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Studies on serum lipoprotein in the neonatal period

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With 4 figures and 2 tables

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Introduction

The serum content of lipids in newborns is determined partly by the maternal-fetal transport (5, 6), and partly by the lipid synthesis by the newborn (30). Cholesterol seems to be formed in the newborn (30) but it may also pass the placental barrier (7, 40). On the other hand, the low content of unesterified fatty acids in serum of newborns and the high content of these acids in pregnant women supports the theory that free fatty acids cannot pass the placenta. This may be explained by the binding of fatty acids to albumin (2, 31). This theory is furthermore supported by data indicating that the human fetus covers its energy requirement by carbohydrate (41). Corresponding to this the newborn have low levels of serum lipoproteins (33) and cholesterol (23, 39). The determination of lipids and lipoproteins in the newborn period by means of the uptake of lipophilic dyes applied to serum on filter paper, has given contradicting results with regard to the time dependent changes in the serum lipoproteins (1, 12, 22, 33, 34). Furthermore, older paper electrophoretic methods (32, 33) do not make it possible clearly to seperate and identify the pre-ß-lipoprotein from the main fractions consisting of *a*-1- and *ß*-lipoprotein, as well as the chylomicrons. We have therefore found it relevant to elucidate the neonatal changes in serum lipoproteins in more detail, both by means of recent methods enabling an optimal separation of serum lipoproteins (15) and also by the recently developed quantitative radial immunodiffusion technique, which in the present paper has been used for quantitative estimates of *ß*-lipoprotein (10). This method seems more direct and accurate than the indirect assay based upon the determination of serum cholosterol (23, 39).

Materials and methods

Patient material

The sera from newborns and their mothers all originate from the neonatal and obstetric departments, Rigshospitalet, Copenhagen. The blood samples were taken 1–2 hours after the morning meal. At birth, blood was taken from the mothers by puncture of a cubital vein and from the newborn as cord blood. After birth the blood was taken from the infants as heel blood. The blood was centrifuged after coagulation and the serum samples were analysed within three hours.

Data from 18 children with a birth weight above 2500 g were compared with data from 7 children with birth weight below 2500 g. All children were healthy and thriving well, they

were fed mothers milk supplemented with „half skimmed milk" Eledon (E). The data of these two groups were finally compared with those from 3 children with birth weight above 2500g fed exclusively, (E).

Chemicals

Unless stated otherwise, the chemicals used were of the highest obtainable purity form British Drug Houses, Dorset, England.

Methods

Separation and semi-quantitative estimation of serum lipoproteins was made by electrophorezing the serum proteins in albumin containing agar-agarose-gel. (35) (0.26 g agarose + 0.30 g DIFCO-special Noble agar (DIFCO, Ltd., Chicago, USA) + 0.25 g bovine albumin (Beringwerke, Marburg an der Lahn, Germany) / 100 ml buffer). The agar and agarose were suspended in 0.05 M Na-barbital buffer (pH 8.6) and slowly heated in a water bath to 95°. After complete solubilization of the agar the mixture was cooled down to 45° and albumin was added during agitation. The homogeneous mixture was cast on microscope slides (1 × 7 cm), 1 ml per slide. In order to avoid migration of the proteins between the agar-gel and the glass surface, the slides were coated with aqueous agar prior to application of the electrophoretic gel. This was done by pouring a 95° warm aqueous agar solution (0.50 g DIFCO special noble agar / 100 ml distilled water) on the slides (about 1 ml per slide). After drying off the slides at room temperature (22°) the slides were coated with a thin, firmly attached layer of agar and they were now ready for casting the electrophoretic medium (vide supra). The electrophoretic slides were stored at 4° until used.

A systematic study of the optimal conditions for separation of serum lipoproteins in the electrophoretic equipment recommended by Wieme (45), which allows fast separation below light petrol ether, revealed that:

1. the light petrol ether did not disturb the electrophoretic separation and distribution of lipoproteins if the transverse through (1.5 cm × 1 mm) for the applied serum sample (20 µl) was sealed prior to electrophoresis with a 50° warm agar-buffer.
2. the maximal separation of alpha-, pre-beta, beta- and chylomicrons was obtained by application of the sample 1 cm from the cathodic edge, i. e. 2 mm from the cathodic agar block of WIEME'S apparatus (43).
3. the electrophoretic time was 32 min. at 7.5 volt per cm in a 1.0 mm thick agar layer.

After an electrophoretic separation for 32 min. at 28°, the slides were fixed for 5 min. in a mixture of 70 vols. ethanol + 25 vols. distilled water + 5 vols. glacial acetic acid. Hereafter, the slides were dried below filter paper at room temperature (22°). The lipoproteins were stained in a solution containing: 200 mg Sudan Black suspended in 100 ml 60% aqueous ethanol heated to 90°, cooled to 22° and filtered prior to use. (The staining solution was stable for one week). The background on the slides was finally destained (10 min.) in 50% aqueous ethanol.

The amount of staining material bound to the lipoprotein fractions was estimated by scanning the electrophoretic slides in a Vitatron scanning equipment consisting of photometer (UFD 100, Vitatron Ltd., Dieren, Holland), a scanning unit equipped with photomultiplier no 200/21, and slit-with 0.25 mm and finally recorder UR 100, equipped with an integration unit. The scanning was performed at 604 mm. By application of increasing dilutions of serum in 0.9% (w/v) NaCl and of increasing dilutions of purified beta-lipoprotein (vide infra), it was possible to demonstrate a linear relationship between peak-area and the reciprocal value of the dilution. However, this linear relationship showed a different slope for the different lipoprotein fractions (cf. fig. 1.) and furthermore, the chylomicron curve showed a typical assymptotic feature at higher concentrations of this fraction. The percentage distribution of the lipoprotein patterns visualized below, therefore give only approximate relative percentages but they can, in agreement with fig. 1. be corrected for difference in staining capability.

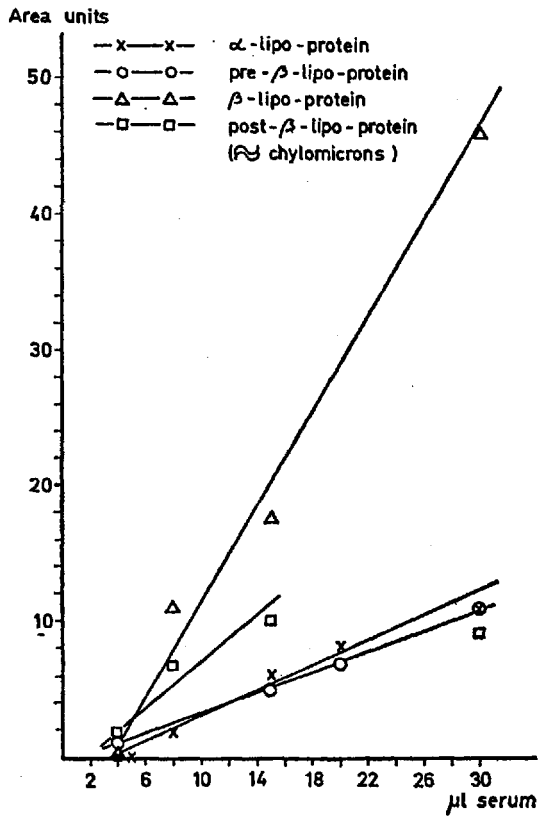

Fig. 1. The uptake of the lipid stain, Sudan Black, to the serum lipoproteins: alpha-, pre-beta, and beta-lipoprotein (as well as to the post beta- fraction, identical with the chylomicrons).

The Lipoproteins were electrophoretically separated in albumin containing agar-agarose gel (cf. the text) and, after fixation and drying of the electrophoretic slide, stained with Sudan Black (cf. the text). The slides were scanned at 604 nm in a Vitatron scanning apparatus. *Ordinate:* Peak area (arbitary units). *Abscissa:* µl normal human serum applied in the gel prior to electrophoresis.

It is seen from the figure that approximately a straight line relationship was obtained between the amount of lipoprotein present in the gel and the stain uptake (eveluated from the peak area). Only the chylomicrons show a more assymptotic feature of the stain binding curve at a higher amount of this fraction applied in the gel.

Quantitative immuno-chemical estimation of beta-lipoprotein was performed by means of the quantitative radial immuno-diffusion (29) under the experimental conditions described by CLAUSEN (10). A standard solution of immuno-chemical pure beta-lipoprotein was prepared from 100 ml pooled normal human serum by precipitation with dextran sulphate in the presence of $CaCl_2$ (8). The precipitate, mainly consisting of beta-lipoprotein, was dissolved in 3.02% (w/v) Na-citrate and finally dialyzed against 0.15 M Na-phosphate buffer (pH 6.5). The lipoprotein was re-precipitated, dialyzed once more and finally purified by filtration on Sephadex G-200. 10 ml dialyzed fraction (about 0.5 g protein/100 ml) was applied on a Sephadex G-200 column (40 × 2 cm). The column was made by heating the Sephadex G-200 powder for 20 min., to 90° in 0.15 M Na-phosphate buffer (pH 6.5) and then cooled

to room temperature (room temperature, 22°). During the elution procedure the buffer remained 10 cm above the Sephadex layer. With the LKB fraction collector (LKB Ltd., Stockholm, Sweden) type 3000 Ultro Rac, equipped with a 10 ml Siphon stand, the beta-lipoprotein was collected in the first 40 ml. The total protein content on every step of the isolation procedure was estimated by means of the LOWRY method (27) (cf. 26).

The purity of the beta-lipoprotein was controlled by micro-immuno-electrophoresis of 1.5 µl 5% (w/v) lipoprotein solution using either 100 µl polyvalent horse antiserum against normal human serum or 100 µl mono-specific anti-beta-lipoprotein from rabbits. The experimental conditions used were those given by CLAUSEN (10). The first mentioned antiserum was obtained from the central laboratory of the Dutch Red Cross, Amsterdam (batch no. PHO13 P10). The last mentioned anti-serum was made in the laboratory by protracted immunization of rabbits with 250 µl 5% (w/v) lipoprotein solution (10). The antiserum obtained by immunization for three months, was mono-specific against beta-lipoprotein when tested against normal human serum in micro-immuno-electrophoresis.

Initial studies revealed a linear relationship between the amount of beta-lipoprotein applied (1.5 µl solution) in the radial immunodiffusion plate and the diameter of the circular diffusion area (cf. fig. 2). The recovery was estimated to 95% when purified beta-lipoprotein was added to normal human serum.

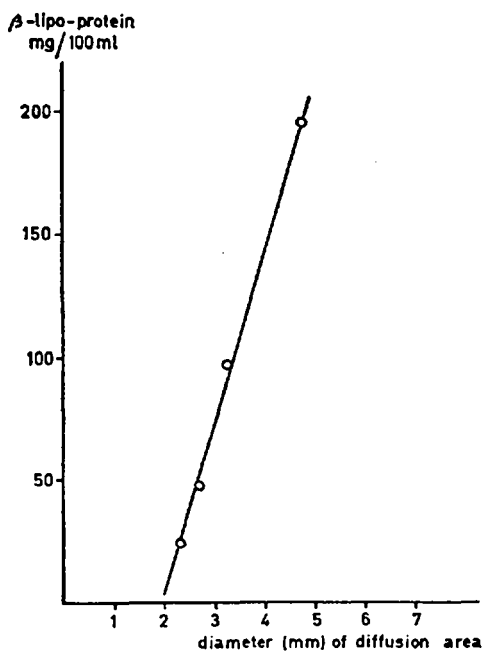

Fig. 2. The standard curve for the quantitative assay of serum beta-lipoprotein by means of the quantitative radial immuno-diffusion. *Ordinate:* diameter (mm) of the diffusion area after two days immuno-diffusion. *Abscissa:* concentration of beta-lipoprotein applied in the immuno-diffusion plate (sample volumne 1.5 µl).

Statistical evaluation

of data consisted of determination of the standard deviation (SD) and calculation of the regression curve for the relationship between the data estimated at different ages and the

Table 1. Concentration of β-lipoprotein in serum of newborns. Relationship to age and diet.
The data indicated in the table are obtained by regression analysis (cf. the text) of the function relating the serum content of beta-lipoprotein at different times after birth as function of age (days). The significance of the slope of the regression curve is indicated.

	Number of points on regression curve.	Initial concentration at birth.	Daily change	P-level of significance
Birth weight > 2500 g Diet: Mothers milk supplemented with (E). N = 18	31	141.3 mg%	20.6 mg%/day	0.1%
Birth weight ≦ 2500 g Diet: Mothers milk supplemented with (E). N = 7	15	95.2 mg%	7.3 mg%/day	0.1%
Birth weight > 2500 g Diet: E* N = 3	14	169.9 mg%	20.9 mg%/day	not significant

*) E∼ Eledon (Half skimmed milk). (cf. Z. Ernährungswissenschaft 1969: 9: 353).

corresponding age. Also, the normal distribution of the data was controlled by estimation of the degree of „Skewness" (11). The statistical evaluation was made on Olivetti's computor: Programma no. 101, using the programs no. 1.50, 1.52 and 3.40 made by WILLIAMS (40).

Results

Table 1 demonstrates on a basis of the statistical evaluation, a significant increase in β-lipoprotein of serum with increasing age, apart from children fed (E). The highest daily increase of β-lipoprotein was shown in the group of children fed mothers milk supplemented with (E) and this value was found to be lowest in the group with a birth weight below 2500 g. In the group fact (E) the increase of *β*-lipoprotein was not significant.

Fig. 3 demonstrates the relationship between age and the mean values of β-lipoprotein in the fullborn infants fed mothers milk supplemented with (E), the mean value of serum *β*-lipoprotein increased in the days after birth. However, prior to the seventh day of life, the mean did not reach the mean values of the mothers at birth (435 ± 219 mg/100 ml) (cf. fig. 3).

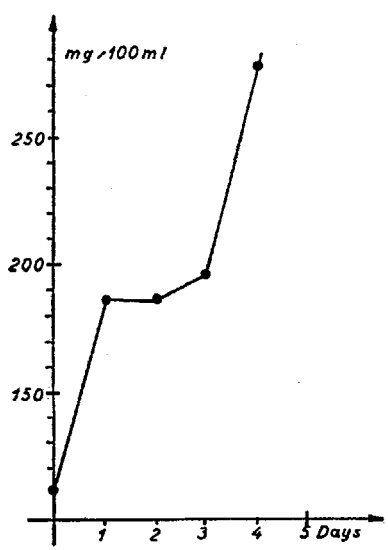

Fig. 3. Mean concentration of beta-lipoprotein in cord serum and serum from infants at different times after birth. *Ordinate:* Mean values. *Abscissa:* Age in days.

A semi-quantitative electrophoretic estimation of the serum lipoprotein pattern revealed great individual differences. Fig. 4 shows individual results of the changes in the lipoprotein pattern after birth. Apart from case No 190669 (Fig. 4) a lipoprotein fraction with mobility as pre-*β*-lipoprotein was the predominating fraction at birth, relatively this fraction decreased during the following days. In the same period the *α*- and *β*-lipoproteins, as well as the chylomicrons, increased thus supporting the data obtained by the radial immuno-diffusion, mentioned above.

Table 2. The relative distribution of serum lipoproteins and changes after birth in a group of infant fed mothers milk supplemented with (E).

		α-l-lipoprotein	pre-β-lipoprotein	β-lipoprotein	chylomicrons
Mother's serum at birth. N = 16	Mean: %	19.1 ± 7.1	12.3 ± 6.0	39.1 ± 14.2	29.5 ± 15.7%
	Range:	0.0 — 27.6	0.0 — 56.4	12.0 — 60.7	7.5 — 65.0
Cord Serum N = 8	Mean: %	< 1.0	75.8 ± 22.8	22.0 ± 22.0	1.0%
	Range:		38.9 — 100.0	0.0 — 61.2	0.0 — 8.1
Infant serum one day after birth N = 9	Mean: %	< 1.0	55.7 ± 30.1	44.3 ± 30.1	< 1.0%
	Range:		13.6 — 100.0	0.0 — 63.5	
Infant serum two days after birth N = 4	Mean: %	< 1.0	55.0 ± 31.3	41.2 ± 26.8	3.9 ± 0.7%
	Range:		23.1 — 86.0	14.0 — 66.7	0.0 — 15.4
Infant serum three days after birth N = 6	Mean: %	3.3 ± 0.7	32.2 ± 17.1	61.3 ± 16.2	3.8 ± 0.6%
	Range:	0.0 — 20.0	15.0 — 59.7	40.3 — 85.0	0.0 — 16.7
Infant serum five to six days after birth N = 6	Mean: %	2.7 ± 1.0	33.8 ± 23.3	57.1 ± 22.1	3.3 ± 0.8%
	Range:	0.0 — 16.2	18.8 — 79.3	20.7 — 79.4	0.0 — 20.0

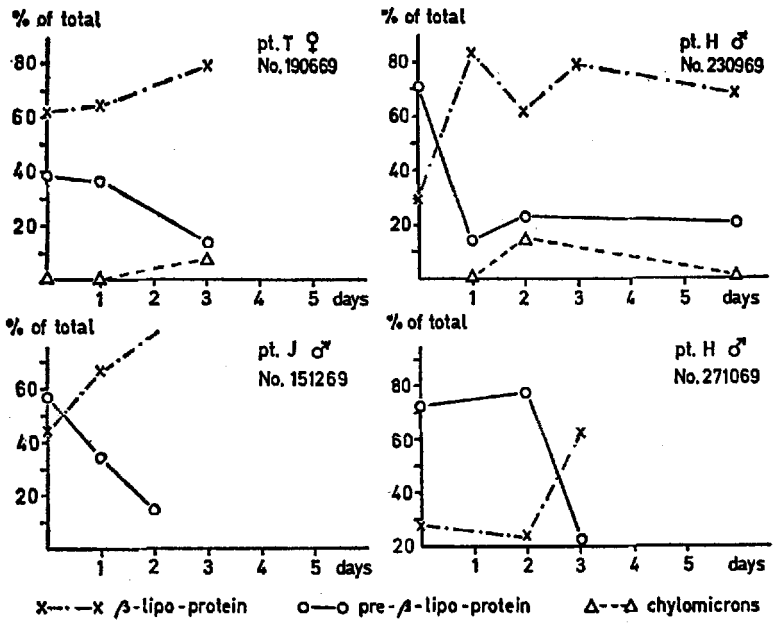

Fig. 4. Shows the relative distribution of serum lipoproteins in blood from newborns during the first week of life. The lipoproteins were separated by means of electrophoresis in albumin containing agar-agarose gel, fixed and stained with Sudan Black (cf. the text). Distribution was estimated by scanning. *Abscissa:* Time in days. *Ordinate:* Percentage of lipoprotein content.

Table 2 shows a comparison between the mean values of the relative distribution percentages of the four lipoprotein fractions under study. The data of the mothers at birth are compared to those of children fed mothers milk supplemented with (E) at different times after birth. It is obvious from table 2 that although great standard deviations occur, the pre-β-lipoprotein fraction is relatively the predominant fraction and that relatively, this fraction decreases after birth. This relative decrease is associated with a relative increase in the three other fractions, but mainly in the β-lipoprotein. Even on the sixth day of life, the α-1- and the chylomicron fractions are relatively seven and nine times lower, respectively, than the corresponding fractions of the mothers at birth. As the total serum lipid increases after birth (34), it may be tempting to suggest, that all serum lipoproteins increases absolutely, and therefore also the pre-beta-lipoprotein in spite of a relative decrease.

Discussion

As mentioned in the introduction, the neonatal period is associated with pronounced changes in serum proteins and lipids. The present demonstration of preponderance of a lipoprotein fraction with mobility as the pre-β-lipoprotein at birth corresponds to studies, showing that the fetus may have its main part of energy demand covered by carbohydrate (41) since this lipoprotein fraction is rich in triglycerides, the level of which is influenced by the supply of carbohydrates (15). As β-lipoprotein

does not pass the placental barrier (17) the β-lipoprotein found in the newborn must have been synthesized in the fetal or newborn organism.

As cholesterol and its esters are mainly transported in the β-lipoprotein fraction, the increase in serum β-lipoprotein during the first weeks of life, found in the present communication, thus corresponds closely to SEARCY's demonstration (39) of an age dependent increase in serum cholesterol in the neonatal period. The rising lipoprotein level after birth is presumably correlated to the increasing amount of fat which the infant recieves when feeding has been initiated, particulary in those given human milk. (16). This may be supported by the data of the present communication showing the highest daily increase in β-lipoprotein in children fed mothers milk. This theory is also supported by studies of kwashiorkor (12, 38, 40), showing that this protein deficiency syndrome is associated with hyperlipidemia, which is eliminated by adequate protein supply. On the other hand, the low level of β-lipoprotein at birth may also be linked with the high level of oestrogens at birth, because it is known that oestrogen medicamentation decreases serum lipids and β-lipoprotein (24, 37).

With regard to the question as to which concentration of serum cholesterol and β-lipoprotein is the optimal one, from a nutritional point of view, no definite answer can be given. Thus recent studies of newborns (29) indicate that atheromatic streaks may form in the neonatal period. The atheromatogenous deposits seem partly to be irreversible accumulations (4) and as our recent studies (20) indicate, it is possible to increase serum linoleic acid by high intake of this acid, and as this acid decreases serum level of cholesterol (14,25), it would seem reasonable from these points of view, to enrich the diet of newborns and infants with linoleic acid. The presence of linoleic acid in the diet of infants also seems imperative, as this acid is a necessary precursor for the synthesis of long-chained unsaturated fatty acids, which are deposited in biomembranes for instance in the central nervous system (18, 19). However, this essential acid is also necessary for the formation of hormones, belonging to the group of prostataglandins. (cf. the review by BERGSTRØM (3)). On the other hand, an enrichment of the diet with linoleic acid cannot be made without proper regard to the vitamin-E content of the diet. In conditions with relative Vitamin-E deficiency the polyunsaturated fatty acids undergo peroxidative decomposition (6, 42), which in the neonatal period may may give rise to weakening of the plasma membrane of the erythrocytes and thereby to hemolysis (6, 36). In a following work, we intend to elucidate these facts in further detail and to discuss whether newborns in Europe, as is the case in USA (6, 36), are in danger of a deficiency in vitamin-E and/or essential fatty acids, or whether abnormalities in these components are more related to physiological than dietary factors. (CLAUSEN & FRIIS-HANSEN to be published).

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Zusammenfassung

Durch eine quantitative radiale Immunodiffusion und Elektrophorese in albuminhaltigem Agar-Agarose-Gel wurden die Verschiebungen in den Serum-Lipoproteinen in der Fetalperiode der Neugeborenen verfolgt. Bei der Geburt konnte ein Übergewicht (relativ) von einer Fraktion mit Mobilität wie Prä-Beta-Lipoprotein und ein niedriges Alpha- und Beta-

Lipoprotein nachgewiesen werden. Letztes stieg in der ersten Woche nach der Geburt fast linear mit dem Alter in Tagen, erreichte aber nicht den Serum-Lipoproteinwert der Mutter.

Beim Vergleich der Konzentration von Serum-Beta-Lipoprotein bei Neugeborenen mit Geburtsgewicht und Kost, konnte der niedrigste tägliche Zuwachs bei Neugeborenen mit Geburtsgewicht unter 2500 g gefunden werden. Bei Kindern (Geburtsgewicht über 2500 g) mit Muttermilch unter Zugabe von „half skimmed milk" (E) ernährt, ergab sich der höchste tägliche Zuwachs von Beta-Lipoprotein.

Die Ergebnisse wurden auf Grundlage der vorliegenden Literatur über die Physiologie der Lipid Metabolisme der Geburtsperiode besprochen.

Summary

The daily changes in the distribution and serum level of lipoproteins in newborns were elucidated by means of quantitative radial immuno-diffusion and electrophoresis in an agar-agarose gel, containing albumin.

At birth, a relative preponderance of a lipoprotein fraction with mobility as pre-beta-lipoprotein was found associated with low values of the alpha- and beta-lipoproteins. The beta-lipoprotein fraction increased approximately linearly with age after birth during the first weeks of life. However, in that period, the adult values were not met.

The lowest cord serum β-lipoprotein values were found in infants with a birth weight below 2500 g.

By a correlation of the serum beta-lipoprotein values in newborns to diet and birth weight, the lowest daily increase in the beta-lipoprotein was found in newborns with a birth weight below 2500 g. On the other hand, the babies fed mothers milk supplemented with half-skimmed milk, E, (birth weight above 2500 g) revealed higher daily gain in serum concentration of beta-lipoprotein.

The results are discussed on the basis of the literature available on the physiology of the lipid metabolism of the neonatal period.

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