STUDIES ON THE SPECIFIC ROTATION OF MILK : ESPECIALLY ON THE CAUSE OF THE LOW SPECIFIC ROTATION OF HUMAN MILK

TAKEUCHI, Toshifumi

(Citation)
The Kobe journal of the medical sciences,5(2):1-16

(Issue Date)
1959

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/0100489277

STUDIES ON THE SPECIFIC ROTATION OF MILK: ESPECIALLY ON THE CAUSE OF THE LOW SPECIFIC ROTATION OF HUMAN MILK

Toshifumi TAKEUCHI

(Received for publication June 2 1959)

In 1939, Malyoth and Kirimlidis[1] presented a treatise, emphasizing the importance of β-lactose in infant nutrition, in which they stated that lactose in human milk exsisted mainly as β-form, according to a factor which shifted the state of equilibration of lactose toward β-side, and by adding β-lactose to cow's milk formula, it was able to increase the per cent of Lactobacillus bifidus in the bacterial counts of intestinal flora of bottle-fed infants to the extent of that of breast-fed infants. Therefore, they designated β-lactose as " Körpernahe Zucker ".

On the other hand, Habild[2] in 1949, and Sasaki et al[3] in 1952, insisted that lactose in human milk existed in the same state of equilibrium between α- and β-form as in the aqueous solution, based upon the facts that no mutarotation was observed after milking and the values of specific rotation showed no significant difference among human milk, cow's milk and lactose solution in water (invariably plus 55.2°).

While Malyoth's work is rather speculative and lacking the actual evidence, Habild's or Sasaki's work appears to be methodologically doubtful, because they did not estimate the true value of lactose content in milk.

To settle this controversy, investigations were undertaken in our laboratory to determine the carbohydrate content and the optical rotation of various milks. As a result, certain new facts have been disclosed. The present paper deals with the characteristic feature of the specific rotation of human milk and its cause.

I. SPECIFIC ROTATION OF MILK SERUM

Materials and Methods

Human milk, human colostrum and cow's milk were collected from various sources. All samples were raw. Careful attention was paid to keep them as fresh as possible. Each sample was centrifuged for 20 min. at 3,000 r. p. m. and after cooling to 5 °C, the solidified cream was removed. The skimmed milk was treated by the following three different methods: (1) centrifuged for 1 hour at 37,500 to 40,000 r. p. m. with Spinco L type ultracentrifuge, (2) deproteinized by Somogyi's[4]

竹内敏文
From the Department of Pediatrics, Kobe Medical College (Director: Prof. Dr. Y. HIRATA)

method or (3) by Sharp & Doob's[5] method. For convenience, the deproteinized filtrate of skimmed milk was designated as milk serum. For desalting, ion-exchangers (Amberite IR-120 in H-form and IR-4B in OH-form) were used. Amount of lactose in milk serum was determined by (1) Bertrand's method,[6] (2) colorimetric method by Nelson-Somogyi[7] and (3) anthrone method.[8] Optical rotation was determined with a Franz Schmidt and Haensh's polarimeter and the specific rotation was calculated by the following equation :

$$[\alpha]_D = \frac{\alpha \times 100}{c \times l}$$

α : Optical rotation expressed in degrees.
l : Length of tube in decimeters.
c : Concentration of sugar in grams per 100 ml.

It must be pointed out that the values of the specific rotation were calculated by the equation with the values of sugar contents expressed as lactose, hence, the values obtained were the apparent specific rotation.

Results

1. Specific Rotation of Human Milk Treated with Ultracentrifuge.

This procedure was carried out to avoid denaturation of milk as perfectly as possible.

Table 1. Specific Rotation of Ultracentrifuged Human Milk.

Number of Mother	$[\alpha]_D$ (plus, in degree)						
	Time after Milking (hrs.)						Average
	3	4	5	6	7	9	
1(a)			31.5				31.5
1(b)				37.3	36.5	36.9	36.9
1(c)		39.6	39.0	38.1	38.9		39.2
2						41.6	41.6
3					45.3		45.3
4					49.4		49,4
5				45.0			45.0
6	47.9	48.0	48.0	48.0			48.0
7	48.7						48.7
8	48.0						48.0
9	47.1	47.5	47.5	47.6			47.4
10	48.3						48.3
11	43.7						43.7
12	41.9						41.9
13		48.0	47.4	47.7	47.1		47.6
						Average	44.1

As shown in table 1, it was observed that the mean value of $[\alpha]_D$ of human milk was plus 44.1°, which was significantly lower than that of lactose solution in water.

and that the values of $[\alpha]_D$ revealed individual, daily and diurnal variations. Mutarotation was not observed after milking.

2. Specific Rotation of Milk Serum Deproteinized by Somogyi's Method.

Ultracentrifuged human millk mentioned above gave a positive ninhydrin test and still contained lactoalbumin and inorganic salts. Therefore, in order to detemine the true $[\alpha]_D$ due merely to carbohydrates contained in milk, deproteinization by Somogyi's method and desalting by ion-exchangers were carried out. Samples so treated showed no more positive ninhydrin test and were considered to be composed of only carbohydrates of original milk. The results were summarized as follows:

(1) As shown in Table 2, the values of specific rotation of human milk sera, especially of human colostral sera were definitely lower than those of cow's milk sera and of the aqueous solution of lactose in a state of α–β equilibrium.

Table 2. Specific Rotation of Milk Sera.

	Lactose-solution	Cow's Milk	Human Milk	Human Colostrum
$[\alpha]_D$ (plus, in degree)	55.5	54.4	39.0	42.8
	55.4	57.1	41.2	30.9
	55.3	53.1	43.9	40.9
	55.8	52.8	40.1	33.3
	55.7	52.3	40,4	37.9
	55.2	51.1	48.1	39.1
	55.1	52.0	46.9	41.1
	55.9	55.4	49.2	39.3
	55.3	53.9	42.1	31.5
	55.6	54.5	45.9	35.4
Average	55.5	53.7	43.7	37.2
u^2 *	0.071	3.153	13.275	17.711

* u: unbiased variance.

(2) Table 3 shows that the values of $[\alpha]_D$ of human milk sera reveal individual, daily and diurnal variations. As seen in Table 4, however, no mutarotation

Table 3. Individual, Daily and Diurnal Variations of $[\alpha]_D$ of Human Milk Sera.

Samples of Human Milk	Time of Milking	$[\alpha]_D$ (plus, in degree)	
		14, March	18, March
K.	10° a.m.	48.3	
	12° a.m.	50.0	47.7
	3° p.m.	50.0	—
S.	11° a.m.	50.7	—
	12° a.m.	53.0	49.0
	3° p.m.	49.2	—

Table 4. Stability of $[\alpha]_D$ of Human Milk Sera.

	$[\alpha]_D$ (plus, in degree)	
	Sample 1	Sample 2
Immediately after Milking	49.6	50.0
24 hrs. after Milking	49.3	50.7
After Heating for 30 min. at 100 °C	49.1	50.0
After Desalting	49.0	49.8

was observed after milking at room temperature and the values of $[\alpha]_D$ were thermostable and not influenced by the desalting procedure.

(3) There was no correlation between $[\alpha]_D$ and mother's age or her living condition.

(4) The values of $[\alpha]_D$ were not altered by oral or parenteral administration of thiamine, riboflavin, niacin or ascorbic acid to mother.

(5) As seen in Table 5, no change of $[\alpha]_D$ was observed by various methods of deproteinization such as Somogyi, Folin-Wu, Benedict, Sharp & Doob,[5] or the methods using trichloroacetic acid, cadmium hydroxide, metaphosphoric acid and uranyl acetate.

Table 5. Change of $[\alpha]_D$ of Human Milk Sera due to Various Deproteinization Methods.

Methods of Deproteinization		$[\alpha]_D$ (plus, in degree)
$ZnSO_4$-Ba $(OH)_2$	(Somogyi)	38.5
Sodium tungstate	(Folin-Wu)	38.9
Sodium tungstate et molybdate	(Benedict)	38.9
Mercuric chloride	(Sharp & Doob)	38.3
Trichloroacetic acid		38.9
Cadmium hydroxide		38.9
Metaphosphoric acid		39.0
Uranyl acetate		39.2

3. *Comparison of Sugar Quantities in Milk Sera between Anthrone Method and Sharp & Doob's Method.*

The determination of sugar content was made by anthrone method on the samples of milk sera deproteinized by mercuric chloride used in the Sharp & Doob's method, and these values were compared with those of Sharp & Doob's method,[5] which was calculated on the assumption that only lactose was concerned with the optical rotation of the filtrates and existed in the same state of α-β equilibration as in aqueous solution.

As seen in Table 6, in case of human milk, especially of human colostrum, there were remarkable differences of sugar quantity between the values obtained by anthrone method and by polarimetry (Sharp & Doob's method), while, in the case of

cow's milk there were no significant differences between them. These evidences suggest that the assumption in the Sharp & Doob's method is not available in case of human milk and human colostrum.

Table 6. Comparison of Sugar Contents Determined by Anthrone Method and by Sharp & Doob's Method.

	Optical Rotation (plus, in degree)	Anthrone Method		Sharp & Doob's Method	
		Sugar g/dl)	$[\alpha]_D$ (plus, in degree)	Sugar (g/dl)*	$[\alpha]_D$ (plus, in degree)
Human Colostrum	0.62 0.61	1.75 1.70	35.4 36.5	1.12 1.11	
Human Milk	0.76 0.65 0,55 0.76	1.78 1.46 1.29 1.78	42.1 44.5 42.6 42.7	1.38 1.18 0.99 1.38	55.2
Cow's Milk	0.56 0.49	1.02 0.95	54.9 51.6	1.01 0.89	

* The values were calculated by the equation to which the values of optical rotation and the theoretical value of $[\alpha]_D$ of lactose in α-β equilibration (+55.2°) were applied.

II. CAUSE OF THIS LOW SPECIFIC ROTATION OF HUMAN MILK SERUM

1. Theoretical Consideration.

On the basis of theoretical consideration, any one of the three occasions mentioned below would be expected for a better understanding of the fact that $[\alpha]_D$ of human milk serum revealed such a low value.

(a) Coexistence of a substance(s) which makes the equilibration of lactose shift toward β-side by reacting to acetal-OH.

(b) Coexistence of a substance(s) which may be fixed with a part of lactose to form a β-derivative, its reducing power being equal to that of lactose.

(c) Coexistence of a potent levorotatory substance(s) which induces the specific rotation to be decreased.

Table 7. Mutarotation of α- or β-Lactose Solution, when Sodium Bisulfite was added.

Time after Adding of Sulfite	$[\alpha]_D$ (plus, in degree)	
	5% α-Lactose plus 5% Na-bisulfite	5% β-Lactose plus 5% Na-bisulfite
10 min.	—	37.8
20	70.0	37.4
30	—	36.8
40	57.4	37.0
50	53.0	37.2
60	49.0	37.0
90	44.0	37.2
120	43.0	37.2
24 hrs.	36.8	37.0

As the possibility of (a), sulfite or borate was considered, as pointed out by Habild [2]. For instance, as seen in Table 7, when sodium bisulfite was added to aqueous solution of α-lactose or β-lactose, mutarotation had occurred and the equilibrium was shifted apparently to β-side.

However, it would be unreasonable to consider the presence of inorganic salts in such a high concentration in milk as an etiologic factor, since this low specific rotation of human milk was observed even on the samples which were freed of inorganic salts by ion-exchangers.

As the possibility of (b), sugar phosphates were suspected. Actually, it is reported by Malpress[9] and Gauder[10] that at the last step of synthesis of lactose in the mammary gland of guineapig, lactose was liberated from lactose-1-phosphate by the action of phosphatase. So, the determinations of inorganic phosphate, △7p, △30p and total phosphate were carried out by Fiske & Subbarow's method[11] on the deproteinized (by Somogyi's[4] method) and desalted samples of human milk, but no phosphate was found. This result suggests that the deproteinized and desalted samples of human milk do not contain any amount of inorganic phosphate or sugar phosphates.

In conclusion, it seemed probable that (c) would be most likely to occur.

2. *The Possibility of Coexistence of Oligosaccharide-like Substance(s) in Human Milk Serum besides Lactose.*

Methods

Desalted and deproteinized samples of milk sera were analyzed for sugar content by Bertrand's method[6] and by anthrone method[8], and the values obtained were compared with each other.

Results

As shown in Table 8, it is apparent that the values obtained by anthrone method are higher than those by Bertrand's method and that the differences between them are inclined to become less in order of human colostrum, human milk and cow's milk. It would be reasonably expected, therefore, that human milk serum, especially human colostral serum, contains a small amount of oligosaccharide-like substance(s) which can react with anthrone reagent to produce blue color, after being hydrolyzed by the strong acid.

Table 8. Determination of Sugar in Milk by the Two Methods; Anthrone Method and Bertrand's Method.

Kinds of Milk	Number of Samples	Sugar Content (Average)		Difference (g/dl)
		Anthrone (g/dl)	Bertrand (g/dl)	
Human Colostrum	12	1.16	0.96	0.20
Human Milk	9	1.02	0.90	0.12
Cow's Milk	5	0.73	0.64	0.09

3. *Existence of N-containing Substance(s) in Human Milk Serum besides Protein and Free Amino Acid.*

Methods

Nitrogen contents were determined by the micro-Kjeldahl method on the desalted

samples of human milk serum, after being proved to be negative to ninhydrin reagent.

Results

As shown in Table 9, milk sera of various sources contained a small amount of N-containing substance(s) besides protein and free amino acid. It is of special interest that human colostrum contains the substance(s) several times as much as cow's milk.

Table 9. Nitrogen Content of Desalted Milk Serum Determined by Micro-Kjeldahl Method.

	Nitrogen Content		
	Human Colostrum (mg/dl)	Human Milk (mg/dl)	Cow's Milk (mg/dl)
1	30.0	12.6	4.5
2	27.0	10.2	5.0
3	25.8	9.6	5.0
4	27.0	9.6	4.5
5	23.4	9.6	5.5
6	24.0	6.5	4.0
Average	26.2	9.7	4.8

4. Paperchromatography of Milk Serum.

Methods

For materials, pooled human milk sera were condensed under reduced pressure and dried. Hydrolysates of milk sera were obtained by hydrolyzing those materials with 0.5N-H_2SO_4 for 6 hours at 100 °C. Paperchromatography [12, 13] was performed by ascending procedure, using Toyo-Roshi No. 50 and No. 51 for filter paper, pyridine-1-butanol-water (4 : 6 : 3 v/v) as solvent, and ammoniacal $AgNO_3$ and Elson-Morgan's reagent [13] (para-dimethylaminobenz-aldehyde) as color reagent.

Results

(1) *Milk Sera.*

As a preliminary experiment, unhydrolyzed milk sera of various sources were analyzed by paperchromatography. On the paperchromatograms of human colostrum, human milk and cow's milk, in all cases, the spot of lactose was detectable constantly. Furthermore, besides the spot of lactose, two spots were detected frequently in case of human colostrum, rarely in case of human milk and none in case of cow's milk. Of these two spots, one spot was identified as fucose, however, the other spot could not be identified. The unknown spot was located under the spot of lactose and considered to be the reducing substance(s) of higher molecule than lactose.

(2) *Hydrolysates of Milk Sera.*

Paperchromatography of the hydrolysates resulted in the spots shown in Fig. 1.

In case of human milk or human colostrum, six spots were detected, among which five spots were identified, from the top downward, as fucose, glucose, galactose,, glucosamine and lactose. The lowest spot was always observed faintly and had the same Rf as the unknown spot of unhydrolyzed human milk serum. The spot of lactose was not always detectable and very faintly, even when it was recognized. The fourth spot was confirmed as glucosamine by its Rf and development of pink color with Elson-Morgan's reagent. On the contrary, raw cow's milk revealed only the spots of glucose and galactose. No spot of fucose, glucosamine, lactose or unknown was detected. It was, therefore, supposed that human milk or human colostrum contained a small amount of oligosaccharide-like substance(s), which liberated such sugars as fucose and glucosamine when hydrolyzed.

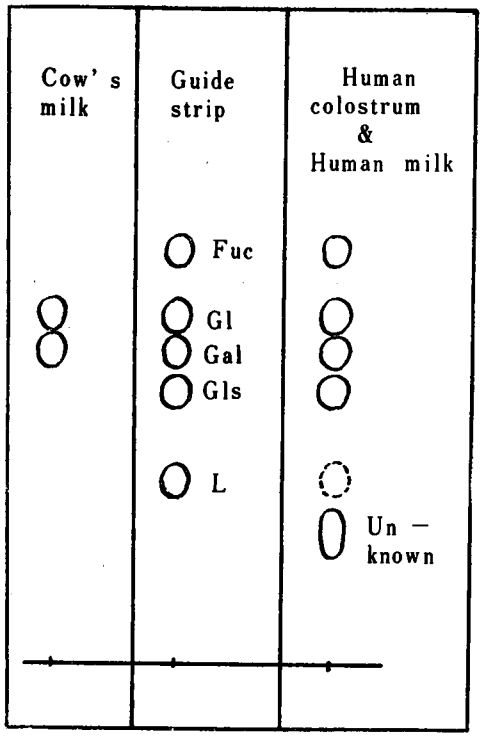

Fig. 1. Paperchromatogram of Hydrolysates of Various Milk Sera: Ascending procedure.
Solvent; pyridine-butanol-water.
Color reagent; ammoniacal $AgNO_3$.
Fuc=Fucose, Gl=Glucose, Gal=Galactose. Gls=Glucosamine, L=Lactose.

5. Total Glucosamine Content of Milk Sera and its Relation to the Specific Rotation.

Methods

For the determination of reducing sugar and glucosamine, Nelson-Somogyi's[7] and Rondle-Morgan's[14] methods were respectively used. As it was well established that reducing sugar interfered with the Elson-Morgan's reaction, all samples were diluted so as to contain 1 mg. of reducing sugar and determined on their gulcosamine contents, using the standard curve obtained by the known amounts of glucosamine to which 1 mg. of reducing sugar was added. The colorimetric measurement was made with an Evelyn's photoelectric colorimeter, using filter No. 515 or No. 540.

Two designations are used as follows: Free glucosamine means glucosamine determined on the unhydrolyzed samples, and total glucosamine means glucosamine determined on the hydrolyzed samples with 0.5N-H_2SO_4 at 100 °C for 6 hours, over which time no increment of glucosamine is observed even if hydrolysis is continued. The

difference between free and total glucosamine, therefore, means the amount of glucosamine, which exists in combined state and is liberated by acid hydrolysis.

Results

(1) The amounts of free glucosamine contained in raw cow's milk, human milk and human colostrum were all less than 5 mg. per 100 ml.

(2) As seen in Table 10, the amounts of total glucosamine in the hydrolysates of cow's milk sera were less than 20 mg. per 100 ml, whilst human milk sera, especially human colostral sera, contained a larger quantity of total glucosamine.

(3) The obvious correlation was observed between the $[\alpha]_D$ of various milk sera and their quantities of total glucosamine. The coefficient of correlation was −0.78 (Fig. 2).

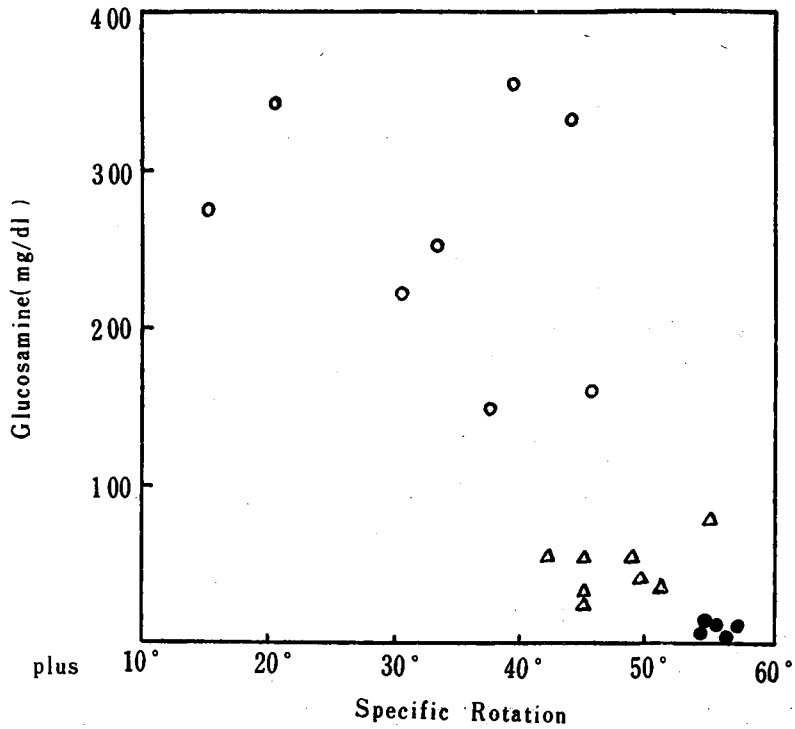

Fig. 2. Correlation between the $[\alpha]_D$ of Various Milk Sera and their Quantities of Total Glucosamine.
(○: Human Colostrum, △: Human Milk, ●: Cow's Milk.)

Table 10. $[\alpha]_D$ and Total Glucosamine of the Hydrolysates of Various Milk Sera.

	Reducing Sugar as Glucose (g/dl)	$[\alpha]_D$ (plus, in degree)	Glucosamine (mg/dl)	Average of Glucosamine (mg/dl)
Cow's Milk	4.5	56.0	less than 20.0	less than 20.0
	4.5	57.3		
	4.3	54.1		
	4.3	55.2		
	4.4	56.7		

Human Milk	7.6 6.0 6.2 7.2 6.0 8.5 8.2 8.9	54.8 45.0 45.2 52.5 49.0 45.4 44.1 47.7	81.6 33.6 24.0 36.0 40.8 57.6 48.0 55.2	47.1
Human Colostrum	5.8 6.5 5.8 6.1 7.7 3.5 5.0 7.7	31.3 38.5 43.8 45.5 15.6 37.6 21.4 33.6	220.8 354.2 330.2 159.8 273.6 146.4 339.8 248.6	259.2

6. *Isolation of N-containing Oligosaccharide-like Substance(s) and its Microbiological Activity.*

Materials and Methods

The pooled samples of human milk sera were desalted by ion-exchangers, condensed under reduced pressure and dried for storage. These samples were fractionated by the method essentially similar to that described by Kuhn & György [20] and Whistler & Durso.[22] That is, the dried material was dissolved in water, stirred with charcoal (Norit A) for 1 hour at room temperature, and after washing the charcoal with distilled water several times, it was eluted with 20% acetic acid. The fraction eluted with 20% acetic acid was adsorbed on the Carboraffin-Celite column (Darco G-60 and Celite No. 535) and the column was eluted with distilled water and ethanol of various concentrations.

On all the fractions of each step of this process, determination of reducing sugar and of total glucosamine, estimation of $[\alpha]_D$ and paperchromatographic identification were undertaken. The techniques used were the same as previously mentioned, except that hydrolysis for total glucosamine determination was carried out with N-HCl for 15 hours at 100 °C and $[\alpha]_D$ was calculated basing on the dry weight.

For the bacteriological examination, the regular strains of L. bifidus isolated from the feces of healthy breast-fed infants were inoculated into Plotho's medium [23]

Table 11. Composition of K-medium.

Lactose	3.0	g.
Casein Hydrolysate	1.0	ml.
Pepton	0.5	g.
Yeast-extract	0.05	g.
Cystine or Cysteine	0.02	g.
Salt-Mixture *	0.5	ml.
Water	to 100	ml.

PH 6.5-6.8;
Autoclaved for 10 min. at 120°.

* Salt-Mixture:

$MgSO_4 \cdot 7H_2O$	1.0	g.
$FeSO_4 \cdot 7H_2O$	0.5	g.
NaCl	0.5	g.
$MnSO_4 \cdot 2H_2O$	0.337	g.

Dissolved in 250 ml. water.

or K-medium, the composition of which is shown in Table 11. The growth of L. bifidus was measured by production of acid, the amount of which was estimated by titration with N/10 NaOH and expressed as ml. of the consumed alkali to neutralize the acidity produced.

Results

(1) *Fractionation of Human Milk Serum by Charcoal.*

As shown in Table 12, the values of $[\alpha]_D$ appear to decrease in proportion to the increase of total glucosamine and to the decrease of reducing sugar. This suggests that the low specific rotation of human milk serum is concerned with the amount of N-containing substance(s), which contains glucosamine as one of the constituents.

(2) *Fractionation of Charcoal-Elute by Carboraffin-Celite Column.*

Table 13 shows the properties of each fraction eluted from Carboraffin-Celite column with ethanol of various concentrations. The levorotatory fraction was successfully isolated by elution with 30% ethanol. The reducing sugar of this fraction showed the lowest value (approximately 16% of dry weight) and, in contrast, the total glucosamine revealed the highest value (approximately 18% of dry weight). It was, therefore, concluded that human milk serum contained a small amount of levorotatory N-containing substance(s), including glucosamine.

Table 12. Fractionation of Human Milk Serum by Charcoal.

Fractions		Human Milk Serum	Charcoal-Filtrate	Charcoal-Elute
Dry Weight	(g.)	2.485	2.186	0.222
Volume of Solution	(ml.)	25.0	25.0	12.5
Concentration	(%)	9.940	8.744	1.770
α (at 24°)	(plus, in degree)	3.77	3.73	0.20
Reducing Sugar:				
as Glucose	(g/dl)	8.60	5.00	0.39
as Lactose	(g/dl)	12.30	7.30	0.57
$[\alpha]_D^{24°}$	(plus, in degree)	37.9	42.7	11.3
Glucosamine:				
Free	(mg.)	0	0	3.1
Total	(mg.)	84.0	29.0	32.5
Free/Total	(%)	0	0	9.5
Total/Dry Weight	(%)	3.4	1.3	14.6

Table 13. Fractionation of Charcoal-Elute by Carboraffin-Celite Column.

Eluting Fractions		Aq. dest.	5%-Ethanol	15%-Ethanol	30%-Ethanol
Dry Weight	(g.)	0.663	0.595	0.979	1.367

Volume of Solution	(ml.)	50.0	50.0	25.0	50.0
Concentration	(%)	1.326	1.190	3.916	2.734
α (at 24°)	(in degree)	+0.16	+0.07	+0.12	−0.07
Reducing Sugar:					
as Glucose	(g/dl)	0.30	0.24	0.49	0.22
as Lactose	(g/dl)	0.44	0.35	0.70	0.31
$[\alpha]_D^{24°}$	(in degree)	+12.0	+5.9	+3.1	−2.6
Glucosamine:					
Free	(mg.)	6.2	5.3	5.9	7.7
Total	(mg.)	77.0	68.0	138.0	240.0
Free/Total	(%)	8.1	7.8	4.3	3.2
Total/Dry weight	(%)	11.6	11.4	14.1	17.6

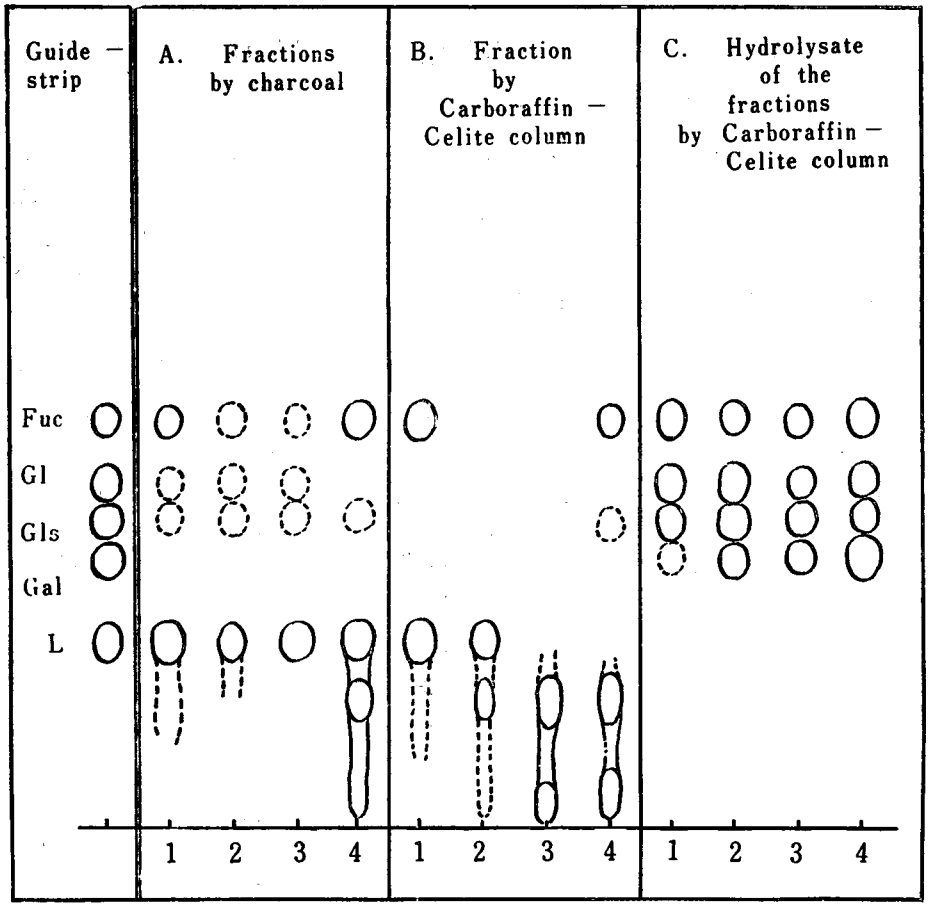

Fig. 3. Paperchromatogram of Fractions Obtained by Charcoal or Carboraffin-Celite Column and of their Hydrolysates.

Ascending procedure. Solvent; pyridine-butanol-water. Color reagent; ammoniacal $AgNO_3$. Abbreviation; same as in Fig. 1, except the following.

Part A: 1. Human colostrum, 2. Filtrate of charcoal, 3. Wash water of charcoal. 4. Elute by acetic acid.

Part B and C: 1. Elute by water, 2. Elute by 5%-ethanol, 3. Elute by 15%-ethanol, 4. Elute by 30%-ethanol.

(3) *Paperchromatography of Each Fraction.*

The Paperchromatography of each fraction, which was eluted from charcoal and Carboraffin-Celite column, resulted in the spots shown in Fig. 3. As seen on the figure, lactose was removed by the treatment and the last elute from Carboraffin-Celite column with 30% ethanol showed only the faint spot of fucose and the slow-moving tailing spot(s), which was possibly attributable to the reducing substance(s) of higher molecule than lactose. The paperchromatogram of the hydrolysates of this fraction revealed four spots distinctly, which were identified as fucose, glucose, galactose and glucosamine. This evidence indicates that human milk contains a reducing substance(s) of oligosaccharide-like nature, which is composed of at least four monosaccharides mentioned above.

(4) *Change of* $[\alpha]_D$ *of Cow's Milk Serum by Addition of the* 30% *Ethanol Fraction.*

The fraction eluted with 30% ethanol from Carboraffin-Celite column was added to cow's milk serum in various concentrations and the $[\alpha]_D$ of the serum was measured.

As shown in Table 14, it was confirmed that cow's milk serum revealed the $[\alpha]_D$ to be nearly equal to that of human milk serum by adding 30% ethanol fraction in concentration of approximately 4% to original milk.

Table 14. $[\alpha]_D$ of Cow's Milk Serum, when the 30% Ethanol Fraction was added.

Amount of the Fractiou added to Original Cow's Milk (%)	$[\alpha]_D$ (plus, in degree)
26.7	21.7
4.0	44.3
2.0	46.4
1.0	49.5
0.25	52.0

(5) *Bifidus Activity of the* 30% *Ethanol Fraction.*

As indicated in Table 15, when the fraction eluted with 30% ethanol was added to the culture media, regardless of anaerobic or aerobic culture, L. bifidus showed a marked increase in contrast to the control group. In other words, this fraction possessed the growth-stimulating activity for L. bifidus which was isolated from the feces of breast-fed infants.

Table 15. Bifidus activity of the 30% Ethanol Fraction.

Culture	Media		Acid Production (ml.)		
			Strain 1	Strain 2	Strain 3
Anaerobe	Plotho	30% EtOH-Frac.	16.25	15.15	16.05
		Control	8.45	7.75	8.15
	K.	30% EtOH-Frac.	14.25	14.85	15.90
		Control	8.25	7.45	8.05
Aerobe	K.	30% EtOH-Frac.	14.20	10.25	11.50
		Control	7.75	6.85	8.25

DISCUSSION

In 1939, Malyoth [1] stated: "Man findet in der Literatur verschiedentlich angegeben, dass das Verhaeltnis von α-Lactose zu β-Lactose in der Frauenmilch zugunsten der β-Lactose weitgehend verschoben sei, waehrend das Verhaeltnis in der Kuhmilch sich gerade umgekehrt zeige." But, there is no concrete proof on the α–β equilibration state of lactose in milk, and it is interpreted that his statements mean only a speculative opinion to support the experimentally better influences of the administration of pure β-lactose than α-lactose to bottle-fed infants.

In 1949, Habild [2] denied the existence of the factor in human milk which induces the equilibration of lactose to shift toward β-side, since no mutarotation was observed on the aqueous solution of lactose, even when the filtrates or dialysates of human milk obtained by various methods were added to it. Moreover, he observed that no mutarotation occurred after milking in case of human milk and also of cow's milk, and concluded: "In frischer Frauenmilch kommen α- und β-Lactose im gleichen Verhaeltnis vor wie Lactose in waesserigen Loesungen. Auch in ganz frischer Frauenmilch laesst sich ein vermehrter Gehalt von β-Lactose nicht nachweisen."

According to my study, also, in which the ultracentrifuged fraction or deproteinized and deionized filtrates by various methods were used, milk serum of various sources did not show mutarotation after milking. So, regarding the mutarotation of milk, I came to concur with the Habild's opinion. However, it is not reasonable to conclude that lactose in milk exists in the same state of α–β equilibrium as in aqueous solution, only because no mutarotation was observed. The reason is as follows:

Generally, the specific rotation is defined according to the following equation:

$$[\alpha]_D = \frac{\alpha \times 100}{c \times l}$$

Hence, for the determination of $[\alpha]_D$, it is necessary to evaluate α and c (l is known length of the tube used). Futhermore, the equation is applicable to a solution of one substance, and when two or more substances with optical rotation coexist, the observed α is a sum of rotation of coexisting substances. Substances without opitical rotation, too, are able to have some influences on the value of α, when they exist mixedly. Therefore, even when no mutarotation is observed, the equilibrium of lactose may be shifted toward β-side, if any substance(s) which can fix the acetal-OH of lactose molecule on β-configuration coexists in the solution. Inversely, even when lactose in milk exists in the same equilibration as in aqueous solution, the apparent $[\alpha]_D$ may be different far from that of lactose in aqueous solution, if strongly levorotatory substance(s) coexists even in a small quantity.

Under these circumstances, it is not reasonable to assume that lactose exists in the same state of equilibrium as in aqueous solution, only because no mutarotation is observed. The similar objection is applicable to Sasaki's [3] work and it may be unreasonable to determine the $\alpha : \beta$ ratio of lactose in the deproteinized filtrates of

milk without evaluating the sugar content, unless the evidence that the filtrates do not contain any substance except lactose is given.

From the foregoing point of view, milk of various sources were skimmed, deproteinized and deionized by various methods. The optical rotation and the sugar content of the filtrates were determined and the values of calculated specific rotation were compared with each other. It was found that the apparent $[\alpha]_D$ of milk sera of human origin was significantly lower than that of cow's origin.

According to literatures, in 1931, Polonovski and Lespagnol[15] reported that human milk contained, besides lactose, two other glucosides which were designated as allolactose (6-[β-d galactosido]-d-glucose) and gynolactose (levorotatory, N-containing substance), and in 1953, György and Kuhn et al[16,17,18,19,20,21] isolated the N-containing polysaccharides ($[\alpha]_D^{25°} = -10°$ to $-40°$) from human milk and indicated that those were the essential growth factor for L. bifidus var. Penn. to which the name of "Bifidus Factor" was assigned.

Then, the cause of this lower $[\alpha]_D$ of human milk was investigated, and as it was assumed that human milk contained, besides lactose, oligosaccharide-like substance(s), it was tried to isolate the substance(s), which was not yet purely separated, but was proved to be composed of fucose, glucose, galactose and glucosamine and to be levorotatory. This oligosaccharide-like substance(s) could induce the apparent specific rotation of cow's milk to shift toward β-side to the extent nearly equal to that of human milk by adding it in concentration of 4% to the original cow's milk.

The substance(s) was also proved to have growth-stimulating effect for L. bifidus. These facts suggest that the substance(s) is rich in the component(s) of the bifidus factor described by György and Kuhn et al.

In conclusion, the apparent specific rotation of human milk serum is significantly lower than that of cow's milk, and this low specific rotation is at least in part due to the N-containing levorotatory oligosaccharide(s) which is composed of fucose, glucose, galactose and glucosamine and is possibly identical to one of the bifidus factor(s) described by György and Kuhn. However, the state of α-β equilibration of lactose in milk is not yet clarified by the present work and it will remain unsettled until the method to obtain lactose fraction of milk without changing the original state of α-β equilibration is found.

SUMMARY

It was observed that the apparent specific rotation of human milk serum, especially of human colostral serum, was significantly lower than that of cow's milk or aqueous solution of lactose in α-β equilibrium and was rather invariable and thermostable. Further investigations revealed that human milk contained a small amount of N-containing oligosaccharide-like substance(s). This substance(s) showed a rather marked degree of levorotation and was composed of fucose, glucose, galactose and glucosamine and would be the most causative factor for the low specific

rotation of human milk serum. Unexpectedly, it was further confirmed that this substance(s) would possibly be the same as the component(s) of "Bifidus Factor" which was isolated from human milk by György and Kuhn.

ACKNOWLEDGEMENT

The author wishes to express his profound appreciation to Prof. Yoshiho Hirata and Dr. Teruo Kondo for their incessant encouragement and helpful suggestions during the course of this work. The author is also indebted to Mr. T. Haraguchi and Mr. S. Ikeda for their technical assistance.

The present work was supported in part by Grant-in-Aid from the Ministry of Education. The results were reported briefly at the 61st Annual Meeting of the Japan Academy of Pediatrics in 1958, by Prof. Yoshiho Hirata.

REFERENCES

1. G. Malyoth: Z. Kinderheilk., **56**, 590, (1934); G. Malyoth & St. Kirimlidis: Klin. Wschr., (1939), II, 1240.
2. G. Habild: Z. Kinderheilk., **67**, 206, (1949).
3. R. Sasaki, M. Kondo, S. Morishige & T. Sato: J. Jap. Soc. Food & Nutrition, **5**, 139, (1952).
4. M. Somogyi: J. Biol. Chem., **160**, 74. (1945).
5. P. F. Sharp & Doob: J. Dairy Sci., XXIV, 589, (1941).
6. H. Yoshikawa: Rinsho-Ikagaku, P. 129.
7. N. Nelson: J. Biol. Chem., **153**, 375, (1944).
8. J. H. Roe: J. Biol. Chem., **212**, 335, (1955).
9. M. G. McGeown & F. H. Malpress: Biochem. J., **52**, 606, (1952).
10. J. E. Gauder, W. E. Peterson & P. D. Boyer: Arch. Biochem & Biophys., **60**, 259, (1956).
11. C. H. Fiske & Y. Subbarow: J. Biol. Chem., **66**, 375, (1925); **81**, 629, (1929).
12. R. Block, E. Durrum & G. Zweig: A Manual of Paper Chromatography and Paper Electrophoresis, P. 128, Acad. Press Inc. New York, (1955).
13. S. M. Partridge: Biochem. J., **42**, 238, (1948); **42**, 251, (1948).
14. C. Rondle & W. Morgan: Biochem. J., **61**, 586, (1955).
15. M. Polonovski & A. Lespagnol: Chem. Z., (1931), I, 2078; (1931), II, 1712; (1931), II, 2024.
16. P. György: Pediatrics, **11**, 98, (1953).
17. P. György, R. F. Norris & C. S. Rose: Arch. Biochem. & Biophys., **48**, 193, (1954).
18. P. György, R. Kuhn, C. S. Rose & F. Zilliken: Arch. Biochem. & Biophys., **48**, 202, (1954).
19. P. György, J. R. E. Hoover, R. Kuhn & C. S. Rose: Arch. Biochem. & Biophys., **48**, 209, (1954).
20. A. Gauhe, P. György, J. R. E. Hoover, R. Kuhn, C. S. Rose, H. W. Ruelius & F. Zilliken: Arch. Biochem. & Biophys., **48**, 214, (1954).
21. R. M. Tomarelli, J. B. Hassinen, E. R. Eckhardt, R. H. Clark & F. W. Bernhart: Arch. Biochem. & Biophys., **48**, 225, (1954).
22. R. L. Whistler & D. F. Durso: J. Amer. Chem. Soc., **72**, 667, (1950).
23. G. Malyoth, A. Bauer & E. Serick: Z. Kinderheilk., **73**, 93, (1953).