Glycoproteins from Mature Human Milk Whey

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Extract

Two milk-specific glycoprotein fractions and serum orosomucoid
were isolated from mature human milk. One of the milk-specific
glycoproteins was homogeneous and was termed glycoprotein A. Its
sedimentation and diffusion constants were 2.6 S and 6.3 F,
respectively, giving a molecular weight of 29,500. It contained some
70% carbohydrate consisting of sialic acid, galactose, fucose,
glucosamine, and galactosamine. It was not immunologically re-
lated to serum orosomucoid. The other glycoprotein, called glyco-
protein B, was heterogeneous with an average molecular weight of
near 4,000, and carbohydrate comprised some 75% of its weight.
Both glycoprotein fractions supported the growth of *Lactobacillus
bifidus* var. *Penn*. Removal of sialic acid enhanced the growth-pro-
moting activity of glycoprotein B but not of A.

Speculation

Mature human milk whey contains a number of glycoproteins
that stimulate the growth of *L. bifidus* var. *Penn*. These glyco-
proteins may act in concert with the well known oligosaccharides to
maintain an acid environment in the infant's gastrointestinal tract.

Mature human milk contains a number of carbohydrate-con-
taining proteins (glycoproteins) possessing a diversity of biologic
functions. Perhaps the best characterized of such proteins is
lactoferrin, a non-heme iron-binding protein. It has some 7.2% of
carbohydrate (16). Another glycoprotein isolated from human
milk is κ-casein, an α-casein-stabilizing factor similar to cow's
milk κ-casein (13). Of serum glycoproteins, human milk contains
considerable amounts of immunoglobulins (6), and immunologic
testing has revealed the presence of trace amounts of other
glycoproteins, such as transferrin, haptoglobin, and orosomucoid
(11).

Human colostrum has, in addition, been shown to contain a
number of acid glycoproteins with *L. bifidus* growth-promoting
activities (12, 15). These have generally contained high amounts of
carbohydrate (near 75%). Bovine colostral acid glycoproteins (3)
do not possess the *L. bifidus* growth-promoting activity (15).

Both human mature milk and colostrum were shown to contain
various oligosaccharides that promote the growth of *L. bifidus*
(10); however, no reports seem to be available regarding the
presence in milk of acid glycoproteins of the type seen in the
colostrums of human beings and of cows. In view of the alleged
importance of the *L. bifidus* growth factors in regard to infant
health and nutrition (8, 9), it seemed worthwhile to investigate the
possible existence of acid glycoproteins in human mature milk,
which, in addition to the well known oligosaccharides, might
support the growth of *L. bifidus*. The present paper describes the
isolation of one such glycoprotein, as well as serum orosomucoid,
and shows the presence in human milk of other glycoproteins with
similar chemical properties.

MATERIALS AND METHODS

HUMAN MILK

Mature human milk was obtained from private donors or from a
milk bank. The individual samples were defatted immediately after
collection and frozen for purposes of storage. When required, the
samples were thawed at 4°, and the whey was prepared by
acidifying the skim milk as described previously (3).

Orosomucoid was prepared from human serum as described
previously (5). Its antiserum (rabbit) was purchased from Hyland
Laboratories (21). Sephadex gel chromatography media were
acquired from Parmacia Corp. (22) and CM-cellulose was bought
from Sigma Chemical Co. (23). Agar used in agar gel electropho-
resis and Ouchterlony-type double diffusion experiments was
obtained from Difco Laboratories (24).

All common laboratory chemicals were of reagent grade quality
and were obtained from Fisher Scientific Co. (25).

FRACTIONATION PROCEDURES

Ammonium sulfate fractionations of whey proteins were per-
formed in an ice bath at or near neutral pH. All precipitates were
removed by centrifugation in a Sorvall RC-2B centrifuge at 8,000
rpm. Each precipitate was redissolved in water, dialyzed exhaus-
tively against distilled water, and freeze-dried.

Gel filtration with Sephadex G-25 was carried out at 4° with
water as the eluant using a column (2.5 × 50 cm). The rate of flow
was 12 ml/hr with 6-ml fractions being collected every 30 min. The
void volume was 70 ml. Gel filtration on Sephadex G-200 was
performed as above, except that 0.1 M NaHCO₃ was used as the
eluant, and the flow rate of 6 ml/hr with 3-ml fractions being
collected every 30 min. The void volume of the Sephadex G-200
column was also 70 ml.

Chromatography on CM-cellulose was done as previously
described (3, 5).

Every fraction eluted was tested for carbohydrate by the
phenol-H_2SO_4 method (7) and for absorption at 280 nm.

ANALYTICAL METHODS

Agar gel electrophoresis was performed by the method of
Zschocke (20), where agar concentration was 1.25%. Moving
boundary electrophoresis was done in an Antweiler microelectro-
phoresis apparatus as described previously (2). Protein concentra-
tions were 3% in 0.05 M sodium Veronal-0.035% ethylene diamine
tetra-acetic acid buffer at pH 8.6. Ultracentrifugation was carried
out in a Spinco model E centrifuge in synthetic boundary cells at
protein concentrations of 3-10 mg/ml using 0.1 M NaHCO₃ as a
solvent. The temperature was 20°. Sedimentation coefficients,
calculated according to the method of Schachman (17), were
plotted against concentrations, and the curve was extrapolated to
zero concentration to obtain the sedimentation constant. The

diffusion constant was determined in a Spinco model H apparatus, equipped with interference fringe optics, at 2.7° and also at several protein concentrations (0.8–3 mg/ml) using the 0.1 M $NaHCO_3$ solvent. The diffusion coefficients were plotted against concentrations and curve was extrapolated to zero concentration to obtain the diffusion constant. Partial specific volumes were determined from composition as described by Schachman (17). Molecular weights were calculated by the sedimentation-diffusion method (17). Other physical methods used were those described previously (14), as were quantitations of amino acids and carbohydrates and the method for identification of hexoses.

Immunochemical assays for orosomucoid were done by the Ouchterlony-type double diffusion procedure as described previously (19).

LACTOBACILLUS BIFIDUS VAR. PENN. GROWTH

The growth-promoting activities of the various glycoproteins were tested as described previously (15). Results were expressed in terms of the number of milliliters of 0.1 N NaOH required to titrate the acid produced by the bacterial growth (40 hr at 37°) in 5 ml medium in the presence of 100 μg material tested. The growth response curve was linear in that glycoprotein concentration region.

RESULTS

ISOLATION OF GLYCOPROTEIN-CONTAINING FRACTIONS

Human milk whey was fractionated by ammonium sulfate using volumes of 200–400 ml whey each. A total of seven whey lots from three different donors were fractionated with substantially identi-

Human milk whey (260 ml)
|
88 g $(NH_4)_2SO_4$; dilute to 330 ml
with water; adjust pH to 7.0; let stand overnight

Precipitate (ppt. I)   Supernatant
|
30 g $(NH_4)_2SO_4$;
let stand overnight

Precipitate (ppt. II)   Supernatant
|
30 g $(NH_4)_2SO_4$;
let stand overnight

Precipitate (ppt. III)   Supernatant
|
30 g $(NH_4)_2SO_4$;
let stand overnight

Precipitate (ppt. IV)   Supernatant (pH 6.6)
|
Dialyze in 4 cm tubing for
3 days against 10–15 vol
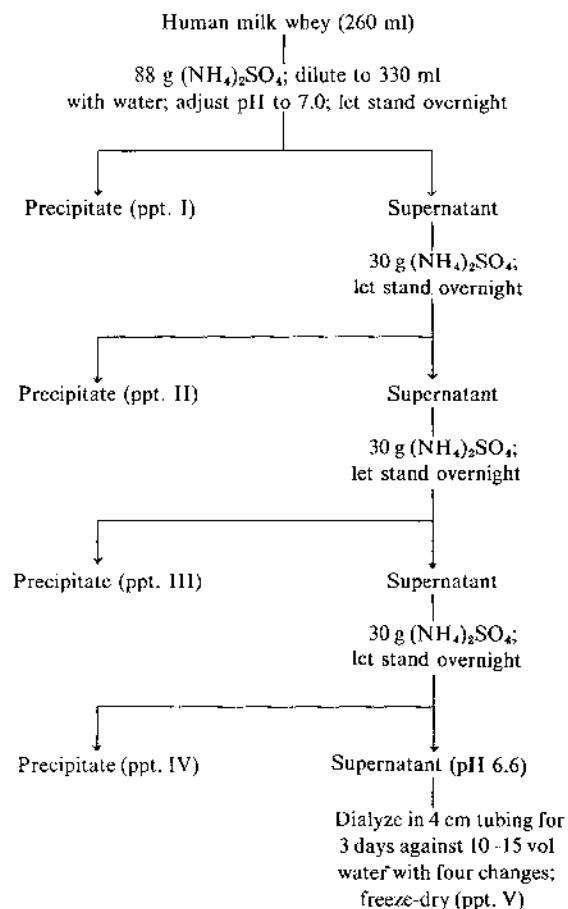water with four changes;
freeze-dry (ppt. V)

Fig. 1. Fractionation of a representative sample of human milk whey with ammonium sulfate. All salt additions were done in an ice bath, and centrifugations, dialyses, and "standing" were performed at 4°.

Table 1. *Properties of ammonium sulfate fractions of human milk whey*[1]

Fraction	Yield, mg from 260 ml whey	Hexose + fucose, %[2]
Ppt. I	823	5.8
Ppt. II	584	4.2
Ppt. III	263	4.8
Ppt. IV	46	7.8
Ppt. V	254	51.0

[1] Precipitate (ppt.) I was precipitated at 2 M concentration, whereas ppt. II was precipitated at 2.6 M $(NH_4)_2SO_4$. Ppt. IV was precipitated at near saturation of $(NH_4)_2SO_4$, and ppt. V is nondialyzable material soluble in saturated $(NH_4)_2SO_4$ solution.

[2] By the orcinol reaction (18).

cal results. A representative process is produced in Figure 1.

All fractions thus obtained were subjected to agar gel and moving boundary electrophoresis and carbohydrate analysis (orcinol reaction, Reference 18). These results are summarized in Table 1.

Electrophoretic analyses of these fractions revealed that precipitate (ppt.) I consisted largely of material remaining at the origin and some material moving cathodally. Ppt. II consisted of lactoferrin and α-lactalbumin, whereas ppt. III had α-lactalbumin, serum albumin, and some lactoferrin. Ppt. IV contained some α-lactalbumin, a component moving like serum α_1-globulin and material moving faster than serum albumin. The components of ppt. V were stained very faintly, and this fraction was submitted to moving boundary electrophoresis. The latter showed the presence of two components: one moving like the α_1-globulin (orosomucoid), the other migrating like serum β-globulin at pH 8.6.

Table 1 indicated that ppt. IV and V were rich in carbohydrate, and this paper is concerned with the components of these two fractions.

OROSOMUCOID

Orosomucoid has been detected in mature human milk by immunochemical means (11), and from our previous experience with this protein (4), it was expected that orosomucoid would appear in ppt. IV. This fraction (120 mg) was, therefore, chromatographed on CM-cellulose as described previously (4, 5), and the breakthrough fraction (24 mg) was subjected to various analyses to ascertain its nature.

This component of ppt. IV moved like orosomucoid both in agar gel and moving boundary electrophoretic systems. Its carbohydrate composition was identical with that of orosomucoid, and in the Ouchterlony double diffusion, it gave a precipitin line with orosomucoid antiserum, which coalesced with that given by authentic orosomucoid. It was concluded that the protein isolated from ppt. IV was indeed serum orosomucoid.

ISOLATION OF TWO MILK-SPECIFIC COMPONENTS OF GLYCOPROTEIN NATURE FROM PPT. V

Ppt. V was dissolved in water and subjected to gel filtration on a column of Sephadex G-25 using water as the developing solvent. The results are given in Figure 2. The fraction indicated was rechromatographed in the same system, and was then subjected to gel filtration on Sephadex G-200 column using 0.1 M $NaHCO_3$ as the developing solvent. Two carbohydrate-rich fractions were separated, and termed A and B. Both were rechromatographed on Sephadex G-200 medium under the above conditions, dialyzed against distilled water, and freeze-dried.

Glycoprotein A was homogeneous in the ultracentrifuge by the method of agar gel electrophoresis and by moving boundary electrophoresis at pH 8.6. Its electrophoretic migration at that pH was identical with that of human serum orosomucoid. Glycopro-
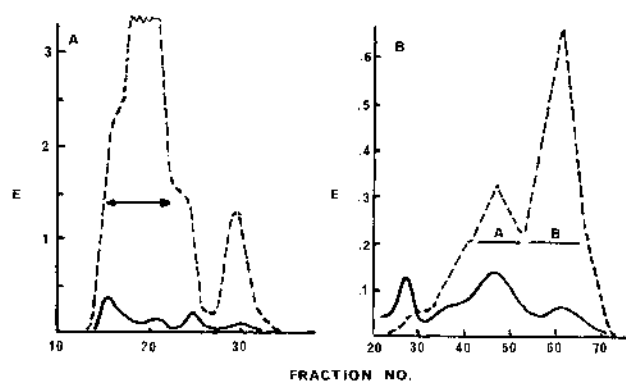
Fig. 2. Gel chromatography of the precipitate (ppt.) V fraction of human milk whey. Broken lines indicate the distribution of carbohydrate, whereas the continuous lines indicate absorption at 280 nm. *Frame A:* chromatography of ppt. V on Sephadex G-25 using water as irrigant. Amount applied was 225 mg, amount recovered in fraction indicated by arrows was 166 mg. After rechromatography under the same conditions, 150 mg were recovered. *Frame B:* chromatography of the fraction indicated in Frame *A* on a Sephadex G-200 column. Amount applied was 168 mg, amounts recovered in fractions (glycoproteins) A and B were 48.0 and 75 mg, respectively.

tein B also showed a single peak in the ultracentrifuge; however, upon agar gel electrophoresis, it showed the presence of at least two components migrating faster than human serum albumin, and upon prolonged moving boundary electrophoresis, three components were observed, whose migration was that of β-globulin, slow β-globulin, and γ-globulin.

The physical properties of the two components of ppt. V are summarized in Table 2, and their compositions are given in Table 3. It is seen that the physical parameters of glycoprotein A were very similar to those of a colostrum glycoprotein previously isolated (14); however, it did differ from the colostrum glycoprotein in its amino acid and carbohydrate composition.

The nature of the carbohydrate moieties of the two glycoproteins was further investigated by mild acid hydrolysis. After hydrolysis with 2 M HCl for 10 hr at 100°, the samples were placed on a "medium" column of the amino acid analyzer, and the glucosamine to galactosamine ratios were determined. These proved to be 8:1 for glycoprotein A and 50:1 for glycoprotein B.

The nature of the hexose was determined by first hydrolyzing the glycoproteins with 1 M HCl for 4 hr at 100° (2-mg sample in 1 ml acid) then subjecting the dried sample to paper chromatography in the butanol-pyridine-water (4:3:2) system. Glycoprotein A showed galactose only, whereas glycoprotein B showed galactose and

Table 2. *Physical properties of two glycoproteins isolated from human milk whey precipitate (ppt.) V*[1]

	Glycoprotein	
	A	B
Sedimentation constant ($s^0_{20,w}$)	2.6	0.95
Diffusion constant ($D^0_{20,w}$)	6.3	15.9
Partial specific volume ($\bar{V}$)	0.662	0.650
Molecular weight[2]	29,500	4,200

[1] Ultracentrifugation was done in a Spinco model E ultracentrifuge using synthetic boundary cells at 20°; the diffusion constant was determined in a Spinco model H apparatus at 2.7°. Solvent in both cases was 0.1 N NaHCO₃. The partial specific volume was calculated from composition, and molecular weight was calculated by the sedimentation-diffusion method.

[2] Apparent weight average molecular weight for glycoprotein B, as it is a mixture of three components.

Table 3. *Amino acid and carbohydrate composition of two glycoproteins isolated from human milk whey precipitate (ppt.) V*[1]

	Glycoprotein		
	A		B
Amino acid or monosaccharide	g/100 g	Residues/ molecule	g/100 g
Lysine	0.6	1	1.0
Histidine	0.6	1	0.9
Arginine	0.6	1	0.2
Aspartic acid	2.9	7	4.9
Threonine	4.0	12	0.8
Serine	2.3	8	3.7
Glutamic acid	2.5	6	4.5
Proline	4.0	12	0.8
Glycine	1.9	10	1.7
Alanine	2.1	9	1.7
Valine	2.6	8	1.0
Methionine	0.0		0.5
Isoleucine	0.9	2	0.7
Leucine	0.9	2	0.9
Tyrosine	0.6	1	0.3
Phenylalanine	0.6	1	0.3
N-Acetylneuraminic acid	5.5	6	6.7
Hexose	28.0	51	32.0
Methylpentose	8.1	16	11.0
Hexosamine[2]	28.5	42	26.5

[1] There was no half-cystine or tryptophan in either glycoprotein. Hydrolysis was performed in 5.8 M HCl at 110° for 24 hr in sealed tubes under N₂ atmosphere. Amino acid values were corrected by the method of Anderson *et al.* (1). Carbohydrates were determined as described in *Methods*. All analyses were done in triplicate and the results averaged.

[2] Glucosamine to galactosamine ratio for glycoprotein A was 8:1.

traces of glucose, but no mannose. Both glycoproteins showed the presence of fucose (Fig. 3).

Neither glycoprotein fraction A nor B gave a precipitin line with human serum orosomucoid antiserum.

BIOLOGIC PROPERTIES OF MILK WHEY GLYCOPROTEINS

Both glycoproteins A and B were tested for their ability to promote the growth of *L. bifidus* var. *Penn.* The results are given in Table 4. It is apparent that glycoprotein A gave a considerable bacterial growth-promoting activity, which was at the same level as those of the colostrum glycoproteins described previously (15). Glycoprotein B, on the other hand, have a very low *L. bifidus* growth-promoting activity that was, contrary to that glycoprotein A, increased upon desialization.

DISCUSSION

This paper indicates that mature human milk contains relatively large quantities of serum orosomucoid, as well as milk-specific glycoproteins with carbohydrate content much greater than that of either orosomucoid or cow's milk and colostrum glycoproteins. In many respects, glycoprotein A was very similar to the human colostrum glycoprotein previously isolated (14); both had molecular weights of near 30,000 and some 70% total carbohydrate, and both had *L. bifidus* growth-promoting activities. In addition, both had nearly identical number of hexose, fucose, and hexosamine residues per molecule of glycoprotein, although the milk protein had only half as much sialic acid, and its glucosamine to galactosamine ratio was 8:1 instead of 3:1 as in the colostrum material. There were differences in amino acid composition values, especially in regard to aspartic acid, threonine, and glycine.
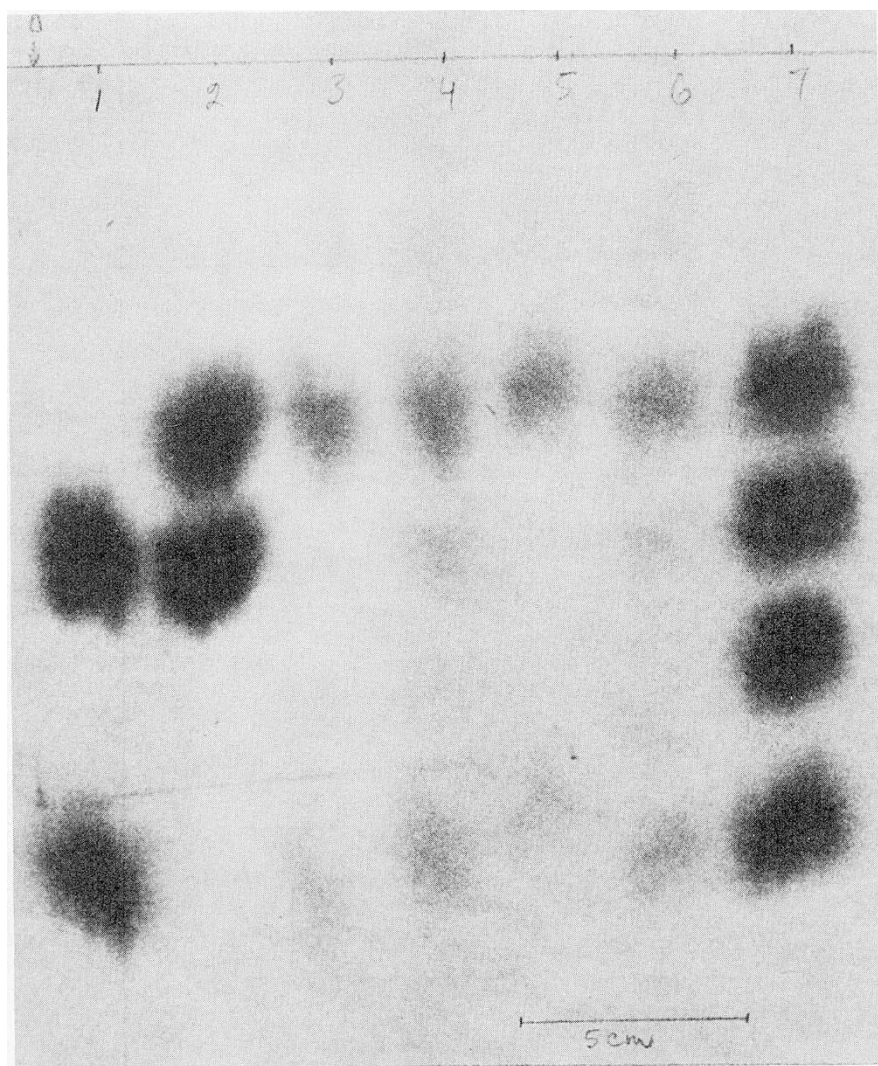
Fig. 3. Descending paper chromatographic analysis of monosaccharides from glycoproteins A and B. In descending order, *channel 1* had glucose and fucose; *2*, galactose and glucose; *3* and *5*, glycoprotein A; *4* and *6*, glycoprotein B; *7*, galactose, glucose, mannose, and fucose. Actual migration of the glucose spot was 25 cm in this experiment.

Table 4. *Lactobacillus bifidus* var. *Penn. growth-promoting activities of human whey glycoproteins A and B*[1]

Compound	Native material	Desialyzed material
Glycoprotein A	4.4	4.9
Glycoprotein B	0.7	1.2
Dialyzed human milk solids	0.3	

[1] The micro-organisms were grown in a synthetic medium as described in Nichols *et al.* (15) where extent of growth was determined by titration of acid produced. Extent of growth is expressed on the basis of the number of milliliters of 0.1 M NaOH used to back-titrate the acid produced by bacteria grown in 5 ml medium for 40 hr at 37° in a N_2-CO_2 atmosphere in the presence of 100 μg material tested.

Glycoprotein B was apparently a mixture of several small moleuclar weight components. Because of this heterogeneity, the determination of the "molecular weight" of the fraction is devoid of a precise meaning. However, these experiments were done and are reported herein simply to indicate that the components of glycoprotein fraction B were much smaller in size than was glycoprotein A. Because glucose was found in glycoprotein B (Fig. 3), it is not unreasonable to assume that one of the components of this fraction was the nondialyzable oligosaccharide studied by György *et al.* (10), which was a poor *L. bifidus* growth promoter in the native state, but acquired such growth-promoting activity upon being desialized. Glycoprotein B exhibited precisely the same behavior.

This paper then supports our previous suggestion (15) to the effect that human milk and colostrum contain nondialyzable *L. bifidus* growth-promoting factors of at least two types: those that stimulate the bacterial growth in their native state, and those that do so when the sialic acid is removed. The former are glycoproteins in nature, whereas the latter are oligosaccharides.

SUMMARY

Two homogeneous glycoproteins were isolated from mature human milk whey. One proved to be identical with human serum orosomucoid, the other was apparently specific to milk. The latter glycoprotein had a molecular weight of 29,500, and contained near 70% carbohydrate consisting of galactose, glucosamine, galactosamine, fucose, and sialic acid. It was also a potent promoter of the growth of *L. bifidus* var. *Penn.* Human milk whey was shown to contain additional glycoproteins, which were isolated as a mixture, and whose average molecular weight was near 4,000. This mixture of glycoproteins was a poor growth-promoting agent for *L. bifidus* var. *Penn.*, although its activity increased after desialization.

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Decanoic acid   placenta
fatty acids   protein binding
hexanoic acid

Transfer across Perfused Human Placenta. IV. Effect of Protein Binding on Free Fatty Acids

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Extract

The effect of protein binding on the rate of placental transfer of hexanoic (C 6) and decanoic (C 10) acids was investigated in an in vitro perfusion system of human placenta. As much as 30% of transferred C 6 was converted to more polar compounds, so that the observations related to the combined effects on transfer and metabolism. Less than 10% of C 10 was similarly metabolized. Both fatty acids are soluble in buffered salt solutions at the concentrations used (40 μM) and both are bound to serum albumin, C-10 having higher association constants (K' for C 6, 1.48 × 10^4; for C 10, 1.03 × 10^5). When the placenta is perfused with buffered salt solution, the transfer of C 6 is 22% more rapid than that of C 10. It is suggested that binding within the placenta retards C 10 more than C 6. The addition of 1 g/100 ml bovine serum albumin to the maternal perfusate reduces the transfer rate of C 10 by 80%, whereas 2 g/100 ml serum albumin has a more moderate effect on C 6 (a reduction of 50%). The addition of 1 g/100 ml serum albumin to the fetal perfusate increases transfer rate of both free fatty acids (FFA), C 6 by about 25% and C 10 by about 250%. With equivalent concentrations of serum albumin in maternal and fetal perfusates, the transfer rate of C 10 was reduced by 65%, whereas there was no detectable effect on transfer of C 6 in two of three experiments.

The transfer rates of FFA increase logarithmically with progressive shortening of the carbon chain from C 16 to C 8 when maternal and fetal perfusates contain serum albumin. Protein binding is apparently the determining factor. The rate of transfer falls off at C 6 and C 4, even though protein-binding continues to decrease. The determining factor may be the hydrophilic nature of these molecules.

Speculation

If the observations on FFA have general applicability, the overall effect of protein binding on placental transfer of a particular substance may relate to its water solubility and protein binding. If water solubility is not a limiting factor, protein binding may decrease transfer rate sharply, as occurs with decanoic acid (C 10). With substances that have very limited water solubility, protein