Effects of N-(4-Hydroxyphenyl)retinamide Supplementation on Vitamin A Metabolism

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ABSTRACT

The efficacy of the retinoid N-(4-hydroxyphenyl)retinamide (4-HPR) has been demonstrated in the inhibition of cancers in a variety of tissues. Moreover, toxicity effects following administration of 4-HPR have been found to be reduced or absent when compared to other retinoids. Pharmacokinetic studies in both animals and humans have focused on the metabolism of 4-HPR and its metabolites, and relatively little information has been published detailing the effects of long-term administration of 4-HPR upon normal endogenous vitamin A metabolism. Thus, the present study was carried out to examine the effects of long-term administration of 4-HPR upon plasma and tissue vitamin A kinetics. Male Sprague-Dawley rats were fed either a control diet sufficient in vitamin A (CON group; 1.0 retinol (ROH) equivalents/g diet) or a CON diet supplemented with 4-HPR (CON+4HPR group; 1173 μg 4-HPR/g diet). Following IV injection of a physiologically radiolabeled dose of ROH, ROH tracer and tracee kinetics were monitored in plasma and tissues over a 41-day period. Kinetic parameters were determined using the SAAM/CONSAM computer modeling programs to carry out graphical analysis of the tracer concentration curves. Mean plasma ROH levels measured for the CON+4HPR group were reduced to one-third of those of the CON group. Most of the kinetic parameters calculated were found to be significantly altered by the inclusion of 4-HPR in the diet. The fraction of the plasma ROH being catabolized per day (fractional catabolic rate) was nearly twice as high in the CON+4HPR treated group (3.61 ± 0.49 day⁻¹; mean ± SD) as compared to the CON group (2.00 ± 0.68 day⁻¹). The amount of time that vitamin A molecules spent in the body before being lost irreversibly from the system (system residence time) was decreased by half in the CON+4HPR group (19.20 ± 7.13 days) versus the CON group (38.63 ± 9.62 days). Despite the increased catabolic rates and decreased system residence times measured for the CON+4HPR group, the estimated vitamin A use in these animals (11.01 ± 3.10 μg/day) was 33% less than that used by the CON group (16.31 ± 2.47 μg/day). Studies investigating the mechanisms by which 4-HPR alters vitamin A kinetics are presently under way in our laboratory. Nevertheless, these results suggest that long-term administration of 4-HPR markedly perturbs normal vitamin A metabolism in rats. Whether 4-HPR similarly alters human vitamin A metabolism with untoward clinical consequences deserves careful evaluation.

INTRODUCTION

In normal epithelial tissue, retinoids play a major role in the promotion of growth and differentiation (1). A lack of growth control and cellular differentiation are important components of the carcinogenic process, and thus the ability of retinoids to suppress growth and induce or enhance differentiation underlie the rationale for their use in the prevention or inhibition of certain types of cancer (1, 2). The efficacy of the retinoid 4-HPR² has been demonstrated in the inhibition of a variety of cancers (3). In animal studies, it has been shown to be particularly effective as a preventive agent for treatment of breast cancer (4, 5). Most recently, promising results have also been demonstrated in animal studies for both the prevention (6) and treatment (7) of prostate cancer. The toxicity effects following administration of 4-HPR, particularly as related to hepatic accumulation, are reported to be greatly reduced or absent when compared to other retinoids (4). The potential effectiveness of 4-HPR in the prevention of mammary tumorogenesis has led to its present use in a series of clinical trials to assess the feasibility of long-term administration of this compound for the purpose of preventing mammary cancer in humans (8, 9). Although no major toxicity symptoms have been reported thus far, a substantial lowering of plasma ROH and RBP has been observed (8, 10—12) in addition to several reported cases of impaired visual function (13). Whereas preliminary pharmacokinetic studies in both animals and humans focused primarily on the metabolism of 4-HPR and its metabolites, the present study was carried out to examine in more detail the effects of long-term feeding of 4-HPR upon plasma and tissue vitamin A kinetics.

MATERIALS AND METHODS

Isotopes and Chemicals. Radiolabeled ROH (11,12-[3H](N)-vitamin A alcohol-specific activity, ~175 μCi/μg; New England Nuclear, Boston, MA) was used as received (see below). All chemicals and solvents were of reagent or HPLC grade. The 4-HPR was provided to the National Cancer Institutes Division of Cancer Prevention and Control by McNeil Pharmaceutical Company (Spring House, PA). Unlabeled retinoids used as standards (ROH, retinyl acetate, and retinyl palmitate) were obtained commercially (Sigma Chemical Co., St. Louis, MO) or synthesized (retinyl steaerate) according to published methods (14). Retinyl palmitate added to diet preparations was purchased from Teklad (Madison, WI). All procedures involving retinoids were carried out under gold fluorescent lighting.

Animals and Diets. Weaning male Sprague-Dawley rats were fed a vitamin A-free diet (Teklad) until their plasma ROH levels declined to <10 μg/dl and their liver levels to <30 RE. They were then switched to a diet that contained vitamin A as retinyl palmitate (0.15 RE/g of diet). This diet served as a “maintenance” diet which maintained plasma levels in the range of 10 μg/dl and allowed little if any tissue storage of the vitamin. Donor animals were kept on this diet until they were used for preparation of the radiolabeled dose to be injected into recipient animals. Recipient animals were fed the vitamin A-free diet for 3 weeks after weaning, at which time they were fed a control diet containing vitamin A (0.8 RE/g diet as retinyl palmitate) for 7 weeks. At approximately 10 weeks after weaning, recipients with an average weight of 369 ± 11 g were randomly assigned to one of two dietary groups; either they were continued on the control diet (CON group; n = 4) or were fed the control diet supplemented with 4-HPR (CON+4HPR group; 1173 μg 4-HPR/g diet; n = 5). Recipients were then adapted to their respective experimental diets for 6—7 days prior to injection with donor plasma and were fed these diets for the remaining 41-day experimental period. Additional groups of recipients treated in a similar manner were studied simultaneously but for shorter periods of time (0.5, 2, and 6 h and 1, 3, 7, 15, and 25 days). Body weights, food consumption, and plasma vitamin A levels were monitored throughout the study.
The resulting donor plasma containing the $[^{3}H]\text{ROH:RBP:TTR}$ complex was prepared (4°C) overnight. Donor rats were anesthetized with methoxyflurane (Pitman-Moore, Washington Crossing, NJ), and the labeled preparation was injected into the portal vein which had been exposed by a short midline incision. Following injection, the incision was sutured, and rats were allowed to recover for 90–100 min, which based on pilot studies done in our laboratory was the approximate time at which the plasma radioactivity peaked. At this time, they were reanesthetized, and a large terminal blood sample was collected by cut down each major vessel puncture into syringes containing sodium heparin. The resulting donor plasma containing the $[^{3}H]\text{ROH:RBP:TTR}$ complex was stored at —70°C under nitrogen atmosphere and protected from light until use. Aliquots of plasma were stored under nitrogen at 4°C and used for injection into recipient rats within 24 h of collection. The plasma from two donors injected with the labeled carrier solution was pooled and used for injection into the 41-day group recipients described in the present study.

**In Vivo Turnover Studies.** During an early portion of their light cycle (0700–1000 h) a baseline ($I_0$) blood sample was collected from the orbital sinus of nonfasting recipient rats. Weighed aliquots (averaging 0.8 g) of $[^{3}H]\text{ROH:RBP:TTR}$-labeled donor plasma (specific activity, ~85 μCi/μg) were then injected into the tail vein, and serial blood samples were collected from the orbital sinus at geometrically increasing intervals from 10 min postinjection until the rats were killed at 41 days. Similarly treated groups of recipients fed the CON and CON+4HPR diets were killed at 0.5, 2, and 6 h, and 1, 3, 7, 15, and 25 days postinjection, and liver and kidney vitamin A mass data from these animals is provided in the present report for comparison purposes. Optimal times of sampling and the length of studies were based on data collected from preliminary experiments done in our laboratory and computer simulations of the expected plasma and tissue kinetics. We estimated that ~40 days would be sufficient to ensure that the slope of the terminal portion of the plasma tracer concentration curve would be fairly constant and that, in the tissues, the tracer ($[^{3}H]\text{ROH}$) would have sufficient time to "equilibrate" with the tissue stores of tracee (unlabeled vitamin A). Aliquots of plasma were stored at —70°C under nitrogen atmosphere and protected from light until analysis. Following removal of a terminal blood sample, rats were anesthetized with methoxyflurane. The whole body was then perfused by gravity feed (1.9 ml) of 0.9% saline containing 0.2% d-isosorbic acid (Sigma) as an antioxidant infused into the right auricle. After perfusion was complete, tissues were removed, blotted dry, weighed, put into Whirl-Pak bags for storage, purged with nitrogen, and frozen (~70°C), along with the remaining carcass. Retinoids were analyzed by liquid scintillation spectrometry (LS 5000TA; Beckman Instruments, Fullerton, CA). Dried sample aliquots were solubilized in scintillation cocktail (Ready-Organic; Beckman) and counted to a 2-sigma error of 1% or a minimum counting time of 100 min. Sample cpm were converted to dpm using the instrument single label dpm program.

**Kinetic Analyses and Nomenclature.** We first estimated the plasma volume [body weight (g) × 0.035 = plasma volume (ml)] and then calculated the plasma tracer concentration at the time of injection ($t_0$, dpm/ml = dpm injected/estimated plasma volume). For kinetic analysis of the plasma response curves, the plasma radioactivity data for individual animals were normalized to the fraction of injected dose at the 10-min collection point. A geometric mean fraction of dose for each time point was calculated for recipients in both the CON and CON+4HPR groups. Using the SAAM/CONSAM computer modeling program (17, 18), the group mean observed data were fit by a weighted, nonlinear, least-squares regression to a multicomponent exponential equation of the general form:

$$y_i = \sum_{i=1}^{n} \frac{I_i e^{-t_i}}{t_i}$$

where $y_i$ is the fraction of the dose in plasma at time $t$, $I_i$ and $g_i$ (day$^{-1}$) are constants equal to the intercept and fractional slope, respectively, of each component and $n$ is the number of exponential terms in the equation. The intercept terms were used to calculate normalized exponential constants ($H_i$) as follows:

$$H_i = \log \frac{I_i}{\sum_{i=1}^{n} I_i}$$

Using SAAM/CONSAM, kinetic parameters of interest were calculated (19–21) based on the area under the plasma tracer response curve (AUC) which was determined by:

$$\text{AUC} = \sum_{i=1}^{n} H_i / g_i$$

where $M_p$ is the mean plasma ROH mass during the turnover study. The fractional catabolic rate (FCR, day$^{-1}$), the rate of irreversible utilization of vitamin A expressed as a fraction of the plasma ROH pool, is obtained by:

$$\text{FCR} = 1/\text{AUC}$$

Transit times ($t_p$, h) or the time, on average, that a molecule of vitamin A spends in the plasma during a single transit, were calculated by:

$$t_p = \int_0^\infty \text{AUC} / \text{area}$$

Plasma equivalent residence times ($t_{eq}$, h) represent the total amount of time, on average, that a molecule of vitamin A spends in the plasma prior to
irreversible loss. Thus, $\bar{T}_p$ is the residence time of a one compartment system
having an equivalent FCRp, and is calculated by:

$$\bar{T}_p = \frac{1}{FCR_p}$$

The recycling number (RNp), the total number of times a vitamin A
molecule cycles through the plasma before it is irreversibly lost, was calculated by:

$$RN_p = (\bar{T}_p/i_p) - 1$$

The mean system residence time (SRT; days) is the total time, on average,
that a vitamin A molecule spends in the body following introduction into the
plasma, prior to irreversible loss:

$$SRT = \sum_{i=1}^{\infty} H_i/g_i$$

The mean recycling time (RT; days) is the amount of time, on average, that
a molecule of vitamin A spends outside the plasma before cycling back to the
plasma:

$$RT = (SRT_p - \bar{T}_p)/RN_p$$

Statistical Analyses. Coefficients of variation or fractional SD (SD divided
by mean) were calculated as part of the SAAM/CONSAM routines that were
used. Student’s t test (22) was used to determine significant differences (α
level of 0.05 or less) between the mean values for plasma and tissue vitamin
A masses and kinetic parameters calculated for the recipient groups.

RESULTS

Recipients grew normally and appeared to be in good health throughout the entire study period. Although the body weights of both
groups were similar to begin with, the CON+4HPR group gained
significantly more weight during the course of the turnover study as
compared to the CON group (66.1 ± 9.9 @g/dl). The liver and
kidney vitamin A mass values for the 0.5-, 2-, 6-, and 24-h
samples are shown in Table 1. Since, as might be expected, the
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liver vitamin A masses and kinetic parameters calculated for the recipient groups.

Table 1 Liver and kidney vitamin A mass values for times sampled throughout the course of the turnover study

<table>
<thead>
<tr>
<th>Time</th>
<th>Liver (n=12)</th>
<th>Liver (n=3)</th>
<th>Kidney (n=3)</th>
<th>Kidney (n=11)</th>
</tr>
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<tbody>
<tr>
<td>0–24 h</td>
<td>236.94 ± 44.01d</td>
<td>232.55 ± 11.42d</td>
<td>1.34 ± 0.21d</td>
<td>1.39 ± 0.42d</td>
</tr>
<tr>
<td></td>
<td>(n=12)</td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>3 days</td>
<td>322.55 ± 11.42d</td>
<td>312.26 ± 99.46d</td>
<td>1.31 ± 0.25d</td>
<td>1.43 ± 0.28d</td>
</tr>
<tr>
<td></td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>7 days</td>
<td>312.26 ± 99.46d</td>
<td>297.31 ± 29.31d</td>
<td>1.43 ± 0.28d</td>
<td>1.48 ± 0.14d</td>
</tr>
<tr>
<td></td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>15 days</td>
<td>297.31 ± 29.31d</td>
<td>293.03 ± 48.46d</td>
<td>1.43 ± 0.28d</td>
<td>1.48 ± 0.14d</td>
</tr>
<tr>
<td></td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>25 days</td>
<td>293.03 ± 48.46d</td>
<td>275.30 ± 32.19d</td>
<td>1.40 ± 0.25d</td>
<td>1.48 ± 0.14d</td>
</tr>
<tr>
<td></td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>41 days</td>
<td>275.30 ± 32.19d</td>
<td>236.94 ± 44.01d</td>
<td>1.41 ± 0.32d</td>
<td>1.53 ± 0.21d</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td>(n=12)</td>
<td>(n=12)</td>
<td>(n=11)</td>
</tr>
</tbody>
</table>

* Expressed as mean ± SD total RE in tissue.  
* Means in the same column not having a common superscript letter (i.e., d and D) are significantly different (P < 0.05) from one another.  
* Pooled samples collected at 0.5, 2, 6, and 24 h.

Fig. 1. Response curves for plasma ROH kinetics. Data presented as group average
(±SEM) expressed as observed (■ CON; △ CON+4HPR) and calculated (lines) geo
metric mean fraction of injected dose ([3H]ROH:RBP:TTR) donor plasma. Normalized exponential intercepts (H) and fractional
rate constants (g) were obtained by weighted, nonlinear, least squares regression analysis (17, 18) and are shown in Table 2. Although there
was an initial rapid decline of tracer in both groups, this was more
pronounced in the 4-HPR supplemented group. The curve of the
CON+4HPR group began to diverge from that of the CON group at
less than 5-h and started to plateau ~25–30 h later than the CON
group. From approximately 200 h, both groups continued a gradual
decline throughout the remainder of the study period.

Kinetic parameters calculated for individual recipients are pre
sented in Table 3. The fraction of the plasma ROH pool being used
irreversibly each day, the FCRp, was significantly higher in the
4-HPR supplemented group as compared to the CON group, averag
ing 3.61 ± 0.49 and 2.00 ± 0.68 day−1, respectively. The amount of
time that a vitamin A molecule remained in the plasma during each of
its passages through the plasma was similar in both groups as indic
ated by the mean transit times (i_p) measured for the CON
(1.19 ± 0.033 h) and CON+4HPR groups (1.03 ± 0.06 h). However,
the residence time values ($\bar{T}_p$), which include all of these passages or
41-day point, the CON group liver values were similar (7- and 15-day
points) or tended to be higher (to 24-h, 3-day, and 25-day points) than
those of the CON+4HPR group. The vitamin A content of the kidneys
for the CON and CON+4HPR groups was similar throughout the
course of the turnover study.

Plasma tracer response curves are presented in Fig. 1. All data are
presented as geometric group mean fraction of injected dose in plasma
versus time postinjection of the dose of labeled ([3H]ROH:RBP:TTR)
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4114
cycles of a vitamin A molecule through the plasma, indicated that vitamin A remained in the plasma of the CON group (12.86 ± 3.42 h) for a significantly longer period of time than the CON+4HPR group (6.70 ± 0.79 h). The level of recycling (RN) of vitamin A in the plasma was also different between groups with the CON group having a higher level of recycling. Vitamin A molecules recycled, on average, 9.81 ± 2.83 times before irreversibly leaving the plasma as compared to 5.54 ± 0.82 times in the CON+4HPR group. The disposal rate (DR), which is an estimate of whole body utilization of vitamin A, was also different between groups with the CON group having 3.99 ± 0.53 days in the basic metabolism of vitamin A and how this is affected by the administration of different retinoids is essential to help assure the efficacy of such studies in this area. The mean recycling time (RT), which is an estimate of whole body utilization of vitamin A, plasma and tissue levels of vitamin A will help to clarify the role of retinoids as preventive agents in carcinogenic processes. In contrast to much of the previous work in this area, we have focused on the present paper, not on 4-HPR metabolism or pharmacokinetics, but rather on how chronic long-term administration of 4-HPR affects normal vitamin A kinetics and subsequent metabolism of the vitamin. As chemoprevention and chemotherapy involving retinoids are increasingly being carried out on a long-term basis, information involving basic metabolism of vitamin A and how this is affected by the administration of different retinoids is essential to help assure the efficacy of such therapies.

Although the normal control mechanisms that regulate the levels of vitamin A in plasma, liver, and extracellular tissues have yet to be clearly defined, they appear to be part of a tightly regulated system that is able to establish and maintain a homeostatic setpoint for plasma vitamin A levels (26, 27). In the present study we included 4-HPR in the diet to mimic the chronic administration regimen used in human clinical studies investigating the anticancer properties of this retinoid.

| Table 2 Normalized exponential intercepts (H) and fractional rate constants (g) for control and control+4HPR groups |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Control         | Control+4HPR    |
| H₁              | H₂              | H₃              | H₄              | H₅              | H₆              | H₇              | H₈              |
| 1.620E-01       | 1.2759E+00      | 3.7629E-01      | 1.5897E-01      | 1.7105E-01      | 4.5335E-02      | 4.9677E-03      | 5.4495E-04      |
| 7.3714E+01      | 1.1143E+00      | 3.1950E-01      | 1.3674E-01      | 6.0965E-02      | 4.1514E-02      | 2.6845E-03      | 7.5973E-04      |
| 5.7876E-01      | 1.2964E+00      | 3.3028E-01      | 1.6051E-01      | 1.4643E-01      | 4.8050E-02      | 4.0336E-03      | 5.3684E-04      |

* Normalized intercepts and fractional rate constants were derived from individual animal data using SAAM/CONSAM to fit the plasma fraction of injected dose versus time curves. The values of H are unitless and the units for g are in terms of day⁻¹.

** DISCUSSION **

The present study was designed to examine the effects of the synthetic retinoid 4-HPR on ROH metabolism in both plasma and tissues. Our interest in 4-HPR stems in part from the possible cancer chemopreventive and therapeutic potential of this particular retinoid. Additionally, the ability of 4-HPR to lower plasma levels of ROH enabled us to investigate the homeostatic mechanisms involved in the regulation of plasma and tissue levels of vitamin A, particularly as they relate to the kinetics and overall metabolism of the vitamin. Although evidence from some epidemiological studies (23, 24) has suggested that higher levels of plasma ROH levels might be associated with decreased risk of developing certain types of cancer, not all studies in this area are in agreement (25). Nevertheless, a better understanding of the basic mechanisms involved in regulating both plasma and tissue levels of vitamin A will help to clarify the role of retinoids as preventive agents in carcinogenic processes. In contrast to much of the previous work in this area, we have focused on the present paper, not on 4-HPR metabolism or pharmacokinetics, but rather on how chronic long-term administration of 4-HPR affects normal vitamin A kinetics and subsequent metabolism of the vitamin. As chemoprevention and chemotherapy involving retinoids are increasingly being carried out on a long-term basis, information involving basic metabolism of vitamin A and how this is affected by the administration of different retinoids is essential to help assure the efficacy of such therapies.

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| Table 3 Kinetic parameters for ROH metabolism in control and 4-HPR supplemented groups |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Control         | Control+4HPR    |
| FCR’ (day⁻¹)   | iₜ’ (h)         | Tᵣ’ (h)         | RN’ (µg/day)    | DR’ (µg/day)    | SRT’ (days)    | RT’ (days)     |
| 1.52            | 1.18            | 15.75           | 12.36           | 13.82           | 44.52          | 3.60           |
| 3.00            | 1.15            | 8.00            | 5.94            | 19.68           | 24.50          | 4.12           |
| 1.84            | 1.23            | 13.07           | 9.59            | 16.33           | 44.88          | 4.68           |
| 1.65            | 1.16            | 14.63           | 11.38           | 15.42           | 40.62          | 3.55           |
| Mean ± SD      | 1.99 ± 0.033    | 12.86 ± 3.42    | 9.81 ± 2.83     | 16.31 ± 2.47    | 38.63 ± 9.62   | 3.99 ± 0.53    |

* Methods of calculation of kinetic parameters are found in the text; data presented as mean ± SD.

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Although the normal control mechanisms that regulate the levels of the vitamin in plasma, liver, and extracellular tissues have yet to be clearly defined, they appear to be part of a tightly regulated system that is able to establish and maintain a homeostatic setpoint for plasma vitamin A levels (26, 27). In the present study we included 4-HPR in the diet to mimic the chronic administration regimen used in human clinical studies investigating the anticancer properties of this retinoid.
The effects of feeding 4-HPR upon plasma vitamin A levels and overall vitamin A kinetics were striking. The average plasma ROH values for the CON+4HPR group were nearly three times lower than those of the CON group. The kinetic parameters we measured demonstrated that 4-HPR administration had profound effects on the homeostatic controls that normally regulate vitamin A metabolism. Values for the fractional catabolic rate, the fraction of the plasma ROH pool being catabolized per day, were found to be nearly twice as high in the 4-HPR treated group as compared to the control group. The residence time values, which include all cycling through the plasma, indicated that prior to being irreversibly lost from the system, vitamin A molecules stayed in the plasma of the 4-HPR-treated group only about one-half as long the control group. As might be expected, these differences were also reflected in the lower number of recyclings of vitamin A through the plasma of the 4-HPR-treated group. Thus, a molecule of vitamin A in a control group animal recycled an average of about 10 times before it was used or lost from the system, whereas it only recycled 6 times in a 4-HPR-treated animal. The mean system residence time, an estimate of the total amount of time a molecule of vitamin A entering the plasma spends in the body before it is lost from the system, was twice as high in the control group as compared to the 4-HPR-treated group. This meant that, despite having a lower apparent utilization rate, vitamin A appeared to be staying in the body of the treated animal for a shorter length of time. Taken together, these changes in kinetic parameters suggest that 4-HPR alters the basic kinetics of vitamin A in such a way as to result in a larger portion of the exchangeable pool of the vitamin leaving the system on a daily basis. The effects of altering the movement of the vitamin through the plasma and the tissues in such a manner on a short or long-term basis remain to be clarified, as well as whether these effects are primary or secondary in nature. Whether normal tissue functions are being compromised by these changes in vitamin A kinetics was not directly examined in the present experiments; however, several recent studies have demonstrated that 4-HPR is able to interfere with the normal functioning of several enzymes important in overall vitamin A metabolism (28, 29). *In vitro* studies with 4-HPR have indicated that it might interfere with the esterification of ROH in rat liver and mammary tissue (28). It would be of interest to examine whether or not esterification in the 4-HPR group has been affected similarly *in vivo* in our studies. Dew et al. (29) have recently reported that several retinoids, including 4-HPR, were found to inhibit both intestinal and liver lecithin-retinol acyltransferase activities, providing further evidence that 4-HPR is affecting primary components involved with normal vitamin A metabolism.

Although the absolute amount of vitamin A mass moving through the plasma was considerably less in the treated group than the control, this change was primarily related to the lower plasma values of the former group (only one-third that of the controls). It is possible to obtain an estimate of the amount of vitamin A turnover through the plasma for both groups [i.e., mass of plasma ROH * (1/ * 24)]. Thus, on a daily basis, the 4-HPR-treated group was turning over only about 40 μg of vitamin A, whereas the CON group was turning over nearly 190 μg. The daily utilization of vitamin A by the 4-HPR group was also considerably less as reflected in the disposal rate values calculated, with the treated group using only about two-thirds of the vitamin that the control group was using on a daily basis. When the disposal rate values are expressed as a percentage of plasma vitamin A turnover, once again striking differences are apparent. The 4-HPR-treated group was using ~28% of what was turning over through the plasma (with ~72% being recycled), whereas the control group was using only ~8% (with ~92% being recycled), respectively. In other words, a much greater portion of the vitamin A in the plasma of the 4-HPR-treated group was being utilized and much less recycled in comparison to the control group.

In the face of this altered metabolism tending to remove vitamin A from the system, there are several ways one might expect the system to respond, which include increasing the mobilization of hepatic and/or extrahepatic stores to maintain homeostasis and/or down-regulating certain vitamin A requiring components of the system in an attempt to conserve the stores of the vitamin. In either case, one might predict that such responses would be reflected in the tissue stores of vitamin A in the 4-HPR-treated group. In the present study, the liver vitamin A levels of the 41-day animals were higher in the CON+4HPR group as compared to the CON group. However, in all of the other groups we examined between 0.5 h and 25 days, as well as in a 40-day preliminary experiment done in preparation for the present study, the CON+4HPR group liver vitamin A values were similar or tended to be lower than those of the CON group. These findings would be in agreement with the early work done by Moon et al. (30) in which they found that 4-HPR did not alter liver vitamin A even when administered over a 6-month period. Thus, the possibility that the elevated liver levels of vitamin A observed in the 41-day 4-HPR-treated group might be an anomaly needs to be examined and verified in future work. There could be a 4-HPR-related interference with the normal secretion of vitamin A from the liver without an apparent effect on liver stores of the vitamin. The suggestion that 4-HPR might be interfering with the normal hepatic secretion of ROH:RBP has been made earlier (10, 12, 31). However, these studies were either carried out for short periods of time or not provide any information on hepatic or extrahepatic tissue levels of vitamin A following long-term administration of 4-HPR. For example, Berni and Formelli and colleagues (10, 12), in contrast to the *in vivo* metabolism approach we have used in the present study, have used an *in vitro* approach to study possible mechanisms that might be involved in the 4-HPR-related depression of plasma vitamin A levels. Initially they carried out protein binding studies examining the ability of RBP to bind to a variety of retinoids other than retinol as well as the ability of different retinoid:RBP complexes to bind TTR (10). They found that 4-HPR was able to bind to RBP with a relatively high affinity. But this complex was unable to bind to TTR to form the normal trimolecular plasma transport complex. They hypothesized that the reduced plasma levels of ROH associated with 4-HPR administration might be a result of interference with normal ROH:RBP formation, resulting in a reduction of the normal ROH:RBP:TTR complex and an increase in a 4-HPR:RBP. Consequently, the inability of the latter complex to bind TTR might result in it being cleared more rapidly from the plasma via glomerular filtration. A subsequent study involving a series of competition studies demonstrated that *in vitro* 4-HPR was able to displace ROH complexed to RBP only minimally, whereas ROH was able to displace 4-HPR to a greater extent (12). Based on their results, they suggest that it is unlikely that the depression in plasma ROH levels observed with 4-HPR administration is caused by a competition of 4-HPR with ROH for the RBP binding site. Thus, if 4-HPR is interfering with normal ROH:RBP complex formation, it would more likely be occurring in hepatic and/or extrahepatic tissues from which ROH:RBP is mobilized into the circulation. Smith et al. (31) have suggested that 4-HPR administration might be associated with a partial blockage of the ROH:RBP complex, which could in turn result in a decreased concentration of ROH and RBP in the plasma. However, their study was carried out for only relatively short periods of time (either 5 or 24 h) and involved an i.v. bolus dose of 4-HPR as compared to the longer-term chronic feeding regimen we used in the

3 Unpublished observations.
present paper. A more recent study by the same group involved both a feeding experiment in which 4-HPR was included in the diet and a short-term experiment designed to study acute effects of 4-HPR administration (32). In contrast to their earlier work, the results of their latter study suggested that, at least in the liver, 4-HPR might actually be inducing secretion of RBP into the circulation. Since vitamin A liver masses for the animals they studied were not reported, it was not possible to evaluate how possible alterations involving the secretion of RBP affected the level of hepatic stores of the vitamin. The role of the liver as well as extrahepatic tissues in relation to the mechanisms involved in the depressed plasma vitamin A levels in 4-HPR treated animals remains to be clarified.

As mentioned earlier, the data presented herein is a precursor to a larger-scale, more detailed study of 4-HPR and ROH metabolism we have recently undertaken. When completed, this larger data set will, by design, aid in the development of a more detailed compartmental model to more fully describe vitamin A kinetics in individual tissues in both control and 4-HPR treated animals. The present studies have suggested that such a model, describing the effects of 4-HPR upon normal vitamin A metabolism, is necessary both to verify and extend our present findings as well as to further delineate the chemopreventive and chemotherapeutic properties of this retinoid. In summary, our results indicate that the long-term administration of 4-HPR is associated with significant perturbations of normal vitamin A metabolism. Of particular interest is the finding that the fraction of the plasma ROH pool being catabolized per day was found to be nearly twice as high in the 4-HPR-treated group as compared to the control group. However, since the plasma vitamin A concentrations in the treated group were considerably lower than those of the control values, the absolute amount of vitamin A mass moving through the plasma and tissues was considerably less than the control. Since our studies have been carried out for relatively long periods, they have particular relevance to long-term human trials being conducted to evaluate the chemopreventive potential of 4-HPR. Whether or not basic kinetic and related metabolic alterations similar to those we have observed in the present study might be associated with untoward clinical consequences in humans warrants further investigation.

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Effects of N-(4-Hydroxyphenyl)retinamide Supplementation on Vitamin A Metabolism

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