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Effect of *N*-(4-Hydroxyphenyl) Retinamide on Food Intake, Growth, and Mammary Gland Development in Rats (41736)

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Abstract. Food intake and growth were depressed during the first week of feeding the anticarcinogenic retinoid *N*-(4-hydroxyphenyl) retinamide (HPR) at a concentration of 782 mg/kg diet to female rats. Food intake was normalized thereafter, but body weight did not reach that of control animals until 40 days later. The use of a pair-fed group demonstrated that weight depression in HPR fed animals was entirely due to reduced food intake. Mammary glands from HPR-fed animals showed decreased ductal branching and decreased end bud proliferation relative to control glands. Total hepatic retinol and retinol concentration were lower ($P < 0.05$) for HPR fed animals than for controls. The effects of HPR on mammary development and retinol storage were attributable to dietary HPR per se. HPR was detected in mammary gland and body fat at concentrations of 27 and 53.7 nmol/g, respectively.

Retinoids are a set of molecules comprising vitamin A and its synthetic analogs. They have been used to prevent chemically induced carcinogenesis in the bladder (1), skin (2), and mammary gland (3). The synthetic retinoid *N*-(4-hydroxyphenyl) retinamide (HPR), a derivative of retinoic acid (Fig. 1), has been shown to inhibit *N*-nitroso-*N*-methylurea-induced mammary carcinogenesis. HPR also exhibits an antiproliferative effect on rat mammary epithelium that may be involved in HPR inhibition of mammary carcinogenesis (3). In previous studies in our laboratory we have observed a transient reduction in body weight gain in female rats receiving dietary HPR. Thus, we were interested in determining whether the effect on body weight gain was related to food intake and whether or not these factors influenced the antiproliferative effect of HPR on the mammary epithelium.

Materials and Methods. *Animals and Diets.* Three groups (designated A through C) of seven 58-day-old female Sprague–Dawley rats (ARS/Sprague–Dawley, Madison, Wisc.) were housed individually in stainless-steel metabolism cages. Food intake and body weight were recorded daily. Two diets were used: a placebo diet and an HPR-containing diet. The placebo diet was prepared by mixing 12.5 g ethanol with 36.4 g trioctanoin, 0.5 g *dl*-α-tocopherol, and 0.6 g "Tenox 20" (Eastman Chemical Products Incorp., Kingsport, Tenn.) (contains weight percent): tertiary butyl hydroquinone, 20; anhydrous citric acid, 10; propylene glycol, 70). After mixing for 15 min, these constituents were made to 1 kg with "Wayne Meal" (Allied Mills Incorp., Chicago, Ill.). All ingredients were then thoroughly mixed in a Patterson–Kelly blender. The HPR diet was prepared similarly, except that 782 mg of HPR was included in the trioctanoin–ethanol mixture. Diets were stored at −20°C.

Group A received the HPR diet *ad libitum*. The other two groups were fed the placebo diet. Group B was fed *ad libitum* whereas animals in group C were pair-fed to the intakes of corresponding animals in group A. Initial mean body weight of the groups ranged from 176 to 180 g. After 157 days on experiment, animals were anesthetized with diethyl ether, decapitated, and blood, liver, perirenal fat, and abdominal mammary glands taken. Serum was prepared by centrifugation of blood at 2000 rpm for 20 min at 5°C. Tissues and serum were stored at −60°C.

Preparation of mammary glands for whole mounts. Whole mounts of right abdominal–inguinal mammary glands were prepared from each animal as described previously (3).

Determination of tissue HPR levels. Samples (0.6 g) of left abdominal–inguinal mammary gland and body fat were homogenized in methanol and left to stand at 5°C for 2 hr. Following filtration, the methanol extract was mixed, at twice its volume, with chloroform and the chloroform–methanol mixture washed with 0.88% KCl. The washed extract was evaporated to dryness under nitrogen, redis-

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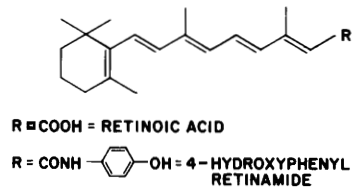

FIG. 1. Retinoid structures.

solved in chloroform, and spotted onto Silica Gel G plates (0.2 mm thickness), previously activated at 110°C for 20 min. Plates were developed in an atmosphere of nitrogen using acetone:petroleum ether (bp 35–48°C) 18:82 (v/v) as the developing solvent system (4). After development and visualization under UV light, bands corresponding to HPR (R_f 0.11) and a metabolite of HPR (R_f 0.32) were scraped off the plates, and extracted with chloroform:methanol 4:1 (v/v). Aliquots of extract were evaporated to dryness, redissolved in chloroform, and reacted with trifluoroacetic acid (TFA). Both compounds gave a bright red color with TFA. Optical densities were read at 564 nm—the λ_{max} of the reaction for HPR with TFA (3).

Determination of retinol in liver, serum, and body fat. Livers were ground with 2.5 parts by weight of anhydrous sodium sulfate and extracted overnight at 5°C with 100 ml chloroform. The extract was filtered, made up to original volume, and aliquots taken for determination of retinol content using TFA. The optical density of the resultant bright blue complex was read at 620 nm (5). Serum (1.5 ml) was hydrolyzed under nitrogen with 1.5 ml ethanol and 0.6 ml 11 *N* KOH at 60°C for 30 min. Distilled water (3 ml) was then added and retinol extracted with 12 ml *n*-hexane, which was removed under a stream of nitrogen. The residue was dissolved in chloroform and retinol quantitated with TFA (5). Body fat and mammary gland (0.6 g) were heated under nitrogen for 30 min at 89°C with 4 ml of freshly made 5% ethanolic pyrogallol, 0.75 ml of 11 *N* KOH, and 50 mg L-ascorbic acid. After hydrolysis, 6 ml of distilled water was added and retinol extracted with *n*-hexane and quantitated as above.

Results. *Food intake and body weight.* Voluntary food intake was lower in animals fed the HPR diet than for those fed placebo diet from 58 to 65 days of age. Thereafter, it was similar for both these groups (Fig. 2). Dietary HPR resulted in an initial body weight depression, followed by weight recovery and resumption of growth (Fig. 3 and Table I). Rats fed the placebo diet *ad libitum* weighed significantly more than the other two groups from 60 until 66 days of age ($P < 0.05$). However, nonsignificant differences in body weight between the animals fed the placebo diet and the other two groups persisted after this time (Table I and Fig. 3). Final mean body weights of groups A to C were 278, 281, and 282 g, respectively (Table I). The weight of pair-fed animals was similar to those of animals fed the HPR diet throughout the study.

Mammary gland development. Mammary glands of both groups fed the placebo diet were morphologically similar, but glands of HPR fed animals showed a decrease in ductal branching and end bud proliferation (Figs. 4A–F).

Serum and tissue retinoid levels. Serum levels of retinol were similar in all groups. Total liver retinol content and concentration of retinol were significantly lower for animals fed the HPR diet than for those fed the placebo diet (Table II).

HPR level was significantly ($P < 0.01$) lower in mammary gland than in body fat (Table III). A metabolite of HPR (R_f 0.32) was also detected in body fat and mammary gland. This compound, like HPR, gave a bright red color with TFA. Pair-feeding had no effect on retinol concentration in either body fat or mammary

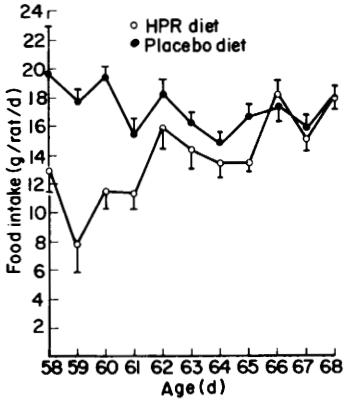

FIG. 2. Voluntary food intake from 58 to 68 days of age in female Sprague–Dawley rats fed placebo diet and HPR diet. Each point represents the mean ± SEM of seven observations.

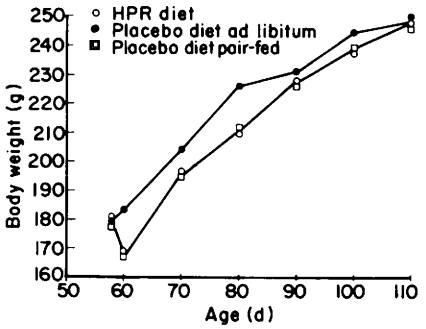

FIG. 3. Body weight from 58 to 110 days of age in female Sprague–Dawley rats. Each point represents the mean of seven animals.

gland. Retinol levels could not be determined in body fat and mammary glands from HPR-fed animals since the material contained in the hexane extract of hydrolyzates of these tissues gave a bright red color with TFA (probably due to hydrolytic breakdown products of HPR and HPR metabolites) rather than the characteristic blue color that retinol gives with TFA. Molar concentration of retinol in mammary gland and body fat in placebo-fed animals (Groups B and C) were much lower than those of HPR in tissues from animals fed diet supplements with HPR.

Discussion. This study shows that the initial reduction in body weight seen in HPR-fed animals is due to a depression in food intake. The reduction in body weight does not affect the antiproliferative actions of HPR on mammary epithelium which suggests that the HPR-mediated reduction in weight gain is, most likely, not involved in its anticarcinogenic action on the mammary gland. The exact mode of action of HPR on mammary gland morphogenesis is not known. However, HPR inhibits prolactin-induced differentiation of mammary glands *in vitro* (6) and the morphology of the cultured glands is similar to that of glands of animals given dietary HPR (6). These observations suggest that either HPR or HPR metabolites exert their action directly upon the mammary epithelium.

It is possible, however, that the actions of HPR on the mammary gland is not direct, but that HPR affects mammary gland development by bringing about changes in the hormonal milieu of animals and these, in turn, influence gland development. However, results from the studies of Moon and colleagues suggest that this is unlikely; when they administered retinyl acetate to rats at levels that caused changes in mammary gland morphology similar to those observed here, no effects were observed on either the estrus cycle or serum prolactin levels (7, 8).

Plasma retinol levels were unaffected by HPR feeding, but total hepatic content and concentration of retinol were lower for HPR fed animals than for either the placebo or pair-fed group. The retinol values for the latter two groups were similar (Table II). Thus, it would appear that the effect on hepatic vitamin A storage is due to dietary HPR per se. Whether the decreased hepatic storage is due to decreased absorption, increased metabolism, or

TABLE I. CHANGES IN BODY WEIGHT WITH AGE

AGE (days)	Group A (HPR diet *ad libitum*)	Group B (placebo diet *ad libitum*) body weight (g)	Group C (pair-fed placebo diet to the intake of Group A)
58	180 ± 2.2[a]	177 ± 2.5	176 ± 2.8
60	168 ± 4.1[c]	183 ± 2.0	166 ± 2.7[c]
66	186 ± 5.2[b]	200 ± 2.3	178 ± 4.4[c]
70	196 ± 3.6	204 ± 3.6	194 ± 2.7
80	212 ± 5.1	226 ± 4.5	212 ± 5.2
90	228 ± 3.9	231 ± 4.5	228 ± 1.3
100	238 ± 3.9	245 ± 4.5	240 ± 1.1
110	248 ± 4.2	250 ± 4.4	245 ± 1.3
215	278 ± 6.3	281 ± 4.7	282 ± 4.1

[a] Mean ± SEM ($N = 7$).
[b] Significantly different from group B, $P < 0.5$ by analysis of variance.
[c] Significantly different from group B, $P < 0.01$ by analysis of variance.

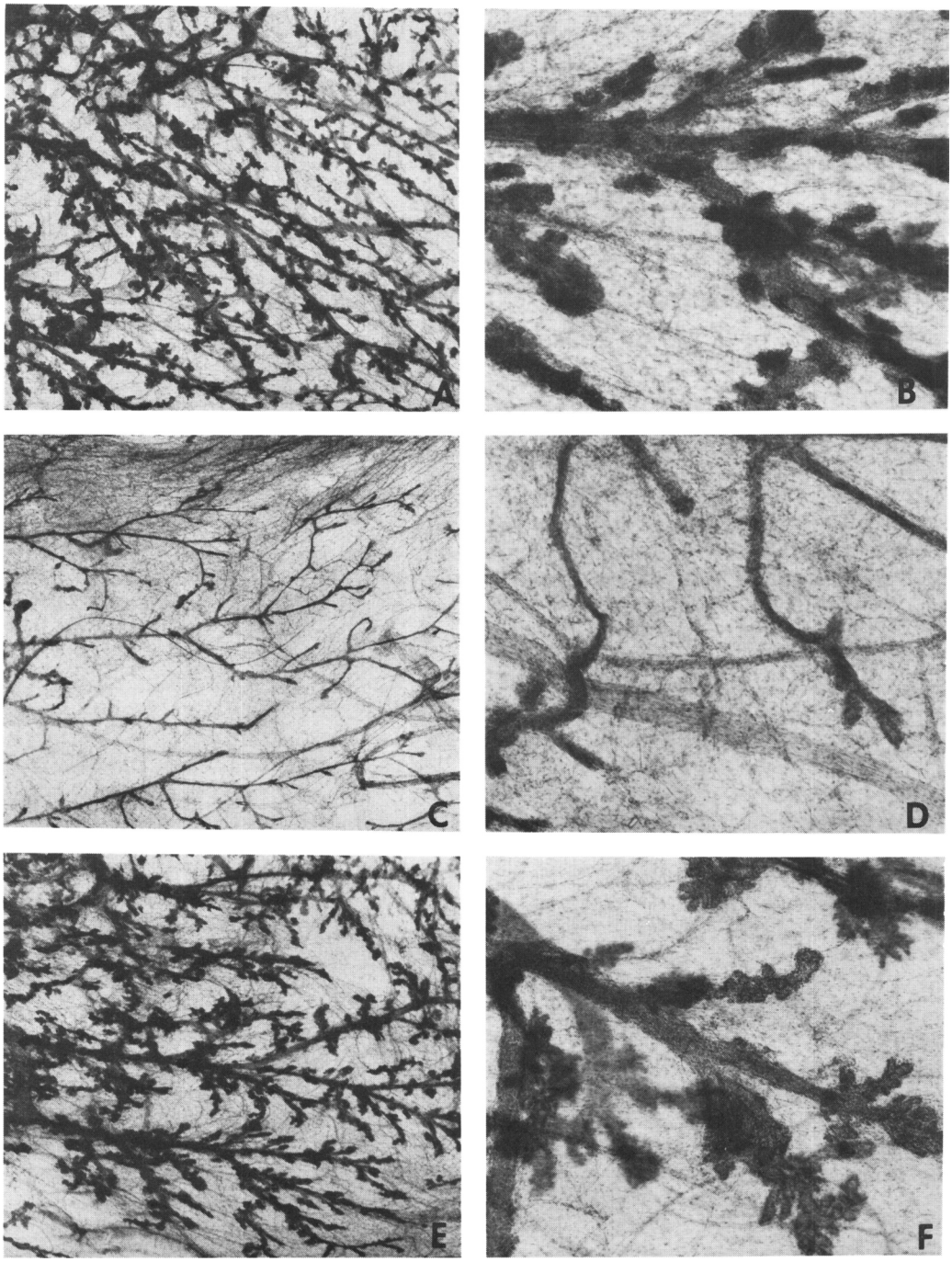

FIG. 4. Representative areas of whole mounts of mammary glands of rats fed diets beginning at 58 days of age continuing until sacrifice at 215–216 days of age. Photographs are representative of histological sections from all animals in each group and all sections of mammary tissues. (A) Placebo diet *ad libitum*. ×5. (B) Same as A. ×40. (C) *N*-(4-hydroxyphenyl) retinamide (782 mg/kg) *ad libitum*. Note paucity of end buds and ductual branching. Compare to A. ×5. (D) Same as C. Compare to B. ×40. (E) Placebo diet pair-fed to intake of C. Note structural similarity to A. ×5. (F) Same as E. ×40.

TABLE II. HEPATIC AND SERUM LEVELS OF VITAMIN A

Group	Treatment	Total hepatic retinol (μmole)	Hepatic concentration of retinol (μmole per g)	Serum retinol (nmole per dl)
A	HPR diet *ad libitum*	14.6 ± 0.94[a]	1.33 ± 0.07	150 ± 40
B	Placebo diet *ad libitum*	19.8 ± 0.77[b]	2.58 ± 0.072[c]	124 ± 13
C	Pair-fed placebo diet to the intake of Group A	19.3 ± 0.91[b]	2.50 ± 0.058[c]	143 ± 24

[a] Mean ± SEM (N = 7).
[b] Significantly different from group A, $P < 0.05$ by analysis of variance.
[c] Significantly different from group A, $P < 0.01$ by analysis of variance.

increased extrahepatic storage of retinol is not yet known. Little is known of the way in which synthetic retinoids affect the absorption and metabolism of retinol, but this study suggests that research along these lines may be worthwhile.

Liver weights were similar for the two groups of animals fed the placebo diet, being (Mean ± SEM): 7.68 ± 0.20 and 7.72 ± 0.18 g for *ad libitum* and pair-fed groups, respectively. Both of these values were significantly ($P < 0.01$) lower than that for HPR-fed animals (11.1 ± 0.25 g). The reason for the hepatomegaly induced by HPR remains to be elucidated. It is not known whether HPR induces hepatic compositional changes other than that in retinol level. A more detailed analysis of livers (e.g., for protein, lipid, and DNA) from HPR and placebo fed animals would be desirable in future studies involving the use of this compound.

The concentration of HPR was higher in body fat than mammary gland (Table III) and this may reflect the higher lipid content of body fat (9). HPR was not detected in liver and this confirms previous studies showing that HPR is stored principally in extrahepatic tissues (3). The nature of the HPR metabolite, R_f 0.32, is not yet known. If the molar extinction coefficient of this compound with TFA is similar to that of HPR then its molar concentration is about twice as high as that of HPR in both mammary gland and body fat (Table III). High performance liquid chromatography (HPLC) analyses of ethereal extracts of mammary glands from HPR-fed animals indicate that the most important storage form of HPR is a metabolite of HPR rather than the parent-compound itself. This metabolite was found to have similar elution characteristics to *N*-(4-methoxyphenyl) retinamide (3). Whether or not the HPR metabolite

TABLE III. RETINOID LEVELS IN MAMMARY GLAND AND BODY FAT

Group	Treatment		Mammary gland	Body fat
A	HPR Diet *ad libitum*	Concentration of HPR (nmole per g)	27.0 ± 1.2[a]	53.7 ± 4.6
		Ratio of O.D. units developed in TFA assay by material in band R_f 0.32 to O.D. developed by material in band R_f 0.10 (HPR).	2.00	2.00
B	Placebo Diet *ad libitum*	Concentration of retinol (nmole per g)	3.48 ± 0.13	3.41 ± 0.052
C	Pair-fed placebo diet to the intake of Group A	Concentration of retinol (nmole per g)	3.61 ± 0.094	3.31 ± 0.104

[a] Mean ± SEM (N = 7).

isolated by thin layer chromatography in this study is the same as that isolated by HPLC remains to be established. Further dietary studies with HPR, in particular studies on the nature of HPR metabolites in mammary gland, may yield valuable insights into the actions of retinoids in cancer prevention.

Sylvester *et al.* (10) restricted food intake of 7,12-dimethylbenz[α]athracene-treated female Sprague–Dawley rats to 50% of *ad libitum* values from 7 days before, until 30 days after, carcinogen administration. Tumor incidence and number of tumors per tumor-bearing animals were reduced by 60 and 70%, respectively. The food intake depression seen in HPR-fed rats used in this experiment was only of 8 days' duration and mean daily depression in intake was only 40%; furthermore, the HPR-induced changes in mammary gland morphology were independent of this food intake depression. However, this does not rule out the possibility that food intake depression per se is involved in HPR's inhibition of chemically induced carcinogenesis. Studies to address this possibility are in progress.

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