, the average κ-casein content in milk from both SDM and RDM was significantly lower. As samples from Jersey were collected only once, the comparison was made only on the basis of the mean value of the SDM and RDM breeds from the first investigation (August 17).

A regression analysis showed that the κ-casein content (mg κ-casein per ml skim milk) and the protein content (total protein percentage in skim milk) were positively correlated in the three breeds investigated. For SDM, RDM and Jersey, the correlation factors were found to be 0.58, 0.62 and 0.71 respectively. The results of the individual κ-casein determinations (κ-casein as percentage of the total protein content in skim milk) from SDM and RDM breeds were also compared with the estimations from each animal of the percentage fat in milk, the milk yield (kg per day) and the time after calving. These calculations showed that the relative κ-casein content was not dependent on any of these quantities.

DISCUSSION

Immunological methods for quantitative determinations of β-lactoglobulin and α-lactalbumin have previously been described (*Larson & Twarog* 1961, *Larson & Hageman* 1963). Similar procedures for immunoquantitation of other milk protein components have not been published. One of the reasons might be the difficulties involved with the isolation of proteins pure enough for production of specific antibodies. *Parry & Carroll* 1969 have, in connection with investigation of the micelle structure, produced an anti-κ-casein serum which is stated to be 95 % pure. Neither the anti-κ-casein used in the present investigation is strictly specific. However, this does not influence the accuracy of the method, as the major (measured) precipitate is completely separated from weaker precipitates originating from antibody impurities. Fig. 1 shows that the antigen-antibody precipitates due to the reaction between the antibody and the κ-casein in milk are not so well-defined, as those originating from the pure κ-casein. The reason for this is not known, but it was considered of minor importance, as the reproducibility of the method, ± 4.1 %, was satisfactory. Estimation of κ-casein by means of Laurell-electrophoresis is simple to set up, and the results can be read after 16 hrs. Therefore, the present procedure can easily be developed into a suitable method for routine-series analyses of the κ-casein concentration in milk samples.

As early as 1956 *Rolleri et al.* determined the content of α-, β-, and γ-casein in individual samples. The method used did not differentiate between α_s- and κ-casein; but the investigation

showed that milk from Holstein-Friesian cows contained significantly less α-casein than milk from Jersey, Brown Swiss, Guernsey and Ayrshire cows. The Danish cattle breeds SDM and RDM are most related to Holstein-Friesian cattle. In accordance with this, the content of κ-casein in milk from SDM and RDM was found to be significantly lower than in Jersey milk. *Larson & Kendall* (1957) extended the previous investigations and found that except for the first few days the relative α-casein content was constant during the lactation period. The absolute amount of α-casein was, on the other hand, decreasing during the last two thirds of the lactation period. In the present investigation it is shown that the relative κ-casein content in milk from SDM and RDM does not vary significantly during the investigated part of the lactation period (1—11 months). There was a weak positive correlation ($r_{SDM} = 0.46$, $r_{RDM} = 0.36$) between the absolute amount of κ-casein and the time after calving. As the κ-casein only constitutes a part of the α-casein complex, this result is not necessarily contradictionary to the findings of *Larson & Kendall*. The κ-casein content in colostrum and milk from the first month after calving was not determined in the present investigation.

As already mentioned under results the amount of κ-casein was positively correlated to the total content of protein in milk. Such a relation could be expected because of κ-caseins action as a stabilizer for the casein micelles.

The content of κ-casein as percentage of total protein in skim milk of Danish cattle (Table 1) is higher than the previously reported results. Using DEAE-cellulose chromatography *Rose et al.* (1969) found 14.8 % κ-casein in alkylated whole casein, which is equal to about 12 % of the total protein content in milk. *Ribadeau Dumas* (1968) used determinations of C-terminal amino acids, cleaved from whole casein by carboxypeptidase A, for estimations of the κ-casein content. He found 11.4 %, equal to about 9 % κ-casein, expressed as percentage of the total protein content in milk. The present investigation shows that there are great, individual, variations in the κ-casein concentration. It is, therefore, difficult to compare the present results with earlier findings, as the previous investigations include only few individual samples. Moreover, differences between the investigated cattle breeds as well as methodical differences can contribute to the non-uniform results. *Manning et al.* (1971) investigated a somewhat larger number of samples (25 individual milk

samples). By determining the sulfhydryl groups in reduced whole casein, an average of 22.5 % κ-casein was found. Expressed as percentage of the total protein content in milk, this is equal to about 18 %, which is of the same magnitude as the average content of κ-casein found in milk from Danish cattle breeds.

As mentioned above no significant correlation could be found between the κ-casein concentration and the lactation period. However, for the individual cows rather great variations occurred from day to day (Table 1). For future studies of genetically determined variations in the κ-casein content and the possible consequence for milk technology, it will, therefore, be necessary to estimate the κ-casein content at least twice during the lactation period, to establish the characteristic κ-casein level of individual cows.

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SAMMENDRAG

Kappa-kasein i mælk. En immunkemisk undersøgelse af κ-kaseinindholdet i mælk fra danske kvægracer.

κ-kaseinindholdet i mælk fra SDM, RDM og Jersey blev bestemt ved kvantitativ immunelektroforese i antistofholdig agarosegel.

Metoden, der bestemmer det totale κ-kaseinkompleks, er velegnet til rutinemæssige undersøgelser. Reproducerbarheden var ± 4,1 %.

Der blev i procent af total protein fundet signifikant lavere κ-kaseinindhold i mælk fra SDM (21,5 %) og RDM (21,3 %) end i mælk fra Jersey (23,9 %). Undersøgelsen sandsynliggør, at κ-kaseinet udgør en større del af mælkens totalprotein end tidligere antaget.

κ-kaseinmængden var positivt korreleret med mælkens proteinindhold. Derimod var der ingen relation mellem det procentiske κ-kaseinindhold og fedtprocenten, ydelsen og tidspunktet i laktationen.

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