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Biochem. J. (1964) **91**, 130

Studies on Human Casein Preparations from Single Milk Samples

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(Received 14 August 1963)

The reported values for the sialic acid content of human casein vary widely. Malpress (1961, 1962) has given a maximum value of 2.1% for samples prepared from milk collected from the fourth to the eighth day after parturition, and a range of 0.30–0.38% for samples from mature milk. Other values given for mature milk are 0.76% (Johansson & Svennerholm, 1956), 0.8 and 0.37% (Alais & Jollès, 1962*a, b*).

Discrepancies are also present in reports on the extent of rennin action on casein samples: Malpress (1962) found the release in combined form of 30% of the total sialic acid by this enzyme; Alais & Jollès (1962*b*) obtained a glycopeptide, containing sialic acid, from some but not all of their casein preparations as the result of rennin action.

These variations could be the result of the different procedures used for preparing casein. Alternatively they might indicate true individual variations in caseins from different mothers, or from the same mother at different stages of lactation; the complexity of human casein shown by its heterogeneity on electrophoresis in the presence of urea (Malpress, 1962; Alais & Jollès, 1962*a*) suggests possibilities of variation in support of this alternative view.

The problem has now been further studied by an investigation of caseins, prepared by a standard procedure, from 80 single milk samples taken from 47 mothers and covering all stages of lactation.

MATERIALS AND METHODS

Preparation of casein. Milk samples (3–100 ml.) were stored at 2° and used for the preparation of casein within 1 week of collection. (In a few exceptional cases the samples were kept frozen for up to 1 month before use.) The milks were centrifuged at 10 000*g* at 5° for 15 min. to remove fat. The skimmed milks (pH 6.5–7.5) were filtered through cotton wool, adjusted to pH 4.7 with HCl and kept at 2° overnight; they were then warmed to 25° for 30 min. (cf. Maeno & Kiyosawa, 1962) and centrifuged at 35 000*g* at 10° for 30 min. The precipitated caseins were dispersed and recentrifuged twice in 0.01*M*-acetate adjusted to pH 4.7 with *N*-NaOH. The volume of the skim-milk supernatants was measured; they were then filtered through cotton wool and retained for the estimation of 'non-casein sialic acid'.

The washed casein precipitates were dissolved in water with minimum amounts of 0.1*N*-NaOH, care being taken to keep the pH less than 10, and the solutions (50–100 ml.) were filtered through a cotton-wool plug and adjusted to pH 4.7 with HCl. After storage overnight at 2° the caseins were collected by centrifuging at 15 000*g* at 10° for 15 min. They were washed twice with ethanol-ether (1:1, v/v) and twice with ether, and dried in air.

In the present paper 'casein' is defined as the protein prepared from human milk samples by the foregoing method, and 'mature casein' refers to the preparations derived from milk taken on or after the tenth day of lactation.

Starch-gel electrophoresis. This was carried out at pH 8.6 by using the discontinuous tris-citrate-borate buffer system of Poulik (1957) in the presence of 7*M*-urea as described by Wake & Baldwin (1961). All analyses were made at 2° in a

Perspex tray (21.5 cm. $\times$ 10.5 cm. $\times$ 1 cm.) which allowed four samples to be run simultaneously. Samples, each representing about 2 mg. of protein, were put into the slots as 0.3% solutions in 0.076M-tris-citrate buffer, pH 8.6, brought to a glue-like consistency by the addition of small amounts of starch. The voltage gradient was about 4v/cm. and the analyses were stopped after about 24 hr. when the brown boundary had travelled 10–15 cm. from the origin. The gels were stained with 0.01% Nigrosin in methanol-acetic acid-water (5:1:4, by vol.).

Estimation of sialic acid. The thiobarbiturate method (Warren, 1959) was used. *N*-Acetylneuraminic acid (L. Light and Co. Ltd., Colnbrook, Bucks.) was used as a standard and gave a molar extinction coefficient of 70 000 at 549 m μ . 'Non-casein sialic acid' values were obtained after a twentyfold dilution of the supernatants. Unhydrolysed-blank estimations were carried out on all solutions.

Action of rennin. Crystalline rennin (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) was used in a final concentration of 0.001% to determine the extent of rennin action on casein preparations. The incubations were carried out for 2 hr. at 37° in unbuffered solutions of casein (0.1–0.2%) adjusted to pH 5.8; under these conditions rennin action was usually complete in less than 1 hr., the change in pH during incubation being negligible. The enzyme was destroyed by the addition of an equal volume of 24% (w/v) trichloroacetic acid, the solutions were centrifuged at 35 000g at 10° for 30 min. and the clear supernatants were extracted three times with an equal volume of ether. The aqueous layers were evaporated to dryness over P₂O₅ *in vacuo* and the dry residues were dissolved in 0.1N-H₂SO₄. Control solutions were incubated without rennin, the enzyme being added after the addition of trichloroacetic acid.

The sialic acid released as glycopeptide soluble in 12% trichloroacetic acid was measured by the difference in sialic acid values of the test and control solutions, prepared in this way, after hydrolysis at 80° for 1 hr. It is referred to as the rennin-sensitive fraction of the casein sialic acid.

RESULTS

Eighty casein preparations were made, from milk samples given by 47 mothers; they represented stages of lactation from the first day to the eleventh month after parturition.

Yields varied from 20 to 500 mg./100 ml. of skim milk for samples taken on or after the tenth day of lactation (non-colostral milks); these values may be compared with values given by Macy (1949), who quotes a range of 140–680 mg. of casein/100 ml. of human whole milk, obtained from 166 samples by a method which involved no reprecipitation of the casein. In the present work the wide variation in yields showed no correlation with either the stage of lactation at which the sample was taken or with the sample volume. There were differences between samples in the readiness with which casein was precipitated at pH 4.7 and 25° from the skimmed milks, and it is therefore possible that in the present method yields were influenced at this stage. To

Table 1. *Relative mobilities of the bands shown by human casein in starch-gel electrophoresis*

Experimental conditions are given in the text. The distance from the origin to the discontinuous boundary was 12 cm. The mobilities are related to that of band 1, which is taken as 1.0.

Main bands	Relative mobility	Subsidiary bands
—	0.33	7
—	0.45	6'
6	0.56	—
—	0.62	5'
5	0.71	—
—	0.76	4'
4	0.83	—
—	0.86	3'
3	0.89	—
2	0.95	—
1	1.00	—
—	1.04	<i>a</i>
—	1.06	<i>b</i>
—	1.08	<i>c</i>
—	1.13	<i>d</i>
—	1.36–1.40	Boundary smudge
—	1.43	Boundary

minimize the danger that a low yield might be associated with uncharacteristic properties in the isolated casein, an arbitrary exclusion of all samples representing less than 100 mg. of casein/100 ml. of skim milk has been made in the present paper.

Starch-gel electrophoresis. The characteristic band pattern given by the human casein samples consists of six well-defined bands, all of which move towards the anode more slowly than the two main bands given by cow casein under similar conditions (Fig. 1). The bands are here numbered 1–6 in order, band 1 being that which migrates farthest from the point of origin. Table 1 gives the measure of the relative mobilities of the bands (that of band 1 being taken as 1.00). In addition values are given for a number of other bands which occur both more rarely and, when present, with far less intensity. Of these, bands *a*, *b*, *c* and *d* have in the present study been mainly, but not exclusively, associated with milk samples taken during the first month of lactation; they were present in only a few of the samples from this period, however, and were not seen in any sample taken earlier than the tenth day. Bands 3', 4', 5' and 6' were rarely detectable with the casein concentrations normally used (0.3%); they were sometimes present when this concentration was doubled. Band 7 was seen only once, in association with an uncommon pattern (type C). Other features of the stained gels were a weak and confused smudge, running almost with the boundary, given by casein from samples taken early in lactation, and a weak generalized staining extending from the origin to approximately the band 6

position which was not always detectable but was found in samples at all stages of lactation. This last observation gains in significance from the occurrence of a similar diffuse and weakly staining area in electrophoretic patterns of cow casein, where it has been found to represent the rennin-sensitive κ -casein fraction (Wake & Baldwin, 1961). In the present work it has been found that the six-band electrophoretic pattern of human casein is unchanged by the action of rennin and it is clear that the heterogeneity of human casein is greater than this pattern implies.

A limited study of caseins prepared from colostrum milks secreted during the first week of lactation gave a consistent picture of the order of band appearance, but suggested that the stage of lactation at which any given band appeared might vary. One series is shown in Fig. 2: in this case milk samples were obtained from the third to the sixth day of lactation and the caseins isolated showed bands 1 and 2 increasing in intensity throughout; bands 3 and 4 appeared in trace amounts on the fourth day, and increased in intensity on the following days; band 5 appeared in trace amounts on the sixth day; band 6 was absent throughout.

A second series, started on the first day of lactation, showed bands 1 and 2 appearing for the first time on the third day: two other series showed no bands at all after 4 and 6 days respectively.

The full six-band pattern was seen at its earliest in the present work in a casein sample from milk taken on the tenth day after parturition; in general the results indicated that for most mothers whose lactation was graded as 'good' the pattern was established soon after this time, but exceptions were encountered in which the appearance of bands, 5 and 6 in particular, was delayed.

Caseins from 32 out of 43 mothers whose milk was sampled on or after the tenth day of lactation gave a band pattern with intensities conforming to type A (Fig. 3), in which the intensities decreased in the order: 2; 4; 1 and 3; 5; 6. During the first month of lactation a modification of this pattern was sometimes seen in which band 1 was more intense than band 4; it is thought that this was a consequence of the serial development of the bands during this period, in which band 4 appears later than band 1; other minor and temporary differences might also be expected during this period for the same reason, and in assessing the electrophoretic analyses nine such small variations, occurring during the first month of lactation, were classified as 'developmental type A' patterns and included in the type A total.

Seven of the mothers gave caseins classified as type B (Fig. 3), in which the intensities decreased in the order: 2; 1; 4; 3; 5; 6; band 1 had a markedly greater intensity than band 4, and bands 5 and 6

were either weak or absent; in these cases the variation from type A persisted beyond 1 month and could not be regarded as an anomaly of the developmental period. Four of these mothers gave samples at more than one stage of their lactation, but the type B pattern showed no tendency to transpose to type A as the lactation advanced.

Of the remaining four cases two gave electrophoretic patterns of type C (Fig. 3), in which the intensities decreased in the order: 4; 2; 3, 5 and 6; 1. In both cases this pattern occurred when further caseins were prepared from later milks from the same mothers. Lastly, two caseins showed a complete absence of bands although the lactations were at 12 and 21 days respectively and subjectively assessed as 'fair' and 'good'; later samples were not available from these subjects.

Two mothers (type B and type C) gave samples simultaneously from both breasts. In both cases the characteristic pattern (type B or type C) was found in one sample whereas the second showed properties intermediate between type A and the characteristic form; these 'transitional' patterns are referred to in the Discussion section.

One mother (type A) gave samples immediately before and after suckling; the casein band patterns were identical.

The six-band pattern was not dependent on the presence of urea in the starch-gel preparation. In the complete absence of urea shrinkage occurred and the bands, although present, and in their correct relative intensities, were less well-defined; with 2M-urea the electrophoretic analysis was identical with that given in the presence of 7M-urea.

Non-casein sialic acid. The sialic acid in combined form remaining in the skim-milk super-

EXPLANATION OF PLATE 1

Fig. 1. Starch-gel electrophoresis in tris-citrate-borate system of: human casein (bands 1-6); cow casein (two bands, the faster travelling almost with the boundary). The experimental conditions are given in the text; 2 mg. of casein was applied in each case.

Fig. 2. Starch-gel electrophoresis in tris-citrate-borate system of human caseins from milks sampled in early lactation from the same mother at the following times after parturition: 3 days; 4 days; 5 days; 6 days. The experimental conditions are given in the text; 2 mg. of casein was applied in each case. Band 5, which was just present on the sixth day, cannot be seen in the photograph. The prominent bands near the origin (at 3 and 4 days) are not found in the electrophoresis of mature caseins.

Fig. 3. Starch-gel electrophoresis in tris-citrate-borate system of human casein types A, B and C. The experimental conditions are given in the text; 2 mg. of casein was applied in each case. In the type B example bands *b* and *c* (Table 1) can also be seen.

Fig. 1

Fig. 2

Fig. 3

natants after precipitation of casein at pH 4.7 fell from 237 mg./100 ml. on the first day of lactation to 6.5 mg./100 ml. after 11 months (Fig. 4). Since the sialic acid associated with casein is very small

Fig. 4. Sialic acid, in combined form, in human skim-milk samples from which casein had been removed by precipitation at pH 4.7.

Fig. 5. Sialic acid in human casein preparations isolated from milk samples taken at different stages of lactation.

compared with the non-casein sialic acid, except in advanced lactation when the latter values are very low, the distribution of samples in the scatter diagram would be negligibly affected by any incomplete precipitation of casein at the isoelectric point.

Casein sialic acid. The sialic acid in the casein preparations fell from about 1.0 % for samples from milk taken in the first few days of lactation to about 0.5 % after 1 month. The decline continued more slowly to the end of lactation (Fig. 5).

A positive correlation existed between the casein sialic acid and the sialic acid in the casein-free supernatants (Fig. 6). Since the electrophoretic evidence suggested that mature casein is not present until the tenth day of lactation, regression equations have been calculated separately for periods before and after this time, in addition to the equation covering all samples. The equation for mature samples gave a minimal intercept value of 0.34 % for the sialic acid content of casein (Table 2).

An isolated value of 0.10 % for a casein sample prepared from milk taken immediately after suckling is referred to in the Discussion section.

Action of rennin. The amount of the total sialic acid in casein which was released as glycopeptide by the action of rennin increased from less than 10 % during the first few days of lactation to values

Fig. 6. Relationship between casein sialic acid and the non-casein sialic acid of human skim milks. ○, Milks sampled before the tenth day of lactation; ●, milks sampled on or after the tenth day of lactation. The best-fit straight regression line is given for the latter (mature) samples only.

Table 2. Regression equations relating casein sialic acid, and the rennin-sensitive and rennin-insensitive fractions of casein sialic acid, to the non-casein sialic acid of casein-free skim-milk supernatants

In the equations shown, x is non-casein sialic acid (mg./100 ml.), y the fractions shown (g./100 g. casein), t the time from parturition (days), and n the number of samples.

y	t	n	Regression equation	r
Casein sialic acid	≥ 10	36	$y = 0.34 + 0.0042x$	+0.72
Casein sialic acid	< 10	13	$y = 0.58 + 0.0020x$	+0.57
Casein sialic acid	All periods	49	$y = 0.37 + 0.0035x$	+0.82
Rennin-sensitive fraction	≥ 10	32	$y = 0.15 + 0.0024x$	+0.60
Rennin-sensitive fraction	< 10	13	$y = a^* - 0.0007x$	-0.38
Rennin-insensitive fraction	≥ 10	32	$y = 0.17 + 0.0021x$	+0.57
Rennin-insensitive fraction	< 10	13	$y = a^* + 0.0028x$	+0.59

* Calculation of 'a' in these cases leads to unrealistic values

Fig. 7. Action of rennin on human casein preparations isolated from milk samples taken at different stages of lactation. The mean value $\pm$ s.d. of the rennin-sensitive fraction for 32 samples taken on or after the tenth day of lactation is $48.8 \pm 8.8\%$.

mostly falling between 40 and 60 % when lactation had been established; the mean value $\pm$ s.d. for 32 samples from milk taken on or after the tenth day was $48.8 \pm 8.8\%$ (Fig. 7).

In view of the correlation between the sialic acid content of casein samples and the sialic acid in the supernatants from which the casein had been removed (Table 2 and Fig. 6), it was decided to analyse this effect further by considering the rennin-sensitive and rennin-insensitive fractions of the casein sialic acid separately.

Regression equations for these fractions are given in Table 2; they show that, for caseins prepared from milk sampled on or after the tenth day of lactation, the positive correlation obtained for whole casein sialic acid is a property shared by both the rennin-sensitive and rennin-insensitive fractions. By contrast, for immature caseins the positive correlation found with whole casein sialic acid is shown to be the resultant of entirely dif-

Fig. 8. Relationship between the rennin-sensitive fraction of casein sialic acid and the non-casein sialic acid of human skim milks. O, Milks sampled before the tenth day of lactation; ●, milks sampled on or after the tenth day of lactation. The best-fit straight regression line is given for the latter (mature) samples only.

ferent relationships in the two fractions: a negative correlation with the non-casein sialic acid being observed for the rennin-sensitive part, and a positive correlation for the rennin-insensitive part (Figs. 8 and 9).

DISCUSSION

The results presented above were subject to a number of uncontrolled factors. Milk samples were taken from mothers representing all strata of an urban society; this could imply different standards both of maternal nutrition and of the care with which lactation was established and maintained. No attempt was made to assess differences of this kind. Further, the pH of milk samples when received in the laboratory varied from 6.5 to 7.5, but no evidence was obtained to suggest that this initial value was influencing the properties examined in any way.

More serious objections to comparison of the casein preparations arise, however. First, the variation in casein yields might lead to the supposition that some preparations were incomplete

Fig. 9. Relationship between the rennin-insensitive fraction of casein sialic acid and the non-casein sialic acid of human skim milks. O, Milks sampled before the tenth day of lactation; ●, milks sampled on or after the tenth day of lactation. The best-fit straight regression line is given for the latter (mature) samples only.

and possibly uncharacteristic. This objection has been met by the rejection of all samples giving a casein yield of less than 0.1 % [the mean value for casein content of human milks quoted by Macy & Kelly (1961) is 0.37 %], and by the evidence that, whenever a further small casein preparation was obtained from a 'casein-free supernatant', the properties of the second yield differed very little from the main preparation. Secondly, in most cases it was not known at what period after the previous suckling samples were taken. In one case where comparable and extreme data were available from one mother, the sialic acid content of the casein prepared from milk taken immediately after suckling, 0.10 %, was much lower than that found in the casein from milk taken before suckling began, 0.35 %. The value, in the context of the present work, was uniquely low and suggests that the time during which milk rests in the gland may be an important factor in determining the sialic acid content of the isolated protein. Since, however, only three of the mature caseins examined in the present work were prepared from milk samples with a volume less than 20 ml., it is thought that this effect could not have been seriously damaging.

The results suggest that human casein is a protein having variable properties, and that it can be influenced both by the composition of the milk from which it is derived and, more fundamentally,

by variations in the relative activities of certain enzymes of the mammary alveolar cell responsible for its synthesis.

The latter view arises from the electrophoretic evidence. The basic pattern of six bands given by casein on starch gel at pH 8.6, which represents part or all of the rennin-insensitive fraction of the protein, has been shown to vary with respect to the relative intensities of the bands. Three types of pattern, A, B and C, have been recorded; they are easily distinguishable and suitably classify all the caseins found in the present study, except two in which the band pattern was completely absent. Within each type minor variations occurred which were not thought large enough to warrant further type differentiation. An important aspect of the differentiation was that, whenever casein samples were prepared from one individual at more than one stage of the lactation, the characteristic band pattern for the individual remained the same. This was of special interest in those mothers showing the less frequently occurring patterns (type B and type C), one of whom for example gave the type B pattern with caseins obtained on four occasions from the fifteenth to the sixty-third day of her lactation.

The existence of different band patterns in mature caseins seems to be best explained on the supposition that the formation, or possibly the interconversion, of these casein subfractions is a function of specific enzymes in the alveolar cell, and that for different individuals these enzymes have different relative activities. The existence of minor variations from the main types, and also, as a special case, of the 'transitional' types noted in the Results section, might then be reasonably expected.

One particular interpretation may be cited: the near resemblance of the type B pattern to the pattern given by certain immature caseins derived from milk taken in the first week of lactation, especially the enhanced prominence of band 1 and the weakness or absence of bands 5 and 6, suggests that this type is the result of a deficiency in at least one enzyme normally involved in producing the full heterogeneity of the casein molecule, and that this results in a permanently immature type casein in such individuals.

The influence of the milk on human caseins is demonstrated by the direct relationship which exists between the sialic acid in the isolated protein and the non-casein sialic acid in the milk. Both decrease throughout lactation and from the present experiments the sialic acid values for mature samples of casein, obtained by using the derived regression equation (Table 2 and Fig. 6), might be expected to fall to between about 0.70 and 0.35 %.

For such caseins, isolated on or after the tenth day of lactation, when from the electrophoretic

evidence and from the development of maximum sensitivity to rennin (Fig. 7) there is a reasonable presumption that the protein is 'mature', the correlation with the non-casein sialic acid is a property shared in an equal degree by both the rennin-sensitive and the rennin-insensitive fractions of the protein (Figs. 8 and 9). As a consequence of this the proportion of sialic acid found to be sensitive to the action of rennin in such specimens is independent of the time of sampling, and in the present study was found to be about half of the total sialic acid present, though the divergence from the mean value could be very large in individual cases.

In casein prepared from cow's milk, Marier, Tessier & Rose (1963) have shown that all the sialic acid is present in the κ -casein (micelle-stabilizing fraction; Waugh & Hippel, 1956; Waugh, 1958), which has a sialic acid content of about 2.3%. The sialic acid content of whole cow casein ranged from 0.26 to 0.59% in the work referred to (Marier *et al.* 1963), and this variation is ascribed entirely to differences in the κ -casein content of the whole protein, from 11 to 26%.

No fraction, having the high sialic acid content or showing the specific physicochemical properties of κ -casein, has yet been obtained from mature human casein, although Alais & Jollès (1962*b*) have noted that the release of a glycopeptide relatively rich in sialic acid by rennin action is evidence in favour of the existence of a fraction in human casein similar to κ -casein. It is possible therefore that the effects outlined in the present paper will ultimately be found to refer more specifically to variations in the proportion of one fraction having a constant high sialic acid content, and forming only a part of the human casein aggregate, rather than to a progressive change in the casein aggregate as a whole. The unchanging character of the electrophoretic band pattern throughout a lactation gives support to the view that for a given individual much of the casein is well defined and invariable.

SUMMARY

1. Specimens of human casein representing all stages of lactation have been prepared by a standard procedure from 80 milk samples given by 47 mothers.

2. Electrophoresis of human casein on starch gel at pH 8.6 gives a six-band pattern. Three casein types (A, B and C) have been characterized, which differ in the relative strengths of the bands within this main pattern.

3. The results suggest that for any one individual the type does not change during the course of a lactation.

4. The sialic acid content of casein preparations fell during lactation from about 1.0 to 0.2%; it showed a positive correlation with the amount of sialic acid present in the skim milk after casein had been removed.

5. About half the casein sialic acid was sensitive to the action of rennin.

6. It is concluded that the nature and properties of human casein may be influenced by the relative activity of certain enzymes in the mammary alveolar cell, and by the composition of the milk from which it is derived.

The authors acknowledge the help of Dr H. A. Warnock and his staff at the Belfast Maternity and Child Health Division in obtaining milk samples. They also thank Professor D. C. Harrison for his helpful interest in the work, and Mr J. McGrath for skilled technical assistance.

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