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STUDIES ON PARTURIENT PARESIS IN DAIRY COWS

V. ON THE COMPOSITION AND CALCIUM BINDING CAPACITY OF TWO BOVINE SERUM PROTEIN FRACTIONS, WITH SPECIAL REGARD TO PARTURIENT PARESIS

By

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In an earlier paper in this series (*Carlström* 1961a) it was shown that a change of the protein probably takes place in conjunction with calving, resulting in changed calcium binding conditions. In cows afflicted with parturient paresis this change is more pronounced and also seems to be of a different nature. The change probably takes place in the albumin.

In the present study the serum protein was divided into two fractions, of which one consisted mainly of albumin and the other of globulin, and the calcium binding capacity of these fractions was determined. They were also subjected to a partial amino acid analysis.

MATERIAL

The material consisted of blood samples from dairy cows of Swedish red-and-white breed (a few were Holstein-Friesian). All samples were collected within an area of about 30 km from the town of Skara in western Sweden. The material was divided into three groups:

A. Samples from cows outside calving period. The samples were collected at the earliest one month after calving and at the latest one month before calving, i.e. during the period when the cows may be calculated to be least affected by an imminent or

past calving. The cows had calved at least two or three times, except for a few which had calved only once.

B. Samples from cows which had recently calved without complication either before or after calving. The material contained no first calver and only a few second calvers. The samples were collected within 72 hrs. of calving, i.e. in the period when parturient paresis usually occurs.

C. Samples from cows with parturient paresis. The diagnosis was made by the veterinarian who treated the cow, and the sample was collected prior to treatment. The diagnosis was confirmed by calcium analysis, and only cases with a total calcium content below 7.5 mg per 100 ml were included.

The samples consisted of whole blood without additive. Some of the blood was tapped off under a film of paraffin oil.

The samples in groups A and B were drawn by the research station personnel and were centrifuged at the latest a few hours after collection. The samples in group C were drawn by the veterinarian in charge of the cow. They were centrifuged as soon as possible, at the latest 24 hrs. after collection.

The separated serum samples were kept in a refrigerator at +5°C prior to analysis. Serum for electrophoresis and the separated protein fractions were kept deep-frozen prior to analysis.

METHODS

The serum samples were analysed for total calcium, inorganic phosphorus and total protein, and paper electrophoresis was performed. After centrifugation, pH and free calcium ions were determined on serum from the samples taken under paraffin oil.

Total calcium was determined with an atomic absorption spectrophotometer (Perkin-Elmer 303) according to the method set forth in the book of instructions.

Determination of inorganic phosphorus was made by the method of *Fiske & Subbarow* (1925) as described by *Hawk et al.* (1954).

The total protein content was determined by the biuret method as described by *Reiner* (1953).

Paper electrophoresis was done by the method described by *Carlström* (1961a).

The content of free calcium ions was determined according to *Carlström* (1955).

Table 1. Errors of methods.

Analysis	n	$\bar{x}$	Variation	s	v
Ca mg/100 ml	12	10.49	9.8—11.5	0.20	1.89
Inorganic P mg/100 ml	13	6.08	4.5— 8.2	0.13	2.14
Protein g/100 ml	13	8.63	7.9— 9.6	0.28	3.24

Symbols, see text (pp. 91 and 92).
v: error of determination in per cent of mean.

The pH was measured with a Radiometer PHM3i.

Table 1 shows the results of an analysis of the errors of the methods for determination of total calcium, inorganic phosphorus and total protein. The error of the method was calculated on duplicate samples according to the formula $s = \sqrt{\frac{\Sigma d^2}{2n}}$, where d is the difference between the duplicate samples and n the number of duplicate samples.

The investigation of the error of the electrophoretic method was carried out by *Carlström* (1961a), of the method for the determination of free Ca^{2+} ions by *Hallgren et al.* (1959), and of the method referred to below for the determination of amino acids in serum proteins by *Carlström* (1968).

The separation of the serum protein into two fractions was done as follows.

To 20 ml serum was added 32.6 ml saturated solution of ammonium sulphate, whereby the solution became 62 % saturated. Thereby a fraction, consisting mainly of albumin, was precipitated. The mixture was filtered and the precipitate washed free from soluble fraction with 55 ml ammonium sulphate solution saturated to 62 %. Filtrate and washing fluid, which contained a protein fraction, consisting of most of the serum globulin, were transferred to a dialysis hose of cellophane (Wisking, 5 cm dia.), which was sealed at both ends. The precipitate was dissolved in 0.5 % sodium chloride solution and the resulting solution was transferred to a dialysis hose. The salt content of the two protein solutions was lowered by dialyzing each hose against 2×1 l of water for 2×1 day at refrigerator temperature.

The contents of the dialysis hoses were pervaporated. This procedure, introduced by *Kober* (1917) and later described by *Farber* (1935), was done by suspending the hoses under a powerful ventilation fan. Water and low-molecular substances filtered out through the hose, and on the outside the water evaporated in the air stream, while the solid substances settled on the outside, where they either fell off or blew away in the powerful air stream. The procedure was carried out in a cold room during the cold season of the year. On some occasions the solution froze in the hose, which admittedly delayed but did not prevent the pervaporation. Freezing was avoided, however, as the hoses were liable to burst at a subzero temperature.

When the content of the hose had been reduced in this way to 5—10 ml, the hose was softened by rapid moistening with water. The volume of the hose was reduced to below 18 ml, whereupon the solution was dialyzed against 4×200 ml of a diemal buffer made according to *Michaelis* (1931) with ionic strength 0.15 and the same pH as the serum from which the protein derived. The dialysis continued for 4×1 day.

125 mg $CaCO_3$ was dissolved in the smallest possible quantity of 1 N hydrochloric acid and the solution was evaporated to dryness on a water-bath. The residue was dissolved in 100 ml of the Michaelis buffer. This solution contained 50 mg Ca per 100 ml.

After the dialysis the hose was opened and to its contents were added 2 ml of the Ca solution in Michaelis buffer, plus the buffer (without Ca) to a total volume of 20 ml. This solution now contained the same concentration of its respective protein fraction as the original serum, at the same pH and same ionic strength, and also 5 mg Ca per 100 ml.

On the resulting protein solutions determinations were made of the total Ca content and pH (as check) and of the content of free Ca^{2+} ions, and hydrolysis and gas chromatographic determination of amino acids were performed by the method of *Carlström* (1968).

The results of all analyses were statistically analysed by means of Student's t-test. The following statistical symbols were used: n = number of analyses, $\bar{x}$ = mean value, ε = standard error of mean. Significance is indicated by asterisks: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Table 2. Analytical results and statistical calculation.

Analysis	A		B		C		A-B	A-C	B-C
	n	$\bar{x}\pm\varepsilon$	n	$\bar{x}\pm\varepsilon$	n	$\bar{x}\pm\varepsilon$	t	t	t
Ca, total mg/100 ml	20	10.52±0.13	16	9.74±0.26	25	5.29±0.23	2.852**	18.462***	12.495***
Free Ca^{2+} ions mg/100 ml	20	4.41±0.51	16	5.11±0.28	25	2.62±0.19	1.129	3.577***	7.573***
Ca bound (calc'd) mg/100 ml	20	6.11±0.45	16	4.63±0.30	25	2.67±0.13	2.576*	7.958***	6.756***
Inorganic P mg/100 ml	20	5.66±0.26	16	4.98±0.31	24	1.24±0.13	1.695	16.217***	12.450***
pH	20	7.614±0.009	16	7.656±0.016	25	7.628±0.016	2.357*	0.701	1.168

Symbols, see text (pp. 89 and 92).

Table 3. Protein, electrophoresis. Analytical results and statistical calculation.

Analysis	A		B		C		A-B	A-C	B-C
	n	$\bar{x}\pm\varepsilon$	n	$\bar{x}\pm\varepsilon$	n	$\bar{x}\pm\varepsilon$	t	t	t
Total protein g/100 ml	20	8.34±0.12	16	8.15±0.13	25	7.98±0.14	1.057	1.900	0.854
Albumin % of total	20	57.45±1.33	16	55.99±1.32	25	58.49±0.64	0.767	0.748	1.884
α-globulin % of total	20	9.53±0.51	16	11.13±0.42	25	11.10±0.35	2.342*	2.623*	0.055
β-globulin % of total	20	8.47±0.28	16	9.15±0.53	25	9.19±0.36	1.197	1.506	0.065
γ-globulin % of total	20	24.66±1.05	16	23.81±1.25	25	21.31±0.69	0.523	2.754**	1.897
Total globulin % of total	20	42.66±1.33	16	44.09±1.32	25	41.60±0.65	0.753	0.763	1.876
Albumin g/100 ml	20	4.79±0.12	16	4.55±0.09	25	4.67±0.10	1.486	0.773	0.833
α-globulin g/100 ml	20	0.79±0.04	16	0.90±0.03	25	0.89±0.03	2.058*	1.976	0.208
β-globulin g/100 ml	20	0.71±0.02	16	0.75±0.05	25	0.73±0.03	0.784	0.485	0.366
γ-globulin g/100 ml	20	2.07±0.11	16	1.95±0.12	25	1.70±0.06	0.738	3.155**	2.039*
Total globulin g/100 ml	20	3.56±0.13	16	3.61±0.14	25	3.32±0.07	0.254	1.736	1.971

Symbols, see text (pp. 89 and 92).

Table 4. Bound calcium in serum and in serum protein solutions, in per cent of total calcium. Results and statistical calculation.

Analysis	A		B		C		A-B	A-C	B-C
	n	$\bar{x}\pm\varepsilon$	n	$\bar{x}\pm\varepsilon$	n	$\bar{x}\pm\varepsilon$	t	t	t
Serum	20	58.5±4.7	16	47.5±2.6	25	51.2±2.2	1.916	1.510	1.083
Fraction P	19	42.3±3.3	14	21.5±4.3	25	23.3±3.5	3.889***	3.857***	0.319
Fraction F	19	4.9±1.6	14	4.9±2.1	25	6.0±1.5	—	0.483	0.417
Fractions P + F	19	47.3±3.8	14	26.4±4.9	25	29.3±4.7	3.149**	3.361**	0.382
Serum — Fractions P + F, t		1.840		3.499**		4.238***			

Symbols, see text (pp. 89, 92 and 98).

Table 5. Fraction P, amino acid residues in moles per cent of the amino acids determined. Results and statistical calculation.

Amino acid	A		B		C		A-B	A-C	B-C
	n	$\bar{x}\pm\varepsilon$	n	$\bar{x}\pm\varepsilon$	n	$\bar{x}\pm\varepsilon$	t	t	t
Alanine	20	10.89±0.26	17	10.55±0.36	20	11.34±0.26	0.789	1.228	1.810
Aspartic acid	20	12.13±0.15	17	12.54±0.20	20	12.01±0.20	1.636	0.484	1.874
Glutamic acid	20	16.11±0.31	17	16.64±0.27	20	16.08±0.39	1.255	0.140	0.934
Glycine	20	6.48±0.36	17	7.27±0.46	20	8.48±0.57	1.376	2.977**	1.613
Isoleucine	20	5.94±0.55	17	4.99±0.39	20	6.20±0.55	1.361	0.334	1.740
Leucine	20	13.88±0.13	17	13.91±0.29	20	13.43±0.30	0.101	1.377	1.145
Lysine	20	13.80±0.52	17	12.29±0.68	20	11.32±0.62	1.783	3.047**	1.049
Phenylalanine	20	5.75±0.07	17	6.03±0.13	20	5.77±0.11	1.951	0.149	1.497
Proline	20	6.45±0.10	17	6.72±0.15	20	6.77±0.17	1.555	1.623	0.220
Valine	20	8.56±0.15	17	9.05±0.34	20	8.51±0.27	1.394	0.163	1.259

Symbols, see text (pp. 89, 92 and 98).

Table 6. Fraction F, amino acid residues in moles per cent of the amino acids determined. Results and statistical calculation.

Amino acid	A		B		C		A-B	A-C	B-C
	n	$\bar{x}\pm\varepsilon$	n	$\bar{x}\pm\varepsilon$	n	$\bar{x}\pm\varepsilon$	t	t	t
Alanine	20	10.41±0.37	17	9.27±0.37	20	9.79±0.33	2.150*	1.248	1.052
Aspartic acid	20	11.53±0.13	17	11.81±0.26	20	10.97±0.27	1.011	1.848	2.219*
Glutamic acid	20	13.89±0.26	17	14.18±0.30	20	13.66±0.39	0.741	0.491	1.031
Glycine	20	10.78±0.41	17	12.96±0.87	20	14.83±1.06	2.385*	3.581***	1.336
Isoleucine	20	7.60±0.69	17	6.06±0.53	20	7.76±0.58	1.720	0.177	2.133*
Leucine	20	11.61±0.12	17	11.33±0.80	20	10.96±0.26	1.061	2.267*	1.006
Lysine	20	9.58±0.47	17	8.91±0.52	20	7.91±0.39	0.959	2.740**	1.553
Phenylalanine	20	4.66±0.06	17	4.70±0.14	20	4.70±0.13	0.275	0.278	—
Proline	20	8.11±0.14	17	8.45±0.18	20	8.22±0.27	1.510	0.363	0.681
Valine	20	11.84±0.18	17	12.32±0.39	20	11.23±0.37	1.170	1.491	2.037*

Symbols, see text (pp. 89, 92 and 98).

RESULTS AND DISCUSSION

The results of the serum analyses, except in respect of protein content and electrophoresis, are presented in Table 2. Table 3 shows the results of total protein determination and electrophoresis.

In the Tables 4, 5 and 6, and in the text, the protein fraction obtained by precipitation with ammonium sulphate as described above is called fraction P, and the soluble fraction is called fraction F. Fraction P contains mainly, but by no means exclusively, albumin, and the following remarks about this fraction must therefore not definitely be taken as relating to serum albumin. Fraction F contains most of the serum globulin.

Table 4 shows the results of the addition of Ca to solutions of fraction P and fraction F. The results are presented as bound Ca as percentage of total Ca. The corresponding figures for serum are tabulated for comparison.

Tables 5 and 6 show the amino acid analysis of the protein fractions.

Largely speaking, the results of the analyses in blood serum (Table 2) were similar to those found earlier (*Hallgren et al.* 1959, *Carlström* 1961a, b). In the present material the lowering of the Ca content which occurs in conjunction with normal calving relates entirely to bound Ca, while according to *Carlström* (1961a) a small reduction occurs also in the fraction of free Ca^{2+} ions.

The slight rise in pH at normal calving was not recorded on cows with parturient paresis.

Characteristic of the paper electrophoretic pattern of the serum proteins (Table 3) was, as earlier reported (*Carlström* 1961a), that only small changes in size of the protein fractions and in their mutual relations occurred in conjunction either with normal calving or parturient paresis.

Table 4 shows the results of the addition of Ca to fraction P and fraction F solutions. In serum of cows outside the calving period about 58 % of the total Ca was bound. The protein in the same serum could directly bind about 47 %. About 11 % therefore was bound otherwise than directly to protein. The corresponding figures for normal recent calvers were 47, 26 and 21 %, for cows with parturient paresis 51, 29 and 22 %. In recent calvers, accordingly, the protein had a greatly inferior capacity for direct binding of Ca than in cows outside the calving period,

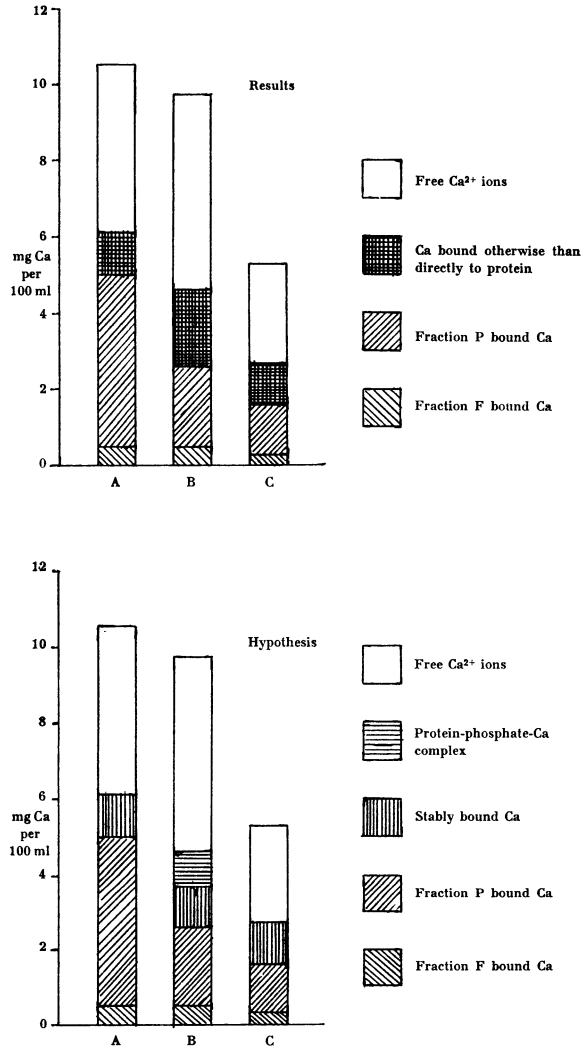

Figure 1. Symbols, see text (pp. 89, 92 and 98).

while no appreciable difference in this respect existed between healthy recent calvers and cows with parturient paresis. The change is entirely referable to fraction P (see Fig. 1).

This means that recent calvers have a very much greater proportion of Ca bound otherwise than directly to protein than cows outside the calving period. Nothing definite can be said about the nature of this fraction, but certain speculations may

be made on the basis of the results in this and earlier studies. The simplest assumption, of course, is that this Ca is bound in a complex of unknown nature, which would explain the figures in Table 4 but would not agree with the results of an earlier study (*Carlström* 1961a). The latter showed that the relation between Ca and protein within each of the three groups of cows could be expressed by a fairly simple formula, but that the formulae for cows after normal calving and for cows with parturient paresis were clearly different. The assumption of a complex does not tally with these results.

Assume instead that in cows outside the calving period the non-direct protein-bound portion is fairly stably bound and that this fraction does not change in conjunction with calving or parturient paresis. In cows outside the calving period the fraction constitutes about 11 % of the total content of Ca, i.e. about 1.2 mg per 100 ml. In healthy recent calvers 1.2 mg per 100 ml constitutes about 12 % of the total Ca, and about 9 % of the total content would thus be bound in a third manner. In cows with parturient paresis, on the other hand, 1.2 mg per 100 ml constitutes about 22 % of the total Ca, and this is precisely the quantity bound otherwise than directly to protein. This means that the difference between healthy cows after calving and cows with parturient paresis is that in the former there exists a Ca compound which is lacking in the latter (see Fig. 1).

In parturient paresis there is a marked reduction of the content of inorganic phosphorus. It is conceivable therefore that the said hypothetical compound is a loosely bound protein-phosphate-calcium complex. The complex would break down in the drastic treatment (precipitation of protein with trichloroacetic acid) in the current method used for determining "inorganic phosphorus" according to *Fiske & Subbarow* (1925), and the phosphate would thus be included in this determination. At least a part of the "inorganic" phosphate in serum would thus be loosely bound to protein, and in some cases — as when the ability of serum protein to directly bind Ca is reduced — be able to form a complex with Ca. This would coincide closely with the results presented above. It would also explain that calcium phosphate apparently exists in supersaturated solution in serum.

In order to establish whether any difference existed in amino acid composition of the fractions P and F in the three groups of cows, a determination was made of the content of 10 amino acids.

The result for fraction P is shown in Table 5. Differences exist in so many points between cows outside the calving period and after calving, and between healthy cows after calving and cows with parturient paresis, that one may say with a considerable measure of assurance that the fraction P is not the same in the three groups. Especial attention may be directed to glycine, the content of which is higher after calving than in cows outside the calving period, and still higher in parturient paresis; and also to lysine, for which the conditions are reversed. The differences between normal calvers and cows with parturient paresis in respect of alanine, aspartic acid and isoleucine are also worthy of note.

As regards fraction F it appears even more certain that the three groups differ (Table 6). This is hardly remarkable in view of the heterogeneity of this fraction and, as already noted, the fact is of no great interest in connection with Ca.

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SUMMARY

In healthy cows unaffected by imminent or recent calving the protein in serum can directly bind the overwhelming proportion of the bound calcium. In recent calvers this capacity is considerably less. When adding ammonium sulphate to blood serum to 62 % of total saturation a protein fraction precipitates which is mainly albumin. This fraction has a far greater calcium binding capacity than the soluble fraction, which contains most of the serum globulin, and the lowering of this capacity after calving is entirely referable to the former fraction. No difference has been found in these respects between normal cows after calving and cows with parturient paresis.

An analysis of 10 amino acids in the two protein fractions described above showed that the amino acid composition of both exhibits differences between recent calvers and cows outside the calving period, and likewise that each of the two fractions differs in composition between healthy cows after calving and cows with parturient paresis.

SAMMANFATTNING

Studier över kalvningsförlamning hos mjölkkor.

V. Om sammansättning och kalciumbindningsförmåga hos två bovina serumproteinfraktioner, med speciell hänsyn till kalvningsförlamning.

Hos friska kor som icke påverkats av strax förestående eller nyss genomgången kalvning kan proteinet i serum direkt binda den allra största delen av det bundna kalciet. Hos kor som nyss kalvat är denna förmåga avsevärt mindre. Då man till serum sätter ammoniumsulfat till 62 % av fullständig mättning, så utfaller en proteinfraktion, som huvudsakligen består av albumin. Denna fraktion har en avsevärt större kalciumbindningsförmåga än den lösliga fraktionen, som innehåller större delen av serumglobulinet, och sänkningen i bindningsförmågan efter kalvning kan helt hänföras till den förra fraktionen. Ingen skillnad i dessa avseenden har konstaterats mellan friska nykalvade kor och kor med kalvningsförlamning.

En analys av 10 aminosyror i de två ovannämnda proteinfraktionerna visade att aminosyresammansättningen hos båda visar skillnader mellan nykalvade kor och kor utanför kalvningstiden, och likaså att var och en av de två fraktionerna uppvisar skillnader i sammansättningen mellan friska nykalvade kor och kor med kalvningsförlamning.

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