Vitamin A combined with retinoic acid increases retinol uptake and lung retinyl ester formation in a synergistic manner in neonatal rats

A. Catharine Ross,[1,*,†] Namasivayam Ambalavanan,[§] Reza Zolfaghari,[*] and Nan-qian Li[*]

Department of Nutritional Sciences* and Huck Institute for Life Sciences,[†] Pennsylvania State University, University Park, PA 16802; and Department of Pediatrics,[§] University of Alabama at Birmingham, Birmingham, AL 35249

Abstract **Vitamin A (VA) is stored in tissues predominantly as retinyl esters (REs), which provide substrate for the production of bioactive retinoids. Retinoic acid (RA), a principal metabolite, has been shown to induce postnatal lung development. To better understand lung RE storage, we compared VA (given as retinyl palmitate), RA, and a nutrient-metabolite combination, VARA, given orally on postnatal days 5–7, for their ability to increase lung RE in neonatal rats. VARA increased lung RE significantly [~14, 2.4, 2.1, and <1 nmol/g for VARA, VA, RA, and control (C), respectively; $P < 0.001$]; the increase by VARA was more than additive compared with the effects of VA and RA alone. Lung histology and morphometry were unchanged. In a 6 h metabolic study, providing [^{3}H]retinol with VARA, compared with VA or C, increased the uptake of newly absorbed ^{3}H by 3-fold, indicating that VARA stimulated the uptake of [^{3}H]retinol and its retention as [^{3}H]RE in neonatal lungs. After cessation of VARA, lung RE remained increased for 9 d afterward, through the period of alveolar development. In conclusion, VARA, a 10:1 nutrient-metabolite combination, increased lung RE significantly compared with VA alone and could be a promising therapeutic option for enhancing the delivery of VA to the lungs.**—Ross, A. C., N. Ambalavanan, R. Zolfaghari, and N-q. Li. **Vitamin A combined with retinoic acid increases retinol uptake and lung retinyl ester formation in a synergistic manner in neonatal rats. *J. Lipid Res.* 2006.** 47: **1844–1851.**

Supplementary key words lung retinyl ester storage • retinol esterification • liver retinyl ester • newly absorbed retinol • neonatal vitamin A status

Retinyl esters (REs) are the most abundant form of vitamin A (VA) in most tissues (1). The hydrolysis of RE generates retinol, which, through oxidative metabolism, yields retinoic acid (RA) (2), a retinoid hormone capable of activating nuclear retinoid receptors and thereby inducing or repressing the transcription of many genes (3–5). Among its many physiological actions, RA regulates cell proliferation, differentiation, and cell-cell interactions (6). For the lungs, retinoids are essential for normal morphogenesis in the fetal period, for maturation and remodeling in the perinatal and postnatal periods, and for maintenance of the fully matured lungs (7–10). In humans, the lungs undergo septation of the thick-walled air saccules in the perinatal period, beginning at approximately week 28 of gestation, with a 2- to 4-fold increase in the gas exchange surface area by weeks 30–40 (7, 11, 12). In rats and mice, septation and the formation of pulmonary alveoli occur mainly in the first 2 weeks of postnatal life (7, 10, 12–14). Whereas a deficiency of dietary VA for 6 weeks resulted in an increased size of rat lung airspaces and reduced elastin in the parenchyma (15), the administration of RA to neonatal rats from postnatal day 3 to 14 increased the septation of the lungs (16, 17). RA also attenuated the oxygen-induced inhibition of alveolarization in rats reared during the period of septation in a hyperoxic environment (18). Retinol has shown promise in reducing pulmonary dysfunction in a lamb model of preterm lung injury (19) and in clinical studies of preterm human infants susceptible to bronchopulmonary dysplasia (20–25).

The neonatal rodent is a useful model in which to study the biochemical and morphological development of the lungs in the perinatal period. Previous studies have characterized the ontogeny of RE storage in the lungs and liver of the perinatal rat (26). In the VA-adequate state, lung RE, mainly as retinyl palmitate, increased between gestational day 14 and 18, then declined abruptly before birth and remained relatively low from day 2 to 21, whereas liver RE increased before birth and increased even higher in the postnatal period (27). The offspring of VA-supplemented mothers showed the same temporal pattern, but their lung

Manuscript received 7 February 2006 and in revised form 13 April 2006.
Published, JLR Papers in Press, May 9, 2006.
DOI 10.1194/jlr.M600061-JLR200

Abbreviations: RA, retinoic acid; RE, retinyl ester; VA, vitamin A.

[1] To whom correspondence should be addressed.
e-mail: acr6@psu.edu

and liver RE concentrations were 1.7- to 7.1-fold higher (28). However, in the absence of maternal VA supplementation, neonates have very low reserves of VA compared with healthy adults (1), as a result of the very limited transfer of VA across the placenta (29). The postnatal period is important for the accrual of VA reserves, as shown in animal and human studies (30–34), but this is dependent on an adequate intake of VA.

Given that RA is a positive regulator of lung maturation and that VA given to the mother can increase lung RE content in neonatal lungs (28), we considered whether providing both VA and RA together, directly to the neonate, might be more effective than providing either retinoid alone. In the present investigations, we tested this nutrient-metabolite combination, VARA, and found it to be severalfold more effective in increasing lung RE than the same amounts of VA or RA given separately. In metabolic studies, VARA increased the distribution of newly absorbed [^{3}H]retinol to the lungs, where it was stored as [^{3}H]RE. The effect of VARA on lung RE was apparent within hours of treatment, and after cessation of treatment, an increased lung RE concentration persisted throughout the postnatal period of lung septation. These results suggest that VARA could be a promising nutrient-metabolite combination for increasing the delivery and storage of VA in the lungs during the postnatal period of lung development.

METHODS

Materials

VA (all-*trans*-retinyl palmitate) and all-*trans*-RA were purchased from Sigma-Aldrich (St. Louis, MO). For oral administration, VA was diluted directly in canola oil to a concentration of 0.1 nmol/g oil (2× concentrate). For RA, 50 μl of ethanol was added to 3 mg of RA, then oil was added to 1 g to achieve a concentration of 0.01 mmol/g (2× concentrate). An oil vehicle control (C) was prepared in the same manner. To ensure that the combined dose of VA plus RA would closely match the sum of the individual doses, the 2× concentrates of VA and RA were mixed 1:1 (w/w) with oil to form the 1× doses for administration or 1:1 with each other to form the VARA dose (0.05 mmol of VA and 0.005 mmol of RA per gram of VARA dose). Retinoids were shielded from ultraviolet light and stored at 4°C in foil-wrapped vials.

Animals, dosage, and experimental designs

Animal procedures were approved by the Institutional Animal Use and Care Committee of Pennsylvania State University. Pregnant Sprague-Dawley rats were purchased from Charles River (Wilmington, MA) or females were mated in our animal facility. In the first experiment, six litters of rats were adjusted on day 4 to 10 neonates per litter (day 1 = <24 h after birth). Four neonates in each litter were randomly assigned to the main study, and four others were assigned to a nested kinetic study. The main study included four treatments: C, VA, RA, and VARA. The dose of VA given to neonatal rats was based on a VA supplement of 50,000 IU (1 IU = 0.3 μg of retinol or 0.56 μg of retinyl palmitate) previously given to human infants (35, 36). We estimated this dose as ~6 mg retinol/kg, equal to ~210 nmol of retinol for a 10 g neonatal rat. Our dose of RA was selected based on the amount used previously by Massaro and Massaro (16) to induce postnatal lung alveolarization. In their study, 500 μg/kg all-*trans*-RA (1.67 μmol/kg) was administered intraperitoneally on days 3–14, whereas we adjusted the dose upward to 625 μg/kg to compensate for an absorption efficiency of ~80% and delivered the dose of RA orally. This resulted in a dose of RA of ~6.25 μg (21 nmol) for a 10 g neonate. VARA was the exact combination of the VA dose and the RA dose and thus had a molar ratio of 10:1 retinol/RA. For each treatment, the appropriate dose was delivered directly into the rat pup's mouth with a small micropipette. For the nested kinetic study, tissues were collected from one C and one VARA neonate from each litter on day 6, after one treatment, and from another pair on day 7, after two treatments.

At the end of each experiment, neonates were individually euthanized with carbon dioxide and weighed. Blood was collected from the vena cava or heart, and the lungs and liver were removed, blotted, weighed, and immediately frozen in liquid nitrogen for storage at −80°C before retinoid analysis.

Retinoid analysis

Plasma retinol was determined on pooled samples (n = 2 neonates/treatment in each pool) after saponification, extraction, and reverse-phase HPLC (37). Lung and liver retinol and RE were quantified by HPLC using methods similar to those described by Zachman, Kakkad, and Chytil (26). Briefly, portions of each tissue were weighed and the samples were extracted overnight in 20 or more volumes of chloroform-methanol (2:1, v/v) by the procedure of Folch, Lees, and Sloane Stanley (38). The tissue extracts were filtered, rinsed, and washed (38). A known amount of an internal standard of trimethylmethoxyphenyl-retinol (provided by M. Klaus, Hoffmann-La Roche, Basel, Switzerland) was added to aliquots of the total lipid extracts, and the samples were dried under argon and reconstituted in ~100 μl of chloroform-methanol (1:1) for HPLC analysis. To obtain enough lung extract from C neonates and to reduce the number of samples for analysis, we analyzed three pools from two neonates per pool in most studies (as indicated in the figure legends), as this number provided sufficient power for statistical testing. Portions of each sample, generally 18–22 μl, were injected onto a C-18 reverse-phase HPLC column (Symmetry C18 2.1 × 150 mm column; Waters Corp., Milford, MA) and eluted with a gradient from 90:10 methanol-water to 100% methanol at a flow rate of 1.5 ml/min for 5 min, followed by 100% methanol at 2.2 ml/min for 15 min, followed by reequilibration with 90:10 methanol-water before the next injection. The eluate was monitored at 325 nm with a Waters 960 photodiode array detector, and the areas of the peaks for trimethylmethoxyphenyl-retinol, unesterified retinol, and all detectable REs were integrated using Millenium-32 (Waters) software. Unesterified retinol in lungs and liver (data not shown) constituted <5% of total VA.

Histology

Lung samples from a parallel set of neonates treated for 3 days with C, VA, RA, and VARA (n = 4/group) were inflated and perfusion-fixed with neutral phosphate-buffered 10% formalin (39). Five micrometer thick sections were prepared from the base to the apex of each lung, and sections were stained with hematoxylin and eosin. Six random fields were evaluated per slide for each variable by an observer masked to slide identity. The software package MetaMorph (Universal Imaging Corp., Downingtown, PA) interfaced with a Nikon Labophot microscope equipped with a QiCam Fast Cooled high-resolution charge-coupled device camera (1,392 × 1,040 pixels = 4.15 MB TIF file per image) was used for image analysis. Epithelial injury and hemorrhage were assessed at a magnification of 400× using a

scale of 0 (normal) to 5 (most), based on predefined definitions [e.g., a hemorrhage score of 1 corresponds to red blood cells in septae, whereas a score of 2 corresponds to scattered intra-alveolar red blood cells (modified from 40)]. Tissue density and mean linear intercept (41) were assessed at 100×. Tissue density is the proportion of the field occupied by tissue (area occupied by tissue/area occupied by lung tissue plus alveoli). Mean linear intercept is the average distance between alveolar septae in micrometers, an indicator of maturation. As alveolar septation occurs, the distance between septae decreases, and hence the mean linear intercept decreases with maturation.

Study of [^{3}H]retinol metabolism

To evaluate the distribution of retinol in the postabsorptive period, 9 day old rats were treated with an oral dose of oil (C), VA, or VARA equivalent to the doses described above. The next day, the same dose containing ~2 μCi of [^{3}H]retinol {[11,12-^{3}H(N)]retinol; Perkin-Elmer} was given orally. Six hours later, tissues were collected as described above. Total ^{3}H in plasma was determined by liquid scintillation spectrometry, and total ^{3}H per plasma volume was calculated by estimating plasma volume as 3.5% of body weight. Lung and liver tissues were extracted with chloroform-methanol as described above, and the organic phase lipid extracts were washed (38). The aqueous washes were pooled, and a portion was counted to assess the formation of aqueous ^{3}H-labeled polar metabolites of retinol. The organic phase lipid extracts were divided into several portions that were counted directly, used for RE analysis by HPLC, or separated on columns of aluminum oxide into [^{3}H]RE and unmetabolized [^{3}H]retinol (42). The fractions thus obtained were dried and counted by liquid scintillation spectrometry. Aliquots of each labeled dose were also counted, and the percentage of the oral dose in each tissue fraction was then calculated.

Statistics

Data are presented as means ± SEM. The results were analyzed by ANOVA, followed by the least-squares means test to determine statistically significant differences among groups (SuperANOVA software; Abacus, Berkeley, CA). When the variance terms were unequal among groups, $\log_{10}$ transformation was performed before statistical testing. P values are reported; differences with $P > 0.05$ were considered nonsignificant.

RESULTS

Growth and plasma retinol

The first experiment included four treatments, vehicle (C), VA, RA, and VARA, which were administered on postnatal days 5–7. The rate of growth and organ weight (liver and lung weight adjusted for body weight) were not affected (data not shown). Plasma total retinol concentration was within the normal range for all groups, averaging 1.4, 1.8, 1.4, and 2.2 μM for C, VA, RA, and VARA neonates, respectively.

Lung RE concentration

Lung RE (>95% of lung total VA) was increased by ~3-fold in the VA group (**Fig. 1A**). Lung RE was also increased in the RA group, to nearly the same extent as in the VA group, even though RA is not reduced to form retinol in vivo (43). Lung RE in the VARA group averaged >14 nmol/g, compared with 2.4 and 2.1 nmol/g in the groups that received VA alone and RA alone, respectively, and <1.0 nmol/g in the normal C group. These data indicate that VA and RA combined in the VARA preparation acted synergistically, as the increase by VARA was more than the sum of the increases attributable to its individual components provided in the same amounts.

The molecular species of lung REs were similar for all treatment groups (Fig. 1B). The major peak was retinyl palmitate with a shoulder of retinyl oleate, followed by a second peak of retinyl stearate. In the lungs of VARA-

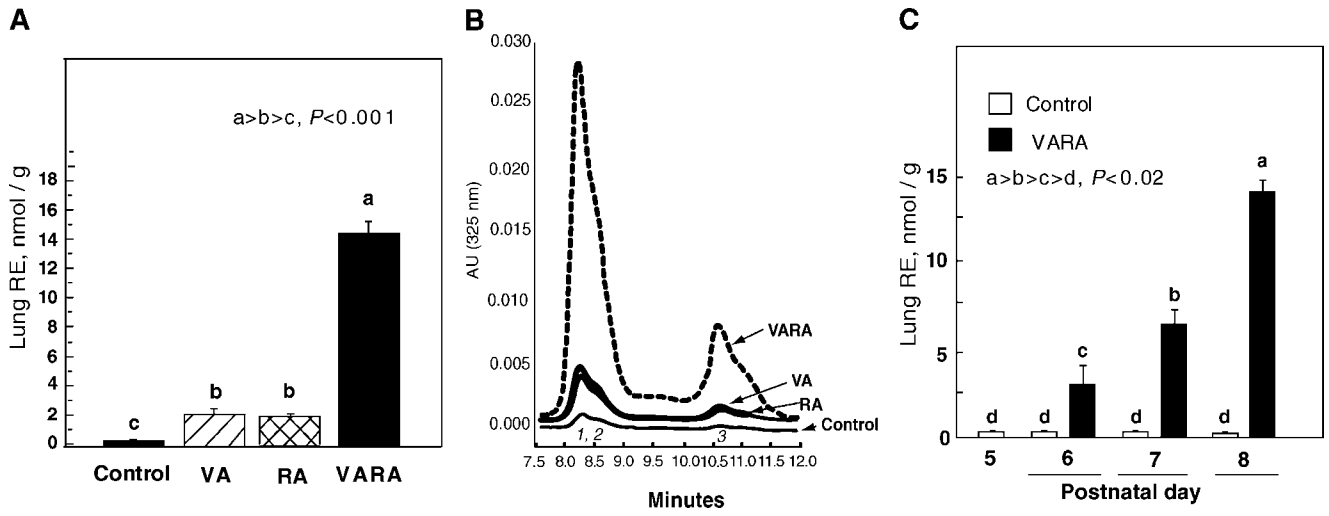

Fig. 1. Lung retinyl ester (RE) concentration in neonatal rats treated on postnatal days 5, 6, and 7 with vitamin A (VA) alone, retinoic acid (RA) alone, or VARA. A: Lung RE on day 8. Results shown are means ± SEM. Groups with different letters are significantly different (a > b > c) by the least-squares means test. B: Typical chromatogram tracings illustrating major peaks of retinyl palmitate (peak 1) with a shoulder of retinyl oleate (shoulder 2), followed by retinyl stearate (peak 3). AU, absorbance units. C: Kinetics of RE accumulation in the lungs of control (C) neonates and neonates treated with VARA once, twice, or three times. Lung RE concentration was determined on the postnatal day indicated, which was the day after the last dose.

treated neonates, several minor REs (data not shown) were detectable in the region before the large peak of retinyl palmitate/oleate. Overall, VARA increased lung RE concentration markedly without altering the relative proportions of the various REs.

In a nested subset of the first experiment, the accumulation of RE in the lungs was examined daily in C- and VARA-treated neonates to determine how quickly the change in lung RE concentration occurred. RE was increased after a single treatment with VARA (Fig. 1C, day 6) and increased again after each additional daily dose (Fig. 1C, days 7, 8).

Histology

To assess whether the treatments produced any gross abnormalities, the lungs of neonates treated on days 5–7 with C, RA, VA, and VARA were examined on day 8 by low- and high-power light microscopy. There were no significant differences in the parameters measured: lung injury score, tissue density, and mean linear intercept (**Table 1**). There were no unusual findings by low-power or high-power light microscopy (data not shown). We did not observe any acceleration or attenuation of alveolar development in any of the treatment groups at this time.

Liver RE is increased equally by VA and VARA

The liver is the major site of RE storage, and newly absorbed VA in chylomicron remnants is delivered rapidly to the liver (44). In the experiments described above, liver RE was analyzed in parallel with lung RE. As expected from previous studies (26–28), liver RE concentration exceeded lung RE concentration by ~20-fold. However, in contrast to the greater effect of VARA, compared with VA alone, on RE in the lungs, RE in the liver was increased equally by VARA and VA alone (**Fig. 2**). Consistent with this, RA by itself had no effect on liver RE, compared with the C group. Thus, liver RE storage increased in response to VA, with or without RA, whereas RA alone had no effect on liver RE, either in the absence or presence of VA.

The distribution of newly absorbed retinol into the lungs is increased by VARA

Because VARA increased lung RE within a short period of time after oral feeding, we next considered that VARA might increase lung RE content by promoting the uptake of newly absorbed retinol into the lungs. Therefore, a metabolic study was conducted in which neonatal rats were treated with an unlabeled dose of C, VA, or VARA and, the next day, with the same dose including [^{3}H]retinol to monitor the uptake of newly absorbed VA into the lungs and the liver. Tissues were analyzed 6 h after oral dosing, a time chosen so that the absorption of [^{3}H]retinol would not yet be complete and differences in the rate of its uptake among the treatment groups might still be apparent. At 6 h, <2.2% of the oral dose of [^{3}H]retinol was present in the plasma compartment, and there were no differences among treatment groups. However, in the lungs (**Fig. 3A**), there was significantly more ^{3}H in the lipid extract in the VARA group, compared with either the VA or C group, and the VA and C groups did not differ from each other. Therefore, even though the doses of VA and VARA contained the same amount of [^{3}H]retinol and the same mass of unlabeled VA, more than twice as much lipid-soluble ^{3}H was present in the lungs of the VARA-treated neonates than in the VA-treated neonates. Very little ^{3}H was present in the aqueous phase of the lung extract, indicating that there had been little metabolism of [^{3}H]retinol to aqueous metabolites or that, if produced, metabolites did not accumulate in the lungs.

The fraction of ^{3}H present as [^{3}H]RE, determined by column chromatography, was higher in the lungs of the VARA and VA groups than in the C group (Fig. 3B) but was not different between the VARA and VA groups. From this result, it can be inferred that the RA component of VARA did not affect the rate of conversion of [^{3}H]retinol to [^{3}H]RE in vivo. At 6 h, ~45% of the newly absorbed ^{3}H dose in the lungs was present as [^{3}H]RE. Thus, the fraction of retinol converted to RE was similar for the VARA and VA groups (Fig. 3B), indicating that there was no differential effect of VARA, compared with VA, on conversion. However, as a consequence of the greater amount of total ^{3}H in the lungs of the VARA group (Fig. 3A), there was significantly

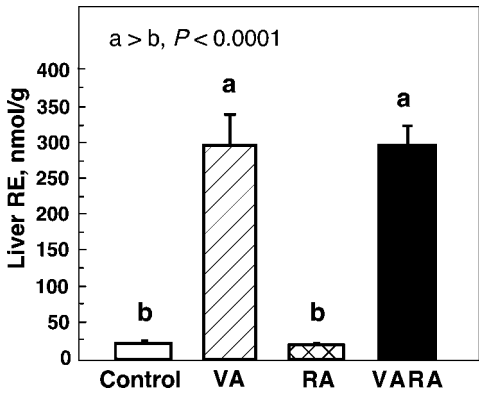

Fig. 2. Liver RE concentration in neonatal rats treated on postnatal days 5, 6, and 7 with VA alone, RA alone, or VARA. Liver RE was determined on day 8. Results shown are means ± SEM for three pools per treatment. Groups with different letters are significantly different.

TABLE 1. Lung injury and morphometry scores in neonatal rats treated with retinoids for 3 consecutive days

Treatment Group	Epithelial Injury Score (Range, 0–5)	Tissue Density	Mean Linear Intercept
		% of space occupied	*μm*
Control	0.29 ± 0.08	27 ± 3	62 ± 4
VA	0.44 ± 0.06 (3)	28 ± 2	63 ± 7
RA	0.29 ± 0.08	29 ± 4	57 ± 5
VARA	0.41 ± 0.09 (3)	32 ± 4	65 ± 1
P	0.46	0.70	0.65

RA, retinoic acid; VA, vitamin A. Lungs were perfusion-fixed and examined on day 9 after treatment on days 6, 7, and 8. Values shown are means ± SEM (n = 4/group except where indicated in parentheses). Hemorrhage score, another index of lung injury, also showed no significant differences.

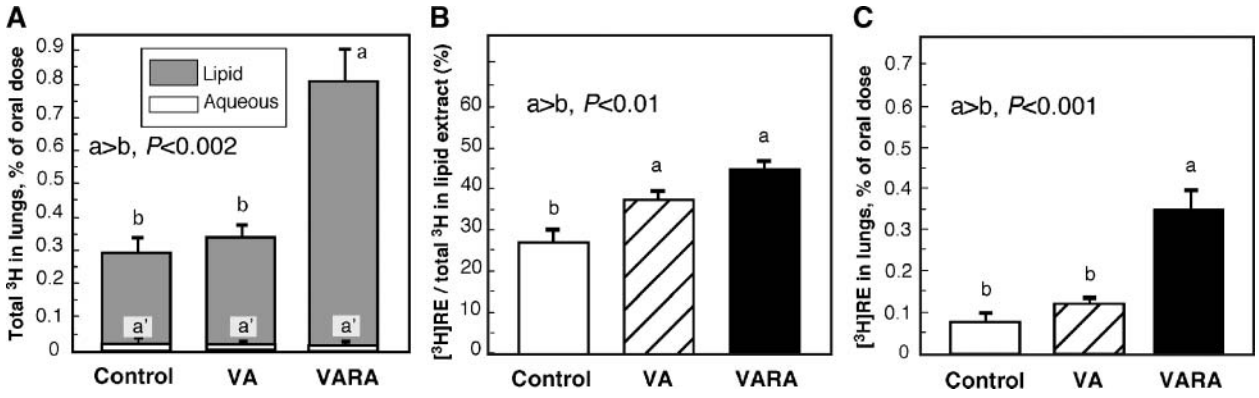

Fig. 3. Metabolism of [^{3}H]retinol present in an oral dose of oil (C), VA, or VARA. Neonates were treated on postnatal day 7 with an oral dose of oil (C), VA, or VARA as described in Methods. The next day, they were treated with the same oral dose containing [^{3}H]retinol. Tissues were collected and analyzed for ^{3}H contents 6 h later. Data shown are means ± SEM (n = 3/group). A: Percentage of the ^{3}H oral dose present in the lungs in the total lipid extract (shaded upper portion of bar), the aqueous phase (open lower portion of bar), and combination (total bar) after total lipid extraction and washing as described in Methods. Groups with different letters are significantly different (a > b) by the least-squares means test. B: Percentage of ^{3}H in the organic phase as [^{3}H]RE. C: Percentage of [^{3}H]retinol in the oral dose recovered as [^{3}H]RE in the lipid phase of the lung extracts.

more [^{3}H]RE in the lungs of VARA-treated neonates at 6 h, compared with the VA and C groups (Fig. 3C).

Similar analyses were performed on the liver (data not shown). Consistent with the results illustrated in Fig. 2 for liver RE mass, there was more [^{3}H]RE in the liver of both the VA and VARA groups compared with the C group, but there was no significant difference between the VARA and VA groups. Therefore, the same pattern of results, with higher but equal levels of RE in the liver of the VARA- and VA-treated groups compared with the C group, was observed for newly absorbed [^{3}H]retinol during the absorptive period and for steady-state RE mass measured after 1–3 days of VARA or VA treatment.

VARA-derived RE is retained during the period of postnatal lung alveolarization

The experiments described above were designed to examine the response to VARA over relatively short times. Because alveolar development extends during the first 2–3 weeks after birth in the rat, it was of interest to determine whether the increase in lung RE seen in 8 day old VARA-treated neonates would persist into postnatal week 3. Therefore, we treated neonates with oil (C) or VARA on days 5, 6, and 7 and analyzed lung and liver RE in half of the neonates in each treatment group on day 8 and in the remaining neonates on day 16, without additional treatment after day 7. **Figure 4** shows that RE mass was higher in the lungs of VARA versus C neonates on day 8, similar to previous experiments. Although the RE content of the lungs declined between days 8 and 16, it nonetheless remained higher in the VARA group. Therefore, the accumulation of lung RE by day 8 resulted in a sustained increase during the second postnatal week, without further treatment. In the liver, RE was approximately seven times higher in VARA-treated rats than in the C group. Although RE concentration per gram declined as the neonates grew, the mass of RE per total liver did not change significantly in VARA neonates (Fig. 4B), whereas it doubled in C neonates. These differences are consistent with significant retention of the accumulated REs in the liver of the VARA-treated neonates and, concomitantly, with the storage of diet (milk)-derived retinol as RE, which was evident by the increase in liver RE in the C group.

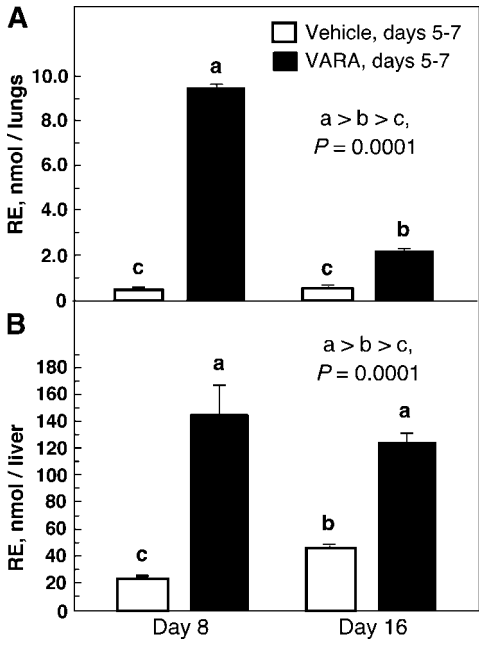

Fig. 4. Retention of RE in the lungs and liver of VARA-treated neonates after cessation of dosing. A: Lung RE in neonates treated on postnatal days 5, 6, and 7 with oil (C) or VARA and analyzed on day 8 or 16. There was no intervening treatment from day 7 to 16. B: Liver RE in the same neonates. Data are expressed per organ; the same statistical differences were obtained for data expressed as nmol RE/g tissue. Data shown are means ± SEM (n = 3 rats/group on day 8 and n = 4 rats/group on day 16). Groups with different letters are significantly different.

DISCUSSION

VA deficiency has long been associated with impaired cell differentiation and abnormal tissue morphology (6, 45). Randomized, controlled intervention studies conducted in populations in which a nutritional deficiency of VA is a public health problem have demonstrated that VA can reduce all-cause and disease-specific mortality in preschool-age children and newborns (35, 36, 46). It is widely believed that the beneficial effects of VA may be attributable to its ability to promote the maturation and functions of epithelial tissues, including those of the immune system, intestine, and lungs, such that VA-adequate children are better able to survive common infectious diseases. Strategies to improve the VA status of infants in the immediate postnatal period are of clinical as well as public health interest. In extremely low birth weight infants hospitalized for the treatment of bronchopulmonary dysplasia and respiratory distress, VA as retinol improved clinical outcome (21, 22). The observations by others that RA given in the postnatal period can promote lung septation in normally nourished rodents (16) raises the possibility that the physiologically low levels of VA present in tissues at birth may be marginal, and could possibly be rate-limiting, for the development of the lungs postnatally.

Because neonates are especially vulnerable to VA deficiency, and because retinoids have been shown to affect lung maturation in postnatal rats, we decided to compare the ability of VARA, VA, and RA to stimulate RE formation in a neonatal rat model. The amount of VA was based on the dose previously used for VA supplementation in newborn humans (35, 36), scaled to the body weight of young rats (see Methods), whereas the dose of all-*trans*-RA used in our studies was similar to that shown previously to increase lung alveolar septation in neonatal rats (16). VARA was formulated as the exact combination of these two doses, and on this basis it contained 10 mol of retinol (as retinyl palmitate) to 1 mol of RA. It is well established that RA is not reduced to retinal and, then, retinol in vivo; therefore, RA itself is not a precursor of REs (43).

None of the retinoid treatments affected growth, and plasma retinol was only slightly higher in the VARA group, but still within the normal range. Thus, the increase in lung RE in VARA-treated neonates was not a reflection of differences in plasma retinol. An evaluation of lung histology, conducted after 3 days of retinoid treatment, revealed no unusual findings, and by morphometry there were no differences in indicators of lung injury or maturation (Table 1). Thus, VARA, like VA and RA alone, was well tolerated. We did not expect to see a difference in alveolar septation by day 8, and none was evident by the criteria used. In previous studies, which had shown increased alveolar septation after treatment with RA, RA was administered intraperitoneally daily from postnatal day 3 to 14, when lung maturation was evaluated (16). Additional research will be needed to determine whether VARA affects lung maturation and/or function in neonatal rats.

Several findings emerged from these studies. First, we observed significant increases in lung RE concentration in neonates supplemented with either all-*trans*-RA or VA alone (Fig. 1). Because RA is not converted to retinol in vivo, the increase in RE in the lungs of the RA-treated group must reflect differences in the distribution and metabolism of retinol, derived either from the diet (milk) or from other tissues. Indeed, kinetic studies carried out in adult rats have indicated a regulatory role of RA on the metabolism of retinol (47), as the fractional uptake of [^{3}H]retinol from plasma into the lungs was increased in rats fed RA in the diet, as was the VA content of the lungs. Similarly, lung RE was higher in adult rats treated with a water-soluble metabolite of RA, retinoyl-β-glucuronide, or RA for 12 days (48). However, to our knowledge, an oral supplement of VA combined with RA has not been studied previously. VA supplementation alone, given to pregnant rats on gestational day 19, had been shown to increase the RE concentration in the lungs and liver of their fetuses, newborns, and neonates (26–28). In our studies, VA alone, given directly orally to neonates, also significantly increased the concentration of RE in the lungs and liver (Figs. 1, 2). From both of these results, it can be inferred that RA has the potential to regulate the metabolism of retinol and thus increase RE content modestly, but VA (retinol) itself is rate-limiting for the storage of higher levels of RE in the lungs. Collectively, these studies show that the lungs of neonates have a greater capacity to form and retain RE than is apparent in the absence of VA supplementation.

Second, the nutrient-metabolite combination, VARA, increased lung RE more effectively than either VA or RA alone and was significantly more effective compared with the sum of the two retinoids given individually (Fig. 1A). This result indicates that the VA and RA components of the VARA mixture act in a synergistic manner, from which it may be inferred that each of these retinoids plays a different but interacting role in the promotion of lung RE concentration. Although VARA greatly increased lung RE concentration, the normal pattern of REs was maintained (Fig. 1B). VARA increased lung RE every day, dose by dose, over the course of a 3 day treatment (Fig. 1C). Therefore, it is apparent that the lungs of neonates, although structurally immature, are capable of taking up and storing much more RE than is usually present. In contrast to the lungs, the RE concentration in the liver responded only to VA or to the VA component of VARA (Fig. 2). The reasons for the lack of effect of RA, alone or combined with VA, on RE storage in the liver are not known, but they might be related to the liver's ability to rapidly oxidize RA (49). Future studies on RA catabolism are needed to understand the difference between the effect of RA on lung RE and, simultaneously, the lack of effect of RA on liver RE storage. Additionally, the functional consequences, if any, of increasing RE storage in the lungs of neonates are unknown at present. The possibility that the lungs of VARA-treated neonates might be protected under conditions of lung injury, such as from exposure to high oxygen, is of interest to us. Previously, daily treatment with

RA attenuated the inhibition of lung alveolar septation induced by exposure to 95% oxygen in neonatal rats (18). Experiments in progress with neonatal mice suggest that VARA can increase neonatal lung RE levels, even during exposure to high oxygen (M. James et al., unpublished data) (50).

Because the effect of VARA on lung RE was observed to be rapid, we next compared the metabolism of newly absorbed [^{3}H]retinol in oil- (C-), VA-, or VARA-treated neonates. This experiment revealed that just 6 h after [^{3}H]retinol was given orally, more ^{3}H was present in the lipid extract of the lungs of VARA-treated neonates compared with either the C group or the VA group. However, the VA group and the C group did not differ from each other. Therefore, the mass of VA being absorbed was not a determining factor for the percentage of [^{3}H]retinol taken up by the lungs. The fraction of [^{3}H]retinol esterified (Fig. 3B) did not differ between the VA and VARA groups, suggesting that the rate of retinol esterification did not drive the increase in [^{3}H]RE. However, with the increased distribution of newly absorbed [^{3}H]retinol into the lungs of VARA-treated neonates (Fig. 3A), there was significantly more [^{3}H]RE in the lungs of VARA-treated neonates compared with both VA and C neonates (Fig. 3C). These results imply that the presence of RA in the VARA combination, compared with VA alone, modifies the distribution of newly absorbed [^{3}H]retinol into the lungs. At the 6 h time point, the uptake and esterification of newly absorbed [^{3}H]retinol probably had not reached a steady state, because the fraction of total [^{3}H]retinol that was in the [^{3}H]RE eluate was ~45%, less than the steady-state fraction of RE mass (~95%), which included both newly formed and preexisting RE.

Given that the VARA preparation contained only 1 mol of RA for every 10 mol of VA, it is evident that only a small proportion of RA is required to significantly affect the distribution of much larger amounts of VA (retinol). It is not known yet whether the uptake of newly absorbed VA into the lungs of neonates involves the hydrolysis of diet/milk-derived REs in chylomicrons and the reesterification of retinol, as has been shown for adult rat liver (51). Although a more precise explanation for the effect of VARA cannot yet be provided, the results of this metabolic experiment have narrowed the search for mechanisms to the early absorptive period, within the first 6 h after dosing, when the uptake and processing of newly absorbed VA are still ongoing. It is unlikely that intestinal VA absorption was differentially affected by VARA compared with VA, because liver RE was increased equally by both of these supplements (Fig. 2; data for [^{3}H]RE not shown). The amount of oil available for absorption was the same for all groups, so chylomicron formation should not have differed in any groups, and the RE content per chylomicron particle should have been equivalent after treatment with either VARA or VA, both of which contained the same mass of VA. At present, it appears most likely that events related to the postabsorptive trafficking of newly absorbed chylomicron-associated VA to the lungs, and the cellular disposition of newly absorbed VA in the lungs after the uptake of VA, could be promising directions for future investigations. We also considered an induction of the retinol-esterifying enzyme, lecithin:retinol acyltransferase, as a possible mechanism. However, in preliminary studies, lung lecithin:retinol acyltransferase mRNA was not significantly increased by VARA, although lung RE was increased significantly (Fig. 1), and, as shown in Fig. 3B, the fraction of newly absorbed [^{3}H]retinol that was converted to [^{3}H]RE was not increased by VARA compared with VA. Therefore, a higher rate of retinol esterification appears unlikely to explain the increase in RE content in the lungs of VARA-treated neonates.

In summary, VARA was more effective than VA alone or RA alone at increasing RE levels in the lungs of neonatal rats, at least in part because VARA stimulated the trafficking of newly absorbed [^{3}H]retinol to the lungs. Further studies are needed to define the molecular and/or biochemical mechanisms by which VARA promotes lung RE formation and to assess the impact of this treatment on lung retinoid metabolism and lung function. The ability of VARA to increase lung RE more than an equivalent amount of VA suggests that it may be a promising therapeutic option for differentially increasing RE in this organ.

This work was supported in part by National Institutes of Health Grants R01 CA-90214 (A.C.R.) and K08 HD-046513 (N.A.) and by the Children's Center for Research and Innovation (N.A.) and Dorothy Foehr Huck funds (A.C.R.).

REFERENCES

1. Ross, A. C. 2005. Introduction to vitamin A: a nutritional and life cycle perspective. *In* Carotenoids and Retinoids. Molecular Aspects and Health Issues. L. Packer, U. Obermüller-Jevic, K. Kraemer, and H. Sies, editors. American Oil Chemists' Society Press, Champaign, IL. 23–41.
2. Napoli, J. L. 1999. Interactions of retinoid binding proteins and enzymes in retinoid metabolism. *Biochim. Biophys. Acta.* **1440:** 139–162.
3. Lefebvre, P., P. J. Martin, S. Flajollet, S. Dedieu, X. Billaut, and B. Lefebvre. 2005. Transcriptional activities of retinoic acid receptors. *Vitam. Horm.* **70:** 199–264.
4. Privalsky, M. L. 2004. The role of corepressors in transcriptional regulation by nuclear hormone receptors. *Annu. Rev. Physiol.* **66:** 315–360.
5. Balmer, J. E., and R. Blomhoff. 2002. Gene expression regulation by retinoic acid. *J. Lipid Res.* **43:** 1773–1808.
6. Takatsuka, J., N. Takahashi, and L. M. de Luca. 1996. Retinoic acid metabolism and inhibition of cell proliferation: an unexpected liaison. *Cancer Res.* **56:** 675–678.
7. Chytil, F. 1996. Retinoids in lung development. *FASEB J.* **10:** 986–992.
8. Warburton, D., M. Schwarz, D. Tefft, G. Flores-Delgado, K. D. Anderson, and W. V. Cardoso. 2000. The molecular basis of lung morphogenesis. *Mech. Dev.* **92:** 55–81.
9. Schuger, L., J. Varani, R. Mitra, Jr., and K. Gilbride. 1993. Retinoic acid stimulates mouse lung development by a mechanism involving epithelial-mesenchymal interaction and regulation of epidermal growth factor receptors. *Dev. Biol.* **159:** 462–473.
10. Maden, M., and M. Hind. 2004. Retinoic acid in alveolar development, maintenance and regeneration. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **359:** 799–808.
11. Jobe, A. H., and M. Ikegami. 2000. Lung development and function in preterm infants in the surfactant treatment era. *Annu. Rev. Physiol.* **62:** 825–846.

12. Massaro, D., and G. D. Massaro. 2002. Pre- and postnatal lung development, maturation, and plasticity: invited review. Pulmonary alveoli: formation, the "call for oxygen," and other regulators. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **282:** L345–L358.
13. Zachman, R. D., and M. A. Grummer. 2000. Retinoids and lung development. *In* Contemporary Endocrinology. Endocrinology of the Lung: Development and Surfactant Synthesis. R. D. Zachman and M. A. Grummer, editors. Humana Press, Inc., Totowa, NJ. 161–179.
14. McGowan, S. E. 2002. Contributions of retinoids to the generation and repair of the pulmonary alveolus. *Chest.* **121 (Suppl. 5):** 206–208.
15. Baybutt, R. C., L. Hu, and A. Molteni. 2000. Vitamin A deficiency injures lung and liver parenchyma and impairs function of rat type II pneumocytes. *J. Nutr.* **130:** 1159–1165.
16. Massaro, G. D., and D. Massaro. 1996. Postnatal treatment with retinoic acid increases the number of pulmonary alveoli in rats. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **270:** L305–L310.
17. Massaro, G. D., and D. Massaro. 2000. Retinoic acid treatment partially rescues failed septation in rats and in mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **278:** L955–L960.
18. Veness-Meehan, K. A., R. A. Pierce, B. M. Moats-Staats, and A. D. Stiles. 2002. Retinoic acid attenuates O_2-induced inhibition of lung septation. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **283:** L971–L980.
19. Bland, R. D., K. H. Albertine, R. A. Pierce, B. C. Starcher, and D. P. Carlton. 2003. Impaired alveolar development and abnormal lung elastin in preterm lambs with chronic lung injury: potential benefits of retinol treatment. *Biol. Neonate.* **84:** 101–102.
20. Shenai, J. P., K. A. Kennedy, F. Chytil, and M. T. Stahlman. 1987. Clinical trial of vitamin A supplementation in infants susceptible to bronchopulmonary dysplasia. *J. Pediatr.* **111:** 269–277.
21. Tyson, J. E., L. L. Wright, W. Oh, K. A. Kennedy, L. Mele, R. A. Ehrenkranz, B. J. Stoll, J. A. Lemons, D. K. Stevenson, C. R. Bauer, et al. 1999. Vitamin A supplementation for extremely-low-birthweight infants. National Institute of Child Health and Human Development Neonatal Research Network. *N. Engl. J. Med.* **340:** 1962–1968.
22. Darlow, B. A., and P. J. Graham. 2002. Vitamin A supplementation for preventing morbidity and mortality in very low birthweight infants. *Cochrane Database Syst. Rev.* CD000501.
23. Ambalavanan, N., J. E. Tyson, K. A. Kennedy, N. I. Hansen, B. R. Vohr, L. L. Wright, W. A. Carlo, and National Institute of Child Health and Human Development Neonatal Research Network. 2005. Vitamin A supplementation for extremely low birth weight infants: outcome at 18 to 22 months. *Pediatrics.* **115:** E249–E254.
24. Ambalavanan, N., T. J. Wu, J. E. Tyson, K. A. Kennedy, C. Roane, and W. A. Carlo. 2003. Comparison of three vitamin A dosing regimens in extremely-low-birth-weight infants. *J. Pediatr.* **142:** 656–661.
25. Shenai, J. P., M. G. Rush, M. T. Stahlman, and F. Chytil. 1992. Vitamin A supplementation and bronchopulmonary dysplasia—revisited. *J. Pediatr.* **121:** 399–401.
26. Zachman, R. D., B. Kakkad, and F. Chytil. 1984. Perinatal rat lung retinol (vitamin A) and retinyl palmitate. *Pediatr. Res.* **18:** 1297–1299.
27. Shenai, J. P., and F. Chytil. 1990. Vitamin A storage in lungs during perinatal development in the rat. *Biol. Neonate.* **57:** 126–132.
28. Shenai, J. P., and F. Chytil. 1990. Effect of maternal vitamin-A administration on fetal lung vitamin-A stores in the perinatal rat. *Biol. Neonate.* **58:** 318–325.
29. Dann, W. J. 1932. The transmission of vitamin A from parents to young in mammals. *Biochem. J.* **26:** 1072–1080.
30. Davila, M. E., L. Norris, M. P. Cleary, and A. C. Ross. 1985. Vitamin A during lactation: relationship of maternal diet to milk vitamin A content and to the vitamin A status of lactating rats and their pups. *J. Nutr.* **115:** 1033–1041.
31. Tanumihardjo, S. A., Muherdiyantiningsih, D. Permaesih, Komala, Muhilal, D. Karyadi, and J. A. Olson. 1996. Daily supplements of vitamin A (8.4 μmol, 8000 IU) improve the vitamin A status of lactating Indonesian women. *Am. J. Clin. Nutr.* **63:** 32–35.
32. Valentine, A. R., and S. A. Tanumihardjo. 2005. One-time vitamin A supplementation of lactating sows enhances hepatic retinol in their offspring independent of dose size. *Am. J. Clin. Nutr.* **81:** 427–433.
33. Rice, A. L., R. J. Stoltzfus, A. de Francisco, and C. L. Kjolhede. 2000. Low breast milk vitamin A concentration reflects an increased risk of low liver vitamin A stores in women. *Adv. Exp. Med. Biol.* **478:** 375–376.
34. Stoltzfus, R. J., and B. A. Underwood. 1995. Breast-milk vitamin A as an indicator of the vitamin A status of women and infants. *Bull. World Health Organ.* **73:** 703–711.
35. Humphrey, J. H., T. Agoestina, L. Wu, A. Usman, M. Nurachim, D. Subardja, S. Hidayat, J. Tielsch, K. P. West, Jr., and A. Sommer. 1996. Impact of neonatal vitamin A supplementation on infant morbidity and mortality. *J. Pediatr.* **128:** 489–496.
36. Rahmathullah, L., J. M. Tielsch, R. D. Thulasiraj, J. Katz, C. Coles, S. Devi, R. John, K. Prakash, A. V. Sadanand, N. Edwin, et al. 2003. Impact of supplementing newborn infants with vitamin A on early infant mortality: community based randomised trial in southern India. *BMJ.* **327:** 254.
37. Ross, A. C. 1986. Separation and quantitation of retinyl esters and retinol by high-performance liquid chromatography. *Methods Enzymol.* **123:** 68–74.
38. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
39. Auten, R. L., M. H. Whorton, and S. N. Mason. 2002. Blocking neutrophil influx reduces DNA damage in hyperoxia-exposed newborn rat lung. *Am. J. Respir. Cell Mol. Biol.* **26:** 391–397.
40. Simma, B., G. Luz, R. Trawoger, C. Hormann, G. Klima, A. Kreczy, and M. Baum. 1996. Comparison of different modes of high-frequency ventilation in surfactant-deficient rabbits. *Pediatr. Pulmonol.* **22:** 263–270.
41. McGowan, S., S. K. Jackson, M. Jenkins-Moore, H. H. Dai, P. Chambon, and J. M. Snyder. 2000. Mice bearing deletions of retinoic acid receptors demonstrate reduced lung elastin and alveolar numbers. *Am. J. Respir. Cell Mol. Biol.* **23:** 162–167.
42. Ross, A. C. 1982. Retinol esterification by mammary gland microsomes from the lactating rat. *J. Lipid Res.* **23:** 133–144.
43. Dowling, J. E., and G. Wald. 1960. The biological function of vitamin A acid. *Proc. Natl. Acad. Sci. USA.* **46:** 587–608.
44. Ross, A. C., and E. H. Harrison. 2006. Vitamin A and carotenoids. *In* Handbook of Vitamins. 4th edition. D. B. McCormick, R. R. Rucker, J. W. Suttieand, and J. J. Zempleni, editors. CRC Press, Boca Raton, FL. 16–31.
45. Wolbach, S. B., and P. R. Howe. 1925. Tissue changes following deprivation of fat-soluble A vitamin. *J. Exp. Med.* **42:** 753–777.
46. Sommer, A., and K. P. West, Jr. 1996. Vitamin A Deficiency: Health, Survival, and Vision. Oxford University Press, Inc., New York.
47. Cifelli, C. J., J. B. Green, and M. H. Green. 2005. Dietary retinoic acid alters vitamin A kinetics in both the whole body and in specific organs of rats with low vitamin A status. *J. Nutr.* **135:** 746–752.
48. Barua, A. B., S. E. McGowan, K. D. Ivanoff, B. C. Goswami, and J. A. Olson. 2004. Elevation of retinyl ester level in the lungs of rats following repeated intraperitoneal injections of retinoic acid or retinoyl glucuronide. *Pulm. Pharmacol. Ther.* **17:** 113–119.
49. Napoli, J. L., K. P. Posch, P. D. Fiorella, and M. H. E. M. Boerman. 1991. Physiological occurrence, biosynthesis and metabolism of retinoic acid: evidence for roles of cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP) in the pathway of retinoic acid homeostasis. *Biomed. Pharmacother.* **45:** 131–143.
50. James, M. L., A. C. Ross, A. Bulger, and N. Ambalavanan. 2005. Synergistic effect of vitamin A and retinoic acid in reducing neonatal hyperoxic lung injury (Abstract in Pediatric Academic Societies' Meeting 2005. Washington D.C., May 14–17, 2005. http://www.abstracts2002view.com/pas/).
51. Goodman, D. S., R. Blomstrand, B. Werner, H. S. Huang, and T. Shiratori. 1966. The intestinal absorption and metabolism of vitamin A and β-carotene in man. *J. Clin. Invest.* **45:** 1615–1623.