Cellular Retinol-binding Protein-supported Retinoic Acid Synthesis

RELATIVE ROLES OF MICROSONES AND CYTOSOL

(Received for publication, July 27, 1995, and in revised form, December 28, 1995)

Manja H. E. M. Boerman and Joseph L. Napoli†

From the Department of Biochemistry, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, New York 14214

This study shows that microsomal retinol dehydrogenases, versus cytosolic retinol dehydrogenases, provide the quantitatively major share of retinal for retinoic acid (RA) biogenesis in rat tissues from the predominant substrate available physiologically, holo-cellular retinol-binding protein, type I (CRBP). With holo-CRBP as substrate in the absence of apo-CRBP microsomal retinol dehydrogenases have the higher specific activity and capacity to generate retinal used for RA synthesis by cytosolic retinal dehydrogenases. In the presence of apo-CRBP, a potent inhibitor of cytosolic retinol dehydrogenases (IC \(_{50}\) = \(1 \mu M\)), liver microsomes provide 93% of the total retinal synthesized in a combination of microsomes and cytosol. Cytosolic retinol dehydrogenase(s) and the isozymes of alcohol dehydrogenase expressed in rat liver had distinct enzymatic properties; yet ethanol inhibited cytosolic retinol dehydrogenase(s) (IC \(_{50}\) = 20 \(\mu M\)) while stimulating RA synthesis in a combination of microsomes and cytosol. At least two discrete forms of cytosolic retinol dehydrogenase were observed: NAD- and NADP-dependent forms. Multiple retinal dehydrogenases also were observed and were inhibited partially by apo-CRBP. These results provide new insights into pathways of RA biogenesis and provide further evidence that they consist of multiple enzymes that recognize both liganded and nonliganded states of CRBP.

The metabolism of retinol (vitamin A) generates RA, \(^1\) a humoral factor critical to vertebrate development (1, 2). In the developing embryo, RA transcriptionally regulates genes that specify body axis pattern and may help program limb formation (3). In mature vertebrates, RA maintains epithelial tissues (preventing squamous cell metaplasia), contributes to bone remodeling, and sustains reproductive processes, including the estrus cycle, spermatogenesis, and placental growth. RA aberrant in concentration, locus, or developmental stage causes teratism and/or toxicity of the central nervous system and skeleton (4–7). RA acts through ligand-activated receptors that comprise two distinct subfamilies of the steroid hormone superfamily of receptors (8–11). These receptors modify transcription as homodimers or by modifying the effects of other receptors through heterodimerization. These pervasive and fundamental effects of RA, as well as the consequences of its aberrant distribution, imply that its biosynthesis must be regulated closely.

In many tissues, unesterified retinol occurs bound to CRBP. Thus, holo-CRBP may provide the most abundant substrate for RA biosynthesis. Direct transfer of retinol between holo-CRBP and enzymes that catalyze RA synthesis would circumvent uncontrolled diffusion of retinol through the aqueous phase and would participate in controlling the pathways of RA synthesis by protecting retinol from opportunistic reactions catalyzed by enzymes that do not recognize the high affinity, high specificity CRBP. Recent work has outlined a pathway of RA biosynthesis with the first step catalyzed by a NADP-dependent microsomal retinol DH, expressed in liver and in extrapatic tissues, that recognizes holo-CRBP as substrate (12–15). Microsomes have low retinal DH activity and do not convert the retinol produced from holo-CRBP into RA at high rates. Rather, microsome-produced retinol undergoes conversion into RA by cytosolic retinol DHs, which can interact with CRBP-retinol complexes (16).

During initial characterization of microsomal retinol DH, we also observed cytosolic retinol DH activity that was inhibited markedly by apo-CRBP and subsequently showed that the inhibition might not result solely from sequestering free retinol, because of ambiguities in the \(K_d\) value for CRBP (12, 17). Inhibition could result from apo-CRBP interacting with a cytosolic enzyme that recognized holo-CRBP as substrate. This suggested the presence of at least two pathways for generating RA from holo-CRBP: one involving retinal synthesis in microsomes and the other involving retinal synthesis in cytosol. In either pathway, subsequent conversion of the retinal into RA occurs in the cytosol. Ottonello and co-workers have analyzed cytosolic retinol DH activity further and concluded that it catalyzes considerable RA synthesis from holo-CRBP in the absence of apo-CRBP (18). The relative contributions, however, of microsomes and cytosol to overall cellular RA synthesis remain uncertain, as well as the interactions between these two pathways and/or their effects on each other.

This work compares the contributions of microsomal and cytosolic retinol DH activities to RA synthesis and characterizes interactions that occur during RA biosynthesis between cytosol and microsomes. With holo-CRBP as substrate in the absence of apo-CRBP, microsomal retinol DH has the higher specific activity and capacity (60–83% of the total of cytosol plus microsomes, individually) to generate retinal for RA synthesis in the four tissues assayed. In the presence of apo-CRBP, the more likely condition in vivo, the microsomal contribution increased to 80–94% of the total retinal synthesized. In separating the activity of rat liver cytosolic retinol DH from that of the ADH isozymes expressed in rat liver, ADH-2 and ADH-3, we demonstrate further that ethanol inhibits cytosolic retinol
DH activity. We also show that at least two forms of cytosolic retinol DH occur and that apo-CRBP inhibits not only cytosolic retinol DH activity but also conversion of retinal into RA. Thus, we establish here that multiple paths and complex interactions contribute to RA biosynthesis.

**Experimental Procedures**

General—Retinoids were purchased from Eastman Kodak and were purified by HPLC (19, 20). Subcellular fractions from rat liver, kidney, testis, and lung were prepared from male Sprague-Dawley rats (~150 g) as described previously (12). Protein was determined by the dye-binding method with bovine serum albumin as standard (21). Kinetic data were fit to standard kinetic equations with the microcomputer program “Enzfitter” (22).

CRBP—CRBP saturated with all-trans-retinol was prepared and purified from CRBP generated in Escherichia coli with the vector pMONCRBP (23) as described previously (12, 16). Purified holo-CRBP had an A\textsubscript{280}/A\textsubscript{360} ratio of 1.4 and was stored in 20 mM Hepes, 150 mM KCl, 2 mM dithiothreitol, and 1 mM EDTA, pH 7.5 (100 nmol/ml) at 70 °C. Apo-CRBP was prepared and purified just as holo-CRBP, omitting saturation with retinol. The concentration of functional apo-CRBP was determined by saturating an aliquot with retinol, separating free and bound retinol by size exclusion chromatography and determining the A\textsubscript{280}/A\textsubscript{360} ratio.

Retinol DH Assays—Assays were done in duplicate (duplicates were within 10% of their averages) at 37 °C in 250 μl of 10 mM Hepes, 150 mM KCl, and 2 mM EDTA, pH 8, with 2 mM NADP for microsomes and 2 mM each of NADH and NADP for cytosol, the combination of microsomes and cytosol, and for the 10,000 × g supernatant (10kS) for the times specified in the figure legends. The concentrations of holo-CRBP and apo-CRBP were 5 and 2 μM, respectively (these represent physiological concentrations in rat liver), unless noted otherwise. Reactions were quenched with 1 ml of 0.025 N KOH/ethanol. Retinol was extracted with 2.5 ml of hexane (hex-1). To obtain RA, the alkaline phase was acidified to pH 4 in 4N HCl and extracted with 2.5 ml of hexane (hex-2). The solvents from hex-1 and hex-2 were evaporated under a stream of nitrogen. Each residue was dissolved in 100 μl of hexane and analyzed by HPLC (16, 20). Retinol in hex-1 and RA in hex-2 were quantified by HPLC (16, 20). Retinol in hex-1 and RA in hex-2 were quantified by HPLC (16, 20). Retinal in hex-1 and RA in hex-2 were quantified by HPLC (16, 20).

**Inhibition of RA Synthesis in the 10kS by Higher Concentrations of Apo-CRBP**

In the combination of cytosol and microsomes, retinal concentration that inhibited cytosolic RA synthesis 50%, suggested that cytosolic retinol DH activity was not contributing substantially to RA synthesis in the 10kS, i.e. in the presence of microsomes. The relatively apo-CRBP-resistant microsomal retinol DH, therefore, must be providing retinal for RA synthesis by the cytosolic retinol DH component of the 10kS. Inhibition of RA synthesis in the 10kS by higher concentrations of apo-CRBP may result from a combination of inhibition of the microsomal retinol DH (12) and a decrease in the conversion of retinol into RA by cytosol (see below).

Comparison of Microsomal and Cytosolic RH DHs—Retinal (Fig. 2, top panel) and RA (Fig. 2, bottom panel) syntheses were measured in cytosol, microsomes, and a combination of cytosol and microsomes with respect to time. In the absence of apo-CRBP (open symbols), the amounts of retinal produced by microsomes alone and RA produced by cytosol alone increased throughout the two hr of the reaction and were about equivalent. Because the cytosolic protein concentration was 5-fold greater than that of microsomes, however, the rate of microsomal retinol synthesis exceeded that of cytosolic RA synthesis. In the combination of cytosol and microsomes, retinal concentrations reached a plateau in 15 min of ~40 pmol and declined after 60 min (data not shown), and RA production exceeded retinal at all times. This illustrates use of the retinal generated from holo-CRBP by microsomes as substrate for cytosolic RA synthesis. Also, after 30 min, RA production in the combination exceeded RA production by cytosol alone.

In the presence of 2 μM apo-CRBP (Fig. 2, top panel, filled holocrp between 0.1 and 10 μM to be (± S.E.): K\textsubscript{m} (μM) 0.4 ± 0.004, 1.5 ± 0.4, 1.2 ± 0.02 for 0, 0.5 and 2 μM apo-CRBP, respectively; V\textsubscript{max} (pmol/min/mg of protein) 4 ± 0.01, 2.3 ± 0.17, 1.9 ± 0.01 for 0, 0.5 and 2 μM apo-CRBP, respectively.

In contrast to cytosol, 1 μM apo-CRBP did not inhibit RA synthesis by the 10kS, which includes cytosol and microsomes, and 50% inhibition of the 10kS required 5 μM apo-CRBP, a concentration equal to that of the holo-CRBP substrate. Lack of inhibition of RA synthesis in the 10kS by 1 μM apo-CRBP, a concentration that inhibited cytosolic RA synthesis >50%, suggests that cytosolic retinol DH activity was not contributing substantially to RA synthesis in the 10kS, i.e. in the presence of microsomes. The relatively apo-CRBP-resistant microsomal retinol DH, therefore, must be providing retinal for RA synthesis by the cytosolic retinol DH component of the 10kS. Inhibition of RA synthesis in the 10kS by higher concentrations of apo-CRBP may result from a combination of inhibition of the microsomal retinol DH (12) and a decrease in the conversion of retinol into RA by cytosol (see below).
symbols), the amounts of retinal produced by microsomes alone were comparable quantitatively throughout the incubation to the retinal produced by microsomes in the absence of apo-CRBP. RA production by cytosol, however, was below detection limits before 30 min, when it reached a plateau, which on the average was only 13% of the amount of RA observed in the absence of apo-CRBP (Fig. 2, bottom panel). In the combination of microsomes and cytosol, retinal concentrations reached a plateau of ~30 pmol and declined after 60 min (data not shown). RA production by the combination increased throughout the incubation and at all times exceeded RA production by cytosol alone, even though the microsomal protein added to the combination was 5-fold less than that of the cytosolic protein present.

The specific activities of microsomal and cytosolic retinol DHs were also compared in the absence (open symbols) and the presence (filled symbols) of apo-CRBP (Fig. 3). Microsomal retinol DH was linear to 0.1 mg of protein and generated retinal from 5 μM holo-CRBP at rates of ~23 pmol/min/mg protein in the absence of apo-CRBP and ~15 pmol/min/mg protein in the presence of 2 μM apo-CRBP. In the absence of apo-CRBP, cytosolic retinol DH activity was linear to 0.25 mg of protein and functioned at a rate of ~4 pmol/min/mg of protein, i.e. ~6-fold less than the rate due to microsomal retinol DH. In the presence of 2 μM apo-CRBP, cytosolic retinol DH activity was not observed below 0.25 mg of protein but was linear between 0.25 and 1 mg of protein. The rate was ~0.33 pmol/min/mg of protein or ~50-fold less than that of microsomal retinol DH in the presence of apo-CRBP.

RA Synthesis during Titration of Microsomes into Cytosol—Titration of microsomes into cytosol was examined for its impact on RA synthesis from holo-CRBP (Fig. 4, top panel). In the absence of apo-CRBP (open circles), cytosol alone produced 20 pmol of RA at a rate of ~3 pmol/min/mg of protein. The addition of microsomes (10 μg of protein) nearly doubled the quantity of RA produced to 39 pmol. The rate of RA production supported by the added microsomal retinol DH, however, was ~63 pmol/min/mg of protein, ~20-fold higher than that of cytosolic retinol DH. In the presence of apo-CRBP (filled circles), RA production was not detected in cytosol alone. Furthermore, at least 50 μg of microsomal protein were required before RA synthesis was observed, which occurred at a lower rate (~4 pmol/min/mg of microsomal protein) than in the absence of apo-CRBP.

Inhibition of RA Production from Retinal by apo-CRBP—During the titration of microsomes into cytosol, in the absence of CRBP, retinal was not observed until the microsomal protein reached 0.1 mg, (Fig. 4, bottom panel, open circles). From 0.1 mg of microsomal protein to 0.4 mg of protein, retinal steadily increased, indicating that microsomal retinal production outpaced its cytosolic conversion into RA. In the presence of apo-CRBP (filled circles), RA production was not detected in cytosol alone. Furthermore, at least 50 μg of microsomal protein were required before RA synthesis was observed, which occurred at a lower rate (~4 pmol/min/mg of microsomal protein) than in the absence of apo-CRBP.

3 The rate calculation used for cytosolic retinol DH was 20 pmol of RA/30 min/0.25 mg of cytosolic protein. This amount of protein was used in the calculation rather than the 0.35 mg actually present in the experiment because 0.25 mg of cytosolic protein ends the linear range of cytosolic retinol DH (see Fig. 2). The rate calculation used for microsomal retinol DH was 19 pmol/30 min/0.01 mg.
what had occurred in the absence of apo-CRBP. Moreover, markedly more retinal accumulated than in the absence of apo-CRBP. Overall, the data of Fig. 4 suggest that apo-CRBP not only inhibits cytosol retinol DH but also diminishes the conversion by cytosol of retinal into RA.

To test the possibility that apo-CRBP inhibits the conversion of retinal into RA, cytosolic RA production was monitored from retinal bound to CRBP in the presence of increasing concentrations of apo-CRBP (Fig. 5). An apo-CRBP concentration 50% of the CRBP/retinal concentration inhibited <20% (filled circles). An apo-CRBP concentration equal to the CRBP/retinal concentration of 0.5 μM caused the maximum inhibition of 50%. Apo-CRBP up to 9-fold greater than CRBP/retinal resulted in no additional inhibition. These data indicate that CRBP/retinal acted as substrate for RA synthesis, because the concentrations of unbound retinal and the rate of RA synthesis did not coincide, i.e. the rate of retinal synthesis was not dependent on the concentration of unbound retinal. The decrease in RA synthesis with increasing apo-CRBP, therefore, was caused by inhibition of retinal dehydrogenation by apo-CRBP. This phenomenon probably contributes to the inhibition of RA synthesis in the 10kS by the higher concentrations of apo-CRBP (see above).

Relative Contributions of Retinol DHs to RA Synthesis—The specific activities of microsomal retinol DH, assayed in the absence of apo-CRBP ranged from 5- to 22-fold greater than those of cytosolic retinol DH in liver, kidney, lung, and testis (Table I). At least 80% of the total retinol DH units were microsomal in these tissues, except in testis, where 60% of the total retinol DH units were microsomal. In the presence of apo-CRBP, the specific activity of microsomal retinol DH ranged from 13- to 60-fold greater than cytosolic retinol DH. Under these conditions, greater than 90% of the units were microsomal, except in testis where 80% of the units were microsomal. Because ethanol inhibits cytosolic retinol DH but not microsomal retinol DH (see below), it was used to provide further insight into the contribution of retinal generated by cytosol to RA synthesis (Fig. 6). Ethanol (hatched bars) inhibited by 90% cytosolic RA synthesis supported by holo-CRBP (compare bars 1 and 2) but stimulated ~40% retinal synthesis in microsomes (bars 3 and 4) and in a combination of microsomes and cytosol (bars 5 and 6). These data indicate that ethanol inhibits the cytosolic retinol DH, but neither ethanol nor its metabolite acetaldehyde inhibits the cytosolic retinal DHs and also that cytosolic retinol DHs do not discriminate against cycosic or microsomal produced retinal. Most importantly, these data show that in the combination of cytosol and microsomes, inhibition of cytosolic retinol DH does not diminish RA synthesis.

Inhibition of Retinol DHs—Microsomal and cytosolic retinol DHs were affected differently by inhibitors (Fig. 7, Table II). Ethanol, octanol, and pentanol, substrates for various medium chain (or classical) alcohol dehydrogenase isozymes, were potent inhibitors of cytosolic retinol DH activity with IC_{50} values <20 μM; even at 10 mM, however, these alcohols did not inhibit microsomal retinol DH. The nonalcoholic solvents Me2SO and dimethylformamide used in these assays were evaluated to test whether inhibition of cytosolic retinol DH resulted from a solvent effect. Although both solvents did inhibit cytosolic retinol DH, inhibition was never >50% and required a much higher concentration than ethanol inhibition: 0.4% (v/v) ethanol caused >90% inhibition; 2% (v/v) of Me2SO or dimethylformamide caused 50% inhibition. Neither Me2SO nor dimethylformamide inhibited microsomal retinol DH. In contrast to the alcohol substrates, carbenoxolone, an inhibitor of short chain alcohol dehydrogenases (26, 27), was an equally potent inhibitor of both microsomal and cytosolic DHs.

The interactions of these compounds with ADH-2 and ADH-3, the two isozymes of the medium chain alcohol dehy-
Microsomal and Cytosolic Retinoic Acid Biosynthesis

Rates (pmol/min/mg of protein) of microsomal and cytosolic retinol DH activities were calculated from linear amounts of protein and time with 5 μM holo-CRBP in the absence and the presence of 2 μM apo-CRBP. Units were calculated from total protein/subcellular fraction of pooled tissues from 20 male Sprague-Dawley rats (150 g each) and are expressed as pmol/min. The relative contribution of each retinol DH to total retinal production is listed under %.

| Tissue | apo-CRBP | Microsomes | Cytosol |
|--------|----------|------------|---------|
|        |          | Rate       | %       |
| Liver  | –        | 17.8       | 83      |
|        | +        | 14.7       | 93      |
| Kidney | –        | 9.6        | 83      |
|        | +        | 5.1        | 94      |
| Testis | –        | 5.1        | 60      |
|        | +        | 3.5        | 80      |
| Lung   | –        | 8.5        | 80      |
|        | +        | 4.8        | 91      |

**Fig. 6.** Effect of ethanol on retinol metabolism in cytosol, microsomes, and a combination of cytosol and microsomes. Cytosol (bars 1 and 2, 0.2 mg of protein) or a combination of microsomes and cytosol (bars 3 and 4, 0.1 and 0.2 mg of protein, respectively) were assayed for RA synthesis or microsomes were assayed for retinal synthesis (bars 3 and 4, 0.1 mg of protein) from 5 μM holo-CRBP in the absence of ethanol (open bars) or in the presence of 250 mM ethanol (2.5% v/v; striped bars) for 30 min. The error bars represent S.D. of 4 replicates. * all of the ethanol-added groups were significantly different from the control without ethanol (p < 0.001).

**Fig. 7.** Inhibition of cytosolic retinol DH. IC50 values were determined for inhibition of cytosolic retinol DH as described under "Experimental Procedures"; pentanol added in Me2SO (filled triangles); octanol added in Me2SO (open circles); pentanol (open triangles); and ethanol (filled circles). In the assays with pentanol and octanol added in Me2SO, 1 μl of Me2SO was added to each tube. Me2SO was used because octanol was not miscible with water. Because Me2SO itself causes some inhibition, pentanol was tested in the presence and the absence of Me2SO so that the octanol data could be related to the results with pentanol and ethanol. Each point is the average of duplicate experiments.

**Table I**

| Agent          | IC50 (μM) | Km or Ki (μM) |
|----------------|-----------|---------------|
| Ethanol        | none^a    | ~20 μM        |
| Pentanol       | none^a    | ~20 μM        |
| Octanol        | none^a    | ~12 μM        |
| 4-Methylpyrazole| none^a    | 180 μM        |
| Carbenoxolone  | 55 μM     | >10 μM        |

^a None, no inhibition at 10 μM.
^b These are Km or Ki values obtained from Julia et al. (24).
^c NS, not saturable.

**Table II**

| Agent          | IC50 (μM) | Km or Ki (μM) |
|----------------|-----------|---------------|
| Ethanol        | none^a    | ~20 μM        |
| Pentanol       | none^a    | ~20 μM        |
| Octanol        | none^a    | ~12 μM        |
| 4-Methylpyrazole| none^a    | 180 μM        |
| Carbenoxolone  | 55 μM     | >10 μM        |
creasing the production of RA (Figs. 1 and 6). For example, cytosolic retinol DH and microsomal retinol DH without de-

Table III

| Cytosol | NAD | NADP |
|---------|-----|------|
| holo-CRBP | pmol RA/min/mg of protein |
| 5 | 44 |
| 1 + EtOH | 0.8 |
| 2 | 5.8 |
| III | 3.4 |
| IV | 3.4 |

- 2% ethanol (v/v) was added to the standard incubation (340 mM).

similar rates. Although two preparations produced RA with NADP alone with rates 61 and 97% of those supported by NAD, two of the pools had greatly reduced RA production, i.e. 15 and 9% of the rates supported by NAD. Furthermore, retinal did not accumulate in these pools that had low NADP-supported activity, indicating the decreased level of an NADP-dependent cytosolic retinol DH. This inference is consistent with the occurrence of at least two cytosolic retinol DHs with different pyridine nucleotide specificities. Moreover, the cytosolic retinol DH activity supported by NAD was inhibited to a greater extent by ethanol (82% inhibition) than was the NADP-supported activity (30% inhibition). This result provides further support for the presence of two retinol DHs.

The generation of RA in cytosol from holo-CRBP with NAD as the sole cofactor indicated that NADP alone supports cyto-
solic retinal synthesis. Consistent with this, cytosolic prepara-
tions without the NADP-supported cytosolic retinol DH activ-
ity, as well as cytosol with the NADP-supported cytosolic retinol DH activity, converted retinal into RA with NADP as sole cofactor (Table IV). Ethanol had a modest or no effect on the rates of RA synthesis from CRBP/retinal.

DISCUSSION

This work demonstrates the primary contribution quantita-
tively of microsomal retinol DH to RA biosynthesis relative to cytosolic retinol DH activity. Not only was microsomal retinol DH severalfold greater in specific activity than cytosolic retinol DH activity in a comparison of subcellular fractions from four rat tissues, it had a greater capacity to generate retinal (the majority of enzyme units) and was relatively resistant to inji-
tion by apo-CRBP. Most revealing were the data showing that cytosolic retinol DH could be inhibited in a mixture of cytosolic retinol DH and microsomal retinol DH without de-
creasing the production of RA (Figs. 1 and 6). For example, 1 μM apo-CRBP inhibited cytosolic retinol DH 50% but had no effect on the amount of RA produced by the 10kS fraction, whereas ethanol caused 90% inhibition of cytosolic retinol DH but enhanced RA production in a combination of cytosol and microsomes, similar to its effect on retinal synthesis in micro-
somes. These results are consistent with either cytosolic retinol DH not functioning in the presence of microsomal retinol DH or microsomal retinol DH compensating for the quantitatively minor production of retinal by cytosolic retinol DH in cases of cytosolic retinol DH inhibition. Notably, under conditions likely to prevail physiologically, i.e. a mixture of holo-CRBP and apo-CRBP with concentrations in the range of ~5 and ~2 μM, respectively, microsomal retinol DH accounted for >90% of the retinal-generating capacity in three of the four tissues screened.

Because there is a RA response element in the CRBP gene (28) and RA induces CRBP expression in vivo (29), Ottone et al. have proposed that generation of CRBP by RA may provide apo-CRBP as a signal in a feedback loop to inhibit RA synthesis from cytosolic retinol DH (18). This hypothesis has limitations. Firstly, CRBP may affect the amount of retinol sequestered by cells; as CRBP increases, the concentration of holo-CRBP could increase as long as plasma retinol were available (30). Generation of CRBP, therefore, does not necessarily result in elevated concentrations of apo-CRBP. It seems more reasonable that RA acts as an on/off signal inducing constitutive expres-
sion of CRBP rather than acutely regulating relatively modest changes in the concentration of CRBP. The “feedback” loop hypothesis encounters another problem during vitamin A de-
pletion. During times of less than optimal blood and cell retinol concentrations, RA synthesis would need to continue efficiently to generate the active humoral agent, even though substrate concentrations were diminishing. Potent inhibition of cytosolic retinol DH during vitamin A deple-
tion, when apo-CRBP concentrations would increase, appears to be counterproductive to RA generation at a time when RA generation would be needed.

Lack of an obvious role for cytosolic retinol DH in RA gener-
ation, because it would be inhibited under normal conditions by the apo-CRBP present, doesn’t imply cytosolic retinol DH has no role; perhaps cytosolic retinol DH reduces retinal generated by β-carotene metabolism into retinoid. Such a function would make sense with respect to the sensitivity of cytosolic retinol DH to inhibition by apo-CRBP. If cytosolic retinol DH were a reductase, inhibiting it during vitamin A deple-
tion would provide increased retinal from β-carotene for conversion into RA. Additional work will ultimately address these issues.

This and previous works have demonstrated unequivocally that microsomal retinol DH does not belong to the medium chain ADH family (12–15). This work has also distinguished between the two known rat liver ADH isozymes and cytosolic retinol DH. Differences in affinities for apo-CRBP, carbenox-
olone, and 4-methylpyrazole distinguish cytosolic retinol DH and ADH-3, whereas differences in affinities for apo-CRBP, carbenoxolone, and the short chain alcohols (ethanol, pentanol, and octanol) distinguish cytosolic retinol DH and ADH-2. These results are consistent with our previous results obtained using 4-methylpyrazole to study RA synthesis from retinol not bound with CRBP in cytosols prepared from rat tissues and from the tissues of ADH^- and ADH^- deer mice (31–33). 4-Methylpyra-
zole inhibited potent (>94%) the conversion of unbound retin-
ol into RA catalyzed by liver cytosol from rat or ADH^- deer-
mouse. In cytosol from the ADH^- deer mouse, however, which showed only 13% of the retinol DH activity of the ADH^- deer-
mouse, 4-methylpyrazole was a much less effective inhibitor. Thus, these previous data had demonstrated the presence of a quantitatively minor retinol DH activity in liver cytosol that was not affected markedly by 4-methylpyrazole. The present data suggest that the cytosolic retinol DH activity that recog-
nizes holo-CRBP as substrate substantially accounts for this 4-methylpyrazole-insensitive DH. The earlier report by Ot-
tonello et al. (18) had concluded that cytosolic retinol DH was distinct from ADH, in part because 1 mM pyrazole did not
inhibit cytosolic retinol DH; however, 1 mM pyrazole does not inhibit ADH-2 (24). On the other hand, carbonoxolone inhibition characterizes several short chain dehydrogenases/reductases but not the medium chain ADHs (26, 27). Whether cytosolic retinol DH, like microsomal retinol DH, belongs to the short chain superfamily remains to be established.

There has been some speculation that an ADH isozyme may contribute to RA biosynthesis, in addition to metabolizing various xenobiotic and endogenous long and short chain alcohols. The proposed isozyme has been revised, however, as evidence has eliminated candidates. There are convincing arguments against this hypothesis, however, that include the requirement for control and specificity in RA biogenesis; the occurrence of holo-CRBP as the predominant substrate available in vivo; the ability of cytosol of the ADH-2 deermouse mutant to convert retinol into RA (32, 33), and the expression of distinct enzymes (microsomal and cytosolic retinol DHs) with extraordinary built in specificity, i.e. they recognize holo-CRBP as substrate (12–15).

Ethanol inhibits cytosolic retinol DH potently despite differences between cytosolic retinol DH and the known liver ADH isozymes. Thus, this is the first demonstration of ethanol inhibition of a retinol DH, identified by its affinity for holo-CRBP (0.4 μM K_m, rat liver cytosolic retinol DH, this work; 0.8 μM K_m, calf liver cytosolic retinol DH, Ref. 18). The impact, if any, however, of ethanol consumption on the retinoid humoral system cannot be predicted from this observation, especially because ethanol stimulates RA biogenesis in microsomes and in a combination of microsomes and cytosol. Certainly chronic excessive ethanol consumption decreases hepatic vitamin A stores (34, 35), and alcoholism leads to testicular atrophy and loss of testes function (36–38), similar to vitamin A deficiency (39, 40). Because of these observations, the possibility has been proposed that the medium chain ADHs catalyze retinol metabolism in vivo and thereby ethanol may competitively inhibit retinol metabolism (34, 35). For the reasons discussed above, the potential involvement physiologically of an ADH isozyme in retinol metabolism remains in question. But our data suggest an alternative model, namely, that a cytosolic retinol DH, which is not a known ADH, is present and is inhibited by low concentrations of ethanol.

Another significant result was the variability among different batches of rat liver cytosol in the NADP-supported cytosolic retinol DH-catalyzed reaction in contrast to the constancy of the NAD-supported reaction. This suggests two or more cytosolic retinol DHs occur, an NAD-dependent enzyme and a NADP-dependent enzyme. More potent ethanol inhibition of the NAD than the NADP-dependent activity (80 versus 30% inhibition, respectively, with 2% ethanol) supports the occurrence of two distinct enzymes.

Our data also show that either NAD or NADP can support the conversion of retinol into RA. Three isozymes with retinal dehydrogenase activity have been separated by anion exchange chromatography of cytosol from rat liver. The major one, representing ~67% of the total liver cytosolic NAD-dependent units, has been purified and does not use NADP as cofactor (16). The other isozymes, and additional isozymes resolved chromatographically from kidney and testis, have not been tested with NADP and may not differ from the NADP-dependent activity observed here. Nevertheless, this result, along with the observation of cytosolic retinol DH multiplicity, clearly broadens the scope of enzymes and/or pathways involved in RA biogenesis.

In summary, several new insights have resulted from this work. The quantitatively dominant contribution has been demonstrated of microsomal versus cytosolic retinol DH to RA biogenesis, including the ability of microsomal retinol DH to supplement retinol produced by cytosolic retinol DH. At least two cytosolic retinol DHs have been demonstrated: a NAD-dependent DH and a NADP-dependent DH. Intriguingly, ethanol in micromolar concentrations inhibits cytosolic retinol DH-catalyzed RA synthesis, despite the differences between cytosolic retinol DH and the known ADHs, and stimulates RA synthesis by microsomes or a combination of microsomes and cytosol. Finally, the complex nature of retinal DHs has been confirmed and extended. It is becoming more apparent that RA biogenesis involves intricate metabolic pathways that involve multiple enzymes that recognize both liganded and nonliganded forms of CRBP.

Acknowledgments—We are grateful to Daniel J. Kosman for helpful discussions during preparation of this manuscript.

REFERENCES
1. Napoli, J. L., Posch, K. C., Fiorella, P. D., and Boerman, M. H. E. M. (1991) Biomed. Pharmacother. 45, 131–143
2. Blomhoff, R., Green, M. H., Green, J. B., Berg, T., and Norum, K. R. (1991) Physiol. Rev. 71, 951–990
3. Maden, M. (1993) Acta Biother. 41, 425–445
4. Morriss-Kay, G., Ward, S., and Sokolova, N. (1994) Arch. Toxicol. 16, 112–117
5. Kochhar, D. M. (1967) Acta Pathol. Microbiol. Scand. 70, 398–404
6. Siano, D. R., Harnish, D. C., Soprano, K. J., Kochhar, D. M., and Jang, H. (1993) J. Nutr. 123, 367–371
7. Harnish, D. C., Barua, A. B., Soprano, K. J., and Soprano, D. R. (1990) Differentiation 45, 103–114
8. Pfahl, M. (1993) Skin Pharmacol. 6, (Suppl. 1), 8–16
9. Chambon, P. (1993) Gene (Amst.) 135, 223–228
10. Madersdorf, D. J., Kliether, S. A., Nakazuka, A., Umesono, K., and Evans, R. M. (1993) Prog. Horm. Res. 48, 59–121
11. Giguere, V. (1994) Endocr. Rev. 15, 61–79
12. Posch, K. C., Boerman, M. H. E. M., Burns, R. D., and Napoli, J. L. (1991) Biochemistry 30, 6224–6234
13. Boerman, M. H. E. M., and Napoli, J. L. (1995) Biochemistry 34, 7027–7037
14. Chai, X., Boerman, M. H. E. M., Zhai, Y., and Napoli, J. L. (1995) J. Biol. Chem. 270, 3900–3904
15. Chai, X., Zhai, Y., Popescu, G., and Napoli, J. L. (1995) J. Biol. Chem. 270, 28408–28412
16. Posch, K. C., Burns, R. D., and Napoli, J. L. (1992) J. Biol. Chem. 267, 19676–19682