codling and is liberated by autolytic systems after death, in agreement with the work of Tarr (1955) on other species. Codling in different degrees of exhaustion had levels of glucose in muscle of 8–32 mg./100 g. of tissue. Sugar analyses of herring were similar.

Details of changes in the sugar content of muscle during commercial chill storage and processing will be described elsewhere.

SUMMARY

1. Ribose in extracts of herring and codling muscles reacts readily with nitrogenous constituents during application to chromatograms. Glucose is stable.

2. Electrolytic desalting interferes with the estimation of ribose.

3. Sulphonated-polystyrene resins in hydrogen form hydrolyse adenosine at room temperature but the error introduced thereby in muscle analyses is insignificant.

4. Removal of salt and amino nitrogen by suitable mixed ion-exchange resins followed by lyophilizing and quantitative chromatography gave an estimate of glucose and ribose in codling and herring accurate to ± 6 %.

The work described in this paper was carried out as part of the programme of the Food Investigation Organisation of the Department of Scientific and Industrial Research.

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The Oligosaccharides of Human Milk

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(*Received* 18 *September* 1957)

The recognition of L-fucose as a constituent sugar in oligosaccharides obtained from human milk was reported by Kuhn (1952) as resulting from the work of one of his associates, A. Gauhe (see also György, Kuhn, Norris, Rose & Zilliken, 1952). Since that time Kuhn and his colleagues have characterized four fucose-containing compounds from this source: a trisaccharide called fucosido-lactose (Kuhn, Baer & Gauhe, 1955, 1956*a*), two pentasaccharides called lacto-*N*-fucopentaose I and lacto-*N*-fucopentaose II, and a hexasaccharide, lacto-*N*-difucohexaose (Kuhn, Baer & Gauhe, 1956*b*). The last three compounds are all fucose derivatives of a non-fucose compound, β-D-galactosido-(1 → 3)-β-acetylglucosamino-(1 → 3)-lactose, referred to by Kuhn and his associates as lacto-*N*-tetraose (Kuhn & Baer, 1956).

Montreuil (1957) has also reported the occurrence of lacto-*N*-tetraose and a series of fucose-containing oligosaccharides in human milk which, in addition to those identified in Kuhn's laboratory, include a second fucosido-lactose compound and at least five other substances in which fucose is present. These he has characterized by the molecular proportions of their constituent monosaccharides—galactose, glucose, fucose and acetylglucosamine.

As a result of our own independent observations on the presence of fucose in human milk (Hytten, Reithel, Fletcher & Malpress, 1954) and having regard to the possible metabolic importance of the fucose-containing oligosaccharides, we have investigated in this work some aspects of their nature and distribution in milk samples from clinically normal women. Preliminary accounts of parts of this work have been given elsewhere (Hytten & Malpress, 1957; Malpress & Hytten, 1957).

EXPERIMENTAL

Collection and pre-treatment of milk samples. Four hours after a breast feed both breasts were completely emptied of milk by an electric Humalactor breast pump (Ratcliff, 1951; Hytten, 1954); the collection took 15–20 min. The combined milk sample which varied from 39 to 96 ml. was immediately mixed with one-ninth of its volume of 50% (w/v) trichloroacetic acid. All samples were then stored in polythene bottles at −5° for several months. When required for analysis the samples were thawed, filtered through a pad of Celite 535 (L. Light and Co.) and the acid filtrates were extracted four times with 1·5 vol. of ether. The aqueous layers were then neutralized by the cautious addition of N-NaOH and traces of ether were removed by an air current. Small samples were then taken for an estimation of total fucose; to the main solutions ethanol was added to a concentration of 5% (v/v) and the pH adjusted to 6·5.

Chromatographic methods

Column chromatography with carbon–Celite (Whistler & Durso, 1950). The solutions at pH 6·5 were added to columns 1·5 cm. in diameter and 45 cm. high, to the upper ends of which 500 ml. Ehrlenmeyer flasks had been sealed as reservoirs; each column contained a mixture of 7 g. of charcoal (British Drug Houses Ltd., Animal charcoal for decolorizing) with 7 g. of Celite 535, which had previously been washed successively with water and 5% (v/v) ethanol (pH 6·5). Separation of the oligosaccharides was obtained by eluting with increasing strengths of ethanol up to 30% (v/v). Ten fractions were obtained from each initial sample, two 350 ml. portions of the eluent being passed through the column at each of five ethanol concentrations. These fractions were concentrated and the ethanol was removed *in vacuo*. After estimation of their fucose content separately, all the fractions eluted at the same concentration of ethanol were combined, giving five main solutions for subsequent qualitative analysis. These solutions are referred to below as the 5, 10, 15, 20 and 30% ethanol eluates respectively. They were further purified by chromatography on powdered cellulose columns or high-capacity filter paper, or both.

Column chromatography with powdered cellulose. The 5% ethanol eluate was heavily contaminated with lactose, most of which was removed by concentrating the solution to about 100 ml., adding ethanol to a concentration of 95% (v/v) and cooling to 5°. The filtrate from the precipitated lactose, and also the 10% ethanol eluate, were concentrated by vacuum distillation and finally evaporated to syrups *in vacuo* over P_2O_5. These syrups were then added to columns (diameter 2·5 cm., height 70 cm.) containing 60 g. of powdered cellulose which had been added as a suspension in a mixture of butanol–pyridine–water (6:1:1, by vol.; Bächli & Percival, 1952). The columns were treated with 5 l. of the same solvent mixture and afterwards extruded and cut into twelve equal sections. After removal of all the solvent in an air current the sections were extracted twice with 50 ml. of water. Those sections in which fucose-containing oligosaccharides were present were further purified by band chromatography on high-capacity filter paper.

Band chromatography. Fucose-containing extracts from the powdered cellulose columns, and the original 15, 20 and 30% ethanol eluates, after suitable concentration over P_2O_5 *in vacuo*, were purified by band chromatography on Whatman no. 3MM filter paper; several sheets were used for each sample. Two solvents were used: ethyl acetate–pyridine–water (2:1:2, by vol.; Jermyn & Isherwood, 1949), and a modification of this solvent which gave improved movement and separation of oligosaccharides composed of more than five or six sugar units (Table 1). The modified solvent was prepared by adding ethyl acetate to the monophasic mixture of ethyl acetate–pyridine–water–acetone (10:5:10:2, by vol.) in the minimum amount to cause separation into two phases after mixing and to give an upper layer having sp.gr. within the range 0·951–0·953 at 25°, the temperature at which chromatograms were run.

Reducing bands were detected on side and centre strips from each chromatogram by the method of Trevelyan, Procter & Harrison (1950) and the unsprayed portions were cut with these strips as a guide; like sections were bulked and the oligosaccharides present eluted with water. After concentration *in vacuo* the individual eluted fractions were each re-run separately on Whatman no. 3MM paper and again detected, cut and eluted as before. Attempts to crystallize these fractions after concentration to small volume at this stage were unsuccessful except for the fraction representing lacto-*N*-tetraose.

Table 1. *R_T values of oligosaccharides in ethyl acetate–pyridine–water solvent with and without the addition of acetone*

Descending chromatography on Whatman no. 3MM paper. Time 20 hr. For details of solvent see text. On typical chromatograms run at the same time under identical conditions lacto-*N*-tetraose (spot *I*) moved 22·3 cm. in the solvent without acetone and 30·4 cm. with acetone added.

Oligosaccharide	No. of hexose units	R_T*	
		Without acetone	With acetone
A	?	0·08	0·19
B	14	0·13	0·31
C	13	0·17	0·41
D	10	0·28	0·52
E	9	0·43	0·63
F	8	0·55	0·81
G	6	0·64	—
H	5	0·77	0·92
I	4	1·00	1·00
J	3	1·43	1·12

* R_T = ratio of distance run by spot to distance run by lacto-*N*-tetraose (spot *I*).

Further purification. Crystalline lacto-*N*-tetraose was obtained by the method of Kuhn, Gauhe & Baer (1953). The remaining fractions from band chromatography were concentrated to 5 ml. or less and precipitated at 50, 80 and 90% ethanol levels. This fractionation procedure was repeated on the 80% ethanol precipitates (which were the main ones), after redissolving them in water, until successive precipitates showed a constant fucose:'core' ratio (see below). The compounds used in this study may therefore be defined as chromatographically pure and as giving steady fucose:'core' ratios on repeated ethanol fractionation.

Additional chromatographic methods. De-salting after hydrolysis experiments was carried out by the pyridine-extraction method, as modified by McGeown & Malpress (1952). R_T values are defined as the ratios of the distance run by the centre of any oligosaccharide spot to the distance run by the centre of a standard lacto-*N*-tetraose spot.

Estimation of fucose

Method I depended on periodate oxidation of the sugar (Nicolet & Shinn, 1941) and the determination of the acetaldehyde so formed by the Rimini reaction (Rimini, 1898; Waldron, 1952). It could be used for estimating 0·1–0·6 mg. of free fucose, or of combined fucose after hydrolysis; it could not be used for solutions containing more than 5 mg. of lactose in the test sample (Fig. 1). Method II was based on the H_2SO_4–cysteine hydrochloride reaction (Dische & Shettles, 1948). It conveniently measured 1–50 μg. of fucose, both free and combined, and had a special value in the modification described below for the estimation of fucose in amounts of less than 5 μg. in the presence of lactose concentrations up to 200 μg., and in its application to the analysis of purificd fucose oligosaccharides.

Method I. Sodium periodate solution was prepared by dissolving 2·66 g. of $HIO_4,2H_2O$ in water, adjusting to pH 6·8 with *N*-NaOH and diluting to 100 ml. The Rimini reagent was made by mixing 4 vol. of a 5% (v/v) solution of ethylene glycol in ethanol with 3 vol. of a 20% (w/v) solution of piperazine in ethanol and 1 vol of 7% (w/v) aqueous sodium nitroprusside. This reagent is prepared immediately before use and it is essential that aldehyde-free ethanol should be used.

Sodium periodate (1 ml.) was added to 3 ml. of the test solution containing up to 0·6 mg. of free fucose. The solutions were mixed and kept at room temp. for 10 min. Rimini reagent (1 ml.) was then added to the tubes, which were gently shaken, put into a bowl of crushed ice and stored in the dark in a refrigerator at 4°. The blue colour which develops in the presence of fucose was read after 1 hr. in a Spekker absorptiometer with an Ilford orange filter (no. 607; wavelength of peak transmission, 600 mμ). The solutions were removed singly from the refrigerator and the readings recorded immediately since the colour fades rapidly in the light. Estimations of standard L-fucose solutions were carried out with each series of measurements.

Fucose is quantitatively freed from its combination in the oligosaccharides of human milk by hydrolysis with N-HCl for 7 min. at 100°. After neutralization of the hydrolysate with NaOH method I may be directly applied, but the intensity of the colour developed is enhanced by the presence of NaCl in the solution (Fig. 2). Hydrolysis was usually carried out on 2 ml. of the test solutions, to which was added 0·5 ml. of 5N-HCl; after hydrolysis the solutions were neutralized with 0·5 ml. of 5N-NaOH. To the solutions used as standards 1 ml. of a solution containing 146 mg. of NaCl was added and water to a total volume of 3 ml.

Method II. The determination of fucose by the standard CyR 10 method of Dische & Shettles (1948) is not valid for solutions containing lactose, since this sugar gives a positive extinction difference value for $\Delta E_{396-430}$ and so falsifies the estimation of fucose, which depends on this measurement. The extent of this interference may, however, be determined because in control solutions, to which 0·1 ml. of water instead of 0·1 ml. of 3% cysteine hydrochloride is added, lactose gives lasting pink colours which show a peak absorption at 520 mμ, a wavelength at which similarly treated fucose solutions show zero absorption

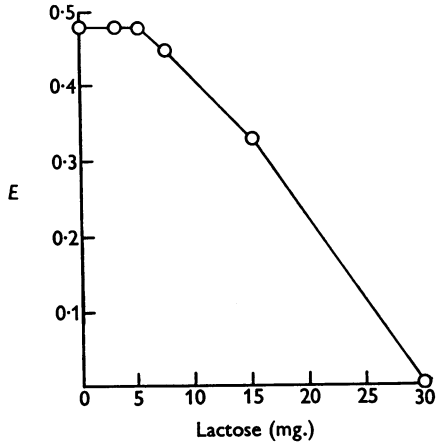


Fig. 1. Fucose estimation (method I). Change in extinction of solutions containing 0·3 mg. of fucose in the presence of increasing amounts of lactose.

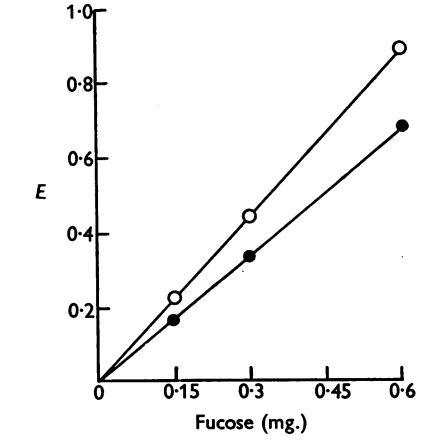


Fig. 2. Fucose estimation (method I). Calibration curves for fucose with (○) and without (●) the addition of NaCl (0·146 g.).

(Fig. 3). Calibration at this wavelength thus permits the direct determination of lactose in mixtures. By coupling this determination with a calibration curve giving $\Delta E_{396-430}$ values for standard lactose solutions, the contribution of lactose to observed $\Delta E_{396-430}$ values in mixed solutions may be found and true fucose values determined. The interference due to lactose is minimized by taking readings 7 hr. after the addition of cysteine hydrochloride to the test solutions or water to the controls. The solutions are kept in the dark at room temp. and the $\Delta E_{396-430}$ values increase for fucose and decrease for lactose, reaching steady values only at the end of this interval (Fig. 4). Lacto-*N*-tetraose shows properties similar to those of lactose.

Calibration (Fig. 5) was checked for each series of estimations by including standard lactose and standard fucose solutions; the results of recovery experiments on various known mixtures of these sugars are given in Table 2. Absorption values were measured on a Unicam SP. 600 absorption spectrophotometer.

Analysis of purified compounds. Both methods described in the previous section were used for the estimation of fucose in the purified compounds obtained in this work. Estimation by the Rimini reaction (method I) was carried out on a known weight of the substance after the necessary preliminary hydrolysis. Estimations made by method II were particularly valuable when applied to purified compounds, for the measurements obtained were the sum of the absorptions due to (i) the total fucose present in the compound and (ii) its fucose-free 'core'. Thus, for the simplest compound, fucosido-lactose, the absorption values were identical with those given by an equivalent equimolecular

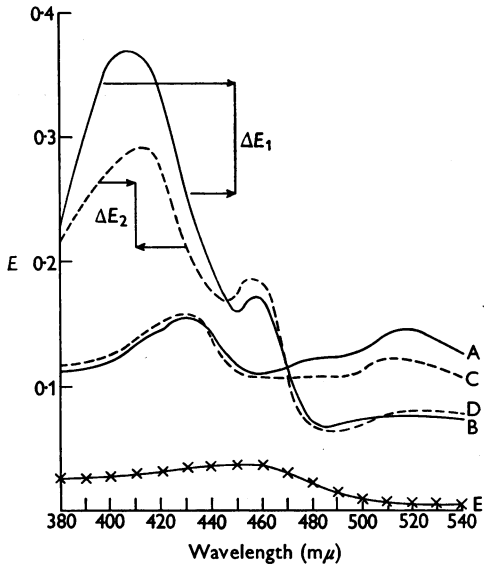


Fig. 3. Fucose estimation (method II). Absorption spectra of fucose (×), lactose (—) and lacto-*N*-tetraose (- - -) solutions. A, 200 μg. of lactose, control solution treated with 0·1 ml. of water; B, 200 μg. of lactose treated with 0·1 ml. of 3% cysteine hydrochloride solution; C, 300 μg. of lacto-*N*-tetraose, control solution treated with 0·1 ml. of water; D, 300 μg. of lacto-*N*-tetraose treated with 0·1 ml. of 3% cysteine hydrochloride solution; E, 50 μg. of fucose, control solution treated with 0·1 ml. of water. A, C and E read against water; B and D read against corresponding control solution. $\Delta E_1 = \Delta E_{396-430}$ for 200 μg. of lactose; $\Delta E_2 = \Delta E_{396-430}$ for 300 μg. of lacto-*N*-tetraose.

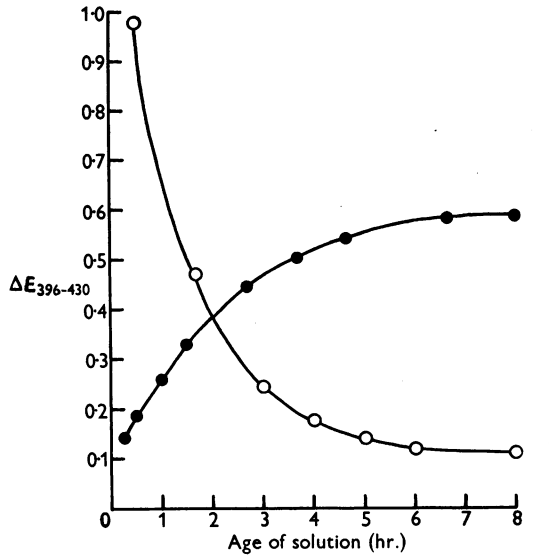


Fig. 4. Fucose estimation (method II). Changes in $\Delta E_{396-430}$ values of solutions on storing. ○, 400 μg. of lactose; ●, 30 μg. of fucose.

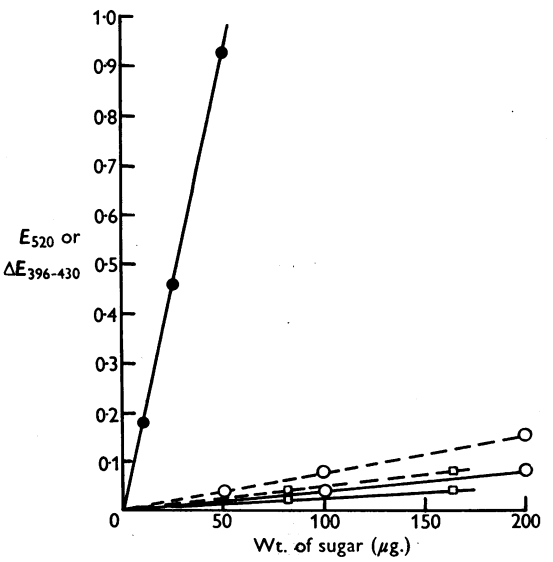


Fig. 5. Fucose estimation (method II). Typical $\Delta E_{396-430}$ calibration curves (—) and E_{520} calibration curves (- - -) for fucose (●), lactose (○) and lacto-*N*-tetraose (□). (E_{520} values for fucose solutions are negligible.)

mixture of the component sugars, fucose and lactose. Provided therefore that calibration curves were available for the various 'core' molecules encountered, so that true fucose values could be derived, the method, in addition to giving an estimate of the amount of fucose in the molecule, also gave fucose:'core' ratios. The 'cores' found in this work were: lactose, lacto-*N*-tetraose, di(lacto-*N*-tetraose) and tri(lacto-*N*-tetraose). Calibration curves for absorption at 520 mμ and for $\Delta E_{396-430}$ were obtained directly from crystalline preparations of the first two compounds, from a non-crystalline but chromatographically pure preparation of di(lacto-*N*-tetraose), and by assessment for tri(lacto-*N*-tetraose).

Estimation of hexosamine and related compounds. *N*-Acetylglucosamine was estimated by the method of Aminoff, Morgan & Watkins (1952), hexosamine by the method of Rondle & Morgan (1955) after hydrolysis in sealed ampoules at 100° for 16 hr. with 0·5N-HCl, and sialic acid by the method of Werner & Odin (1952).

RESULTS

Fucose content of milk samples. Estimation of the total fucose in milk samples before fractionation gave results which were less readily reproduced in duplicate determinations than estimation by summation of the fucose contents of ethanol eluates from the carbon–Celite columns; this was undoubtedly due to the very high lactose:fucose ratio (about 40:1) in the unfractionated samples, and to the presence in them of other impurities. Although both series of results were of the same order, it is the summation method which is taken here as giving the truer values.

The fucose contents of six milk samples taken from each of two women at intervals varying from 3 to 43 days *post partum* are shown in Table 3. Over this period there was no evidence of any change in the level of total fucose values. These figures receive support from the analysis of a bulked sample of six specimens of milk collected from a third woman at intervals of 4–9 days *post partum*, for which the fucose content, estimated before fractionation, was 160 mg./100 ml.; and from a sample collected in advanced lactation (191 days) which showed 117 mg./100 ml. by the summation method and 125 mg./100 ml. before fractionation.

Fractionation of samples of milk on carbon–Celite columns. The separation of the fucose oligosaccharides into fractions eluted from carbon–Celite columns by solutions of increasing ethanol content gave a first insight into the distribution of these compounds in human milk. Figures for a typical elution pattern are given in Table 4. The fall in the fucose content of the second eluate at any given ethanol concentration, and the rise in fucose

Table 2. *Recovery of fucose in the presence of excess of lactose*

The estimations were carried out by method II (see Experimental section).

Fucose in solution (μg.)	Lactose in solution (μg.)	Fucose recovered (μg.)	Fucose recovered (%)	Lactose recovered (μg.)	Lactose recovered (%)
2·5	190	2·3	92	193	102
5·0	180	4·7	94	186	103
6·0	180	5·7	95	180	100
12·0	160	11·8	98	151	94
15·0	150	14·7	98	142	95

Table 3. *Concentrations of fucose in milk samples*

Sample	Day of lactation	Fucose (mg./100 ml.) Before fractionation*	Fucose (mg./100 ml.) After fractionation†
M1	3	139·0	124·7
M2	5	146·4	129·0
M3	7	170·4	144·3
M4	15	159·6	135·6
M5	29	159·7	124·5
M6	43	117·5	112·9
	Mean	148·8 (S.D. ± 18·9)	128·5 (S.D. ± 10·7)
K1	5	99·9	122·1
K2	7	108·4	127·3
K3	15	93·9	116·2
K4	21	105·6	125·1
K5	29	112·8	138·7
K6	36	97·5	139·4
	Mean	103·0 (S.D. ± 7·1)	128·1 (S.D. ± 9·2)

* Estimation by method II (see Experimental section).
† Estimation of 5% ethanol fraction by method II, remainder by method I (see Experimental section).

content immediately the strength of ethanol was increased were possible indications of a separation into fairly well-defined fractions.

Elution patterns for two series of milk samples are shown in Table 5. These suggest that, although the pattern may vary in its detailed characteristics for different women, the oligosaccharides of smallest molecular weight, which are eluted in the 5 and 10% ethanol fractions, are always present in the greatest amount; further, that there is a tendency for the 5% fraction to increase in amount, relative to the others, as lactation advances. The single result from a late lactation period (191 days), when the milk flow was still plentiful, possibly shows this tendency at an extreme point, where over 90% of the total fucose was present in the 5 and 10% ethanol fractions.

Qualitative analysis of ethanol eluates

Ten fractions were isolated in chromatographically pure form from the 5, 10, 15, 20 and 30% ethanol eluates from the carbon–Celite columns. These are referred to as compounds *A* to *J*, and include seven fucose-containing oligosaccharides, two related compounds which contain no fucose and one fraction, compound *A*, which contains fucose but proved of doubtful homogeneity. The distribution of these oligosaccharides in the ethanol eluates is summarized in Table 6.

Analysis of isolated compounds. The results of mild acid hydrolysis, together with fucose estimations on known weights of the purified oligosaccharides, and determination of their fucose:'core' values gave information upon which deductions could be confidently made about the structural types to which the oligosaccharides conformed. The identification of compounds *B*, *C*, *D*, *E* and *F* is based entirely on the results presented in this paper; the structures of compounds *G*, *H*, *I* and *J* have already been established (see Kuhn, 1957) and the confirmation given by our own work is briefly summarized here. Except where otherwise stated all hydrolyses with hydrochloric acid were carried out by heating the solutions for 10 min. in a boiling-water bath.

Compound J; *fucosido-lactose* (Kuhn *et al.* 1955; Montreuil, 1956). Hydrolysis with 0·1N-HCl gave a mixture of fucose and lactose, with traces of glucose and galactose. The fucose:lactose ratio was 0·43 (calc. 0·48).

Compound I; *lacto-N-tetraose* (Kuhn, Gauhe & Baer, 1953, 1954). A crystalline preparation had m.p. 204–205° and was fucose-free. Hydrolysis with 0·1N-HCl showed the original compound largely unchanged; additional spots on the chromatogram were lactose, galactose and glucose, and two other unidentified substances with R_F values

Table 4. *Elution pattern for fucose oligosaccharides*

The fucose-containing fraction from a single sample of milk was eluted from a carbon–Celite column by two successive 350 ml. portions of eluent at each of the five concentrations of ethanol shown.

Concn. of ethanol in eluent (%)	Percentage of total fucose eluted	
	First 350 ml.	Second 350 ml.
5	19·6	3·4
10	37·5	4·8
15	13·9	6·1
20	9·5	2·0
30	3·2	0

Table 5. *Elution patterns of the fucose-containing fractions of milk samples from carbon–Celite columns*

Samples of milk were taken from women at the stated days *post partum*.

Concn. of ethanol in eluent (%)		Percentage of total fucose eluted				
		5	10	15	20	30
Subject	Days *post partum*					
M	3	23·0	42·3	20·0	11·5	3·2
	5	20·0	41·8	20·6	14·5	3·1
	7	34·9	28·8	21·0	12·2	3·1
	15	20·6	38·7	22·8	14·5	3·4
	29	34·6	34·3	18·8	10·3	2·0
	43	41·0	32·5	15·2	9·2	2·1
K	5	51·1	19·4	11·2	13·5	4·8
	7	49·8	22·0	10·1	13·4	4·7
	15	35·5	34·3	20·1	8·1	2·0
	21	51·7	23·6	12·2	9·8	2·7
	29	52·5	28·6	12·3	5·2	1·4
	36	83·7	5·7	4·7	4·2	1·7
R	191	71·0	19·9	7·6	1·5	0

Table 6. *Distribution of oligosaccharides in ethanol eluates from carbon–Celite columns*

+, Compound present; ++, compound present in relatively large amount; −, compound absent.

Compound	Eluate (% ethanol)				
	5	10	15	20	30
A	−	−	−	+	+
B	−	−	+	+	+
C	−	−	+	++	++
D	−	+	++	+	+
E	−	+	++	+	+
F	−	−	+	+	+
G	+	−	−	−	−
H	+	++	−	−	−
I	+	++	++	−	−
J	++	−	−	−	−
Lactose	++	−	−	−	−

between those of lactose and lacto-*N*-tetraose, probably corresponding to the two trioses reported by Kuhn *et al.* (1954) as occurring in hydrolysates of lacto-*N*-tetraose. Glucosamine was not detected on these chromatograms, but was present, in addition to glucose and galactose, after hydrolysis with N-HCl for 30 min.

Compound H; *monofuco-lacto*-N-*tetraose*. Hydrolysis with 0·1N-HCl gave fucose, lacto-*N*-tetraose and traces of hydrolytic products from the latter compound. The fucose:lacto-*N*-tetraose ratio was 0·22 (calc. 0·23). Only one monofuco-lacto-*N*-tetraose was found, and it is not known whether it corresponds to lacto-*N*-pentaose I or II of Kuhn *et al.* (1956*b*).

Compound G; *difuco-lacto*-N-*tetraose* (lacto-*N*-difucohexaose, Kuhn *et al.* 1956*b*). Hydrolysis with 0·1N-HCl gave fucose and lacto-*N*-tetraose with traces of further breakdown products. The fucose: lacto-*N*-tetraose ratio was 0·46 (calc. 0·46).

Compound F. This was a fucose-free compound which, when hydrolysed with 0·1N-HCl gave lacto-*N*-tetraose and small traces of galactose, glucose and glucosamine; hydrolysis with 0·005N-HCl for 2 hr. gave lacto-*N*-tetraose and a second compound (R_T 0·89 in ethyl acetate–pyridine–water) which was not identified. Oligosaccharides are liable to give a range of products on acid hydrolysis, but it was consistent with the chromatographic evidence obtained that compound *F* was primarily convertible into lacto-*N*-tetraose as the one major product. It was therefore tentatively identified as *di*(*lacto*-N-*tetraose*), a hypothesis which was supported by the following observations: (i) the colour developed in the modified Dische & Shettles (1948) reaction had almost exactly half the intensity given by the same weight of lacto-*N*-tetraose and showed a similar absorption curve; (ii) the assumption of a di(lacto-*N*-tetraose) structure led to an intelligible interpretation of the behaviour on hydrolysis, the fucose contents and the fucose:'core' ratios of compounds *B*, *C*, *D* and *E*.

Compound E. Hydrolysis with 0·01N-HCl gave fucose and compound *F*; some unchanged compound *E* was also present and a trace of the compound (R_T 0·89) already noted as occurring after the mild hydrolysis of compound *F*. Hydrolysis with 0·1N-HCl caused the complete disappearance of compound *E* and the liberation of fucose, compound *F*, compound R_T 0·89 and lacto-*N*-tetraose. The analytical figures lent support to the view that this compound was *monofuco-di*(*lacto*-N-*tetraose*): fucose 8·8% (calc. 10·6%); fucose:di(lacto-*N*-tetraose) ratio 0·08 (calc. 0·12).

Compound D. Hydrolysis with 0·01N-HCl gave fucose, compound *E*, compound *F* and a trace of lacto-*N*-tetraose, in addition to some unchanged compound *D*. Hydrolysis with 0·1N-HCl gave fucose, compound *F* and lacto-*N*-tetraose only. This compound was clearly related to compound *E*, but richer in fucose, and the analytical figures agreed fairly well with its identification as a *difuco-di*(*lacto*-N-*tetraose*):fucose 17·0% (calc. 19·4%); fucose:di(lacto-*N*-tetraose) ratio 0·16 (calc. 0·23).

Compound C. Hydrolysis with 0·01N-HCl gave fucose and a compound (R_T 0·23), in addition to unchanged compound *C*. Hydrolysis with 0·1N-HCl gave fucose, compound *E*, a trace of compound *F*, and lacto-*N*-tetraose. The complete absence of compound *D* suggested that compound *C* was a mono-fuco derivative and that the compound (R_T 0·23) might be a new fucose-free 'core'. Since hydrolysis with 0·1N-HCl converted this 'core' into compound *F* and lacto-*N*-tetraose, a tri- or tetra-(lacto-*N*-tetraose) structure seemed likely. The fucose content of compound *C* was 8·5% (calc. for monofuco-tri(lacto-*N*-tetraose) 7·4%; for monofuco-tetra(lacto-*N*-tetraose) 5·6%). The fucose:tri(lacto-*N*-tetraose) ratio (from derived calibration curves assuming the equivalence, in the modified Dische & Shettles (1948) reaction, of 300 μg. of tri(lacto-*N*-tetraose) and 100 μg. of lacto-*N*-tetraose) was 0·08 (calc. 0·08). Compound C was therefore probably a *monofuco-tri*(*lacto*-N-*tetraose*).

Compound B. Hydrolysis with 0·01N-HCl gave fucose, compound *C*, compound (R_T 0·23), compound *D*, compound *E* and lacto-*N*-tetraose, in addition to unchanged compound *B*. Hydrolysis with 0·1N-HCl gave fucose, compound *E*, compound *F* and lacto-*N*-tetraose. Two reactions were probably involved: one giving fucose and compound *C*, the other lacto-*N*-tetraose and compound *D*. The identification of compound *B* as a *difuco-tri*(*lacto*-N-*tetraose*) was supported by the analytical figures: fucose 10·0% (calc. 13·8%); fucose:tri(lacto-*N*-tetraose) ratio 0·13 (calc. 0·16).

Compound A. This fraction gave fucose on hydrolysis. The chromatographic picture was not readily interpretable, however, in relation to the analytical figures obtained, and it seemed probable that 'compound *A*' was a mixture of oligosaccharides.

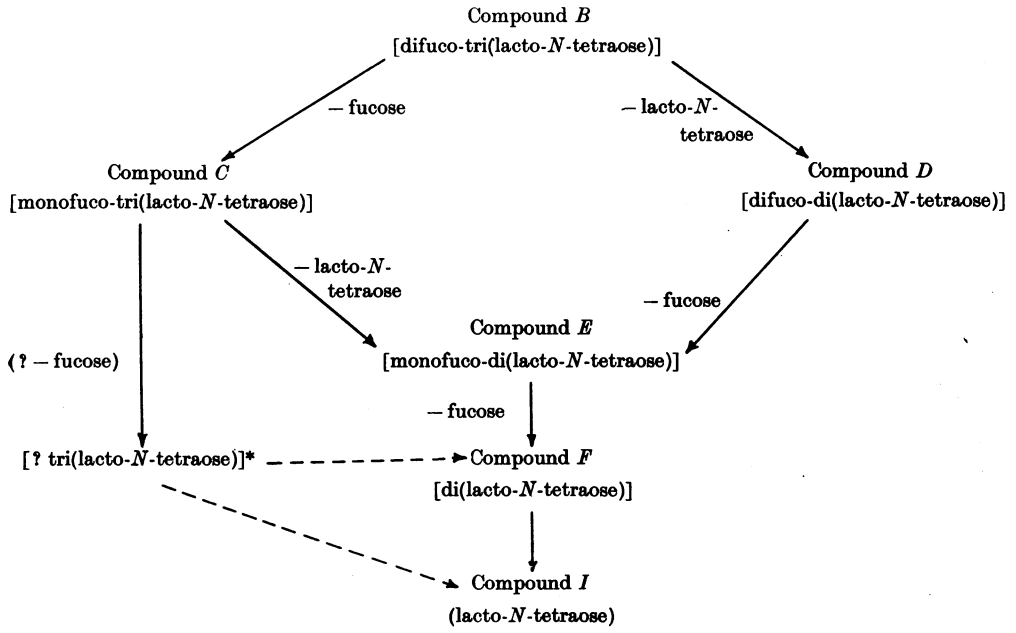
Occurrence of hexosamine and related compounds. Positive values for *N*-acetylglucosamine, hexosamine and sialic acid were given by all the ethanol eluates, the highest values for the first two compounds being recorded in the 10 and 15% eluates and for sialic acid in the 5% fraction. However, sialic acid was absent from the purified fucose compounds reported in this paper, and an attempt to estimate their acetylglucosamine content demonstrated the inapplicability of the standard method

used for this purpose. An excessively high value (182% of the calculated figure) was obtained for the acetylglucosamine content of pure lacto-*N*-tetraose, roughly half the expected values for compounds *E* and *F*, and negligible values for compounds *B*, *C* and *D*. The high value for lacto-*N*-tetraose is in accord with similarly high figures obtained by Kuhn *et al.* (1954) under slightly different experimental conditions.

DISCUSSION

In view of the great lability of fucose in human-milk oligosaccharides the absence of free fucose in the samples when they were fractionated makes it improbable that the conditions of storing had any effect on these compounds; the possibility that some of the smaller oligosaccharides could have arisen from larger compounds by hydrolysis on storing is also most unlikely since they have been isolated by other workers by quite different procedures (Kuhn *et al.* 1953; Montreuil, 1957); also, the sample taken in advanced lactation (see Table 5), which was precipitated with trichloroacetic acid, extracted with ether and fractionated immediately after sampling, showed the presence of rather more of the smaller oligosaccharides than was usual in the stored samples.

The distribution pattern of the fucose compounds on carbon–Celite columns showed that in all samples the smaller-molecular-weight oligosaccharides were present in the greatest amount, particularly fucosido-lactose and monofuco-lacto-*N*-tetraose. These compounds contain 33·6 and 19·2% of fucose respectively, whereas the higher oligosaccharides, present in much smaller amounts, have fucose contents ranging from 7·4 [monofuco-tri(lacto-*N*-tetraose)] to 32·8% (difuco-lacto-*N*-tetraose). It would therefore seem that an estimate of the total fucose-containing oligosaccharides of human-milk samples could be obtained without too great an error by accepting an arbitrary mean fucose content of 25% as representative of these substances. This figure leads to a value of 0·5 g./100 ml., which still takes no account of the presence of related fucose-free compounds such as lacto-*N*-tetraose and di(lacto-*N*-tetraose), the first of which was undoubtedly present in considerable amounts (approximately 0·1 g./100 ml.) in the samples analysed. A total estimate of 0·6 g./100 ml. for the oligosaccharides of human milk seems therefore to be a reasonable estimate of



*Compound having R_T 0·23 in ethyl acetate–pyridine–water (2:1:2, by vol.)

Fig. 6. Summary of the relationships existing between the higher oligosaccharides of human milk as demonstrated by mild hydrolysis with hydrochloric acid. ——→, established changes; - - - →, changes presumed to occur.

true values; this is rather higher than the figure given by Kuhn *et al.* (1955), who assess the 'oligosaccharides excluding lactose' to be 3·0–3·3 g./l., or by Kuhn (1957), who gives the *N*-containing oligosaccharide content as approximately 4 g./l. It is of course possible that the oligosaccharide content of milk samples varies more widely than was found in this study, but it seems important to emphasize that these substances, considered as a group, are in no sense trace constituents of human milk but are present in amounts equal to about 10% of the lactose content.

The tentative identification of five new oligosaccharides, compounds *B*, *C*, *D*, *E* and *F*, was based primarily on the relationships observed to exist between them on mild acid hydrolysis, and also on their relationship to the other, known oligosaccharides (Kuhn, 1957), particularly lacto-*N*-tetraose. The main connexions established in this way are summarized in Fig. 6. The new compounds were not crystalline and the slightly low fucose contents of compounds *B*, *D* and *E* may be due to residual contamination with other substances despite an apparent chromatographic purity. It would be unprofitable at this stage to attempt any comparison between these compounds and those investigated by Montreuil (1957), which he has as yet characterized only by the relative proportions of their component hexose units. It seems probable, however, that at least some of Montreuil's compounds will prove to be identical with those reported here (see also Malpress & Hytten, 1957).

If we exclude the simplest compound, fucosidolactose, a picture emerges from these results of a series of fucose-oligosaccharides in which fucose is attached to a core of one or more lacto-*N*-tetraose units; further, the occurrence of lacto-*N*-tetraose in mild acid hydrolysates of the compounds *B*, *C*, *D*, *E* and *F* suggests that these units are joined by linkages which are relatively more labile than those existing within the lacto-*N*-tetraose molecule itself. Alternatively, since apart from difucolacto-*N*-tetraose [compound *G*; lacto-*N*-difucohexaose (Kuhn, 1957)], the stoicheiometric ratio of fucose to lacto-*N*-tetraose in all these compounds never exceeds unity, we might view the series as consisting of polymerized units of a monofuco-lacto-*N*-tetraose, some of which—presumably in the course of normal metabolism, for practically no free fucose was found in this study—have lost fucose and been converted into compounds having a ratio less than unity. Such a view would give special importance to the monofuco-lacto-*N*-tetraose structure, two forms of which have already been investigated by Kuhn *et al.* (1956*b*); if substantiated it would also simplify the inquiry into the detailed structure of these oligosaccharides, whose 'type' identities presented here cover such a wide range of isomeric forms.

SUMMARY

1. The total fucose content of human-milk samples has been determined at intervals varying from 3 to 43 days *post partum*. The amounts present remained fairly constant over this period and mean values of 128 mg./100 ml. were obtained for each of two series, representing an oligosaccharide content of approximately 0·6 g./100 ml.

2. The distribution pattern of fucose oligosaccharides absorbed on carbon–Celite columns and eluted with increasing strengths of ethanol has been obtained for human milk samples.

3. Ten oligosaccharides from human milk have been purified by chromatographic methods; five of these are tentatively characterized for the first time as, di(lacto-*N*-tetraose), monofuco-di(lacto-*N*-tetraose), difuco-di(lacto-*N*-tetraose), monofuco-tri(lacto-*N*-tetraose), and difuco-tri(lacto-*N*-tetraose).

4. A modification of the ethyl acetate–pyridine–water solvent (Jermyn & Isherwood, 1949) was developed which has special value for the separation of oligosaccharides more complex than a hexasaccharide.

We wish to record our thanks to Professor D. C. Harrison for his interest in this work and to express our indebtedness to Mr R. Dancklefsen for technical assistance.

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Preparation of Crystalline Porphyrin Esters from Bovine Porphyria Urine

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(*Received* 11 *June* 1957)

For studies of porphyrin metabolism in which chromatographic procedures are employed, pure porphyrins are often required as markers. With uro- and copro-porphyrins these are difficult to obtain. A simple method for preparing such markers is therefore desirable.

Preparation of porphyrins from urines very rich in them is simple, as the porphyrins are precipitated on acidification. Recently Ottolenghi-Lodigiani & Serchi (1951) prepared 200–300 mg. of crystalline porphyrin esters from 1 l. of human congenital-porphyria urine in this way; but if the porphyrin concentration is lower, the preparation is much more difficult (cf. Lemberg & Legge, 1949) and varied techniques have been employed. Rimington & Miles (1951) acidified 3 l. of human porphyria urine, collected the precipitate and adsorbed the porphyrins in the filtrate on kieselguhr; but Macgregor, Nicholas & Rimington (1952) preferred adsorption on talc at pH 3–4 and stated that the extraction of the total porphyrin from urine presents considerable difficulty.

The method described below was elaborated during work with bovine porphyria urine (cf. Jørgensen & With, 1955), but has been found to function equally well with human porphyria urines and even normal human urine. In the latter the red fluorescence is quenched, however, and appears only after the chromatographic purification. Volumes of 20 l. have been treated, and special difficulties were encountered because of the alkaline reaction of bovine urine. The only studies on bovine porphyria urine reported previously are those of Rimington (1936), in which the urine was removed from a single animal *post mortem*, and it seems that the porphyrin concentration of this urine was exceptionally high.

Because of the large amounts of porphyrins, current methods of chromatography and crystallization had to be modified. For chromatography on aluminium oxide, mixtures of chloroform and benzene were employed instead of pure benzene, and crystallization was performed at room temperature.

The various steps of the procedure were followed with quantitative and qualitative analyses and the purity of the crystalline esters was checked by paper chromatography, melting-point determination and estimation of the extinction ($E^{1\%}_{1\,cm.}$) at the maximum of the Soret absorption band.

MATERIALS AND METHODS

The urine was from the porphyric steer described by Jørgensen & With (1955). The $CHCl_3$ employed in the procedures was of pharmacopoeia quality (*Pharm. Danica*) unless otherwise noted. The MgO (British Drug Houses Ltd.) employed was the preparation for chromatography, and was found to correspond to grade III according to Brockmann & Schodder (1941). The commercial Al_2O_3 preparations (British Drug Houses Ltd. or Merck Inc.) were too strongly adsorbent and were therefore treated by suspension in water, then sucked dry on a Büchner funnel and dried at 60° for 1–2 days. Before use it was graded by the procedure of Brockmann & Schodder (1941), see also Williams (1946), an adsorbency corresponding to grade III–IV being required. It is important to remove the considerable quantities of non-porphyrin pigment present in all urines, both in porphyria and other conditions. These pigments are presumably dipyrrole pigments like the porphobilin of Waldenström & Vahlquist (1939), the urochrome B described by Stich & Stärk (1953) and the fuscins found in normal as well as pathological faeces (cf. With, 1954). The removal of such pigments was achieved by adsorption on talc, followed by elution with 5–10% (v/v) aqueous pyridine solution or N-HCl–acetone (1:9, v/v). The porphyrin was precipitated from the pyridine on acidification or from the acetone with ammonia; the precipitate was dried and esterified with 5% (v/v) H_2SO_4 in methanol (Fischer & Zerweck, 1924).