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Chromatographic Investigation of the β -carotene Content of Serum of the Newborn Infant

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Several workers have shown that the carotenoid level of the blood from the umbilical cord of newborn infants is considerably lower than that of the mother (Menken, 1934; Gaechtens, 1937; Willstaedt & With, 1939; Byrn & Eastman, 1943; Hoch, 1943 a; Lewis, Bodansky & Shapiro, 1943). On the other hand, the last three of these investigators found that the vitamin A levels are of the same order in both. However, since the serum carotenoids are a mixture of similar substances, it seemed of interest to investigate the concentration of the biologically active β -carotene in the serum of mother and baby.

EXPERIMENTAL

Collection of blood samples. Venous blood was taken from healthy women repeatedly before and after parturition. At parturition, blood was collected from the cord and from the vagina and the samples left to clot in the dark. In a few instances venous blood was oxalated and the plasma used. Contamination of vaginal samples by other fluids was avoided as far as possible; diluted samples were, however, included in this set of analyses, since no serious error was to be expected in the relative proportions of carotenoids. This error and also that due to possible hydraemia may affect the values for the concentration of total carotenoids. In several instances the protein concentration was determined to obtain an estimate of the dilution of the blood.

Method of extraction of carotenoids from serum. 1 ml. of maternal and 1–4 ml. of foetal serum were used. 1.00 ml. of serum (or plasma) was placed in a 15 ml. narrow-necked centrifuge tube and 0.15 ml. of 29% NaOH, 0.5 ml. ethanol and a few drops of light petroleum (boiling range 40–60°) were added in succession with thorough mixing after each addition. About 3 ml. light petroleum and 0.65 ml. ethanol were then added and the mixture shaken up immediately to avoid precipitation of protein, which occurs if an equal volume of ethanol is added directly to serum. Cork stoppers wetted with distilled water were used and the mixtures were shaken by hand for 5 min. The extraction was repeated twice with about 1.5 ml. light petroleum. The pooled extracts were washed three times with distilled water, transferred

to a dry centrifuge tube and, after addition of 0.3–0.4 g. anhydrous Na_2SO_4 , evaporated to dryness in a stream of N_2 (bath temperature 50–55°). The extract was then dissolved in *n*-hexane and transferred quantitatively to a colorimeter tube, made up to exactly 1 ml. and centrifuged (2600 r.p.m.) until completely clear.

Determination of carotenoid content of extract. The extinction of the hexane solution was measured photoelectrically, with an Ilford Spectrum violet filter no. 601. The values of the extinction were obtained by subtracting the $\log_{10}$ (galvanometer reading for the unknown) from the $\log_{10}$ (galvanometer reading for the control (*n*-hexane)). The light intensity was adjusted so as to give a reading for the control between 95 and 105 mm. on the scale. The calibration was carried out with recrystallized chromatographically homogeneous β -carotene. Solutions of this were made up in *n*-hexane. An extinction of 0.1 corresponded to 0.62 μg . β -carotene. Correction was made for the non-linearity of the galvanometer. The deviation from proportionality of concentration and extinction was found to be less than 1% in the range normally used (extinction < 0.45) and greater than 2% only with extinctions above 0.8.

A photoelectric colorimeter was built on the principle of the Evelyn instrument (Evelyn, 1936) and its essential details are given here in so far as they differ from those of the Evelyn model. The light source was a 6 V. 6 c.p. car side-light bulb fed from a car accumulator (60 amp. hr.). The light intensity was varied by means of a series stud resistance. Each step altered the galvanometer readings by about 5%. The maximum current taken was 0.57 amp. No reflector was used. The colorimeter tubes were matched ordinary centrifuge tubes 90 $\times$ 9.5 mm. with an internal diameter of 8 mm. The light beam was limited by a stop to illuminate an area of 10 $\times$ 2 mm. on the colorimeter tube. The distance from the filament to the centre of the tube was 30 mm. A Calorex glass (infra-red filter) of 1.5 mm. thickness (manufactured by Chance Bros. Ltd.) was mounted close to the bulb and the light filters could be placed immediately in front of the photoelectric cell. The latter was an 'EEL' selenium barrier layer cell (manufactured by Evans Electro-selenium Ltd.). The galvanometer used (sensitivity 840 mm./ μa ., 2 sec. periodic time and 175 Ω coil resistance) is a standard type S.S. 6/45 of H. Tinsley and Co.

Chromatography of serum extracts. Very uniform adsorption columns of diameters up to 13 mm. were made up as follows. A 5–10 cm. length of glass tubing was drawn to a short point so as to retain the adsorbent while allowing free passage of hexane. It was filled first with a few grains of coarse $\text{Al}(\text{OH})_3$ and then with Al_2O_3 (standardized according to Brockmann, manufactured by Savory and Moore Ltd.). Hexane was introduced to about half the height of the tube allowing the hexane to wet the Al_2O_3 . A rubber tubing was attached to the upper end of the tube and with the lower end immersed in hexane slight suction was applied and the whole of Al_2O_3 lifted 2–3 cm. When the tube was tapped the Al_2O_3 settled down and the air still contained in it collected into a small bubble which escaped through the layer of Al_2O_3 and hexane.

After estimation of the total carotenoids the serum extract was evaporated to dryness, taken up in a small volume of *n*-hexane (approx. 0.03 ml.) and transferred quantitatively with *n*-hexane to an absorption column, 16–18 mm. long and 2.2 mm. in diameter. For extracts of serum from cord blood a smaller column (1.4 $\times$ 12 mm.) was used. On elution with hexane or 0.5–1% acetone in hexane, usually five coloured bands were formed in the following order (from the bottom upwards): (1) orange-yellow, mainly β -carotene (with lipoids), identified spectroscopically and by mixed chromatogram; (2) brown-orange, usually narrow and faint (in two extracts comparatively large amounts of this pigment were present and absorption

maxima in *n*-hexane were observed (visually)* at 470 and 500 $m\mu$; this pigment was possibly identical with neolycopene A (Zechmeister, LeRosen, Schroeder, Polgár & Pauling, 1943)); (3) rose-red, mainly lycopene; (4) yellow, mainly pigments (with lipoids) with absorption maxima similar to those of α -carotene at 474 $m\mu$ and of β -carotene at 452 $m\mu$; after saponification of these pigments both maxima appeared at positions corresponding to α -carotene. A similarly anomalous position of the 447 $m\mu$ maximum of 'leaf xanthophyll' in the presence of 0.6% butter can be seen in a graph published by Miller (1937). The explanation which is given there for the anomalous extinctions, i.e. scattering of light, is not applicable to the present case, where the solutions were optically clear; (5) yellow, mainly lutein (with lipoids). In some cases a rose-red band of the same colour as lycopene was also observed closely below the band (2). Separation of (4) and (5) into more bands was obtained by development with increasing concentrations of acetone, and, with larger quantities of extract, traces of pigments other than those mentioned were observed. Chromatography of serum carotenoids has been carried out by a number of authors (Dániel & Scheff, 1935; Süllmann, Szécsényi-Nagy & Verzá, 1936; Dániel & Béres, 1936; Willstaedt & Lindqvist, 1936; Van Veen & Lanzing,

* A small direct vision grating spectroscope, manufactured by R. and J. Beck, Ltd., London, was used for the determination of absorption maxima.

Table 1. *Changes in the extinction of carotenoids produced by saponification*

(a) *Saponification of the serum extracts by boiling with 60% KOH in methanol or with NaOH-H₂O-ethanol*

Method of saponification	Extinction		Duration of heating (sec.)	Extinction of β -carotene fraction of		% recoveries of total carotenoids after chromatography of	
	Before saponification	After saponification		Material not saponified	Saponified material	Material not saponified	Saponified material
KOH-methanol	0.245	0.173	20	—	—	—	—
	0.212	0.179	30	—	—	—	—
	0.202	0.149	30	0.076	0.067	97	84
	0.167	0.160	< 30	0.061	0.053	100	93
	0.164	0.140	30	—	—	—	—
	0.172	0.148	30	0.033	0.040	83	86
	0.177	0.132	< 30	—	—	—	—
	0.200	0.158	< 30	—	—	—	—
NaOH-ethanol	0.169	0.139	180	—	—	—	—
	0.143	0.122	180	0.041	0.043	96	100
	0.218	0.148	180	0.051	0.038	95	92

(b) *Saponification of the fractions of serum extracts obtained by chromatography*

Fraction	Method of saponification	Extinction		Duration of heating (sec.)
		Before saponification	After saponification	
1. β -Carotene	KOH-methanol	0.093	0.0915	20
		0.089	0.085	20
Remaining xanthophylls, etc.		0.165	0.116	20
		0.142	0.105	20
2. β -Carotene'	NaOH-ethanol	0.171	0.164	—
		0.192	0.203	—
		0.076	0.076	30
Remaining xanthophylls, etc.		0.131	0.111	120
3. β -Carotene, pure, recrystallized	NaOH-ethanol	0.302	0.302	30

1937; Lanzing, 1938; Willstaedt & With, 1938; Thompson, 1943) and reference to unknown carotenoids (corresponding to positions between bands (1) and (4)) has been made by several of them. A pigment with absorption maxima similar to those of band (2) mentioned above has been described by Willstaedt & Lindqvist (1936).

In this investigation no attempt has been made to separate the small amounts of carotenoids other than β -carotene which accompany the lipoids of the first fraction. The first coloured band was eluted with *n*-hexane and it is designated here as β -carotene. The remainder of the pigments was eluted by adding a small amount of absolute ethanol to the hexane still left on top of the column. Low recoveries may be due to incomplete elution of pigments other than β -carotene and to bleaching of all fractions, including β -carotene. I have, therefore, calculated the extinction which is contributed by fraction (1) as a percentage of the total extinction found both before and after chromatography.

Saponification of extracts. Chromatography of extracts from serum prepared without saponification may be complicated by the presence of esters of xanthophylls and by lipoids which act as eluting agents. The lipoids may also affect the extinction measurements. Chromatography is therefore usually carried out on saponified material (Zechmeister & Chohnoky, 1943). The two main reasons for the choice in this investigation of a technique employing extracts which have not been saponified are (a) the losses in total carotenoids observed under conditions of hot saponification (Table 1) and (b) the possibility of the production of artefacts.

The loss of carotenoids is mainly due to losses of lutein and carotenoids other than β -carotene. Losses of carotenoids on saponification have been previously observed by Clausen & McCoord (1936).

Recrystallized chromatographically homogeneous β -carotene showed no losses in extinction after heat treatment with alkali, but it then contained small amounts of pigment which form a yellow band below the β -carotene (probably this is neo- β -carotene B (Zechmeister & Polgár, 1942)) and some yellow pigment adsorbed above β -carotene. This pigment had a general absorption in the blue and violet from about 500 $m\mu$. No pronounced maxima could be observed visually. A pigment similar in appearance and spectrum was also found in very small amounts in the unsaponified extract of one serum.

The saponification of the β -carotene (plus lipid) fraction of serum extracts led to the formation of a sharp red-orange band above the β -carotene. This effect was also obtained with nearly lipid-free, chromatographically homogeneous β -carotene isolated from serum.

The known xanthophyll esters complicate the chromatogram on the Al_2O_3 column only at positions above the lycopene (Zechmeister & Chohnoky, 1943) and therefore would not be expected to interfere with the separation of β -carotene.

In order to estimate the influence of the lipoids on the extinction measurements, the β -carotene fraction was again chromatographed on an activated Al_2O_3 type H 100/200 (manufactured by Peter Spence and Sons, Ltd.), which is a very strong adsorbent, and the bulk of the lipoids were separated. The β -carotene which appeared orange-yellow when first adsorbed, later changed to pink and after elution with acetone-hexane had the same absorption maxima as crystallized β -carotene. When the lipid fraction was again united with this material the 478 $m\mu$ maximum was shifted

Table 2. *Comparison of the photoelectric and visual (micro-) estimation of carotenoids*

	(μ g. carotenoid, as β -carotene.)		Ratio: visual photoelectric
	Photo- electric	Visual	
Total carotenoids	0.162	0.162	1.00
	0.493	0.490	0.99
	0.083	0.097	1.17
	0.205	0.222	1.08
	0.188	0.198	1.05
	0.244	0.226	0.93
	0.324	0.331	1.02
	0.470	0.518	1.10
	0.580	0.566	0.98
Fraction (1), β -carotene	0.050	0.052	1.04
Fractions (2)–(5)	0.262	0.256	0.98
Fraction (1), β -carotene	0.127	0.12	0.95
Fractions (2)–(5)	0.787	0.76	0.96
		Average	1.02

towards the longer wave-lengths and the solution appeared slightly more orange than pure β -carotene. The effect of lipoids on the extinction in a sample containing 1.2 μ g. β -carotene in 1 ml. *n*-hexane as measured with the Spectrum violet filter was, however, small and well within the experimental error ($\pm 0.5\%$). It seems fair to assume that the error in the estimation of β -carotene caused by the presence

Table 3. *Carotenoids, β -carotene and total proteins in venous serum at different times before and after parturition, and in serum of blood from the vagina at parturition*

Subject	Date	Total protein (g./ 100 ml.)	Total caro- tenoids (μ g./ 100 ml.)	% β -carotene of total	
				After chroma- to- graphy	Before chroma- to- graphy
A.W.	4. ii. 44	6.71	142	33.5	31.0
	13. iii. 44	6.66	186	40.5	37.5
	27. iv. 44	6.56	196	37.0	36.5
	7. v. 44	6.06	163	39.5	38.5
	(parturition)				
	9. v. 44	6.01	154	38.5	35.5
	26. v. 44	6.83	138	39.5	37.0
H.S.*	10. xi. 43	—	123	30.0	34.0
	1. xii. 43	—	100	31.0	27.5
	15. xii. 43	—	121	24.5	22.0
	29. xii. 43	—	144	35.5	35.5
	18. ii. 44	—	175	33.5	31.5
	23. iii. 44	—	199	25.5	25.5
	18. iv. 44	6.48	278	28.5	27.5
	21. iv. 44	6.43	249	29.5	28.5
	(parturition)				
	27. iv. 44	—	163	34.0	32.0
D.S.	4. v. 44	7.44	184	32.5	31.5
	30. xii. 43	6.77	91	40.0	35.5
	9. xii. 43	6.75	134	42.0	39.5
	23. iii. 44	6.23	131	34.0	31.0
	3. v. 44	—	138	37.5	36.0
	(parturition)				
	5. v. 44	6.15	122	38.5	37.5

* First six samples taken by finger prick and analyzed by the visual method using 0.157 ml. serum.

of serum lipoids will be negligible provided the wave band used is not too narrow and measurements are made at a sufficient dilution.

Microtechnique for determination of serum carotenoids. Total carotenoids from cord blood serum were determined both photoelectrically and visually (Hoch, 1943b), while the chromatographic fractions were measured visually since the concentrations were below the limit of the photoelectric method. In two cases it was possible to measure these fractions also photoelectrically (see Table 2) and the proportions of β -carotene found by the two methods were 16 and 17% in one, and 14 and 13.5% in the other case. A serious drawback of the visual micro-estimation of serum carotenoids in extracts prepared without saponification—besides the low accuracy inherent in a visual method which employs artificial standards—is the difference in colour of the β -carotene fraction due to the lipoids present (see above), which is pronounced if the lipoids form an appreciable portion of the total volume (<0.04 ml.) used. Table 2 shows the results on samples estimated by both photoelectric and visual method.

RESULTS

Table 3 shows the levels of carotenoids, β -carotene and proteins in maternal serum at different times before, at and after parturition.

The variation in the proportion of β -carotene near term was small and did not exceed 20% (see also Table 5).

Tables 4–6 show the relation of the total carotenoids, β -carotene, lycopene and pigment (2) in serum of mother and infant at parturition.

The concentrations of total carotenoids found in cord serum are in agreement with earlier work (Menken, 1934; Gaetgens, 1937; Willstaedt & With, 1939; Byrn & Eastman, 1943; Lewis, Bodansky & Shapiro, 1943; Hoch, 1943a).

The proportion of β -carotene in total carotenoids was markedly lower in the foetal than in maternal

Table 4. *Total carotenoid content of maternal and foetal serum*

(The figures in brackets refer to venous blood 2–4 days after parturition.)
Carotenoid content expressed as β -carotene ($\mu\text{g./100 ml. serum}$).

	M.H.	R.	J.F.	E.S.*	F.L.	K.J.	H.S.	D.S.	A.W.
Mother	62 (92)	80 (92)	53 (158)	21 (116)	107 (89)	230 (183)	249 (163)	138 (128)	160 (154)
Infant	13	18	18	8	9	24	29	26	17
Ratio $\frac{\text{infant}}{\text{mother}}$	0.21	0.23	0.34	0.38	0.08	0.10	0.12	0.19	0.11

* Blood sample much diluted.

Table 5. *Proportions of β -carotene in the total carotenoids of maternal and foetal serum*

(a) % β -carotene, of total carotenoids recovered after chromatography. (b) % β -carotene, of total carotenoids measured before chromatography.

	M.H.		R.		J.F.		E.S.		F.L.	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Mother:										
At parturition	35	32 (91)	20.5	15 (72)?	39.5	37 (93.5)	42.5	44.5 (104)	45	43 (95)
2–4 days after parturition	40.5	39.5 (97)	21	20 (95)	38	35.5 (93.5)	52	51.5 (99)	47	44.5 (95)
Infant at parturition	20	19.5 (98)	13.5	12.5 (93)	22	17.5 (80)	30.5	28.5 (93)	24	23.5 (98)
Ratio $\frac{\text{infant}}{\text{mother}}$	0.57		0.66		0.56		0.72		0.53	
	K.J.		H.S.		D.S.		A.W.			
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)		
Mother:										
At parturition	34.5	33 (95)	29.5	28.5 (97)	38	36.5 (96)	39.5	38.5 (98)		
2–4 days after parturition	36	34.5 (96)	34	32 (94)	39	38 (97)	38.5	35.5 (92)		
Infant at parturition	20	16.5 (84)	16	14.5 (91)	14	12.5 (89)	16	15 (94)		
Ratio $\frac{\text{infant}}{\text{mother}}$	0.58		0.54		0.37		0.40			

(The figures in brackets give the recoveries of total carotenoids after chromatography.)

Table 6. *Amounts of brown-orange pigment (2) and lycopene (3) in maternal and foetal serum*

	M.H.		R.		J.F.		E.S.		F.L.		K.J.		D.S.		A.W.	
	(2)	(3)	(2)	(3)	(2)	(3)	(2)	(3)	(2)	(3)	(2)	(3)	(2)	(3)	(2)	(3)
Mother	+	Trace	+	+++	0	++	+	0	0	+	+++	++	++	++	+	+
Infant	+	0	0	++	0	++	?	0	0	0	++	+	0	Trace	0	0

serum in all cases investigated. The ranges were 13.5–30.5 % for foetal and 20.5–42.5 % for maternal serum at parturition. The ratio

$$\frac{\% \beta\text{-carotene in total carotenoids (infant)}}{\% \beta\text{-carotene in total carotenoids (mother)}}$$

was between 0.53 and 0.72 in seven, and 0.40 and 0.37 in two cases. Pigment (2) and lycopene, when present in maternal serum, appeared also in foetal serum in a number of instances. In four instances pigment (2), and in three instances lycopene, was not found in foetal serum, although contained in maternal serum.

DISCUSSION

The difference between the concentrations of total carotenoids of maternal and foetal serum may be explained by assuming a barrier in the placenta, since it is improbable that there is a mechanism in the foetus sufficiently effective to remove the carotenoids from the circulation at the rate at which they would enter it if there were no such barrier.

The difference in the proportions of β -carotene in the carotenoids of mother and infant can be interpreted as the resultant of two functions: (a) the permeability of the placenta and (b) the rate of elimination from the foetal circulation. These are probably different for each individual carotenoid. It is remarkable that the ratio

$$\frac{\% \beta\text{-carotene in total carotenoids (infant)}}{\% \beta\text{-carotene in total carotenoids (mother)}}$$

varies only within fairly narrow limits. One may suppose that a constant ratio would not be brought about by placentae of different permeabilities and that more probably the foetal organism would be mainly responsible for the smaller proportion of

β -carotene in total carotenoids of cord blood serum, e.g. by converting it into vitamin A, or storing it, more quickly than the xanthophylls are being disposed of.

SUMMARY

1. The total carotenoids and the proportion of β -carotene in the total carotenoids were determined in maternal and foetal serum at parturition. The total carotenoids in foetal serum ranged from 8 to 29 $\mu\text{g./100 ml.}$ The proportion of β -carotene in the total carotenoids was markedly lower in the infant's serum in all cases. The ratio

$$\frac{\% \beta\text{-carotene in total carotenoids (infant)}}{\% \beta\text{-carotene in total carotenoids (mother)}}$$

was 0.53–0.72 in seven, and 0.40 and 0.37 in two cases.

2. There was no significant variation of the percentage β -carotene of total carotenoids in the maternal serum during the time near term.

3. A micro chromatographic technique for the separation of the carotenoids of serum is described.

4. The use of extracts without saponification for the determination of serum carotenoids is advocated.

5. Details of construction of an instrument similar to the Evelyn photoelectric colorimeter are given.

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