Effects of dietary retinoic acid on cellular retinol- and retinoic acid-binding protein levels in various rat tissues

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Abstract A study was conducted to explore the effects of retinoic acid, fed to retinol-deficient rats, on the tissue distribution and levels of cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP). Sensitive and specific radioimmunoassays were employed to measure the levels of both CRBP and CRABP. Two groups of six male rats each were fed a purified retinoid-deficient diet supplemented with either: *i*) retinyl acetate (control group); or *ii*) retinoic acid (30 mg/kg diet) (retinol deficient-retinoic acid group). The retinoic acid supplementation was begun after 38 days on the retinoid-deficient diet alone, and was continued for 52–54 days. Analysis of the data indicated that only the CRBP level of the proximal epididymis in the retinol-deficient/retinoic acid group differed significantly from (was lower than) the corresponding control level, at the 1% confidence level. CRABP tissue levels did not differ significantly between the two groups. Thus, a moderately large intake of retinoic acid, as the only source of retinoids, had very little effect on the tissue distribution or levels of either its own cellular binding protein (CRABP) or of CRBP. This study provides further information showing that the tissue levels of the cellular retinoid-binding proteins are highly regulated and maintained in rats, even in the presence of marked changes in retinoid nutritional status.—**Blaner, W. S., K. Das, J. R. Mertz, S. R. Das, and D. S. Goodman.** Effects of dietary retinoic acid on cellular retinol- and retinoic acid-binding protein levels in various rat tissues. *J. Lipid Res.* 1986. **27:** 1084–1088.

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Specific intracellular binding proteins for retinol and for retinoic acid are widely present in tissues throughout the body (1, 2). These cellular retinoid-binding proteins are thought to play important roles in retinoid metabolism and possibly in retinoid function as well (see refs. 1, 3 for recent reviews). Both cellular retinol-binding protein (CRBP) and cellular retinoic acid-binding protein (CRABP) have molecular weights close to 15,000 and single binding sites for one molecule of retinoid ligand (1).

Radioimmunoassay studies have provided information about the distribution and levels of CRBP (2, 4–6) and of CRABP (2, 5, 6) in rat tissues. However, very little information is available about the factors that regulate the levels of the cellular retinoid-binding proteins in different tissues. An extensive study was recently conducted in this laboratory that was designed to address the question of whether the amount of retinoid ligand affects the level of its corresponding binding protein (2). Four groups of male rats were fed diets that differed greatly in the amount and kind of retinoids provided. These groups were comprised of rats that were normal controls, retinoid-deficient, retinoic acid-fed, and excess retinol-fed. Totally retinoid-deficient rats showed reduced tissue levels of CRBP. Similar tissue CRBP levels were found in the rats in the other three diet groups. Tissue CRABP levels showed no diet-dependent differences, except for one tissue, the skin, where a difference was observed (lower CRABP in retinoid-deficient rats). The results of this study (2) suggested that tissue CRBP levels are influenced by diet and retinoid availability, whereas tissue CRABP levels appear to be minimally influenced by the amount or kind of retinoid ligand available.

In this recent study (2), the retinoic acid-fed rats were found to have low levels or retinol in serum at the time when they were killed, suggesting that the tissues of the rats in this group had not been fully depleted of retinol during the study. Thus, the conclusions about the effects of dietary retinoic acid, in the absence of retinol, on CRBP and CRABP levels were not certain. It was stated (2), that "future studies in which rats are maintained in a retinol-deficient state on retinoic acid for much longer periods of time would be of interest." We now report the results of such a study.

Abbreviations: CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; RBP, retinol-binding protein; HPLC, high performance liquid chromatography.

MATERIALS AND METHODS

Diets and animals

Male weanling rats (weights ranging from 40 to 52 g) of the Sprague-Dawley strain (Camm Research Institute, Wayne, NJ) were randomly divided into two groups of six rats each. The first group of rats was fed a purified, retinoid (vitamin A)-deficient diet (7) supplemented with 2.4 mg of retinol equivalents (in the form of retinyl acetate) per kg of diet for 90 days. The second group (henceforth referred to as the retinol-deficient/retinoic acid group) was fed the same retinoid-deficient diet but without any retinoid supplementation for a period of 38 days. This diet was then supplemented with a moderately large amount of retinoic acid (30 mg per kg of diet), and the rats were fed this supplemented diet for an additional 52 to 54 days. Rats were allowed free access to food and water; the weight of each rat in the two dietary groups was measured twice a week, throughout the study. The study was terminated after 90 days.

Preparation of tissues and radioimmunoassays for CRBP and CRABP

Tissues were collected and processed for radioimmunoassay exactly as described previously (2). CRBP and CRABP were measured by sensitive and specific radioimmunoassays, as described in detail elsewhere (2). CRBP and CRABP show no cross-reactivity with each other in these radioimmunoassays.

Retinol and RBP determinations

Serum and liver RBP levels were determined by radioimmunoassay as reported previously (8). Serum retinol was determined by high performance liquid chromatography (HPLC) as described by Bieri, Tolliver, and Catignani (9). The liver content of retinoid (retinol + retinyl esters) was determined by an HPLC procedure similar to that described by Amedee-Manesme, Furr, and Olson (10).

Statistical methods

The existence of diet-dependent differences in the measured tissue levels of CRBP and CRABP was tested by *t*-test (11). Since a large number of *t*-tests were being carried out (21 tissues for each of the two binding proteins), a difference was declared significant only when P was found to be less than 0.01 (1% level of confidence).

Analyses were also carried out to search for possible relationships between the levels of CRBP and CRABP in the different tissues. The results obtained with the 15 nonreproductive organs and tissues and with the 6 reproductive organs were analyzed separately. Within each group the binding protein levels in the individual tissues of each of the six rats were correlated by linear regression analysis, and the significance of the resulting correlation coefficient was determined (11) for the respective number of individual samples.

RESULTS

Retinoid nutritional status of the experimental animals

The two experimental diets used in this study differed greatly with regard to the kind and amount of retinoids they provided to the rats in the two diet groups. The retinoid nutritional status of the rats was assayed by monitoring their growth (**Fig. 1**) and by measuring their serum and liver levels of retinol and of RBP at the time of killing (**Table 1**). The control diet, containing 2.4 mg of retinol equivalents (as retinyl acetate) per kg of diet, supplied sufficient retinol to maintain the rats in normal retinoid status. The retinoid-deficient diet was liberally supplemented after 38 days with retinoic acid (30 mg per kg of diet) and the rats in this diet group were kept on this supplemented diet for another 52 to 54 days. The liver and serum levels of both retinol and RBP are given in Table 1 for the control and for the retinol-deficient/retinoic acid-supplemented rats. The liver and serum levels of both retinol and RBP measured in rats from the control group were within the normal range for these parameters (12, 13). As would be expected for fully retinol-deficient rats (12, 13), the retinol-deficient/retinoic acid-supplemented group pos-

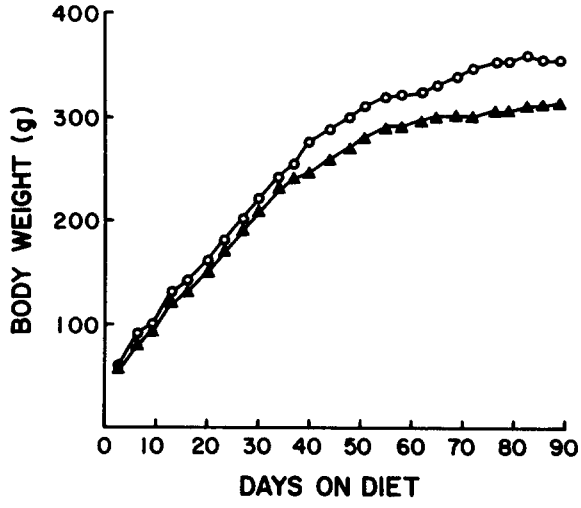

Fig. 1. Mean growth curves of rats from each of the two experimental groups, for the animals assayed for tissue CRBP and CRABP levels. The weights of the retinol-deficient/retinoic acid group were significantly below those of the control rats from day 77 onwards at the 1% confidence level (significant differences at the 5% confidence level were detected from day 62 onwards). (○) Indicates rats maintained on the control diet; (▲) indicates rats maintained on the retinol-deficient/retinoic acid-supplemented diet.

TABLE 1. Retinol and retinol-binding protein levels in serum and liver[a]

	Control	Retinol-Deficient/ Retinoic Acid
Serum retinol (μg/dl)[b]	40.3 ± 9.5	N.D.
Serum RBP (μg/ml)	40.8 ± 13.7	9.4 ± 2.5
Liver retinol (μg/g)[b]	81.5 ± 21.7	0.06 ± 0.01
Liver RBP (μg/g)	19.2 ± 3.7	107.4 ± 20.1
Rat weight (g)	360 ± 22.5	311 ± 26.1

[a]Values represent mean levels ± SD determined for all six experimental animals from each dietary group. Retinol and RBP were determined as described in Materials and Methods; N.D., not detected.

[b]Units of retinol are μg of retinol/dl serum and μg of retinol equivalents/g liver wet weight.

sessed serum retinol levels that were below the lower limits of detection in our assay. Serum RBP levels in these rats were very low, and liver RBP levels were elevated approximately fivefold over the control group. As seen in Fig. 1, the rats maintained on the retinoid-deficient diet began to gain weight less rapidly than the animals on the control diet from about day 28 onwards. During the fairly long period of retinoic acid supplementation, from day 38 onwards, the rats in this group grew at a slower rate than the control group, with significantly lower (at the 1% level) body weights observed starting at day 77 (at the 5% significance level, differences were seen from day 62 onwards). Nevertheless, these rats looked clinically healthy and did grow, albeit at a slower rate, throughout the period of retinoic acid supplementation.

Levels of CRBP in different tissues

The levels of CRBP measured in various organs and tissues from male rats maintained on the experimental (retinol-deficient/retinoic acid-supplemented) diet and on the control diet are listed in **Table 2**. As before (2), all organs and tissues examined, regardless of the diet, were observed to contain measurable levels of CRBP. The organs that were analyzed are grouped as being either nonreproductive or reproductive. The highest concentrations of immunoreactive CRBP found in the rats fed the control diet were in the proximal portion of the epididymis, the liver, and the kidney.

Only the proximal epididymis showed a statistically significant (at the 1% confidence level) effect of diet on CRBP levels. Thus CRBP levels in the proximal portion of the epididymis were significantly reduced (mean values of 19.4 vs. 65.1 μg/g) in rats fed the retinol-deficient/retinoic acid diet compared to controls. Kidney CRBP levels for the control group (mean 57 μg/g) and for the retinol-deficient/retinoic acid-supplemented group (mean 37 μg/g) differed from each other at the 5% confidence level. In view of the number of tissues being compared, however, the significance of a single difference at the 5% confidence level is uncertain (since for 21 *t*-tests, one difference at the 5% level would be expected by chance alone). The remaining tissues showed no diet-dependent differences in CRBP level.

Levels of CRABP in different tissues

The levels of CRABP measured in the tissue homogenates of the control and of the retinol-deficient/retinoic acid-supplemented rats are listed in **Table 3**. Measurable levels of CRABP were found in all organs and tissues examined. The highest concentrations of CRABP (20–70 μg/g wet weight) in the rats fed the control diet were found in the vas deferens, seminal vesicles, skin, and testes.

Statistical analyses of these data were conducted to test for significant diet-dependent differences in tissue CRABP levels. None of the tissues showed differences in CRABP levels between the two groups that were significant at the 1% confidence level. Only the testis showed a difference at the 5% level, between the control (mean 21.1 μg/g) and the retinol-deficient/retinoic acid diet groups (mean 8.6 μg/g). As indicated above, however, in view of the number of tissues being compared, the significance of a single difference at the 5% level is uncertain. None of the small differences in CRABP levels observed between the two diet groups with other tissues were statistically significant at the 5% confidence level.

TABLE 2. Tissue levels or CRBP[a]

Tissue	Control	Retinol-Deficient/ Retinoic Acid
	μg CRBP/g wet weight	
A. Nonreproductive		
Liver	52.2 ± 21.9	63.2 ± 27.3
Kidney	57.0 ± 16.2[b]	37.0 ± 7.9[b]
Lung	20.6 ± 9.7	18.8 ± 6.8
Lymph nodes	15.4 ± 3.4	15.5 ± 4.4
Eye	11.0 ± 6.3	8.3 ± 2.8
Spleen	6.4 ± 2.3	8.8 ± 3.3
Small intestine	10.2 ± 2.8	14.8 ± 4.8
Adrenals	5.7 ± 2.3	6.2 ± 1.0
Thymus	4.1 ± 1.8	4.0 ± 1.7
Brain	4.4 ± 3.4	2.9 ± 1.1
Stomach	4.4 ± 1.7	5.5 ± 1.8
Skin	1.8 ± 0.7	1.6 ± 0.9
Pancreas	2.1 ± 0.5	2.7 ± 1.1
Fat	0.8 ± 0.5	0.8 ± 0.3
Muscle	1.7 ± 1.4	1.0 ± 0.5
B. Reproductive		
Epididymis, proximal[c]	65.1 ± 14.2[d]	19.4 ± 6.6[d]
Testis	20.8 ± 6.6	15.3 ± 6.6
Epididymis, distal[c]	5.5 ± 1.5	5.7 ± 2.1
Prostate	3.4 ± 0.8	5.8 ± 4.9
Vas deferens	2.9 ± 0.8	2.0 ± 0.6
Seminal vesicle	2.1 ± 0.6	2.2 ± 0.6

[a]Values represent means ± SD for six independently determined samples. Samples were treated as described in Materials and Methods.

[b]Levels are different at the 5% confidence level.

[c]Epididymis, proximal, caput and proximal corpus; epididymis, distal, distal corpus and cauda (see Materials and Methods).

[d]Levels are significantly different at the 1% confidence level.

TABLE 3. Tissue levels or CRABP[a]

Tissue	Control	Retinol-Deficient/ Retinoic Acid
	μg CRABP/g wet weight	
A. Nonreproductive		
Liver	1.5 ± 0.5	1.7 ± 0.7
Kidney	2.2 ± 1.0	2.1 ± 1.2
Lung	0.5 ± 0.1	0.5 ± 0.2
Lymph nodes	7.6 ± 4.8	5.5 ± 3.1
Eye	3.1 ± 1.5	5.4 ± 2.3
Spleen	1.1 ± 0.4	1.2 ± 0.6
Small intestine	2.3 ± 1.5	2.2 ± 1.2
Adrenals	1.3 ± 0.8	1.5 ± 0.5
Thymus	3.5 ± 1.9	1.8 ± 0.8
Brain	3.1 ± 1.4	2.9 ± 0.8
Stomach	2.3 ± 1.5	2.3 ± 1.8
Skin	23.1 ± 12.0	24.3 ± 7.8
Pancreas	0.3 ± 0.1	0.4 ± 0.3
Fat	0.1 ± 0.0	0.1 ± 0.0
Muscle	0.2 ± 0.1	0.3 ± 0.1
B. Reproductive		
Epididymis, proximal[b]	0.9 ± 0.3	0.6 ± 0.1
Testis	21.9 ± 10.7[c]	8.6 ± 2.3[c]
Epididymis, distal[b]	13.1 ± 6.4	8.3 ± 2.8
Prostate	1.8 ± 1.1	2.6 ± 1.2
Vas deferens	71.6 ± 44.5	63.9 ± 31.5
Seminal vesicle	65.3 ± 24.6	89.9 ± 16.5

[a]Values represent means ± SD for six independently determined samples. Samples were treated as described in Materials and Methods.
[b]Epididymis, proximal, caput and proximal corpus; epididymis, distal, distal corpus and cauda (see Materials and Methods).
[c]Levels are different at the 5% confidence level.

Correlation between CRBP and CRABP levels

Linear regression analyses were carried out to search for possible relationships between the levels of CRBP and CRABP in the various tissues of the rats in the control and retinol-deficient/retinoic acid diet groups. The results of these analyses are shown in **Table 4**. No relationship was found between levels of the two binding proteins in the nonreproductive tissues. However, as was the case with the study of Kato et al. (2), CRBP and CRABP levels measured in the reproductive organs, of both the control and retinol-deficient/retinoic acid rats, were found to have a significant (at the 1% confidence level) inverse relationship.

DISCUSSION

The present study was designed to extend the recent report from our laboratory (2) on the effects of retinoid status on the tissue levels of CRBP and CRABP in the rat. The general goal of both the present and the previous (2) study has been to obtain information concerning the question of whether the amount or availability of a retinoid ligand affects the level of its corresponding intracellular binding protein. Our recent study (2) examined the effects of a control diet compared to a totally retinoid-deficient diet, a diet with retinoic acid as the only retinoid provided, and a diet with excess retinol (tenfold greater than that in the control diet). No differences in tissue CRBP levels were found in the rats fed the control, retinoic acid, or excess retinol diets. However, totally retinoid-deficient rats showed reduced CRBP levels in most of their tissues and organs. In contrast, tissue CRABP levels showed no diet-dependent differences except for the skin. In a more limited study, Eriksson et al. (6) measured CRBP levels by radioimmunoassay in the liver, kidney, testis, lung, spleen, thymus, and brain of normal and retinoic acid-fed rats. The rats maintained on the retinoic acid diet did not show significantly altered amounts of CRBP, as compared to control rats, in the tissues investigated. Unfortunately, the report of Eriksson et al. (6) did not indicate the age, length of time on the diets, or other data such as the serum and liver retinol levels of their experimental animals so that these two studies cannot be compared directly. Our previous (2) finding of reduced tissue CRBP levels in retinoid-deficient rats could not be clearly interpreted from the data available. One possibility was that in the absence of retinol, the specific ligand for CRBP, CRBP levels decline. An alternative possibility was that the reduced tissue CRBP levels reflected the totally retinoid-deficient state of the rats, rather than a specific response to the absence of retinol. We had hoped that the retinoic acid diet group would shed light on this issue, since the rats in this group were fed a retinoid-free diet supplemented only with retinoic acid. Unfortunately, however, the retinoic acid-fed rats were found not to be fully retinol-deficient at the time when they were killed. Thus, although the liver retinol levels of the retinoic acid group were extremely low, some retinol (mean 10.6 μg/dl) was still present in serum. Hence it was unclear whether tissue CRBP levels would decrease, as they did in totally retinoid-deficient rats, when these rats became fully retinol-deficient or whether dietary retinoic acid could prevent a decrease in tissue CRBP levels.

The results of the present study provide a clear answer to this question. In this study, the experimental group was maintained in a fully retinol-deficient state with an ample supply of retinoic acid in the diet. In general, the tissue CRBP levels of the retinol-deficient/retinoic acid-

TABLE 4. Relationship between tissue levels of CRBP and CRABP[a]

Diet Group, Organ/Tissues	Number of Samples (n)	Correlation Coefficient (r)
Control, nonreproductive	90	−0.099
Control, reproductive	36	−0.414*
Retinol-deficient/retinoic acid, nonreproductive	90	−0.125
Retinol-deficient/retinoic acid, reproductive	36	−0.481*

[a]Linear regression analysis gave the *r* values listed. Values marked * are statistically significant (at the 1% confidence level); the other *r* values were not significant at the 5% confidence level.

supplemented rats did not differ from those of the control rats. A statistically significant (at the 1% level) difference in CRBP levels between the two dietary groups was seen only in the proximal portion of the epididymis. Kidney CRBP levels in the two groups differed from each other at the 5% confidence level. The remaining 19 tissues or organs examined showed no diet-dependent difference in CRBP levels. These data indicate that in almost all tissues, the availability of retinol, the specific ligand which binds to CRBP, does not influence the tissue level of this binding protein. Taken together, the present and previous (2) results suggest that tissue CRBP levels are maintained provided that sufficient retinoids are available to maintain the animal (and the tissue) in a reasonably normal state. CRBP levels in most tissues, hence, appear to be independent of and not regulated by the amount of retinol ligand available.

The present study was also designed to explore whether feeding rather large amounts of retinoic acid to rats that were fully retinol-deficient would influence tissue levels of its binding protein (CRABP). Retinoic acid was provided at a level (30 mg/kg diet) that was 2.5 times that provided in our previous study (2), and at a level at least 30 times that needed to maintain rats in a clinically healthy state (14). Although we did not measure retinoic acid levels in serum or tissues, it is likely that most tissues of the rats on this diet received a relatively substantial supply of retinoic acid. Nevertheless, the tissue CRABP levels of the retinol-deficient/retinoic acid-fed rats did not differ from the levels in the corresponding tissues of the control rats. Thus, none of the tissues showed differences in CRABP levels between the two groups that were significant at the 1% confidence level. Only the testis showed a difference at the 5% level between the two groups. The other tissues or organs examined showed no diet-dependent differences in CRABP level. These results indicate that, in general, tissue CRABP levels are not influenced by the amount or availability of retinoic acid, the specific ligand which binds to CRABP.

A significant inverse relationship was found between CRBP and CRABP levels in the different tissues that comprise the male reproductive tract. This finding has been reported previously by Kato et al. (2). CRBP levels were found to be highest in the testis and proximal portion of the reproductive tract and decreased distally, whereas the opposite was true for CRABP. Although the biological significance of this observation still must be elucidated, this finding suggests that the two retinoid-binding proteins and their respective ligands both serve vital but different physiologic functions within the reproductive tract.

The findings of the present and previous (2) reports indicate that the amount and availability of a specific retinoid ligand (retinol or retinoic acid) does not importantly affect the tissue levels of its corresponding binding protein. Thus, the tissue levels of the cellular retinoid-binding proteins are highly regulated and maintained even in the presence of marked changes in retinoid nutritional status. The nature of the regulatory processes that control the tissue levels of CRBP and CRABP and the biomedical roles that these proteins play inside cells, remain to be determined.

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REFERENCES

1. Chytil, F., and D. E. Ong. 1984. Cellular retinoid-binding proteins. *In* The Retinoids. Vol. 2. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Academic Press, New York. 89–123.
2. Kato, M., W. S. Blaner, J. R. Mertz, K. Das, K. Kato, and D. S. Goodman. 1985. Influence of retinoid nutritional status on cellular retinol- and cellular retinoic acid-binding protein concentrations in various rat tissues. *J. Biol. Chem.* **260:** 4832–4838.
3. Chytil, F., and D. E. Ong. 1979. Cellular retinol- and retinoic acid-binding proteins in vitamin A action. *Federation Proc.* **38:** 2510–2514.
4. Adachi, N., J. E. Smith, D. Sklan, and D. S. Goodman. 1981. Radioimmunoassay studies of the tissue distribution and subcellular localization of cellular retinol-binding protein in rats. *J. Biol. Chem.* **256:** 9471–9476.
5. Ong, D. E., J. A. Crow, and F. Chytil. 1982. Radioimmunochemical determination of cellular retinol- and cellular retinoic acid-binding proteins in cytosols of rat tissues. *J. Biol. Chem.* **257:** 13385–13389.
6. Eriksson, U., K. Das, C. Busch, H. Nordlinder, L. Rask, J. Sundelin, J. Sällström, and P. A. Peterson. 1984. Cellular retinol-binding protein: quantitation and distribution. *J. Biol. Chem.* **259:** 13464–13470.
7. Navab, M., J. E. Smith, and D. S. Goodman. 1977. Rat plasma prealbumin. Metabolic studies on effects of vitamin A status and on tissue distribution. *J. Biol. Chem.* **252:** 5107–5114.
8. Smith, J. E., D. D. Deen, Jr., D. Sklan, and D. S. Goodman. 1980. Colchicine inhibition of retinol-binding protein secretion by rat liver. *J. Lipid Res.* **21:** 229–237.
9. Bieri, J. G., T. J. Tolliver, and G. L. Catignani. 1979. Simultaneous determination of alpha-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. *Am. J. Clin. Nutr.* **32:** 2143–2149.
10. Amedee-Manesme, O., H. C. Furr, and J. A. Olson. 1984. The correlation between liver vitamin A concentrations in micro- (needle biopsy) and macrosamples of human liver specimens obtained at autopsy. *Am. J. Clin. Nutr.* **39:** 315–319.
11. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. The Iowa State University Press, Ames, IA.
12. Muto, Y., J. E. Smith, P. O. Milch, and D. S. Goodman. 1972. Regulation of retinol-binding protein metabolism by vitamin A status in the rat. *J. Biol. Chem.* **247:** 2542–2550.
13. Smith, J. E., Y. Muto, and D. S. Goodman. 1975. Tissue distribution and subcellular localization of retinol-binding protein in normal and vitamin A-deficient rats. *J. Lipid Res.* **16:** 318–323.
14. Goodman, D. S., J. E. Smith, R. M. Hembry, and J. T. Dingle. 1974. Comparison of the effects of vitamin A and its analogs upon rabbit ear cartilage in organ culture and upon growth of the vitamin A-deficient rat. *J. Lipid Res.* **15:** 406–414.