Biological Activity and Metabolism of the Retinoid Axerophthene (Vitamin A Hydrocarbon)

Dianne L. Newton, Charles A. Frolik, Anita B. Roberts, Joseph M. Smith, Michael B. Sporn, Axel Nürenbach, and Joachim Paust

National Cancer Institute, Bethesda, Maryland 20014 [D. L. N., C. A. F., A. B. R., J. M. S., M. B. S.], and BASF Aktiengesellschaft, 6700 Ludwigshafen am Rhein, Germany [A. N., J. P.]

ABSTRACT

Biological properties of axerophthene, the hydrocarbon analog of retinol, have been studied both in vitro and in vivo. In tracheal organ culture axerophthene reversed keratinization caused by deficiency of retinoid in the culture medium; its potency was of the same order of magnitude as that of retinyl acetate. Aixerophthene supported growth in hamsters fed vitamin A-deficient diets although less effectively than did retinyl acetate. Aixerophthene was considerably less toxic than was retinyl acetate when administered repeatedly in high doses to rats. Administration of an equivalent p.o. dose of axerophthene caused much less deposition of retinyl palmitate in the liver than did the same dose of retinyl acetate, while a greater level of total retinoid was found in the mammary gland after administration of axerophthene.

INTRODUCTION

Both natural and synthetic retinoids have recently been shown to inhibit mammary carcinogenesis in the experimental animal (9, 14, 15). Although these studies indicate a new approach to the prevention of breast cancer in human subjects, the potential chronic toxicity of the retinoids that thus far have been used in the experimental studies (retinyl acetate and retinyl methyl ether) precludes their clinical use thus far have been used in the experimental studies (retinyl acetate and retinyl methyl ether) precludes their clinical use in women at high risk for development of this disease. The identification of new retinoids that will have enhanced activity, better pharmacokinetic properties, or lesser toxicity is therefore essential. In a previous study we showed that after p.o. dosing of equivalent amounts the less polar analog, retinyl methyl ether, yielded a greater concentration of retinoid in the breast of the rat when compared to retinyl acetate (20). Retinyl methyl ether is also more effective than is retinyl acetate for inhibition of mammary carcinogenesis induced in Sprague-Dawley rats by 7,12-dimethylbenz(a)anthracene (9). Further studies on relatively nonpolar retinoids therefore appear warranted. One such compound, which has had little biological evaluation, is the retinoid hydrocarbon axerophthene (Chart 1). Early reports on the putative synthesis of the hydrocarbon with the structure shown in Chart 1 were subsequently shown to be incorrect, since these early preparations were found to have the retro structure (10, 11). Although the synthesis of the hydrocarbon compound shown in Chart 1 was subsequently reported in the patent literature (2, 3), there has been no extensive published report dealing with the biological activity and properties of this molecule. In this study we report a detailed investigation of the biological activity and metabolism of axerophthene. Subsequent studies will deal with the possible effectiveness of this compound for prevention of experimental breast cancer.

MATERIALS AND METHODS

Aixerophthene was synthesized as follows: 1100 g (1.75 mol) of crystalline all-E-retinyltriphenylphosphonium bisulfate (16) were dissolved in 1500 ml of dimethylformamide at about 25°. A solution of 140 g (3.5 mol) of sodium hydroxide in 1100 ml of water was added while the temperature of the mixture was kept at about 25°. After being stirred for 3 hr at about 25°, the mixture was extracted with n-hexane (3 × 800 ml). The n-hexane solution was washed twice with 500 ml of 60% aqueous methanol and concentrated in a vacuum to give 660 g of light yellow oil that partially crystallized upon standing overnight at room temperature. The semi-solid material was stirred with 500 ml of methanol at 50° for several min and filtered to obtain 143.5 g (30%) of axerophthene with m.p. 75–77°. An analytically pure sample was obtained by recrystallization from poly(oxyethylene):castor oil (4). This pure sample had the following physical constants: m.p. 81–82° (4); UV (cyclohexane): λ_max 323 nm, E_max = 1850; H-nuclear magnetic resonance (CDCl_3, 220 MHz): 1.03 (1-CH_i, 6H), 1.71 (5-CH_3), 1.76 (14-CH_3, J = 6.6 Hz), 1.95 (9-CH_3), 1.81 (13-CH_3), and 6.11 ppm (7- and 8-H), in accordance with the literature (17).

Methods for assay of reversal of keratinization in hamster tracheal organ cultures have been described in detail (6, 19). The procedure and diet used for measurement of growth promotion in vitamin A-deficient hamsters have been described at length (5). In studies on metabolism and tissue distribution, retinoids were extracted from tissue with peroxide-free ethyl ether after grinding of the tissue with anhydrous sodium sulfate (1). In some experiments, total retinoid in tissue extracts was measured with trifluoroacetic acid (7). For the measurement of total retinoid, 10 parts of reagent (trifluoroacetic acid:chloroform, 1:2, v/v) were added to 1 part of tissue extract (redissolved in chloroform after removal of the ether with nitrogen). Absorbance was then measured at 30-sec intervals at either 574 nm (ε_max for axerophthene, ε = 125,000) or 616 nm (ε_max for retinol or retinyl esters, ε = 160,000). Values used for molar extinction coefficients have been extrapolated to zero time. Molar extinction values for retinol, retinyl acetate, and retinyl palmitate in the trifluoroacetic acid reaction have all been reported to be identical (7).
retinoids by HPLC on Spherisorb ODS columns has recently been described (8). In the present studies methanol:water solutions have been used for elution of columns rather than acetonitrile:water solutions, as reported previously. Use of methanol allows more effective analysis of lipid-rich extracts on Spherisorb columns.

RESULTS

Reversal of Keratinization in Tracheal Organ Culture by Axerophthene. This assay is a highly sensitive measure of the ability of retinoids to control normal epithelial cell differentiation and has been used routinely to measure the relative potency of different retinoids (20). Dose-response curves for both axerophthene and all-trans-retinoic acid are shown in Chart 2. All-trans-retinoic acid has been used as a reference substance in this assay since it is the most potent retinoid found to date for in vitro reversal of keratinization. Although axerophthene completely reversed keratinization at 10^{-6} and 10^{-7} M, it was ineffective in 3 of 17 tracheal cultures at 10^{-8} M and in 15 of 22 cultures at 10^{-9} M, and it was totally ineffective at 10^{-10} M. In contrast, all-trans-retinoic acid was completely effective in reversing keratinization at concentrations as low as 10^{-9} M. The activity of axerophthene found in these studies is approximately equal to that previously reported for retinyl acetate (20), although careful titration in the 10^{-8}- to 10^{-9}-M range would be required for a more exact comparison of these 2 compounds.

Effect of Axerophthene on Survival and Weight Gain of Hamsters Fed Vitamin A-deficient Diets. A comparison of the relative ability of axerophthene and retinyl acetate to maintain survival and weight gain in animals deprived of dietary retinoids is shown in Table 1. When hamsters were given only 0.01 ¿mol of either retinoid per week, retinyl acetate was clearly superior to axerophthene, since hamsters did not survive on this low dose of the hydrocarbon. However, at the higher dosage level of 0.10 ¿mol/week, there was no significant difference in survival or in weight gain between the 2 groups.

Comparative Toxicity of Axerophthene and Retinyl Acetate. In 2 sets of experiments, axerophthene was clearly less toxic than was retinyl acetate when given p.o. to rats. The 2 retinoids were given in high doses to young rats, and inhibition of growth or lethality was measured over a 2-week period (Table 2). At the lower dose level, a significant difference between the 2 retinoids was evident only in terms of growth suppression; by the end of 14 days, the rats treated with retinyl acetate had a much greater inhibition of growth than did those treated with axerophthene. At the higher dose level, the rats treated with retinyl acetate showed a markedly greater suppression of growth as early as 7 days after treatment; by 14 days there were no survivors in the group treated with retinyl acetate, while 5 of 6 of the group treated with axerophthene survived. The differences between axerophthene and retinyl acetate are highly significant statistically; at the higher dose levels, the confidence levels are greater than 99.9%.

Levels of Metabolites in Liver and Breast after Multiple Dosing with Axerophthene and Retinyl Acetate. The availability of new methods for the accurate determination of retinoids and their metabolites in tissue samples has enabled us to determine significant differences between axerophthene and retinyl acetate, both in terms of their relative distribution to the liver and breast and in terms of their
D. L. Newton et al.

Table 2
Comparison of toxic effects of large doses of axerophthene and retinyl acetate given to rats

Four-week-old female Sprague-Dawley rats fed Wayne laboratory chow were randomized into 6 groups, each containing 6 rats, with each group having a mean weight of 58 to 60 g. They were treated 5 times/week (Monday through Friday) P.O., as shown below. The vehicle was cottonseed oil containing 4% CHCl₃. The experiment was terminated at the end of 14 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survivors at 7 days</th>
<th>Survivors at 14 days</th>
<th>Wt at 7 days (g)</th>
<th>Wt at 14 days (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle alone, 0.2 ml, 5 times/wk</td>
<td>6/6</td>
<td>6/6</td>
<td>88 ± 3³</td>
<td>120 ± 4</td>
</tr>
<tr>
<td>Axerophthene, 30.4 μmol, 5 times/wk</td>
<td>6.6</td>
<td>6.6</td>
<td>72 ± 3</td>
<td>95 ± 6b</td>
</tr>
<tr>
<td>Retinyl acetate, 30.4 μmol, 5 times/wk</td>
<td>6/6</td>
<td>4/6</td>
<td>63 ± 5</td>
<td>65 ± 9</td>
</tr>
<tr>
<td>Vehicle alone, 0.3 ml, 5 times/wk</td>
<td>6/6</td>
<td>6/6</td>
<td>86 ± 2</td>
<td>117 ± 3</td>
</tr>
<tr>
<td>Axerophthene, 45.6 μmol, 5 times/wk</td>
<td>5/6</td>
<td>5/6c</td>
<td>67 ± 3c</td>
<td>89 ± 5</td>
</tr>
<tr>
<td>Retinyl acetate, 45.6 μmol, 5 times/wk</td>
<td>5/6</td>
<td>0/6</td>
<td>50 ± 2</td>
<td>0/6</td>
</tr>
</tbody>
</table>

³ Mean ± S.E.
⁴ Significantly different from respective group treated with retinyl acetate; p < 0.05.
⁵ Significantly different from respective group treated with retinyl acetate; p < 0.001.

metabolism in these tissues. Reverse-phase HPLC with Spherisorb ODS columns (8) eluted with methanol:water gradients provides excellent resolution of retinol, retinyl acetate, axerophthene, and retinyl palmitate, as shown in Chart 3. Retention times varied slightly depending on the age of the columns and on the particular Spherisorb ODS column used. Recovery of these retinoids is essentially quantitative with the methods used. Chart 4 shows the profiles of parent substances and metabolites found in either the liver or breast after repeated p.o. dosing of rats with either axerophthene or retinyl acetate. Tissue samples were obtained 24 hr after the last of 9 doses, and extracts were applied to Spherisorb ODS columns. Since liver extracts from both treated and untreated animals contain 10 to 100 times more retinoid than do comparable breast extracts, a smaller aliquot can be applied to columns to give chromatograms that have excellent base lines and sharp, symmetrical peaks, with no interference from tissue lipids or other endogenous tissue constituents. Thus, analysis of liver extracts (Chart 4 D to F) provides chromatograms with resolution essentially identical with that obtained by the direct application of the pure marker retinoids shown in Chart 3. In contrast, breast extracts contain much less retinoid, and larger volumes of sample must be applied to the columns for adequate detection. Lipids in these extracts, as well as the larger sample volumes, caused loss of resolution during HPLC analysis, as shown in Chart 4, B and C.

Liver extracts from rats treated with solvent alone (Chart 4D) showed a small peak of retinol and a major peak of retinyl palmitate, which is in accord with classical studies (12, 13). After repeated dosing of rats with either axerophthene or retinyl acetate, the parent compound was not found in the liver in any appreciable amount (Chart 4, E and F). Treatment with retinyl acetate caused marked elevation of liver stores of retinyl palmitate with only a small increase in liver retinol (Chart 4E). The increase in peak sizes at 18 and 23 min (Chart 4E) probably represents the formation of fatty acid esters of retinol other than the palmitate ester, such as the linoleate, oleate, and stearate esters (A. B. Roberts, unpublished observations). Comparison of Chart 4, E and F, makes it clear that an equivalent p.o. dose of axerophthene caused much less deposition of retinyl palmitate in the liver than did the same dose of retinyl acetate.

A different pattern of metabolites was found in breast extracts. Chart 4A shows that, compared to the liver, there is little endogenous retinoid in the breast. Treatment with retinyl acetate (Chart 4B) caused the appearance of a major peak with retention time identical with retinol, with no peak seen in the retinyl palmitate region of the chromatogram. Anerophthene treatment (Chart 4C) resulted in major deposition of this substance in the breast with 2 much smaller peaks seen in and immediately in front of the retinol region. The identity of this last, most polar peak is unknown. Most of the retinoid found in the breast after axerophthene treatment is axerophthene itself, which is in marked contrast to the situation found in the liver.

The total levels of retinoids found in both liver and breast after treatment with axerophthene were also quantitated with trifluoroacetic acid, and the results are shown in Table 3. Since retinol, retinyl acetate, and retinyl palmitate all yield the same molar absorbance with trifluoroacetic acid at 616 nm (7), the determination of the molar concentration of total retinoid, expressed as retinol and/or retinyl ester,

![Chart 3. Separation of retinoids by HPLC. A solution containing 0.36 μg of retinol, 0.90 μg of retinyl acetate, 0.52 μg of axerophthene, and 4.74 μg of retinyl palmitate in methanol was applied to a 5-μm Spherisorb ODS column (3 x 250 mm). A linear gradient, beginning with 83% methanol in water and ending with 97% methanol in water, was run over a period of 12 min, with a flow rate of 1.2 ml/min. Elution was continued with 97% methanol in water to remove retinyl palmitate from the column. Recorder artifact during the first 3 min, resulting from solvent injection, has not been plotted.](cancerres.aacrjournals.org)
which are associated with retinoids bearing strongly polar toxic properties of retinoids appear to result from either (a) both in vitro and in vivo, as well as toxicological properties treatment with axerophthene. In contrast, axerophthene treatment of rats with retinyl acetate resulted in a 3-fold greater deposition of retinoid in the liver than did equivalent treatment with axerophthene. In contrast, axerophthene was more effective than was retinyl acetate in elevating retinoid levels in the breast, although in this case breast may be validly performed in a tissue in which these compounds are the only retinoids present in significant amount. Treatment of rats with retiny acetate resulted in a 3-fold greater deposition of retinoid in the liver than did equivalent treatment with axerophthene. In contrast, axerophthene was more effective than was retinyl acetate in elevating retinoid levels in the breast, although in this case breast deposition of retinoid in the liver was 3-fold greater than that in the breast. Tissue levels were determined with the trifluoroacetic acid method after extraction of tissue with ethyl ether. Numbers are expressed as total \( \mu \text{mol} \) of retinoid found per g, wet weight, of tissue.

Chromatograms are shown from representative single animals. The original samples of breast and liver tissue were taken and ground with 5 parts of anhydrous sodium sulfate. The dried tissue powders were then extracted with ether (10 ml for breast samples, 20 ml for liver samples), and 4-ml aliquots of the ethereal extracts were taken. The ether was removed with nitrogen, and the extract was redissolved in 400 \( \mu \)l of chloroform to which 500 \( \mu \)l of acetonitrile were subsequently added. Aliquots of these redissolved extracts were then applied to Spherisorb ODS columns; for breast extracts 100-\( \mu \)l samples were applied to the columns; for liver extracts 10-\( \mu \)l samples were used. Gradient elution of columns was performed as shown in Chart 3. Chromatograms are shown from representative single animals. The original amounts of tissue samples taken were as follows: A, 0.23 g; B, 0.18 g; C, 0.37 g; D, 1.28 g; E, 0.84 g; F, 1.22 g. Recorder artifact during the first 3 min, resulting from solvent injection, has not been plotted. Detection for breast samples (A, B, and C) was with a Spectra-Physics Model 8200 detector with a wavelength detector set at 325 nm was used. The larger cell volume in the samples (A, B, and C) was with a Spectra-Physics Model 8200 detector with a wavelength detector set at 325 nm. The larger cell volume in the samples (A, B, and C) was with a Spectra-Physics Model 8200 detector with a wavelength detector set at 325 nm. The larger cell volume in the samples (A, B, and C) was with a Spectra-Physics Model 8200 detector with a wavelength detector set at 325 nm.

It is probable that this is the case from the demonstrated increase in retinyl esters in the liver following axerophthene administration. In particular, the biological activity of axerophthene in tracheal organ culture suggests that enzymatic mechanisms for activation of retinoids are not confined to the liver and that nonhepatic tissues may play an important role in the metabolism of retinoids.

**REFERENCES**

Cancer Research


Biological Activity and Metabolism of the Retinoid Axerophthene (Vitamin A Hydrocarbon)


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/38/6/1734

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.