Ontogeny of two vitamin A-metabolizing enzymes and two retinol-binding proteins present in the small intestine of the rat

David E. Ong,[1] Peter C. Lucas, Bharati Kakkad, and Timothy C. Quick[2]

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232

Abstract The patterns of expression of cellular retinol-binding protein (CRBP), cellular retinol-binding protein, type two [CRBP(II)], lecithin: retinol acyltransferase (LRAT), and microsomal retinal reductase were examined for rat small intestine during the perinatal period. CRBP was present (15 pmole per mg soluble protein) at the earliest time examined, the 16th day of gestation, declining by 70% by birth, maintained to adulthood. In contrast, CRBP(II) appeared 2–3 days before birth, rising to its highest level (500 pmole per mg soluble protein) by day 3 after birth, then declining by 50% during the late suckling period to the adult level. Immunohistochemistry revealed that CRBP(II) initially appeared in the epithelial cell layer in a patchy manner, resolving by birth into an even staining of all villus-associated enterocytes. In contrast, CRBP was evenly expressed in the epithelial cell layer at day 17/18 but was absent by birth. Intestinal LRAT activity increased rapidly in the 2 days prior to birth, then declined at weaning to the adult level. Microsomal retinal reductase was measurable in the intestine at birth, but not detected during the early suckling period, reappearing at day 21. Considerable increase was then observed coincident with weaning, when carotenes, from which retinal is derived, became an important source of vitamin A. The pattern of appearance of these elements appears to prepare the intestine for the necessary processing of vitamin A required after birth. —**Ong, D. E., P. C. Lucas, B. Kakkad, and T. C. Quick.** Ontogeny of two vitamin A-metabolizing enzymes and two retinol-binding proteins present in the small intestine of the rat. *J. Lipid Res.* 1991. **32:** 1521–1527.

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Depending on the dietary form, the absorption of vitamin A requires several metabolic steps (reviewed in reference 1). Provitamin A carotenoids, such as β-carotene, enter the enterocyte where they may be oxidatively cleaved to retinal. The retinal is reduced to retinol which is then esterified with fatty acids. These esters are incorporated into the chylomicrons for export from the gut. Ingested retinyl esters are hydrolyzed in the gut lumen and the liberated retinol is taken up by the enterocyte where it is re-esterified and also incorporated into the chylomicrons.

Research over the last several years has brought forward several candidates to be part of the biochemical machinery necessary for these transformations. The intestine has been shown to contain two retinol-binding proteins, cellular retinol-binding protein (CRBP) and cellular retinol-binding protein, type two [CRBP(II)] (2). CRBP(II) has approximately equal affinity for all-*trans*-retinal and all-*trans*-retinol, while CRBP binds all-*trans*-retinol preferentially (3). CRBP(II) is an abundant protein of the small intestine, comprising approximately 1% of the soluble protein in mucosal extracts, but CRBP is less than 0.005% (2). In addition, immunohistochemistry has revealed that CRBP(II) is restricted to the villus-associated enterocytes while CRBP is found in nonabsorptive cells (4). This distribution and binding specificity of CRBP(II) led to the suggestion that it may act as a substrate carrier for the enzymes responsible for the reduction and esterification of vitamin A that occurs during absorption. In support, all-*trans*-retinal bound to CRBP(II) was found to be reduced to retinol by a novel retinal reductase present in microsomes from the small intestinal mucosa (5). Interestingly, retinal-CRBP(II) was largely restricted from reduction by a second distinct reductase, present in the cytosol, previously suggested as the candidate enzyme for this step because of its ability to reduce unbound retinal (6). The product of the microsomal reductase, retinol-CRBP(II), was found to be esterified in an acyl CoA-independent manner by a novel microsomal enzyme (7). This enzyme, now called lecithin:retinol acyltransferase (LRAT), transfers the fatty acid from position one of phosphatidylcholine to retinol (8). The other candidate enzyme for this esterification

Abbreviations: CRBP, cellular retinol-binding protein; CRBP(II), cellular retinol-binding protein, type two; LRAT, lecithin:retinol acyltransferase; HPLC, high performance liquid chromatography.

[1]To whom correspondence should be addressed.

[2]Current address: Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118.

step, acyl CoA-retinol acyltransferase (9), was ineffective in vitro in esterifying retinol bound to CRBP(II), compared to its activity for free retinol (7). Studies with cells in culture have suggested that LRAT may be the predominant activity for retinol esterification in those cells, under the conditions examined (10, 11). The high levels of CRBP(II) in the absorptive cell may ensure that the great majority of all-*trans*-retinal and retinol would be bound to this carrier protein and restricted to metabolism by the microsomal retinol reductase and LRAT, respectively.

Here we have examined the ontogeny of these two enzymes and of CRBP and CRBP(II) in the small intestine of rat. The patterns observed are consistent with the proposed roles for CRBP(II), microsomal retinal reductase, and LRAT in the absorption of vitamin A.

EXPERIMENTAL PROCEDURES

Animals

Timed pregnant Sprague-Dawley rats were obtained from Sasco (St. Louis, MO). Animals were fed a standard rat chow diet that contained 15 IU of vitamin A/g and 4.5 ppm carotene.

Preparation of tissue samples

Separate collections were made for the determination of binding proteins and for the measurement of enzyme activities. Animals were killed at the appropriate time by decapitation. The whole small intestine was removed as rapidly as possible and placed on ice. In some cases samples were frozen at −70°C for later preparation. Cytosol preparations for determination of CRBP and CRBP(II) by radioimmunoassay were made as previously described (12). Microsomal fractions were prepared as previously described (5).

Samples for immunohistochemistry were prepared by immersing decapitated fetuses in PerFix (Fisher) for about 30 min. The partially fixed torsos were then carefully cut sagittally at the midline with a razor blade to produce two equal pieces, which were placed in PerFix again for 1 h. Samples were then dehydrated in ethanol and embedded in paraffin prior to sectioning.

Immunocytochemical localization of CRBP and CRBP(II)

Localization with polyclonal antibodies to rat CRBP and CRBP(II) was accomplished as previously described (4) with the exception that Triton X-100 was omitted from solutions used for localization of CRBP. The above reference (4) described the specificity of the respective immune reagents and the control reactions employed. The ABC method of Hsu, Raine, and Fanger (13) was used on 5-μm saggital sections of the fetal torso.

Assay of LRAT and microsomal retinal reductase

LRAT was assayed by HPLC analysis of esters formed, as previously described, with the exception that bovine serum albumin was not included in the assay (7). Retinal reductase was assayed as previously described, without change (5).

RESULTS

Ontogeny of CRBP and CRBP(II) in the small intestine

Sensitive, specific radioimmunoassays were used to determine the levels of both CRBP and CRBP(II) in extracts of soluble protein from the whole small intestine collected during the perinatal period. The highest level of CRBP, 15 pmol/mg protein, was observed at the earliest time examined, day 16 of gestation (**Fig. 1**). The level quickly declined to about 5 pmol/mg protein by birth and stayed at this level through 35 days of age. Similar levels were observed in adult animals.

In contrast, no CRBP(II) was detected in the small intestine at day 16 of gestation (Fig. 1). By gestational day 19, CRBP(II) was detectable at about 20 pmol/mg protein, beginning an abrupt rise that peaked at about 500 pmol/mg protein several days after birth. Levels then declined, reaching about 200 pmol/mg protein by day 24 after birth, similar to levels observed in older animals. The patterns of CRBP and CRBP(II) expression observed here essentially mirror the levels of mRNA for the two proteins, reported earlier (14).

Because the gut of the rat undergoes considerable morphological change during the last 5–6 days of gestation, we were interested in the cellular distributions of the two retinol-binding proteins during this critical time. Whole

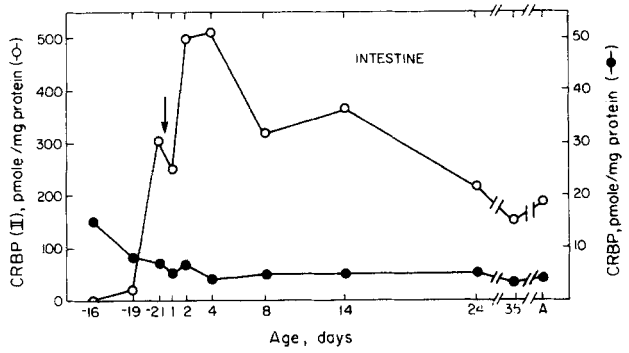

Fig. 1. Ontogeny of CRBP and CRBP(II) in rat small intestine. Amounts of CRBP (●) and CRBP(II) (○) in the soluble protein of whole small intestine were determined by radioimmunoassay. Note the tenfold difference in scale for the two proteins. Values shown are the averages ± SEM for three separate determinations for a single pooled sample at each time point. Birth is indicated by the arrow. Minus numbers refer to gestational age.

body sections of fetus were examined at different gestational ages. The earliest time examined was day 17/18 of gestation. A typical cross-section of small intestine at this stage is shown in **Fig. 2A**. The villi were not yet well formed and secondary lacunae were seen frequently, in addition to the central lumen. The outer layers of smooth muscle were thicker and less defined than observed at later times. In spite of the low abundance of CRBP(II) at this time, staining was readily apparent, distributed in a very patchy fashion in cells in the villus-associated epithe-

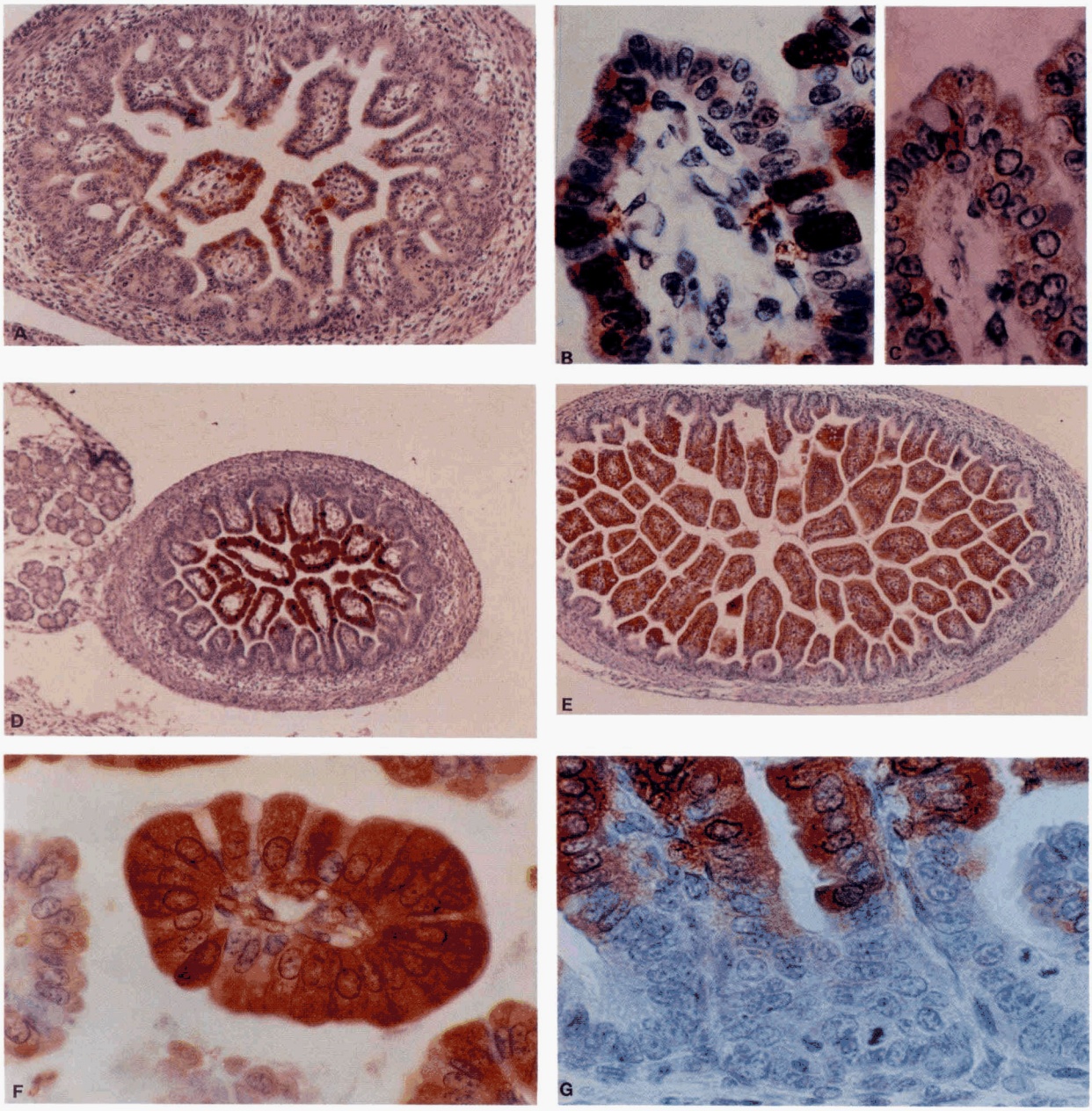

Fig. 2. Immunolocalization of CRBP(II) and CRBP in the fetal small intestine at late gestation. Sagittal sections (5 μm) were prepared from fixed 17–21 day fetuses and stained by the ABC method (13) with either anti-CRBP(II) serum or affinity-purified anti-CRBP IgG (panel C only). Presence of immunoreactive protein is indicated by the brown oxidation product of diaminobenzidine. Sections were lightly stained with hematoxylin to reveal cellular detail. Panel A: Cross-section of proximal small intestine from 17/18 day fetus stained for CRBP(II) (mag 350 ×). Panel B: Higher power view of portion of section shown in panel A (previously published in ref. 16; reproduced by permission of authors). Note the variation in staining intensity of neighboring cells. No brush-border was yet evident (mag 1700 ×). Panel C: Similar section of 17/18 day fetus stained for CRBP. A lighter but more homogenous staining of the epithelial cell layer was noted (mag 1700 ×). Panel D: Section of proximal small intestine of an 18/19 day fetus stained for CRBP(II). Increased staining of a greater number of epithelial cells is apparent compared to day 17/18 (panel A). (mag 170 ×). Panel E: Section of proximal small intestine at day 20/21 (mag 170 ×). Panel F: Coronal section of an individual villus (day 20/21) showing lack of staining of goblet cells. A well-developed brush-border can be seen (mag 1700 ×). Panel G: Higher power view of the base of several villi (day 20/21) showing lack of staining for CRBP(II) (mag 1700 ×).

lial cell layer, predominately toward the center of the lumen. A higher-power view of one emerging villus from this section is shown in Fig. 2B. As can be noted, the appearance of CRBP(II) in the developing enterocyte cell layer was obviously not uniform. Neighboring cells differed markedly in the amount of staining observed. No obvious brush-border was observed, even for cells expressing CRBP(II). In contrast, the staining for CRBP, although considerably fainter reflecting its modest abundance, was apparently homogenous in this cell layer at this time (Fig. 2C). By birth, staining for CRBP was no longer detected in the absorptive cells (results not shown), similar to what we have previously observed for the small intestine of the adult rat (4).

One day later (day 18/19 of gestation), the amount of staining for CRBP(II) had increased considerably (Fig. 2D), as would be expected from the higher levels observed by radioimmunoassay (Fig. 1). Many more cells of the epithelial cell layer now showed staining and, although still somewhat patchy, the pattern was clearly restricted to the villus-associated cells in the central lumen. At this stage, secondary lacunae were rare. By day 20/21 of gestation (Fig. 2E), staining for CRBP(II) was quite homogenous. A close-up view of an individual villus in a coronal section (Fig. 2F) clearly showed that staining was restricted to the enterocyte or absorptive cell; the several goblet cells visible in this view showed little or no staining for CRBP(II). At this stage a well-developed brush-border was clearly evident. In the adult rat, immunostaining indicates CRBP(II) is not present in the differentiating absorptive cells until they migrate from the crypts to the villi (4). True crypts do not develop until after birth, but it was clear that the epithelial cell layer at the villus base (proto-crypt area) was not staining for CRBP(II) (Figs. 2E and 2G). Cell division is now largely restricted to this area and several apparent mitotic figures might be noted in Fig. 2G.

Ontogeny of intestinal LRAT activity

Microsomes obtained from small intestines collected from animals killed during the perinatal period were examined for LRAT activity. The pattern observed is shown in **Fig. 3**. A low level of activity was present at gestational age day 19, but a considerable increase occurred by birth. A further increase of measured activity took place during suckling, peaking at 8–12 days. A steady decline was then noted with adult levels being reached by day 28. For this collection, pups had been weaned at day 21. A completely separate collection of small intestine was made at similar time periods and a similar pattern was observed, including the dip in activity at day 3 (data not shown).

Interestingly, the pattern of esters synthesized by LRAT in vitro was considerably different for microsomes collected during the suckling period compared to the pattern observed for microsomes from fetal intestine or from the weaned animal, as shown in **Fig. 4**. The pattern produced by microsomes of suckling animals was essentially the same at all time points examined, as suggested by the comparison shown for days 1 and 21 (compare tracings B and C). Similarly, little difference was seen between esters synthesized in vitro by LRAT in microsomes from the fetal intestine or from the weaned animal (compare tracings A and D). LRAT will use endogenous phosphatidylcholine as the acyl donor and no exogenous phosphatidylcholine was present for these determinations. The relative proportion of each retinyl ester synthesized in the in vitro assay is shown in **Table 1**. As noted, the pattern for the entire suckling period remained essentially the same and consequently was averaged for the table. During the suckling period greater amounts of unsaturated and shorter chain (C_{12} and C_{14}) fatty acids were noted, at the expense of the normally very abundant saturated fatty acids, palmitate and stearate. The composition was similar to the overall fatty acid composition of milk with the exception of high levels of retinyl myristate, which were comparable to the percentage of retinyl myristate of milk (the composition for milk from dams at day 8 of lactation was selected from reference 15 for comparison purposes). The pattern during the suckling period matches less well with the composition of milk retinyl esters, also taken from reference 15.

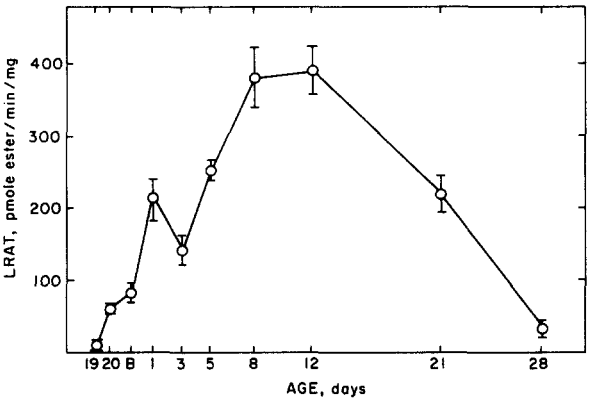

Fig. 3. Levels of LRAT in microsomes from perinatal rat small intestine. Microsomes were prepared from pooled small intestine obtained from animals at the indicated ages (B, birth). Values shown are the averages ± SD of at least three determinations for each sample.

The pattern observed for intestinal microsomal retinal reductase activity was quite different from either LRAT activity or CRBP(II) level (**Fig. 5**). Intestinal microsomes from fetus/pups collected immediately before or after birth had measurable retinal reductase activity. However, the activity was no longer detectable at day 3 and remained absent through day 8. Low levels had returned by day 12, but the considerable increase to adult levels did not begin to occur until weaning. By day 53, the levels ob-

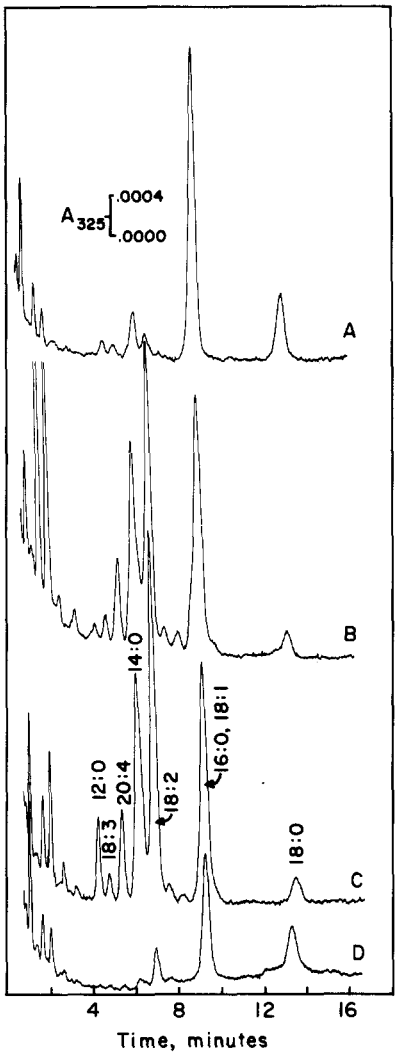

Fig. 4. HPLC profile of retinyl esters synthesized in vitro by intestinal LRAT. Retinyl esters synthesized by LRAT in vitro were resolved by HPLC. Identification, indicated in tracing C, was by reference to the elution position of standards (retinyl esters: 12:0, laurate; 14:0, myristate; 16:0, palmitate, 18:0, stearate; 18:1, oleate; 18:2, linoleate; 18:3 linolenate: 20:4, arachidonate). Age of animal from which intestines were obtained was A, 21 day of gestation; B, 1 day after birth (suckling); C, 21 day after birth (still suckling); D, 28 day after birth (weaned for 1 week).

served approached that previously reported for the adult rat (5).

DISCUSSION

The developmental patterns reported here for CRBP (II), LRAT, and microsomal retinal reductase are consistent with the suggestion that they are involved in the necessary steps of vitamin A absorption. While CRBP was present in the small intestine at highest level at the earliest time examined (day 16 of gestation), CRBP(II) only appeared 2 days before birth, and then rose quite rapidly, increasing about 10-fold by birth. This is consistent with a role for CRBP(II) in the function of the small intestine, while CRBP would appear to have some role in the development of the small intestine.

This was also suggested by the cell-specific location of the two proteins. CRBP(II) first appeared in the epithelial cell layer in an erratic, patchy manner. It appears that individual cells follow their own temporal pattern for differentiation leading to an initial heterogenous appearance of CRBP(II), but this heterogeneity is well resolved to a homogenous staining pattern by birth. At that time all villus-associated enterocytes stained strongly for CRBP(II), goblet cells were negative, and the epithelial cells at the base of the villi (the proto-crypt area) were also negative, as we have observed in the fully developed small intestine of the older animal (4). This lack of early coordination in cellular differentiation between individual neighboring enterocytes has also been observed for two fatty acid-binding proteins, as well as for CRBP(II), but was not evident for two apolipoproteins, which were present in a more homogenous staining pattern similar to what we report here for CRBP (16). However CRBP was also observed in the epithelial cells of the intervillus region, which was not true for the apolipoproteins. Although CRBP was expressed homogeneously in the epithelial cell layer as the small intestine was undergoing considerable morphological change, it was no longer detectable in that layer after well-differentiated villi were formed, suggesting no role for CRBP in the mature absorptive cell.

The developmental pattern for LRAT activity was similar to the pattern for CRBP(II). LRAT activity also increased about 10-fold in the 2 days before birth. A substantial further increase occurred until the highest activity was reached in the mid-suckling period. Activity then declined to adult levels by day 28 post-partum. It should be noted that this measurement of in vitro activity may not accurately reflect the actual amount of enzyme, particularly because the pattern of esters synthesized was markedly different during the suckling period. We have found in other studies (unpublished) that membrane composition appeared to affect LRAT activity. Microsomes that produced a retinyl ester pattern high in retinyl palmitate and stearate, as observed here for the microsomes from the fetal and weaned animal, demonstrated 2- to 3-times more enzyme activity when solubilized and assayed with exogenous phosphatidylcholine than was measured for the intact microsomes (without exogenous phosphatidylcholine). Microsomes that produced an ester pattern enriched in shorter chain or unsaturated fatty acids yielded only an equivalent or lesser activity when solubilized and assayed with exogenous phosphatidylcholine. This may suggest that LRAT has a higher catalytic rate when

TABLE 1. Fatty acid composition of retinyl esters synthesized in vitro by microsomes from the small intestine

Fatty Acid[b]	Retinyl Ester Synthesized			Fatty Acid[a] Composition of Milk	Retinyl Esters[a] in Milk
	Day 20 Gestation (n = 5)	Days 1–21 Suckling (n = 7)	Day 28 Weaned (n = 6)	Day 8 Lactation	Day 8 Lactation
	% of total (mean ± SD)			*% of total*	
12:0	*[c]	3.1 ± 1.8	*	9.1	2.8
14:0	9.8 ± 0.8	27.1 ± 5.3	5.6 ± 2.7	8.2	18.0
16:0	59.6 ± 1.6	14.6 ± 1.8	53.4 ± 3.9	22.8	33.5
18:0	16.9 ± 1.7	3.8 ± 1.2	20.4 ± 3.3	3.0	20.8
18:1	8.9 ± 0.5	16.1 ± 1.9	7.8 ± 0.6	19.3	12.0
18:2	5.1 ± 1.0	28.6 ± 3.2	12.9 ± 2.2	24.3	12.4
18:3	*	1.5 ± 0.4	*	(2.9)	*
20:4	*	5.4 ± 0.8	*		*
Other	*	*	*	*	10.7

[a]Taken from Ross et al. (15).
[b]Chain length: unsaturated bonds.
[c]Less than 1%.

it is present in membranes with greater fluidity, or it may reflect a preference for certain acyl moieties in its phosphatidylcholine substrates. In vitro LRAT activity and ester pattern definitely appeared to be affected by milk ingestion of the microsome donor. When weaning was delayed, a higher in vitro activity was observed for microsomes obtained from animals still suckling at day 28-post-partum compared to weaned animals of the same age; the activity and ester pattern were similar to that observed for microsomes from suckling animals at 21 days post-partum. In contrast, when animals were weaned earlier, at about day 17 post-partum, the in vitro LRAT activity and ester pattern observed for intestinal microsomes subsequently obtained at day 21 post-partum were essentially the same as that observed for microsomes from weaned animals at day 28 rather than that observed for microsomes from suckling animals at day 21 (results not shown). Whether this greater in vitro LRAT activity observed for microsomes collected from animals during the suckling period is also reflected by a greater in vivo LRAT activity was not investigated.

In other studies isolated microsomes have produced a pattern of retinyl esters quite similar to the pattern observed for retinyl ester synthesis by the intact animal (7) or cell (10, 11). In contrast, the retinyl ester pattern produced by everted gut sacs from animals in the mid-suckling period is high in retinyl palmitate and stearate (17), dissimilar to the in vitro pattern produced by microsomes from suckling animals reported here. This indicates that the retinyl esters synthesized by the isolated microsomes may not always accurately reflect the in vivo situation and suggests the pool of phosphatidylcholines used in vivo may be different from that available in vitro, under some circumstances. The possibility of the presence of an additional separate enzyme activity during the suckling period might also be considered.

The similar patterns for ontogenies of CRBP(II) and LRAT are consistent with the perceived physiological needs of the animal. One would expect the intestine to be sufficiently developed at birth to be able to deal with the nutrients present in milk. Milk contains retinyl esters but these cannot be absorbed intact to be incorporated into chylomicrons. Hydrolysis to free retinol is required for absorption; re-esterification then occurs within the absorptive cell. The appearance and considerable increase in CRBP(II) and LRAT in the small intestine shortly before birth would prepare the small intestine for this necessary processing of preformed vitamin A.

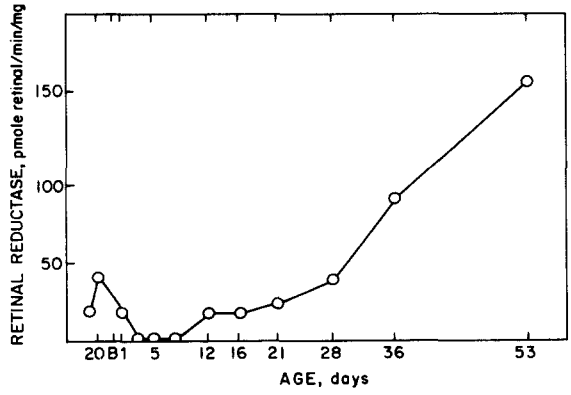

Fig. 5. Levels of retinal reductase in microsomes from perinatal rat small intestine. Microsomes were prepared from pooled small intestine obtained from animals at the indicated ages (B, birth). Values shown are the average of two determinations.

The pattern for microsomal retinal reductase, while much different, also would meet the physiological need of the animal. Microsomal retinal reductase activity was noted in the intestine around the time of birth, but then was undetectable for about 1 week. If this enzyme is solely responsible for the reduction of retinal produced by oxidative cleavage of β-carotene, the intestine would appear not to be competent to utilize this source of vitamin A until the enzyme reappears near the end of week 2, postpartum. But, because the rat is a non-accumulator of dietary β-carotene and does not pass significant quantities through the intestine into the blood (18), little β-carotene would be expected to be present in rat milk. The first β-carotene would only be encountered with ingestion of solid food and the activity of microsomal retinal reductase did begin a considerable increase at the time of weaning. This late rise has been noted for other intestinal enzymes, such as sucrase, that deal with nutrients absent in milk but present in the normal post-weaning diet (19). The reason for the presence of activity in the intestine at the time of birth is unknown. It may be that this was due to a separate microsomal enzyme, not actually involved in vitamin A metabolism in vivo.

In summary, the pattern of appearance of CRBP(II), LRAT, and microsomal retinal reductase match well with the predicted physiological needs of the animal and provide indirect support for the proposition that these proteins are indeed involved in the processing of vitamin A during absorption.

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