Conversion of *all trans* β-carotene to retinal by an enzyme from the intestinal mucosa of human neonates

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The enzymatic conversion of all trans *β-carotene to retinal by an intestinal mucosal enzyme (β-carotene cleavage enzyme, BCC) from autopsy samples of human neonates was demonstrated for the first time. The enzymatic product was characterized as its O-ethyl oxime, which, on high pressure liquid chromatography (HPLC), yielded a sharp peak corresponding to an authentic retinal (O-ethyl) oxime. The enzyme blank and boiled enzyme blank failed to show any significant HPLC peaks corresponding to retinal (O-ethyl) oxime, retinal, or retinol. Based on the observed activities among intestinal samples from 14 premature infants, the BCC enzyme activity ranged from 3.3–1210 pmoles per mg mucosal protein per hr. Studies on the stability of the enzyme using the rat as the experimental animal revealed that as much as 80% of the original activity of the fresh intestine is lost in storage of the dead animal for 8 hr at 25° C followed by storage at 4° C for 16 hr. More importantly, 70% of the fresh enzyme activity is lost after storage of the animals at 4° C for only 8 hr. Thus, the observed activities in the human autopsy samples appear to be markedly underestimated because of the marked loss of enzyme activity from the time of death to the time of assay. Therefore, the true activity of the enzyme can be assessed only after the extent of loss of activity on storage of the human samples can be accurately measured. In spite of repeated attempts, no detectable BCC activity was found in the placentas of pre-term or term infants. Nonetheless, the demonstration of BCC enzyme activity in the intestinal mucosa of human neonates shows that β-carotene can serve as an important source of vitamin A in newborn infants.* (J. Nutr. Biochem. 4:659–663; 1993.)

Introduction

Carotenoids are the sole biological precursors of retinoids. β-carotene is the major precursor of this vitamin. Dietary retinoids in humans are derived primarily from the intake of carotenoids. β-carotene cleavage (BCC) enzyme catalyzes the conversion of *all-trans*-β-carotene into two moles of retinal.[1–3] In addition to β-carotene, several other carotenoids and apocarotenoids are cleaved to yield retinal,[4] and a rough correlation exists between the rate of retinal formation and the biological activity of a given carotenoid.[5] We previously showed that this enzyme is distributed in a variety of herbivorous and carnivorous species, with a notable exception of the cat in which BCC enzyme was virtually absent.[6] Generally it is more abundant in herbivores than in carnivores. More recent studies[7] have shown the existence of carotenoid cleavage enzyme activity where the site of attack on the carotene molecule was other than the central 15,15′-ethylenic bond, resulting in the formation of various β-carotenals. Nonetheless, it appears that the BCC enzyme is a major, if not the sole, enzyme responsible for the conversion of carotenoids to retinoids. Thus, BCC enzyme plays an important role in vitamin A nutrition by serving as a major provider of vitamin A in individuals who are dependent exclusively on dietary carotenoids for their vitamin A requirements.

Recent findings indicate that the serum concentration of β-carotene in the cord blood of term and pre-term infants are one-eighth the concentration in the maternal

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serum.[8] Breast feeding replenishes the plasma β-carotene levels of the infant to normal within 4–6 days because of the high β-carotene content of the colostrum.[8] Furthermore, low-birth-weight infants have lower plasma vitamin A levels than full-term infants.[9–11] Whereas the medium vitamin A concentration of healthy adults in the USA is around 100 μg/g,[12] newborn infants have only 20 μg/g.[13,14] Thus, part of the vitamin A requirements of neonates could be met by the conversion of β-carotene to vitamin A, provided this enzyme is present in the neonatal tissues.

The possibility therefore exists that this key enzyme plays an important role in the vitamin A nutrition of the fetus and the newborn. BCC enzyme is known to be distributed in the intestine, liver, and corpus luteum; the activity being highest in the intestine. Therefore, we have attempted to examine the occurrence of BCC enzyme activity in the autopsy samples of the intestines from neonates and premature infants and in their respective placentas to gain biochemical evidence for the ability of neonates to convert β-carotene to vitamin A.

Methods and materials

Neonatal tissues

After informed consent had been given and surgical pathologic or gross post mortem examination completed, intestinal samples of 14 premature infants who died in the Intensive Care Nursery (ICN) at Pennsylvania Hospital, Philadelphia were collected. Placental samples from seven of these pre-term infants and an additional eight term and nine premature deliveries were also obtained. The proximal portion of the small intestine and the respective placentas were removed and stored at −70° C until assayed for BCC enzyme activity.

Isolation of BCC enzyme

The procedure was essentially according to our earlier method.[15] Briefly, each intestinal segment was washed with 0.154 M ice-cold saline. All subsequent procedures were carried out at 4° C unless otherwise stated. The intestine was slit open longitudinally and the mucosa was scraped into a beaker containing the homogenizing buffer (0.1 M potassium phosphate buffer, pH 7.8, containing 1 mM DTT). The mucosa was homogenized with 5 volumes of the homogenizing buffer and the homogenate was centrifuged at 100,000*g* for 1 hr. The supernatant solution was subjected to 0–60% ammonium sulfate saturation and centrifuged at 16,000*g* for 15 min. The pellet was dissolved in the homogenizing buffer to give a final protein concentration of ~10 mg/mL and stabilized by adding 1 mM reduced glutathione (GSH). The procedure followed for the isolation of the enzyme from the placentas was identical to that of the intestine. Each enzyme fraction was assayed for β-carotene cleavage activity as described below. The protein in various fractions was determined according to Lowry et al.[16]

Enzyme assay

The standard assay was made up as follows: 100 nM β-carotene in 0.1 mL benzene was mixed with 180 μL of 1/10 diluted Tween 20 in water, and the benzene was removed by a gentle stream of nitrogen. To this substrate were added the following components at the indicated final concentrations: potassium phosphate buffer, pH 7.8, 100 mM; GSH, 1 mM; ferrous sulfate, 1 mM; nicotinamide, 15 mM; and the intestinal enzyme fraction, ~7 mg; the final volume was always made up to 2 mL with water. Blank tubes had either no enzyme preparation or an equivalent amount of boiled enzyme fraction (boiled at 100° C for 5 min). After incubation at 37° C for 60 min in a shaking water bath (50 excursions/min) under F40 Gold fluorescent light, the reaction was stopped by adding 2 mL methanol. The *O*-ethyl oxime derivative of the enzymatic product was prepared essentially according to Van Kuijk et al.[17] Briefly 100 μL 0.1 M *O*-ethylhydroxylamine hydrochloride in 0.1 M potassium phosphate buffer, pH 6.5 and 100 μL methanol containing cholesterol (50 μg/mL) were added to the incubation mixture. After 10 min at 25° C, 6 mL water was added, and the whole reaction mixture was thoroughly extracted with 3 × 10 mL portions of light petroleum. The lipid extracts were combined, evaporated to near dryness under a gentle stream of nitrogen, and the residue was finally redissolved in 1 mL methanol.

High pressure liquid chromatography (HPLC)

All HPLC analyses were carried out using a Gilson HPLC automated system equipped with a Kratos Model 783 variable wavelength detector (ABI Analytical Kratos Div., NJ). A 50 μL aliquot of the final methanol extract was subjected to HPLC on a reverse phase 4.6 × 10 cm ODS column (particle size, 3 μm) with the solvent system of 97% methanol:3% water containing 0.5% ammonium acetate as the mobile phase at a flow rate of 1 mL/min. Under these conditions, authentic retinal (*O*-ethyl) oxime distinctly separated from β-carotene with retention times of 4.2 and 27.5 min, respectively. The recovery of added retinal as its *O*-ethyl oxime was 95 ± 3% (n = 4) under these conditions.

Results and discussion

Fourteen premature infants, 11 of whom died soon after birth and three at ages 11 days, 60 days, and 120 days, were studied. Post-conceptual age at the time of death ranged from 22–37 weeks, and the time between death and autopsy ranged from 1–52 hr. *Figure 1* shows the HPLC profile of the enzymatic product from one of the neonatal intestinal enzyme incubations. Unlike the extract from the boiled enzyme incubation (*Figure 1*, top), the extract from enzyme incubation (*Figure 1*, bottom) showed the characteristic retinal-*O*-ethyloxime peak with a retention time of 4.2 min. This clearly shows that the BCC enzyme does exist in the neonatal intestine, and that its product of action on β-carotene is retinal. However, no measurable activity could be detected in any of the human placentas.

The results of the measured intestinal BCC enzyme activity in the autopsy samples of 14 neonates are presented in *Table 1*. The BCC enzyme activity was present in all but two specimens. One of them was from an infant (K-W) born at 31 weeks gestation who died at age 15 days with very severe respiratory distress syndrome and early bronchopulmonary dysplasia (chronic lung disease). The intestinal sample from this infant had leathery texture and looked more like colon than small intestine, raising the question of sampling error. The other intestinal specimen showing no BCC activity was from the second of twins (D-st-B). Death occurred at

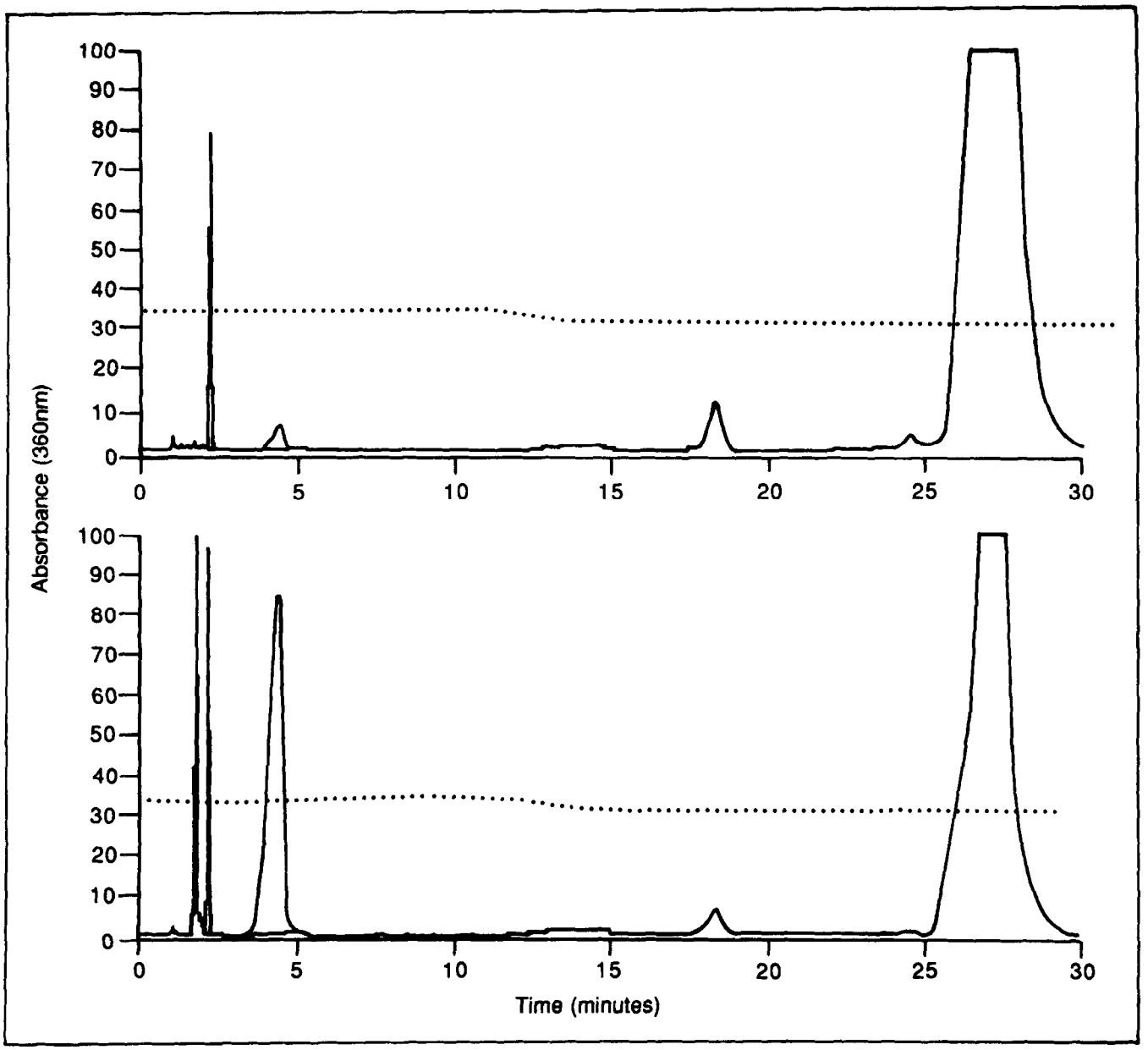

Figure 1 HPLC profile of the lipid extract from the standard assay mixture from a human neonatal intestinal BCC enzyme: top panel: boiled enzyme; bottom panel: native enzyme. See text for details.

postnatal age of 4 hours in Twin A (D-st-A) and at age 6 hours in Twin B (D-st-B). Bacteroides sepsis was present in the mother, and both fetuses and placentas showed severe chorioamnionitis and funisitis. Twin B's intestinal sample had very little mucosa, perhaps indicating autolytic loss. Remarkably, the quantity of intestinal mucosa in Twin A was abundant, and it exhibited a BCC activity of 62 pmoles retinal/mg/hr.

In the remaining 12 samples, the BCC enzyme activity ranged from 3.3–1210 pmoles/mg/hr. Except for one isolated sample, all others showed relatively low activities compared with the reported rabbit intestinal activity of 1000 pmoles/mg/hr[1] and an activity of 350 pmoles/mg/hr in a fresh intestinal biopsy sample of an adult intestine taken at surgery *(Table 1)*. In this regard, it must be emphasized that the time lag between the time of death and the time of storage of the intestinal tissues at −70° C, after the completion of the post mortem exam, varied from 1–52 hours, with 1–4 of these hours being at room temperature in the ICN. This time in the ICN was necessary to help parents in the grieving process.

All placentas were promptly refrigerated after delivery and surgical pathological examination, if done, was completed within 24 hr. Thereafter, all placental samples were stored at −70° C until assayed. In spite of repeated attempts, we could not detect any measurable BCC activity in the placentas of pre-term or term infants. Similarly, no BCC activity could be detected in any of the eight term placentas.

Table 1 β-carotene cleavage (BCC) enzyme activity in human neonates

Subject	BCC enzyme activity (pmoles retinal mg^{-1} hr^{-1})	Gestational age at death (weeks)	Postnatal age at death (days)	Hours post mortem at autopsy (hours)	Cause of death & post mortem findings
A-C	3.3	22	3	6	Immaturity, severe chorioamnionitis and funisitis
B-P	13.0	23	33	52	Immaturity, chronic placental abruption with hypoxia, respiratory distress
C-st-A	133.0	23	4	40	Immaturity, hypoplastic lungs, chronic lung disease
D-st-B	0.0	23	6	40	Immaturity, severe chorioamnionitis, bacteroides sepsis
E-So-A	55.0	24	18	16	Immaturity, severe chorioamnionitis, respiratory distress syndrome, intraventricular hemorrhage
F-st-B	62.0	24	9	40	Immaturity, severe chorioamnionitis, respiratory distress syndrome, intraventricular hemorrhage
G-G	8.1	27	9	4	Immaturity, severe respiratory distress syndrome, severe respiratory failure
H-Cu	100.0	28	4	3	Immaturity, respiratory distress syndrome, bronchopneumonia
I-Sh	1210.0	28	3	4	Immaturity, severe respiratory distress syndrome, birth asphyxia
J-K	45.0	29	16	25	Immaturity, hypoplastic lungs, chronic lung disease
K-W	0.0	31	15	17	Immaturity, severe respiratory distress, early bronchopulmonar dysplasia
L-M	42.0	31	60	6	Immaturity, chronic lung disease, severe respiratory distress
M-I	22.0	35	24	45	Immaturity, severe birth asphyxia, multiple congenital anomalies
N-Mc	33.0	37	4	17	Immaturity, severe respiratory distress syndrome, chronic lung disease
Adult	350.0				Biopsy specimen taken at surgery

To verify whether the BCC activity was influenced by the storage conditions of the intestinal tissue, its activity was measured in rats as a function of time after death and the temperature at which the carcass was stored. The results presented in *Table 2* show that as much as 80% of the original activity of the fresh intestine is lost on storage of the dead animal for 8 hr at 25° C followed by storage at 4° C for 16 hr. These are the general prevailing conditions normally in most hospitals for humans from the time of death to their storage in the morgue. More importantly, 70% of the fresh enzyme activity is lost after storage of the animals at 4° C for only 8 hr. Such a marked loss of the enzyme activity due to storage conditions *(Table 2)* indicates that the observed BCC enzyme activities in the neonatal autopsy samples *(Table 1)* may have been significantly underestimated compared with the enzyme activity in vivo. Therefore, the true activity of the enzyme can be assessed only after the extent of the loss of its activity in storage of the human samples can be accurately measured.

Table 2 Effect of storage under different conditions upon BCC enzyme activity in the rat

Animal storage conditions	Enzyme activity (pmoles retinal mg^{-1} hr^{-1})
fresh	102.0
8 hr at 4° C	29.6
8 hr at 25° C	26.7
8 hr at 25° C and 16 hr at 4° C	19.6
24 hr at 4° C	23.2

Each value is the average of independent determinations from two animals under each condition.

In summary, the results of the present study demonstrate the presence of BCC enzyme activity in the intestinal mucosa of human fetus as early as 22 weeks gestation. β-carotene and other carotenoids are present in newborn infant plasma, though at a much lower concentration than in the mother's blood.[8] In breast-fed infants serum levels of carotenoids increase rapidly within 1 week to normal adult levels because of the high concentration of the carotenes in the colostrum and early breast milk.[8,18,19] Therefore, the demonstration of BCC enzyme activity in human fetal intestinal samples suggests that β-carotene may be an important source of vitamin A nutrition during gestation and early postnatal period.

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