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Toxicity, metabolism and applied uses of 3,4-didehydroretinol

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Toxicity, metabolism and applied uses of 3,4-didehydroretinol

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1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is divided into six sections. In the first introductory section, retinoid deficiency and toxicity are discussed, along with a general background of vitamin A related compounds. The history of A2 and its use in the MRDR assay is reviewed. Section 2 is a manuscript that has been prepared for submission to Teratology. This paper compares the teratogenic potency of three retinoids: all-*trans* isomers of A1, A2, and RA. Five levels of each retinoid were orally administered to pregnant dams on day 8.5 of gestation. Endpoint measures collected from the fetus at day 19.5 of gestation included percent terata, percent embryolethality, length (cm) and weight (g). Amounts of retinoids in tissues of both dams and offspring were also quantified. Section 3 is a manuscript that has been prepared for submission to Neurotoxicology and Teratology. The purpose of this work was to determine if prenatal administration of all-*trans* isomers of A1, A2, and RA effect postnatal behavior of six-week old rats. Comparisons were made between the compounds on the basis of live birth rates, overall growth and development, and significant behavioral changes caused by a range of three dosage levels from each of the three compounds. Section 4 is a manuscript that has been accepted by Nutrition Research for publication. This paper evaluates the vitamin A status of low-income pregnant women in Iowa by use of the MRDR assay. The serum concentrations of β-carotene, α-carotene, lycopene, and α-tocopherol were also measured.

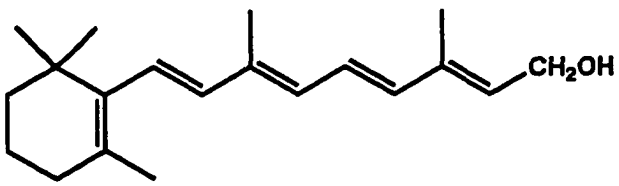
Section 5 is a general summary of the important findings of these papers, and section 6 lists the conclusions that emerge from the thesis work.

Background

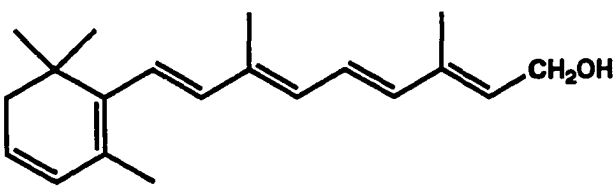
The usage of vitamin A to treat night blindness has been chronicled for three and one-half millennia. Early medical practitioners recommended consumption of ox-liver to ameliorate poor night vision. Others advised the application of liver juices directly to the eye. Modern science has revealed the validity of these methods, with evidence that livers of almost all animals are abundant in vitamin A, which allows dark adaptation of the retina.

In the early 1900's, scientists involved in lipid research realized lipid portions of foods were essential for supporting life. Continued work demonstrated that lipids such as butter and cod-liver oil possessed a much greater ability to promote growth than other fats such as lard and almond oil. An essential growth factor termed "fat-soluble A," which was essential for normal growth and development, was identified. Thus began twentieth century research in the field of vitamin A, or retinol (1).

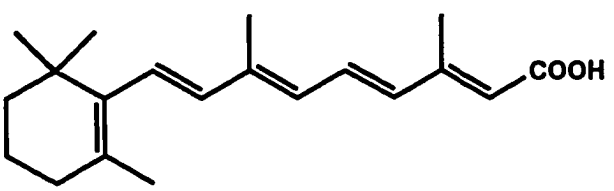
Today the entire family of compounds encompassing retinol, retinaldehyde, retinoic acid, and their many analogs are known as retinoids. The molecular structure of naturally occurring retinoids can be described by dividing the molecule into three parts: a trimethylated cyclohexene ring, a conjugated tetraene side-chain, and a polar carbon-oxygen group which completes the side chain and can exist in three possible oxidation states (Figure 1). However, few synthetic retinoids conform to this definition, since many are tricyclic, non-polyisoprenoid



RETINOL



3,4-DIDEHYDRORETINOL



RETINOIC ACID

Figure 1. Structures of the all-*trans* forms of the naturally occurring retinoids retinol, 3,4-didehydroretinol, and retinoic acid.

compounds. Therefore, a biological definition may be more suitable, as it incorporates all substances in the retinoid family. Sporn and Roberts (2) have proposed that a retinoid be defined as:

> a substance that can elicit specific biologic responses by binding to and activating a specific receptor or set of receptors, with the program for the biologic response of the target cell residing in the retinoid receptor rather than in the retinoid ligand itself (p. 2).

Unquestionably, the discovery of the retinoid receptors, and the knowledge that retinoids regulate gene expression and ultimately the biological activities of all cells, has dramatically transformed the field of retinoid research.

Several analogues and metabolites of retinol have interesting and useful biological effects. Retinoids are commonly used therapeutically for treatments against acne and other skin disorders. Large doses reduce recurrence of some forms of skin cancer. All-*trans* retinoic acid has been shown to be effective in treating acute promyelocytic leukemia. Other retinoids are effective against a variety of chemically induced and spontaneous cancers (3).

One very interesting retinoid caught the attention of scientists when, in 1937, a "new form of vitamin A" was discovered in the livers and other tissues of fresh-water fish. Vitamin A2 was isolated and subsequently characterized (4,5). The compound differed from retinol (A1) only in having one more conjugated double bond at the 3,4 position in the cyclohexene ring. Early work indicated that A2 was able to promote growth in vitamin A deficient rats, although experiments were confounded by the presence of substantial amounts of A1 in the A2 extracts (6). Purer and larger yields of vitamin A became available for use when Farrer and workers (7) successfully synthesized dehydroretinol from methylretinoate by bromination followed by dehydrobromination to the methyl ester of dehydroretinoic acid. Addition of lithium aluminum hydride then yielded dehydroretinol.

Biological studies in rats have indicated that purified A2 possesses 30-40% of the biological activity of vitamin A1 in promoting growth, reproduction, and vision (7-10). Although A2 has been shown to be the predominant form of vitamin A in several species of

Although A2 has been shown to be the predominant form of vitamin A in several species of fish (11), it is present in only small amounts in mammals. A2 accounts for approximately 1-2% of the total vitamin A stored in mouse liver (12) and has been shown to be metabolized to retinol in rats (13), suggesting possible interconversion of A1 and A2 in mammalian systems. A2 has also been identified in normal human epidermis, possibly as a metabolite of A1 (14-17).

Early work indicated that A2 produced a modified form of visual purple in the dark-adapted retina of certain fresh water fish, allowing extra sensitivity to red light (18). Studies were undertaken in the 1940's to determine if A2 could increase the sensitivity of aviator's eyes to the red lights used for military identification. Shantz and workers (8) used a rat model, and discovered that A2 could almost entirely replace A1 in the retina of rats. However, the growth response was depressed for rats dosed at 0.11 μmole of A2 for 12 weeks, when compared to lower doses of vitamin A2 or equivalent doses of A1. Whether this phenomenon was the result of A2 toxicity, or a response to a contaminant in the A2 preparation is not clear. Based on these findings, Millard and workers (19) dosed medical students with 9.75 mg A2 daily for 39 days. Results from this study indicated that A2 increased sensitivity to red light in humans. Subjects were monitored for signs of A2 toxicity, including: eye and skin effects, gastro-intestinal complaints, weight loss, and anemia. Blood levels of A1, calcium, phosphorus, and alkaline phosphatase were also taken. No toxicity of A2 was reported.

Assessment of Vitamin A Status

Despite our increased understanding of vitamin A related compounds, vitamin A deficiency continues to be a serious nutritional problem in many parts of the world. Annual morbidity of xerophthalmia has reached approximately 5 million in children from third world countries. About one quarter million of these children become blind, and annual mortality is approximately 125,000. A series of changes in the eye, usually beginning with night blindness, progress to cause irreversible changes in the cornea, resulting in keratomalacia. Changes in the skin, such as follicular hyperkeratosis and phrynoderma are also evident during vitamin A deficiency. Immune function is also suppressed in deficient states of vitamin A, leading to increased viral and bacterial infections. Protein-calorie malnutrition and zinc deficiency exacerbate the situation.

Within populations, children and pregnant and lactating women tend to be at greater risk for vitamin A deficiency. These periods of development, when proliferative growth and tissue development occur, increase the body's demand for vitamin A. While increased consumption of vitamin A containing foods can meet these demands, many individuals without adequate nutrition develop clinical vitamin A deficiency during these periods of development (20,21).

While overt, clinical signs of vitamin A deficiency allow easy diagnosis of the condition, considerable irreversible damage to the eye may have already occurred. For instance, classical clinical assessment of vitamin A deficiency has included the presence of Bitot's spots with conjunctival xerosis, corneal xerosis, and corneal ulceration. Biochemical

assessment has also been widely used as a diagnostic tool. A serum concentration lower than 0.35 μmol/L (<10 μg/dl) has been used to indicate vitamin A deficiency, while levels in excess of 1.05 μmol/L (>30 μg/dl) are associated with a satisfactory status. Serum concentrations falling between these levels, indicating a marginal vitamin A status, have been difficult to interpret. Subclinical deficiency of vitamin A is correlated with decreased growth and development, increased morbidity of infectious diseases, and increased risk of xerophthalmia and other ocular health problems. Sensitive methods for assessing marginal vitamin A status are needed for use in nutritional surveys of both individuals and populations.

Dietary assessment has been employed to evaluate vitamin A sufficiency. Unfortunately, this method is labor intensive, and produces results that do not correlate well with other measures of vitamin A status. Isotope dilution techniques have been employed as useful techniques in measuring the relative content of total body vitamin A. Unfortunately, this method uses sophisticated equipment and techniques, limiting its usefulness in large surveys or field work (21,22).

A dose-response test for indirectly assessing marginal liver stores of vitamin A was developed for use both in individuals and populations (23). The relative dose response test (RDR) relies on the observation that apo-retinol-binding protein (RBP) accumulates in the liver during less than optimal vitamin A status. When retinyl ester is administered, holo-RBP is released into the serum. Serum retinol (or holo-RBP) is measured initially and then again 5 hours following administration. A standard dose (1.6-3.5 μmole [450-1000 μg]) of retinol equivalents is given orally. Blood samples are taken at time 0 and 5 hours after the dose is

given. Serum concentration is measured by use of high-pressure liquid chromatography (HPLC), or by suitable spectrophotometric or colorimetric methods. The increase in serum retinol concentration, divided by the retinol concentration observed at 5 hours, is the RDR value, and is expressed as a percentage. Values ranging from 20 to 50% indicate a marginal status, while values >50% indicate deficiency. The RDR assay has been validated in humans using direct measures of liver vitamin A (24,25).

While the RDR is an extremely useful tool in assessing marginal vitamin A status, the need to obtain two separate blood samples in a 5-hour interval can pose problems in the field. Also, the need to analyze two blood samples per individual increases time and money spent on each assessment. Possible error is also introduced into the analysis from using two different samples for one calculation.

Due to these limitations, a more refined dose response test was developed. 3,4-Didehydroretinol (vitamin A2) was suggested as an indicator of vitamin A status when researchers observed that the ratio of A2 to retinol (A1) in serum was inversely related to the amount of vitamin A stored in the liver of rats (26,27). Similarly to A1, A2 binds to accumulated apo-RBP in the liver and is released as holo-RBP into the serum (28). Development of the new method, termed the modified relative dose response (MRDR), thus began in earnest (29,30).

The MRDR assay involves giving an oral dose of A2 acetate (0.35 μmol/kg, or 100 μg/kg body weight), dissolved in oil, and then taking a blood sample 5 hours later. At least 200 μl of serum is necessary to perform the assay. The serum is then mixed with an equal

200 μl of serum is necessary to perform the assay. The serum is then mixed with an equal volume of ethanol and an aliquot of retinyl acetate (RAC) is used as an internal standard. Serum is then extracted using hexane, and the amount of A2, A1, and RAC are determined by use of HPLC. Standards of both A1 and A2 are used to quantify the compounds, and a molar ratio of A2 to A1 is calculated. MRDR values ≥ 0.03 are indicative of marginal vitamin A status. This cutoff has been determined by comparison studies with the RDR and other vitamin A assessment techniques. While this cutoff level has been used in the United States, a value of ≥0.06 has been employed to indicate marginal vitamin A status in third-world countries, where the high incidence of protein-calorie malnutrition and other vitamin and mineral deficiencies exacerbate the situation (31). While these cutoff values seem arbitrary, they are based on the following observations: 1) MRDR ratios of healthy children and adults in the U.S. are < 0.03; 2) MRDR ratios of subjects in the U.S. that are >0.03 fall to values <0.03 in all instances following a dose of vitamin A; 3) MRDR ratios in healthy Indonesian children are usually <0.06; and 4) MRDR ratios in Indonesia that are >0.06 fall to values <0.06 following a dose of vitamin A (31).

The MRDR has proven to be a valuable method for use in large field surveys. Synthesized A2 is stable in the dark for at least two years when kept ≤ -20°C, for three months at 10°C, and for at least two weeks at room temperature. The assay itself requires only one blood sample, which can be frozen until analysis by HPLC can take place. HPLC systems and techniques are utilized world-wide, allowing relatively simple analysis of the serum samples.

Retinoid Toxicity

Retinoids are essential for several biological processes, including growth and development, vision, reproduction, cellular differentiation, and pattern formation during embryogenesis. Deficiencies of vitamin A can negatively affect all of these biological processes. The recommended daily allowance (RDA) of vitamin A has been set at a level both to protect most healthy individuals against any of these negative effects and to provide a reserve for up to three months. Chronic doses at least ten times the RDA produce signs of excess vitamin A intake. These chronic toxicity signs include alopecia, ataxia, bone and muscle pain, cheilitis, conjunctivitis, headache, hepatotoxicity, hyperlipemia, hyperostosis, membrane dryness, pruritus, pseudotumor cerebri, various skin disorders, and visual impairment (32,33). Single doses of vitamin A exceeding 0.7 mmol in adults or 0.35 mmol in children produce acute toxicity signs including nausea, vomitting, headache, increased cerebrospinal pressure, vertigo, blurred vision, and muscular incoordination (32,33). Teratogenic effects from excess retinoid consumption occur at much lower doses than those producing acute toxicity symptoms, and at slightly lower doses than those producing chronic toxicity effects. Intake of retinoids early in pregnancy in amounts ten times the RDA have been shown to elicit strong teratogenic effects, including spontaneous abortions or significant malformations of the offspring (33-35).

Retinoids produce a distinct pattern of teratological effects including embryolethality, dysmorphia of the central nervous system (microcephaly, exencephaly, hydrocephalus, encephaloceles, spina bifida), craniofacial defects (agnathia, micrognathia, anotia, microtia,

encephaloceles, spina bifida), craniofacial defects (agnathia, micrognathia, anotia, microtia, astomia, microstomia, cleft palate, teeth malformations), cardiovascular abnormalities (transposition of great vessels, ventribular septal defect, double outlet right ventricle, aortic arch irregularities), eye defects (anophthalmia, microphthalmia, exophthalmos, ablepharon), urogenital problems (missing kidneys) and poorly developed axial skeletal tissues. Most of these abnormalities are noted in all species tested (36). Permanent learning disabilities in the offspring occur from sub-teratogenic doses administered prenatally (37).

The teratogenic "potency" of retinoids may differ by several orders of magnitude and may be related to absorption, distribution, metabolic fate, placental permeability, or to interaction with embryonic intracellular receptors (38). Retinoids act as morphogens capable of altering embryonic cell migration, proliferation, and differentiation. An unsubstituted carboxylic acid group at the end of the tetraene chain (preferably in the all-*trans* configuration as in all-*trans* retinoic acid) seems to be a major structural requirement for the high teratogenic activity of retinoids in rodents (39).

Retinoic acid can fulfill most of the physiologic and pharmacologic functions of retinol. It reverses symptoms of vitamin A deficiency except those related to vision and reproduction (40). Body tissues and cells in cultures possess the metabolic capability to convert β-carotene and retinol into retinoic acid (41-44). Biological effects of retinoic acid may be mediated through its binding to nuclear retinoic acid receptors (45). Accumulated data from nearly two decades has led many researchers to believe that the teratogenic effects of retinol are mediated by the extent of its metabolic conversion to retinoic acid.

The mechanisms by which retinoids exert their teratogenicity has been under intense investigation for several decades. For example, retinoic acid has a profound effect on patterning of the developing chick limb bud (46). A set of interactions involving the polarizing region, a small group of mesenchyme cells at the posterior margin of the limb bud, has been identified in the developing chick. The pattern of structures that develop across the antero-posterior axis of the limb bud is determined by a signal from the polarizing region acting on mesenchymal cells in the progress zone. The progress zone is a region of undifferentiated cells at the tip of the limb. Tickle et al. (46) found that grafts of the polarizing region to the anterior margin of the wing lead to mirror-image duplications of pattern. When retinoic acid is locally applied to the anterior margin, mirror-image duplicated patterns are also produced. The signaling induced by local application of retinoic acid and by the graft of polarizing region tissue is very similar. The effects are dose and position dependent. Extra digits can be produced by increasing retinoic acid concentration or by increasing the length of exposure. The changes that follow retinoic acid treatment appear to be mediated by mesenchymal cells (46,47). Retinoic acid either directly or indirectly specifies the position of mesenchymal cells in the bud, and the interaction of these cells with the those of the apical ridge, which defines morphologic development, is thereby determined. A signal is then produced that controls local cell proliferation in the bud mesenchyme. The acid of A2, namely 3,4-didehydroretinoic acid (ddRA), is also present in the developing chick wing bud, and is generated in situ from retinol through an A2 intermediate (48). RA and ddRA possess equipotent morphogenetic properties in the chick limb.

Lopez and Carrasco (49) have concluded that retinoic acid affects limb development through its effect on neural crest cells, which are believed to establish positional cues in the brachial girdle for limb development. In a study using *Xenopus laevis*, these researchers showed that retinoic acid caused progressive truncation of anterior structures. The most severely affected embryos were devoid of eyes, nasal pits, forebrain, midbrain, and otic vesicles. These animals also possessed an enlargement of the hindbrain that reached the anterior end of the embryo. Spina bifida and deficiencies in elongation were also present. Besides these effects, retinoic acid produced malformations in the brachial arches and disorganization of the pronephros. Temporal and spatial distribution patterns of A1 and A2 tended to coincide during *Xenopus* development (50).

Tickle (51) theorizes that retinoids may be important signaling substances in many parts of the vertebrate embryo where patterns of connective tissue are generated. Specific facial defects and patterning and morphogenesis of the facial primordium that gives rise to the upper beak in chick embryos are inhibited by the application of retinoic acid (52). Local application of retinoic acid to the developing spine can induce the formation of additional vertebral structures (51). Wagner et al. (53) has shown that the floor plate of the neural tube can generate retinoic acid from retinol. This may be the basis of the signaling that regulates some aspects of neuronal differentiation and axonal guidance. Recent studies have shown temporal and spatial patterns of expression of retinoic acid receptors and cellular binding proteins in the face, spine, and nervous system of vertebrate embryos (54-57).

Data accumulated from the analysis of expression patterns, ectopic expression studies, and gene disruption experiments strongly suggests a regulatory role for homeobox-containing genes during all stages of vertebrate morphogenesis (58). The plethora of studies that have tested the response of these genes to various retinoids have been useful in the identification of mechanisms by which retinoic acid associated dysmorphology occurs. However, a basic understanding of the partitioning of retinoids between maternal and embryonic/fetal compartments is also crucial in identifying mechanisms that may produce aberrant development. The developing embryo is dependent on the maternal circulation for vitamin A, since there is no de novo synthesis of retinol or retinoic acid. The flow of retinol across the placenta is bi-directional, but largely favors the fetus (59). Despite the wealth of study in this area, little is understood about how the transport and metabolism of retinoids is involved in teratogenic mechanisms. The function and importance of the various nuclear RAR and RXR receptors and the genes that they regulate in inducing terata also remain unclear. Increased knowledge in these areas may lead to the answers sought in determining mechanisms of retinoid-induced teratogenicity.

Hypothesis

The working hypothesis for the dissertation was that a single oral dose of all-*trans* 3,4-didehydroretinyl acetate (vitamin A2) in oil, when given at the dosage level of 0.14 μmol/kg BW or 2.5 mg for use in the MRDR assay, is safe and effective for women in their third trimester of pregnancy.

While much data has been accumulated regarding teratogenic potency of certain retinoids (such as retinol and retinoic acid), no teratogenic data exists for A2. To test the issue of safety, experiments were designed to evaluate A2 in terms of induced toxicity, teratogenicity and embryolethality. Rat models work well for evaluation of these criteria, as rats are both sensitive and specific models for retinoid induced human teratogenicity. Timed-pregnant Sprague-Dawley rats were dosed with a range of A2 on day 8.5 of gestation. Both dams and pups were evaluated. Comparisons were made between other groups of rats dosed with similar dose ranges of all-*trans* retinyl acetate (A1) and all-*trans* retinoic acid (RA), and to control rats, dosed only with the vehicle. Detailed findings related to this study can be found in Section 2.

. Because retinoids can also induce central nervous system (CNS) deficits in offspring, a second experiment was designed to evaluate the effects of A2 on CNS development in vivo. Similar to the first study, rats were dosed with a range of A2 on day 8.5 of gestation, and compared to control rats and groups of rats dosed with A1 and RA. This study is presented in manuscript style in Section 3.

The effectiveness of the use of A2 (as a part of the MRDR assay) to assess vitamin A status in pregnant women was also tested. Fifty-seven low-income pregnant women from four different ethnic groups were assessed for marginal vitamin A status in the third trimester of pregnancy. Serum concentrations of β-carotene, α-carotene, lycopene, α-tocopherol, total cholesterol and retinol were also determined. The results of the study are presented in manuscript style in Section 4.

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2. COMPARATIVE TERATOGENICITY AND METABOLISM OF THE ALL-*TRANS* ISOMERS OF 3,4-DIDEHYDRORETINYL ACETATE, RETINYL ACETATE, AND RETINOIC ACID IN PREGNANT RATS

A paper to be submitted to Teratology

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ABSTRACT

The teratogenic potencies of the all-*trans* isomers of 3,4-didehydroretinyl acetate (A2), retinyl acetate (A1), and retinoic acid (RA) were compared. Groups of eight timed-pregnant Sprague-Dawley rats were administered single equimolar doses (3.5-352 μmol/kg BW) of the retinoids orally in oil on day 8.5 of pregnancy. A1 induced clear teratogenic effects at only the highest dose, and did not significantly increase embryolethality at any dose. Both RA and A2 were teratogenic and embryolethal at dose ranges of 35-352 μmol/kg BW. While gross terata did not appear at doses lower than 35 μmol/kg BW, embryolethality occurred with both A2 and RA at doses ≥ 11 μmol/kg BW. Dams also exhibited toxic manifestations from doses of A2 and RA above 35 μmol/kg BW. In the liver of dosed dams, the following effects were noted: 1) Liver concentrations of A1 and A2 increased with the dose of A1 and A2, respectively, 2) RA had little effect on A1 except at the highest toxic dose, which induced an increase, and 3) A2 showed a sparing effect on A1. RA, although not detected in fetuses from dams treated with A1, was present in significant concentrations (0.5-

4.1 nmol/g liver) in fetuses from dams treated with A2. The increased teratogenicity and embryolethality of A2 relative to A1 correlates with this enhanced concentration of fetal RA. Therefore, RA and A2 proved to be more toxic and teratogenic than A1, possibly due to differences in transport and metabolism among the retinoids.

INTRODUCTION

Physiological concentrations of retinoids are involved in regulating cell growth and development. At higher concentrations, retinoids possess teratogenic potential that is species dependent (Willhite et al., '89). Humans appear to be the most sensitive species on a body weight basis to retinoid-induced terata (Willhite and Book, '90). The structure of the retinoid is important in determining the embryotoxic and teratogenic potential of the compound (Willhite, '86). Retinoids that possess an acidic terminus group, e.g. 13-cis retinoic acid and all-*trans* retinoic acid, generally have 2-3 fold more teratogenic potential in animals than the alcohol forms of vitamin A, e.g. retinol, and its fatty acyl esters. (Willhite, '90).

3,4-Didehydroretinol (A2) differs from retinol (A1) in that it possesses a double bond at the 3,4 position (Figure 1). Vitamin A2 is a naturally occurring analogue of A1 and is a common component of the food supply (Barua and Nayar, '66). The biological activity of A2 in stimulating the growth of rats is approximately 40% that of A1 (Shantz and Binkman, '50; Sundaresan and Cama, '61; Howell et al., '67).

A2 esters in foods are hydrolyzed to the alcohol (dehydroretinol) in the small intestine, absorbed in micellar form with other lipids, reesterified to the ester in the gut, transported on

chylomicra to the liver, and stored as vitamin A2 ester in the liver (Lederer and Rathman, '38; Goswami and Barua, '86; Tanumihardjo and Olson, '88; Farrer et al., '52; Henbest et al., '55). Vitamin A2 is released from the liver as a complex with retinol-binding protein (RBP) similarly to A1 (Tanumihardjo et al. '87).

A biological metabolite of vitamin A2, all-*trans*-3,4-didehydroretinoic acid (ddRA) has been shown to have equivalent morphogenic properties to its vitamin A counterpart, all-*trans* retinoic acid (RA) (Thaller and Eichele, '90). Further, ddRA was as biologically active as RA using several models of epithelial differentiation, although their metabolism differed depending on the cell type used (Torma et al., '94). However, the toxicity and teratogenicity of A2 in vivo has not been studied previously. We report here the relative teratogenicity of A2, A1, and RA in timed-pregnant Sprague-Dawley rats.

MATERIALS AND METHODS

All-*trans*-3,4-didehydroretinyl acetate was synthesized from retinoic acid (Barua and Ghosh, '72) in our laboratories. The compound was purified twice on a column of 8% water-deactivated alumina. Its purity, as judged by absorption at 350 nm, and determined by HPLC, was 99.9%. All-*trans*-retinyl acetate and all-*trans*-retinoic acid were obtained commercially (Sigma Chemical Co., St. Louis, MO, and BASF Corporation, NJ).

Animals

Timed-pregnant Sprague-Dawley rats (Holtzman, Madison, WI) were housed in accordance with Iowa State University and NIH guidelines in virus-free environments on a

light/dark cycle of 6 am to 6 pm. The animals were fed a diet of standard rat chow obtained from Harlan/Teklad, Madison, WI, containing 14.4 nmol retinol palmitate/g diet.

Study Protocol

Each dam was administered single, equimolar oral doses of the retinoids (3.5-352 μmol/kg body weight) in approximately 250 μl corn oil, pipetted directly into the mouth of each animal by using a Gilson positive-displacement pipette (Rainin Instruments, Woburn, MA) on day 8.5 of pregnancy. Control animals received corn oil only. Gestation was interrupted on day 19.5 by cervical section of etherized dams. Uterine horns were examined, implantation sites counted, and fetuses removed. Maternal serum was collected by cardiac puncture and maternal livers were excised. Fetuses were weighed, measured, and photographed. Thereafter fetal livers were excised, blotted on humid filter paper and frozen at -20°C.

Retinoid Extraction and Analysis

All operations were carried out under yellow light. Extraction of maternal and fetal livers (0.5 g) was accomplished by the method of Barua and Olson ('89). The tissue was ground with 1.5 g anhydrous sodium sulfate exhaustively by use of a pestle and mortar. Methylene dichloride (5 volumes) was added along with internal standard (1.4 μg retinyl acetate; 150 μl), and samples were ground further. Methylene dichloride was filtered, and extracts were pooled and mixed. An aliquot (1 ml) of the extract was evaporated under argon, and the residue redissolved in 200 μl of methanol:methylene dichloride (1:1 v/v) for analysis by HPLC.

Extraction of retinoids from the serum of dams was achieved by treating serum (200 µl), with 100% ethanol (400 µl), 10% acetic acid in water (20 µl), and ethyl acetate (400 µl). Internal standard (1.4 µg retinyl acetate; 250 µl) was added and the mixture was subjected to further extraction by use of hexane (2 X) (Barua and Olson, '89). The extracts were washed, pooled, dried under argon, and resuspended in 100 µl methanol:methylene dichloride (1:1 v/v) for injection onto the HPLC column.

Each sample was applied to a 5 µm C18 Waters Resolve reversed-phase column by use of a WISP autoinjector (Waters, Milford, MA) using the method described by Barua ('90). A Waters 996 photo-diode array (PDA) detector monitored absorbance at 325 nm for retinoic acid, retinol, and retinyl esters, and at 350 nm for 3,4-didehydroretinol and its esters. Two Waters 510 pumps were set at a flow rate of 1.2 ml/min, and operated by use of a Waters automated gradient controller. A gradient solvent system consisted of a linear gradient of solvent A (methanol-water, 68:32, v/v) to solvent B (methanol-methylene dichloride, 4:1, v/v) over 20 min. Solvent B was run for 10 min more, at which time the gradient was changed to initial conditions over a 5-min period. The column is equilibrated for 10 min prior to the next injection. Retention times for retinoic acid, 3,4-didehydroretinol, retinol, 3,4-didehydroretinyl acetate, retinyl acetate, 3,4-didehydroretinyl palmitate, and retinyl palmitate were 12, 18, 21, 24, 25, 31 and 32 min, respectively (Figure 2). The Millennium 2010 software version 1.2, which was developed by Waters for use with their 996 PDA detector, performed data acquisition, processing, and management of chromatographic information. All data were acquired and stored in three-dimensional mode, which allowed

detailed examination of selected spectra, integration of peak areas, and assessment of the purity of compounds.

Statistics

Least squared means, standard errors, means, standard deviations, Student's t-tests and *P* values were determined by use of general linear model procedures of the Statistical Analysis System, version 6.07, Cary, NC.

RESULTS

Toxic effects on dams

Dams dosed with 3.5 μmol/kg BW of both RA and A1 were significantly younger and smaller than other animals used in this study. Throughout the experiment, we perceived that these animals showed a higher level of stress than older animals. Thus, the anomalous data acquired from these two groups may well be a reflection of the animals response to pregnancy rather than to the compounds dosed.

Dams dosed with either 352 μmol/kg RA or A2 showed overt retinoid toxicity, exhibited by loss of hair and hair discoloration. Decreased weight gain which was noted at doses ≥ 35 μmol/kg A2 and ≥ 113 μmol/kg RA, appeared to be a more sensitive indicator of dam toxicity for both retinoids.

Fetal resorption

As summarized in Table 1, control animals experienced no resorption. This is unusual, since average background embryolethality in rodents has been reported to be approximately

4% (Manson, '86). In similar earlier studies in our laboratory, we found 1.8% resorption in control Sprague-Dawley rats (Gunning et al., '93). RA at 352 μmol/kg caused 100% resorption, which decreased in a dose-dependent manner to 3.6% at 3.5 μmol/kg. All doses of A1 gave similar resorption rates of approximately 4%, although others have reported dose-dependent embryolethality in Wistar rats (Langman and Welch, '66). Interestingly, the vitamin A2 dose response curve for resorption was non-linear, with most resorption occurring at the 35 μmol/kg dose.

Terata

RA was the most teratogenic compound, increasing terata significantly ($p \leq 0.0001$) at 113 μmol/kg. Of the terata present, 75% consisted of exencephaly, 17% abdominal protrusion, 8% microcephaly, and 8% bulging crown. No terata was evident for doses of RA ≤ 35 μmol/kg. A2 at 352 μmol/kg induced 5% terata, compared with 3% for A1. Further, the type and extent of terata for the A2 group were more profound than were those caused by Al. Fetuses exhibiting terata at this dose of A2 had bulging crowns (60%) and exencephaly (40%). One stillborn fetus of the A2-treated dams exhibited gross terata, with complete craniofacial malformation, exencephaly, and protrusion of the abdomen. Terata apparent from A1 at the same dose included eye abnormalities (25%) and bulging crowns (75%). Both A2 and A1 also induced terata at the 113 μmol/kg dose; A2 primarily produced malformations of craniofacial features, and A1 generated slight cranial bulges. No terata were evident for doses ≤ 35 μmol/kg of A1 or A2.

Pup length and weight

Growth of the fetus was significantly reduced by doses of RA ≥ 11 μmol/kg. While A1 had no effect on pup length or weight, A2 at lower doses (3.5-11 μmol/kg) somewhat surprisingly tended to reduce pup weight but not length.

Maternal liver

Liver weight/body weight ratios and mean concentrations of retinoids recovered from maternal livers are summarized in Table 2. Liver concentrations of A1 and A2 expectedly increased with the dose of A1 and A2, respectively. RA had little effect on A1 except at the highest toxic dose, which induced an increase of A1, while A2 showed a sparing effect on A1 at higher doses. RA was detected only in the livers of dams treated with 35 μmol/kg of A2.

Fetal liver

Mean concentrations of total A1 in fetal livers tended to decrease with increases in dosing, as summarized in Table 3. A2 was detected in fetal livers from animals dosed with 11-352 μmol/kg A2 with individual values ranging from 0.3-3.9 nmol/g liver. RA was detected in fetal livers from dams treated with the 3.5 and 113 μmol/kg of RA and 11-352 μmol/kg of A2. RA values in individual fetal livers in the RA and A2-treated groups ranged from 0-2.2 nmol/g liver and 0-4.1 nmol/g liver, respectively. RA was not detected in fetal livers from dams treated with any concentration of A1. Furthermore, no A2 was found in fetal livers from dams dosed with any concentration of A1.

DISCUSSION

The maternal toxicity evident in the present study (decrease in weight gain, hair loss, and hair discoloration) is consistent with classic toxic manifestations of retinoid administration. Corroborative of previous studies, RA produced well defined patterns of terata and embryolethality in a dose-dependent manner (Gunning et al., '93; Nau et al. '94). A1 showed teratogenic effects only at the highest dose tested.

Although A2 induced terata also followed a linear dose-response curve, A2 induced embryolethality did not. Embryolethality for A2 at 35 μmol/kg BW was nearly nine times that observed at 352 μmol/kg BW. To validate this finding, the A2 dose at 35 μmol/kg was repeated, in a different group of eight pregnant dams. Similar results were obtained.

The recovery of retinoids from dam livers points to a possible difference in the metabolism of A1 and A2 (Table 2). A2 dosed animals showed a dose-response effect for A2 found in tissues. Likewise, liver storage of A1 increased in a dose-response manner in A1 dosed animals. Compared to control animals, A2 was effective in sparing liver A1 in livers of animals dosed at 35 μmol/kg BW A2. However, the storage of liver A1 in animals dosed at 35 μmolkg BW A2 was roughly twice that of dams treated with the larger dose of 352 μmol/kg BW A2. In fact, for all three compounds administered at the 35 μmol/kg BW dosage level, concentrations of A1 were greatest in the livers of A2 dosed animals. An additional curious finding was the exclusive recovery of RA from the livers of dams dosed with A2 at 35 μmol/kg BW. The fact that RA was not present in tissues of RA dosed animals is not surprising, since tissues were analyzed eleven days after RA was administered. RA is known

to be metabolized quickly, and its inactive metabolites are excreted in the urine and feces within seven days (Roberts and DeLuca, '67). The absence of RA in the livers of A1 dosed animals intimates a difference in the catabolism of A1 versus A2. The unusual ability of A2 at 35 μmol/kg BW to increase liver concentrations of A1 and RA correlates with the increased embryolethality seen at this dose of A2.

In fetal liver tissues, A1 concentrations did not follow a dose-response curve. Indeed, whereas RA dosing had no consistent effect, increasing doses of A1 and A2 were associated with lower total A1 values. In contrast, A2 appears in fetal livers of A2 dosed animals in a dose dependent manner. Interestingly, RA is found in the fetal livers of all A2 dosed animals, with the exception of the lowest A2 dose, but in none of the A1 dosed animals. Even when RA was administered, fetal liver RA at 19.5 days was less than in A2-dosed animals. RA accumulation from an A2 dose was maximal at two mid-range levels of A2 (11 and 113 μmol/kg BW). Due to the high incidence of embryolethality at the 35 μmol/kg BW dose of A2, only one fetal liver was available for analysis of RA concentration. While this liver did contain RA, the RA concentration was not reported since one sample cannot be considered representative of the entire group.

Both A1 and A2 seem to be absorbed and stored well, since both compounds increase in the liver tissues of dams in a dose-dependent manner. However, the accumulation of A1 in fetal livers from a dose of A1 appears to decrease with increasing levels of A1 administered. Conversely, A2 increases in a dose-dependent manner in fetal tissues. Thus, the metabolic regulation and transport of A1 vs. A2 in the pregnant rodent seem to be different. The

detection of RA in the livers of A2 dosed dams and fetuses correlates well with the unconventional response curve of A2 relative to embryolethality. Thus, the increased teratogenicity and embryolethality of A2 relative to that of A1 may be due to the presence of RA in tissues of A2 dosed animals. We hypothesize that at high concentrations, A2 may inhibit conversion of inactive precursors to RA, whereas at slightly lower concentrations, A2 may inhibit catabolism of RA to inactive products. Thus, as the concentration of A2 in the fetus increases in a dose dependent manner, the steady-state concentration of RA will first rise and then fall (Figure 3).

While our data support the above hypothesis, other explanations exist. Retinoid nuclear receptor proteins (RARs and RXRs) are specifically activated by retinoids in a manner unique to each ligand. Consequently, distinct pathways may be initiated by different retinoids, leading to specific developmental, toxic and/or teratogenic effects. 3,4-Didehydroretinoic acid (ddRA) has been shown to be as potent as RA at invoking digit duplications in the chick wing bud (Thaller and Eichele, '90), and the nuclear receptor protein binding profiles for ddRA and for RA in vitro are very similar (Torma et al., '94). Nonetheless, it is conceivable that differences in the metabolism of A1, A2 and their corresponding acids may well lead to differential activation of retinoid nuclear receptors, and thereby account for different toxic and teratogenic effects. Comparative pharmacokinetic studies of A1 and A2 in the pregnant animal and their relative effects on specific reactions in RA metabolism may clarify the unusual outcome data reported here.

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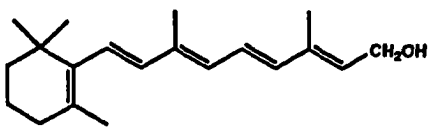
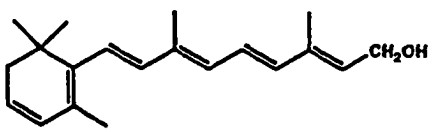
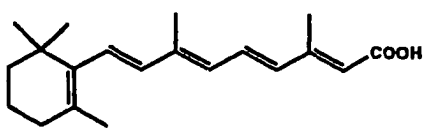


RETINOL



3,4-DIDEHYDRORETINOL



RETINOIC ACID

Figure 1. Formulas of the all-*trans* forms of retinol (A1), 3,4-didehydroretinol (A2), and retinoic acid (RA).

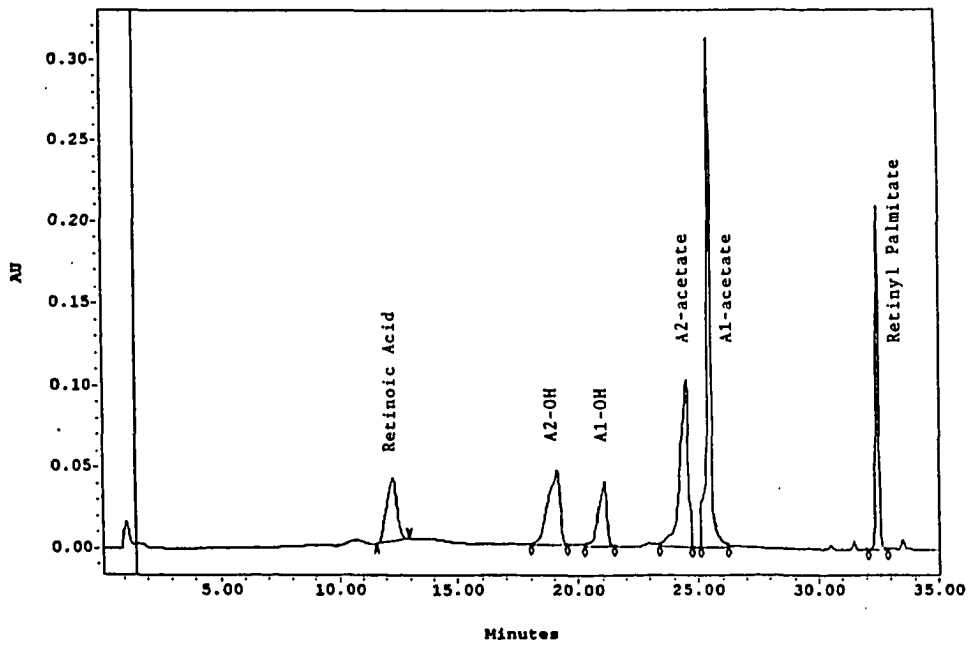


Figure 2. Chromatogram obtained with reversed-phase gradient HPLC of a standard mixture of retinoids.

Table 1. Pregnancy outcome for rats administered all-*trans* retinoids on day 8.5 of gestation

Treatment	Dose in µmol/kg BW	Number of Litters	Implantation Sites	Stillbirths	Percent Resorption	Percent Terata	Mean Pup Weight (g) (Mean±SEM)	Mean Pup Length (cm) (Mean±SEM)
None (control)	0	10	126	0	0	0	2.61±0.09	3.15±0.05
RA	3.5	7	84	0	3.6	0	2.45±0.11	3.29±0.06
	11	6	81	0	7.4	0	2.18±0.12[1]	3.09±0.07
	35	7	98	0	6.1	0	2.34±0.11[1]	3.34±0.06[1]
	113	5	79	1	52.0[4]	15.0[4]	1.66±0.13[4]	2.87±0.07[2]
	352	7	88	--	100.0[4]	--	--	--
A1	3.5	5	50	0	4.0	0	2.34±0.13	3.24±0.07
	11	6	75	0	3.0	0	2.43±0.12	3.35±0.07
	35	8	96	0	4.2	0	2.30±0.10	3.27±0.06
	113	10	103	0	5.8	1	2.53±0.09	3.22±0.05
	352	9	91	0	3.3	3.3	2.45±0.09	3.13±0.05
A2	3.5	7	96	0	1.0	0	2.17±0.11[2]	3.09±0.06
	11	9	108	0	6.5	0	2.21±0.09[2]	3.16±0.05
	35	6	53	0	22.6[2]	0	2.55±0.12	3.22±0.06
	113	8	88	1	11.4	2.3	2.60±0.11	3.27±0.06
	352	9	78	1	2.6	5.1	2.47±0.94	3.15±0.05

[1]Significantly different from control ($p \leq 0.05$).
[2]Significantly different from control ($p \leq 0.01$).
[3]Significantly different from control ($p \leq 0.001$).
[4]Significantly different from control ($p \leq 0.0001$).

Table 2. Liver to body weight ratios, and means ± standard deviations of retinoids recovered from liver tissues of dams (n=3).

Treatment	Dose in µmol/kg BW	Liver/Body Weight Ratios (%)	Total A1 (µmol/g liver)	Total A2 (µmol/g liver)	all-*trans* RA (µmol/g liver)
None (control)	0	3.97±0.07	0.68±0.04	ND	ND
RA	3.5	4.11±0.10	2.18±0.59[2]	ND	ND
	11	4.19±0.10	0.42±0.06	ND	ND
	35	4.26±0.09[2]	0.30±0.09	ND	ND
	113	4.27±0.11[1]	0.42±0.10	ND	ND
	352	3.62±0.09[2]	0.79±0.15	ND	ND
A1	3.5	4.37±0.11[3]	0.71±0.49	ND	ND
	11	3.95±0.10	0.57±0.04	ND	ND
	35	4.06±0.08	1.52±0.40	0.001±0.001	ND
	113	4.19±0.07	1.67±0.15[1]	0.001±0.001	ND
	352	4.12±0.08	2.37±0.13[4]	0.003±0.001	ND
A2	3.5	4.02±0.09	0.61±0.02	0.01±0.003	ND
	11	4.16±0.08	0.35±0.01	0.03±0.01	ND
	35	3.77±0.10	1.83±0.97[1]	0.31±0.12[4]	0.002±0.003[2]
	113	4.10±0.09	1.01±0.94	0.64±0.13[4]	ND
	352	4.15±0.08	1.06±0.50	1.01±0.11[4]	ND

ND = not detected

[1]Significantly different from control ($p \leq 0.05$).

[2]Significantly different from control ($p \leq 0.01$).

[3]Significantly different from control ($p \leq 0.001$).

[4]Significantly different from conrol ($p \leq 0.0001$).

Table 3. Mean concentrations (± standard deviations) of retinoids recovered from liver tissues of fetuses on day 19.5 of gestation (n=9).

Treatment	Dose in µmol/kg BW	Total A1 (nmol/g liver)	Total A2 (nmol/g liver)	all-trans RA (nmol/g liver)
None (control)	0	8.7±1.1	ND	ND
RA	3.5	16.1±1.6[3]	ND	0.23±0.33
	11	9.0±1.0	ND	ND
	35	9.8±0.9	ND	ND
	113	15.7±3.2[2]	ND	0.73±1.03[1]
	352	--	--	--
A1	3.5	21.4±0.7[4]	ND	ND
	11	15.1±1.3[2]	ND	ND
	35	10.7±1.1[1]	ND	ND
	113	13.6±0.7	ND	ND
	352	11.4±3.0	ND	ND
A2	3.5	14.6±2.6[2]	ND	ND
	11	9.8±1.3	0.40±0.03[2]	1.12±0.44[3]
	35	8.8±1.3	0.49±0.11[2]	+*
	113	11.2±1.3	3.36±0.32[4]	4.07±0.035[4]
	352	7.3±1.1	3.52±0.39[4]	0.54±0.005

ND = not detected

[1]Significantly different from control ($p \leq 0.05$).

[2]Significantly different from control ($p \leq 0.01$).

[3]Significantly different from control ($p \leq 0.001$).

[4]Significantly different from control ($p \leq 0.0001$).

*Only one animal was available for this data point. RA was found, but the single value cannot be considered as representative of a group.

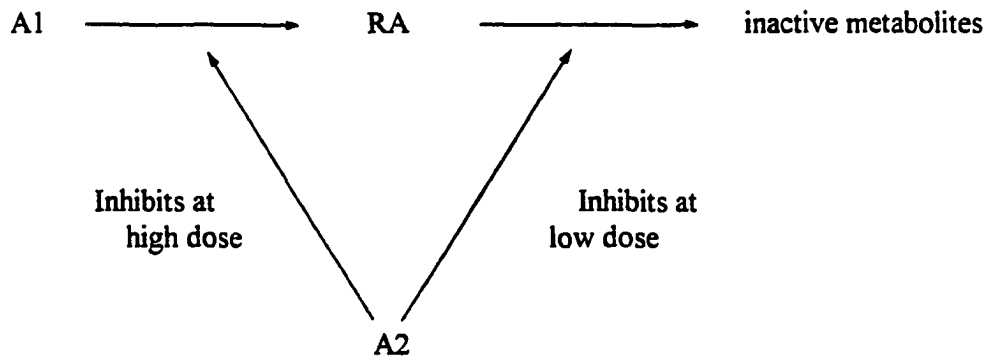


Figure 3. Hypothesized effects of A2 on the formation and synthesis of RA in vivo.

3. EFFECTS OF THE PRENATAL ADMINISTRATION OF THE ALL-*TRANS* ISOMERS OF 3,4-DIDEHYDRORETINYL ACETATE, RETINYL ACETATE, AND RETINOIC ACID ON THE DEVELOPMENT AND SPONTANEOUS MOTOR BEHAVIORAL PATTERNS OF RATS

A paper to be submitted to Neurotoxicology and Teratology

P. K. Duitsman, D.L. Hopper, and J.A. Olson

ABSTRACT

The effects of prenatal administration of the all-*trans* isomers of 3,4-didehydroretinyl acetate (A2), retinyl acetate (A1), and retinoic acid (RA) on pup development and spontaneous motor behavior were evaluated at six weeks postnatally. Groups of 20 timed-pregnant Sprague-Dawley rats were administered single equimolar doses (ranging from 1-11 μmol/kg BW A2 and RA; and 3.5-35 μmol/kg BW A1/kg BW) orally in corn oil on day 8.5 of pregnancy. Dams of control pups received vehicle only. Live birth rates were reduced in groups receiving A2, though no external abnormalities were observed, regardless of the treatment received. Pups from treated dams were paired with pups from control dams and tested using a computer pattern recognition system (RAPID). Significant changes in motor behavior patterns were evident in males whose dams were dosed with 3.5 μmol A2/kg BW and in females whose dams received 1 μmol A2/kg BW, but, surprisingly, not at 3 to 10-fold higher doses of A2. No significant behavioral effects were seen in males in either the A1 or RA groups at the

doses used. The lack of a dose-response relationship in the behavioral effects of A2, which differs from all other retinoids tested, is both interesting and puzzling.

INTRODUCTION

The teratogenic potential of retinoids, in terms of gross malformations, embryolethality and overall development of the fetus, has received much attention. Maternal administration of vitamin A-related compounds has also been shown to effect central nervous system (CNS) function leading to learned behavior dysfunction in rodents (2). Endpoints for classical teratology studies have included fetal death, malformations and growth retardation, which are easily quantified. However, valid methods of measurement of functional deficits in the offspring have not been widely agreed upon. The effects of retinoids on behavioral outcomes are commonly measured by using learned behavior as the endpoint (2). Reported retinoid induced behavioral changes include alterations in righting reflexes, reflex development, response to shock avoidance, performance in Biel maze, puzzle box maze, running wheel, and other pre- and post-weaning activities (4,17,22-24). Doses of retinoids given on day 8.5 of gestation which have produced alterations in behavior have ranged from 11-90 μmol//kg BW (1,3,4,13,17,22,23). Recently, automated methods for evaluating spontaneous motor behavior have been shown to be sensitive, reliable and reproducible (7,9,11).

The teratogenic potential of A2 has recently been shown to be slightly greater than that of A1. Furthermore, the A2 dose response curve for inducing embryolethality is non-linear, compared to linear responses to both A1 and RA (unpublished results, Duitsman and

Olson, 1995). While incidence of malformation, embryolethality and growth development has been evaluated for these retinoids, no information is available on the behavioral effects of A2 on offspring as related to A1 and RA.

The goals for the present study were to determine: 1) if spontaneous behavioral changes could be observed, using a sophisticated computer pattern recognition system, in six week old rat offspring from dams dosed with A2, A1 or RA on day 8.5 of gestation; and 2) if postnatal behavioral effects in males are different from those in females dosed prenatally with A2.

METHODS

Animals and Dosing

A total of 126 timed-pregnant Sprague-Dawley rats were purchased from Harlan Sprague-Dawley, Holtzman Laboratory Animals, Madison, WI. All dams were housed individually in hanging wire cages in accordance with Iowa State University and NIH guidelines and maintained on a 12 hr. day/night light cycle (0700-1900) with food (Teklad Premier, Teklad Inc., Madison, Wisconsin) and water available ad libitum.

All-*trans*-3,4-didehydroretinyl acetate was synthesized in our laboratories. All-*trans*-retinyl acetate and all-*trans*-retinoic acid were obtained commercially (Sigma Chemical Co., St. Louis, MO and BASF Corporation, NJ). Each dam was administered single, equimolar oral doses of the retinoids (1-11 μmol/kg BW for A2 and RA; and 3.5-35 μmol/kg BW for A1), pipetted directly into the mouth of each animal by using a Gilson positive-displacement

pipette (Rainin Instruments, Woburn, MA) on day 8.5 of pregnancy. Control animals received vehicle only. Pups were sexed and culled to eight pups per litter upon parturition. Pups were weaned at 22 days of age and housed separately in hanging wire cages, according to the same protocol mentioned previously for dams.

Measurement of Spontaneous Motor Behavior Patterns

The Rodent Activity Pattern Identification Device (RAPID) system for measuring motor behavior patterns has been described previously (8,9). The observation chamber consisted of a trapezoidal Plexiglas chamber split into two compartments, and divided by a second plate of Plexiglas with six drilled holes to allow paired rats to smell one another. A control rat was placed in one side of the chamber, while a treated rat occupied the other side. The animals were observed simultaneously by two video cameras, one oriented horizontally and the other oriented vertically. Images were taken at one frame per second from each camera for a total of 900 observations per rat over a 15 minute period.

Classification of Activities

The video signals were transferred to a microcomputer for data recording and then to a Digital Equipment Corporation (DEC) 3100 workstation for subsequent pattern analysis and behavioral classification of the data. Two distinct taxonomies were used to classify behaviors: *molecular* (single act) and a *molar* (coordinated action) taxonomies. Sackett (20) defines "molecular taxonomy" as a system that "*defines categories as closely as possible to specific motor actions, postures, gestures, facial expressions, objects, and directions of action*" and "molar taxonomies" as a "*combination of a number of actions, directions, and objects of*

behavior into generic classes defined by the function or outcome of the motor actions". Behavioral acts in both the molecular and molar taxonomies are listed in Table 1 and defined in Table 2. Further details of the testing environment, procedure, taxonomies used, and definitions of behavioral acts have been previously described (8,9). Behaviors are classified using the molecular and molar taxonomies, and the behavioral acts in each taxonomy are analyzed in terms of number of initiations, total time, time distribution and time sequence.

Evaluation of Behavior

Six-week old pups were observed using the RAPID system under red light. For each dosage level of each compound, twenty pairs of six-week old pups were analyzed, with each pair consisting of one treated and one control animal. All animals tested were naive to the environment.

During the period preceding observation, all rats were housed exclusively under diurnal reversal conditions, so that they were exposed only to red light from 0700 to 1900 hours, and only to white light during the remaining 12 hours. Tests were conducted during the period 0900 to 1700 hours under red light. Three measures of spontaneous motor behavior, which are known to contain independent information about CNS control of motor output, were taken. All three were necessary, because for any particular act, significant changes can occur in any one of these parameters for that act while the others remain unchanged (9). These three measures are a calculation of behavioral initiations, behavioral total time, and calculation of time distribution and time sequence of acts.

Behavioral Initiations. Frames in which a particular act began were totaled for the observation period. A mean for each initiated act for both control and treated animals was determined. Student's t-test, with a $p \leq 0.05$ was used to indicate statistical significant differences between the control and treated groups.

Behavioral Total Time. The frame in which a behavior is initiated, and the number of frames for which that behavior is continued, are totaled for the entire observation period. Mean total times were determined, and Student's t tests, with a $p \leq 0.05$, were used to indicate statistical significance.

Time Distribution and Time Sequence. Methods for the analysis of a behavioral act have been adapted from statistical research devoted to analysis of spatial point patterns (5,19). The original spatial techniques are adapted by replacing distance with time. The methods have been previously described (14) and result in a function $K_{\alpha}(t)$ which can be used to analyze the time distribution of a behavioral act, "stand" for example, or in a related form $K_{\alpha\beta}(t)$ to analyze the time sequence of two behavioral acts, for example "stand" and "sit". These methods have been used extensively in prior publications and will not be described in detail here. The time distribution of a specific behavioral act (e.g., "sit", "rear") or sequences of specific behavioral acts ("sit" with "rear") were calculated using equations for K(t) (14). For a time sequence, "sit" with "rear" for example, the time interval for K(t) occurrence begins with the first act and ends with the second act. So the K functions for the sequences of "sit" with "rear" and the opposite pairing "rear" with "sit" should not be identical and, if one is changed by the treatment, this is not necessarily true for the opposite pairing. In addition, K

functions were determined for combined acts (e.g., "attention" or "attention/groom") and sequences of combined acts (e.g., "attention" with "explore" or "attention/explore" with "groom/attention") as described by Kernan et al. (9).

The function K(t) was calculated for each behavioral time distribution or time sequence, and then $\Delta K(t)$ (the difference between K(t) for the exposed animals and matching controls of a particular data set) was calculated for eight time points (2, 5, 10, 20, 30, 45, 100 and 200 s). The bootstrap technique was used to estimate the standard deviation in this measure (9). The *ad hoc criteria* for the significance in the change in a particular time distribution or time sequence between treated rats and matching controls have been described (9). Whenever any time distribution or sequence involved a behavioral act which had an average number of initiations (per animal) less than 10 in either the control or experimental group, the K(t) values were not determined.

When the K function for the treated group is smaller than that for the control group, $\Delta K(t)$, as defined above, is positive; the initiations of that act or pair of joint acts are more dispersed in the treated group than in the controls. If the initiations for the treated group are more clustered than in the controls, the $\Delta K(t)$ value will be negative. For those K functions which are changed by the experimental manipulation, a consistent pattern of more dispersed initiations, positive $\Delta K(t)$ values, has in past experiments been associated with agents known to produce hyperactivity (15), and a pattern of more clustered initiations, negative $\Delta K(t)$ values, has been associated with agents known to produce hypoactivity (10).

The RAPID system, for any given data set, results in up to 154 measures which may each be "changed" or "not changed". Due to the restriction, referred to above, on the average number of initiations of any behavioral act when calculating K(t), the usual number of measures in a typical data set will range from 95 to 126. If the treatment has induced highly stereotypic behavior, the number of measures for that data set may even be considerably less than 95. With this multitude of measures, the first question to be faced is "has the treatment changed the overall behavior of the animals?". Recently Kernan and Meeker (11) have introduced an *ad hoc* statistic, termed RS, which addresses exactly this question. Each of these 154 measures (or more typically, depending on experimental outcome, 95 to 126 measures) can be placed in a vector X that has dimensionality equal to the number of measures. Given such a highly dimensional "signal" vector, there is not a probability model to describe the correlational structure among these observed measures. Thus there is no obvious "best way" to combine X into an overall statistic to test for behavioral change. However, for each measure in X, there exists a criterion to determine whether that particular measure was individually "significantly changed." Using these criteria, the vector X can be mapped into a binary vector, call it Y, with the same dimensionality but where all elements are either 0 (no statistically significant change) or 1 (a statistically significant change). It is useful to recall that part of the information in Y comes from the analysis of the molecular acts and part from the analysis of the molar acts. Differentiating these two parts as being due to Y_1, from the molecular acts, and Y_2 from the molar acts, Y_1 will have a maximum dimension of 106, and Y_2, a maximum of 48. Y_1 and Y_2 will then partition the entire space of the original Y. This

partition is used because every observation of the animals is included in the data used to determine both Y_1 and Y_2. As an example, the molecular acts for the major body positions "walk" and "rear" and the modifier acts "turn" and "sniff" are all included in the molar act "explore". A drug, particularly if it induces hyper- or hypoactivity may change the acts "walk", "rear", "turn", and /or "sniff", or its effects may only be observable when all these are combined into the act "explore". The changes in "walk", "rear", "turn", and "sniff" are contained in Y_1, while any change in "explore" is contained in Y_2. Let S_1 denote the sum of all nonzero elements of Y_1 (remembering that all elements are either 0 or 1) and let T_1 denote the number of elements in Y_1. Y_2, S_2 and T_2 are similarly defined. The conditions of the actual data analysis are such that T_1 is always greater than T_2. Since the original information is, in some sense, summarized in each of these subvectors, it is of interest to use a statistic in which the two are approximately evenly weighted. This can be done by defining the ratio RS as:

$$RS = \sqrt{\left(\frac{S_1}{T_1}\right)^2 + \left(\frac{S_2}{T_2}\right)^2}$$

In their Monte Carlo study of data from the RAPID system, Kernan and Meeker (11) found that, for an experiment or data set having 20 pairs of animals, an RS value greater than 0.138 corresponded to a probability of <0.001 for the control and treatment group to satisfy the null hypothesis. Kernan and Meeker (11) also found, again for 20 pairs of rats, that an RS value of less than 0.067 corresponded to a probability of >0.10 for the control and treated groups to satisfy the null hypothesis. This single statistic, which encompasses all of the data produced in

the study of a particular exposure, indicates whether behavior is changed and at what confidence level that conclusion can be based. It is especially useful in distinguishing low level effects from noise. The RS statistic is an overall indication of change. When a RS value indicates a change in behavior, the details of which measures are changed and how they are changed should be examined, since such an examination often provides important information regarding the specific nature of the behavioral change. With as many as 154 measures for each treatment condition, ordinarily a few of the measures will be described as "changed" when in fact one is only observing the result of statistical chance. We use the RS statistic to decide when observations actually correspond to a statistically significant behavioral change. For completeness however, we will present all measures which are marked as "changed" even in those treatment groups where we conclude these "changes" correspond to "statistical noise" based on the RS value for the treatment group.

RESULTS

Physiological Data

Live birth rates were reduced for litters of animals dosed with A2 (Table 3). Live births were 87%, 91%, and 96% for groups dosed with A2 at 11, 3.5, and 1 μmol/kg BW, respectively. A minor sex effect for live birth rates was evident, with females slightly more vulnerable than males (Table 3). All remaining treatment groups, including control groups, had a 100% live birth rate. Weight gain was reduced in male rats dosed at 3.5 μmol/kg BW A2 and for male rats at all dose levels of RA, though the differences were not statistically

significant from controls (Table 3). Growth of rats from the A1 35 μmol/kg BW group was slightly less than that of control animals, possibly due to a slight teratological effect of A1 at this dose on development. However, animals in groups dosed with A1 from 3.5-11 μmol/kg BW experienced increased growth, with weight gain significantly increased ($p \leq 0.01$) at the 11 μmol/kg BW dose when compared to control animals.

Behavioral Data

RS values for each of the 12 treatments are presented in Table 4. The RS statistic indicates that A1 and RA, at the doses administered on day 8.5 of gestation, do not significantly alter motor behavior of offspring tested six weeks postnatally. However, the RS statistic indicates that A2 produced significantly altered postnatal motor behavior in both male and female rats at six weeks of age. Male animals from the A2 dosed groups experienced profound behavioral changes at the mid-range dose of 3.5 μmol/kg BW (RS = 0.220, $p \leq 0.001$). A2 also produced extreme modification of behavior in female animals dosed at the lowest level of 1 μmol/kg BW (RS = 0.825, $p \leq 0.001$). Surprisingly, the higher and lower doses of A2 given to males and the higher two doses of A2 given to females showed no behavioral consequences.

Table 5 summarizes behavioral modification at six weeks of age following prenatal exposure to A1 and RA. Neither compound produced significant behavioral effects on the offspring in regard to frequency, duration, time sequence, or time duration of acts for either major body position or modifying acts. Table 6 summarizes behavioral modification at six weeks of age following prenatal exposure to A2. The total number of changes in behavior

was greatly increased for female animals dosed with A2 at 1 μmol/kg and male animals dosed with A2 at 3.5 μmol/kg, but not at any other doses. A significant amount of disruption appeared in the time sequence of behaviors for the female animals. The comparative patterns, relative to controls, of behaviors between female animals dosed with A2 at 1 μmol/kg BW and male animals dosed with 3.5 μmol/kg BW A2 are given in Tables 7 and 8.

DISCUSSION

Physiological Data

Toxic effects of prenatal administration of retinoids include birth defects, reduced live birth rates and reduction in growth and development of the offspring (6,12,13,16,22). While no defects were noted for the offspring in this study, reductions in both growth and development and live birth rates were observed. Growth reduction was observed in male rats dosed at 3.5 μmol/kg BW A2 and for male rats at all dose levels of RA, although the reductions were not significantly different from control animals. Reduction of growth and development due to prenatal administration of RA is well documented (6,16). While prenatal administration of A2 has not been shown to reduce growth and development at these levels, reduction of growth for male rats dosed with 3.5 μmol/kg BW A2 coincided with behavioral effects seen in animals from this group. Live births were reduced 13%, 9%, and 4% for groups dosed with A2 at 11, 3.5, and 1 μmol/kg BW, respectively. While the effect of A2 to reduce live birth rates has not been previously reported, A2 has been shown to induce embryolethality at doses ≥1 μmol/kg BW by an as yet undetermined mechanism (unpublished

results, Duitsman and Olson). Animals dosed with A1 and RA prenatally experienced normal birth outcomes, with 100% live birth rates for all litters. The increased teratogenic potency of A2 in regards to live birth rates corroborates our behavioral data, which indicates that A2, while not A1 or RA, induces behavioral changes when given prenatally.

Behavioral Data

Retinoids administered prenatally have also been reported to affect the ability of offspring to perform learned tasks and tasks requiring motor activity (4,17,22-24). However, much discrepancy exists between studies, as discussed previously by Adams (2). Sensitive and reliable methods to assess spontaneous motor behavior following prenatal administration of retinoids have not been previously utilized. The results of the present study indicate no significant changes in the spontaneous motor behavior of animals dosed with A1 (3.5-35 μmol/kg BW) or RA (1-11 μmol/kg BW). Findings from other behavioral studies using similar dosing protocols to the present study (dosing on day 8.5 of gestation) have been inconsistent (1,3,4,13,17,22,23). Approximately one half of all data acquired from dose ranges of A1 (26-105 μmol/kg BW) have indicated alteration of behavior (3,4,13,22,23), while one half have indicated no behavioral modification (3,22,23). The lowest dose of A1 shown to alter behavior in rats postnatally is 11 μmol/kg BW, which altered shock avoidance behavior (22). Open field tests, designed to measure changes in motor activity, did not indicate behavior modification from doses of A1 ranging from 11-42 μmol/kg BW (22). Likewise, in the present study we see no behavioral effect at these dose levels of A1. Similarly, RA has been shown to alter reflex development and preweaning activity behaviors

at 17 μmol/kg when dosed on day 8-10 of gestation (17). To our knowledge, motor activity has not been measured for animals dosed with RA on day 8.5 of gestation. Administration of RA at day 8.5 of gestation in the present study did not alter behavior.

In contrast to A1 and RA, A2 strongly affected behavior of six-week old rats, just as A2 was more potent at reducing live birth rate than either A1 or RA. Profound behavioral changes were observed in six week old females dosed prenatally with 1 μmol/kg BW and males dosed prenatally with 3.5 μmol/kg BW. Curiously, higher doses of A2 for both males and females (11 μmol/kg BW for males and 3.5-11 μmol/kg BW for females), and a lower dose of A2 for males (1 μmol/kg BW) did not induce behavioral abnormalities. Females are more sensitive than males to A2 induced behavioral changes, with behavior modification occurring at the lowest dose of 1 μmol/kg BW for females. The frequency of occurrence, total time, time distribution and time sequence of behavioral acts for both the molecular and the molar taxonomies were all altered by treatment for both of these groups (Table 6). Time sequence is profoundly altered in the female 1 μmol/kg BW treatment group, and less so (although significantly different from control) in the male group dosed with 3.5 μmol/kg BW. Nineteen of the total 39 behavioral changes (49%) in the female group are disruptions of the time sequence of behaviors. Approximately 38% of the behavioral changes identified for the male group are disruptions of the time sequence of behaviors. Sequence of behavior is highly structured and difficult to disrupt (18), and cannot currently be measured with any other automated methodology. While other behavioral measures such as frequency or time

distribution of behaviors may have indicated significant changes if analyzed alone, the time sequence measures identified the greatest area of change.

Table 7 identifies specific behavioral changes between female control animals and those exposed to 1 μmol/kg BW A2. The frequency of occurrence for the molecular acts grooming, head turn, and smelling were significantly different from control animals ($p \leq 0.05$, 0.05, and 0.01, respectively). The total time of individual molecular acts was significantly changed for the behaviors sit, blank, grooming, and head turn ($p \leq 0.05$ for all). Combined acts significantly altered due to exposure of female offspring to 1 μmol/kg BW A2 prenatally included changes in the frequency of the acts groom, explore, and explore/attention ($p \leq 0.05$ for all). Thus, the behavior of the female group dosed with 1 μmol/kg BW A2 appears to be disrupted in a variety of ways. In general, activities requiring whole body displacement were reduced, while activities not related to whole body displacement and time needed for sensory perception and attention tended to increase.

Table 8 identifies specific behavioral changes between male control animals and those exposed to 3.5 μmol/kg BW A2. The frequency of occurrence for the molecular acts walk and smell were significantly different from control animals ($p \leq 0.05$ for both). Frequency of the explore combined acts was also significantly different from control ($p \leq 0.05$). Total time spent on behavior was significantly changed for the molecular acts stand and walk ($p \leq 0.01$ for both), and combined acts explore and attention ($p \leq 0.05$ for both). An increase in overall motor activity is seen for the treated male animals, with a slight decrease in stationary acts.

Thus, unlike the female animals, males seem to be affected in a manner that increases overall activity.

The difference between the effect of A2 on the two sexes then, is an overall affect to decrease activity in females and to increase activity in males. While no other behavioral data exists for effects of A2, sex differences in growth response and liver storage of A2 have been reported (21). Female vitamin A deficient rats, supplemented daily with 0.11 μmol of A2 for 6 weeks, gained only 54% of the weight gained by male rats under the same conditions. When all animals received a normal intake of A1 but then were supplemented with A2 for a two week period (21), female rats stored only 55% as much A2 in the liver as male rats. Thus, males and females seem to metabolize A2 quite differently.

Dose Response

The lack of a true dose response in offspring given A2 during gestation has been observed previously (unpublished findings, Duitsman and Olson). Embryolethality for offspring of pregnant animals dosed with A2 at levels 3.5-352 μmol/kg BW on day 8.5 of gestation peaked at the mid-range dose of 35 μmol/kg BW (unpublished findings, Duitsman and Olson). At higher doses of A2 (113 and 352 μmol/kg BW), embryolethality was less than (50% and 10%, respectively) that observed at the mid-range dose. Unlike A2, RA dosed under the same conditions induced embryolethality in a linear dose response manner. Tissue concentrations of RA on day 19.5 of gestation following a dose of A2 on day 8.5 of gestation did not accumulate in a dose-dependent manner (Duitsman and Olson, unpublished findings). Accumulation of RA in the livers of A2 dosed pregnant animals, and their offspring, tended

to increase at a mid-range dose of A2. Animals dosed with A1 and RA under the same conditions did not contain RA in liver tissue at 19.5 days. Thus, it appears that metabolism and transport of A2 during gestation markedly differ from that of A1 and RA. Also, it seems evident that accumulation of RA in the tissues of these animals is unique to A2 dosed animals.

While behavioral effects identified in the present study occurred at much lower doses than gross effects previously observed as a result of A2 treatment, patterns of the dose response curve were similar. Behavioral changes due to retinoid administration have been shown to occur at levels ten times less than those concentrations inducing gross terata. In the present study, behavioral changes elicited by A2 were remarkable for males at doses 3 times less than doses of A2 which have elicited significant embryolethality. Behavioral changes for female rats in the present study occurred at a dose level of A2 that was 10 times less than concentrations which have produced embryolethality. Both sexes, therefore, are much more sensitive to A2 induced behavioral changes than to gross terata in response to a prenatal dose of A2.

The lack of dose response and increased sensitivity to behavioral changes in the A2 groups was unique among the retinoids tested. However, the large numbers of control animals used to ensure reliable statistical manipulation of the data, and the sensitivity, specificity, and reliability of the methodology used lends credence to our findings. Behavioral data collected from control animals used to test all three retinoids showed no differences in behavior between control animals (n = 240). Therefore, significant differences in behaviors

for females rats dosed with 0.001 mmol/kg BW A2 and males dosed with 0.004 mmol/kg BW A2, when compared with control animals, appear to exist.

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Table 1. Behavioral acts in the molecular and molar act taxonomies.

Molecular Major Body Position	Molar Act Label	Molecular Modifying Act	Molar Modifying Act Label	Molecular Joint Acts	Molar Joint Acts
Stand	A *	Blank	A	Stand-Blank	A-A
Sit	G	Groom	G	Stand-Groom	A-G
Rear	E	Head turn	A	Stand-Head turn	A-A
Walk	E	Look	A	Stand-Look	A-A
Lying Down	G	Sniff	E	Stand-Sniff	A-E
Unclassified		Smell	A	Stand-Smell	A-A
		Turn	E	Stand-Turn	A-E
		Washing Face	G	Sit-Blank	G-A
				Sit-Groom	G-G
				Sit-Head turn	G-A
				Sit-Look	G-A
				Sit-Sniff	G-E
				Sit-Smell	G-A
				Sit-Turn	G-E
				Sit-Wash face	G-G
				Rear-Blank	E-A
				Rear-Sniff	E-E
				Rear-Smell	E-A
				Rear-Turn	E-E
				Walk-Blank	E-A
				Walk-Head turn	E-A
				Walk-Look	E-A
				Walk-Sniff	E-E
				Walk-Smell	E-A
				Walk-Turn	E-E
				Lying down-Blank	G-A
				Lying down-Head turn	G-A
				Unclassified	

* A = attention, E = exploratory, G = grooming.

Table 2. Definitions of behavioral acts in the RAPID behavioral act taxonomy.

	Definition
Major body positions	
Stand	Rat on four feet, not moving compared to previous frame
Sit	Forequarters raised, forepart and hindpart of body almost at right angle, hindpart of body on floor
Rear	Body inclined vertically, forequarters raised
Walk	Rat on four feet and moving compared to previous frame
Lying down	Body ventral surface on floor, not moving (5 frames minimum required)
Unclassified	No major body position can be identified
Modifiers*	
Blank	No modifier can be identified
Grooming	Mouth or paws on body
Head turning	Head moved horizontally more than 15° compared to previous frame
Looking	Head up, not against an object, not moving compared to previous frame
Sniffing	Nose in one of the holes in the observation cage
Smelling	Nose against object, floor or walls and not moving compared to previous frame
Turning	Head and body moved horizontally more than 20° compared to previous frame
Washing face	Forepaws on head

*Note: In all act modifiers movement takes precedence over other modifiers.

Table 3. Live birth rates (%) and mean weight gain/loss of offspring from rats dosed with retinoids on day 8.5 of gestation, when compared to control animals.

Treatment	Dose (μmol/kg BW)	Sex of animal	% Live births	Mean weight (g) gain (+) or loss (-)
A2	11	Male	85%	+2.77
	3.5	Male	96%	-6.92
	1	Male	100%	-1.07
	11	Female	89%	+0.38
	3.5	Female	89%	+2.31
	1	Female	94%	+0.10
A1	35	Male	100%	-1.50
	11	Male	100%	+12.24**
	3.5	Male	100%	+8.43
RA	11	Male	100%	-4.34
	3.5	Male	100%	-5.41
	1	Male	100%	-4.58

**$p \leq 0.01$

Table 4. Value of the RS statistic for the comparison of each of 12 treatment groups with their controls (n=20 for each group).

Treatment	Dose (μmol/kg BW)	Sex of animal	RS
A2	11	Male	0.052
	3.5	Male	0.220***
	1	Male	0.015
	11	Female	0.090
	3.5	Female	0.062
	1	Female	0.825***
A1	35	Male	0.030
	11	Male	0.044
	3.5	Male	0.081
RA	11	Male	0.066
	3.5	Male	0.092
	1	Male	0.032

***$p \leq 0.001$

Table 5. Summary of behavioral act modification in six-week old rats following prenatal exposure to A1 and RA.

Behavioral acts[1]	A1 (μmol/kg BW)			RA (μmol/kg BW)		
	3.5	11	35	1	3.5	11
REGULAR						
NOCC	1	0	0	0	0	1
Time	1	1	0	1	0	2
MBP - TD	0	1	1	0	1	0
MBP - TS	1	0	0	0	1	0
MOD - TD	0	0	0	0	0	1
MOD - TS	1	0	1	0	1	0
COMBINED						
NOCC	0	0	0	0	0	0
Time	1	1	0	0	0	1
TD	0	0	0	0	1	0
TS	1	0	0	1	2	0
TOTAL CHANGES	6	3	2	2	6	5
RS	0.08	0.04	0.02	0.03	0.09	0.06
MOVE	1.02	1.21	0.1	0.84	0.93	0.19

[1]NOCC=number of occurrences (frequency), MBP=major body position, TD=time distribution, TS=time sequence, MOD=modifier acts.

Table 6. Summary of behavioral act modification in six-week old rats following prenatal exposure to A2.

Behavioral acts[1]	A2 Dose					
	(1 μmol/kg BW)		(3.5 μmol/kg BW)		(11 μmol/kg BW)	
	Male	Female	Male	Female	Male	Female
REGULAR						
NOCC	0	3	2	0	1	1
Time	0	4	2	0	0	1
MBP - TD	0	3	1	0	1	0
MBP - TS	0	6	1	1	0	0
MOD - TD	0	0	1	0	0	1
MOD - TS	1	3	3	0	1	2
COMBINED						
NOCC	0	3	1	0	0	0
Time	0	3	2	0	0	0
TD	0	4	1	0	0	1
TS	0	10	2	2	0	0
TOTAL CHANGES	1	39	16	3	3	6
RS	0.01	0.13***	0.22***	0.06	0.05	0.09
MOVE	0.4	1.52	2.37*	0.72	2	0.36

[1]NOCC=number of occurrences, MBP=major body position, TD=time distribution, TS=time sequence, MOD=modifier acts.

*$p \leq 0.05$
***$p \leq 0.001$

Table 7. Frequency and total time of behavioral acts for six-week old female rats exposed to the vehicle alone (control) or to 1 μmol/kg BW A2 prenatally.

	Number of occurrences ± SEM		Time ± SEM	
Act	Control	Exposed	Control	Exposed
Stand	131.3 ± 3.9	141.4 ± 3.3	506.4 ± 11.9	511.6 ± 11.1
Sit	21.6 ± 2.1	22.3 ± 1.6	98.3 ± 17.6	51.6 ± 5.2*
Rear	47.7 ± 3.2	56.7 ± 3.4	143.0 ± 10.5	172.1 ± 11.7
Walk	92.7 ± 3.6	101.4 ± 2.7	147.8 ± 6.1	163.6 ± 5.8
Lying	0.4 ± 0.3	0.2 ± 0.4	4.5 ± 3.4	1.1 ± 0.5
Blank	124.7 ± 3.0	121.3 ± 2.3	327.6 ± 12.0	292.5 ± 8.7*
Grooming	11.2 ± 2.3	5.2 ± 0.7*	20.0 ± 4.8	8.1 ± 1.5*
Head turn	43.2 ± 1.7	50.7 ± 2.7*	48.8 ± 2.1	59.2 ± 3.2*
Looking	2.9 ± 0.4	4.1 ± 0.6	3.0 ± 0.4	4.2 ± 0.6
Sniffing	9.6 ± 1.9	13.3 ± 2.8	14.6 ± 3.0	20.5 ± 4.7
Smelling	108.4 ± 4.0	122.2 ± 3.0**	266.1 ± 13.3	296.6 ± 8.6
Turning	120.6 ± 2.7	121.5 ± 2.1	217.3 ± 5.1	218.2 ± 5.3
Wash face	1.6 ± 0.5	0.6 ± 0.2	2.5 ± 1.0	0.7 ± 0.2
Combined Acts‡				
Groom	11.4 ± 2.4	4.9 ± 0.7*	19.9 ± 4.8	6.8 ± 1.3*
Explore	73.6 ± 3.3	84.3 ± 3.2*	97.1 ± 4.4	110.8 ± 5.3
Groom/Explore	24.0 ± 3.3	17.0 ±1.8	37.5 ± 5.2	23.8 ± 2.6*
Attention	143.8 ± 4.0	151.9 ± 2.7	406.4 ± 9.7	405.5 ± 9.9
Groom/Attention	22.5 ± 3.7	15.9 ± 1.7	48.1 ± 13.1	24.0 ± 3.1
Explore/Attention	146.2 ± 4.3	157.5 ±2.8*	291.1 ± 11.0	328.9 ± 9.1*

*$p \leq 0.05$

**$p \leq 0.01$

‡Combined acts (A,E,G) are composed of several molar acts. Groom is not the same as grooming, which is a molecular modifying act.

Table 8. Frequency and total time of behavioral acts for six-week old male rats exposed to the vehicle alone (control) or to 3.5 μmol/kg BW A2 prenatally.

	Number of occurrences ± SEM		Time ± SEM	
Act	Control	Exposed	Control	Exposed
Stand	130.1 ± 3.6	141.2 ± 4.7	558.9 ± 8.5	527.1 ± 7.8**
Sit	29.8 ± 2.2	26.8 ± 1.6	79.9 ± 8.3	73.8 ± 7.7
Rear	41.0 ± 2.8	41.3 ± 2.4	102.7 ± 8.0	109.4 ± 6.5
Walk	97.6 ± 3.5	110.1 ± 4.4*	157.8 ± 7.3	189.4 ± 8.3**
Lying	0.1 ± 0.1	0.1 ± 0.1	0.6 ± 0.6	0.3 ± 0.3
Blank	120.5 ± 3.7	114.9 ± 2.8	283.6 ± 9.8	267.5 ± 9.4
Grooming	7.6 ± 1.3	5.3 ± 0.7	10.7 ± 2.1	7.6 ± 1.3
Head turn	55.3 ± 2.7	54.8 ± 1.8	63.4 ± 3.2	63.0 ± 2.2
Looking	3.6 ± 0.4	4.5 ± 0.6	3.6 ± 0.5	4.9 ± 0.7
Sniffing	11.8 ± 2.1	11.2 ± 2.1	20.0 ± 4.2	19.6 ± 4.0
Smelling	113.4 ± 3.4	124.3 ± 3.4*	292.4 ± 11.1	295.3 ± 8.7
Turning	125.7 ± 2.8	130.0 ± 2.5	225.3 ± 7.6	241.3 ± 6.9
Wash face	0.8 ± 0.3	0.6 ± 0.2	1.1 ± 0.4	0.7 ± 0.2
Combined Acts‡				
Groom	7.3 ± 1.2	5.1 ± 0.6	9.7 ± 1.7	6.9 ± 0.9
Explore	77.7 ± 4.1	90.4 ± 3.8*	101.5 ± 5.9	123.2 ± 6.4*
Groom/Explore	25.3 ± 2.2	21.5 ± 1.7	35.6 ± 3.3	31.9 ± 3.3
Attention	144.9 ± 2.5	147.0 ± 4.1	448.7 ± 10.0	419.9 ± 9.3*
Groom/Attention	21.6 ± 2.3	21.9 ± 1.9	37.3 ± 4.7	36.7 ± 4.9
Explore/Attention	145.8 ± 3.3	152.3 ± 3.7	267.2 ± 8.0	281.4 ± 7.8

*$p \leq 0.05$

**$p \leq 0.01$

‡Combined acts (A,E,G) are composed of several molar acts. Groom is not the same as grooming, which is a molecular modifying act.

4. VITAMIN A INADEQUACY IN SOCIOECONOMICALLY DISADVANTAGED PREGNANT IOWAN WOMEN AS ASSESSED BY THE MODIFIED RELATIVE DOSE RESPONSE (MRDR) TEST

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ABSTRACT

The vitamin A status of low-income women (n = 57) during the third trimester of pregnancy was assessed by use of the modified relative dose response (MRDR) test. Non-Hispanic White (45), Hispanic (6), Afro-American (5), and Asian (1) women were recruited from public health programs in central Iowa. Serum retinol, β-carotene, α-carotene, lycopene, α-tocopherol, and cholesterol concentrations were also measured. Twenty six percent of the study population were found to be in a marginal vitamin A status with MRDR values ≥0.03, whereas 9% had values ≥0.06. The Hispanic and Afro-American groups seemed to be most at risk, with 50% and 40% (respectively) of the MRDR values ≥0.03 and 33% and 20% (respectively) ≥0.06. Carotenoid values were similar to those found in women in other like studies, except for the Afro-American group, which had mean values less than half those of the other groups. α-Tocopherol concentrations and α-tocopherol/cholesterol ratios of all ethnic groups fell in the normal range. The somewhat elevated cholesterol levels found in all groups can largely be attributed to pregnancy. Clearly, this study identifies a portion of the U.S. population at high risk of vitamin A inadequacy.

INTRODUCTION

An adequate supply of vitamin A from maternal tissue is critical for normal growth and development of the fetus. A marginal or deficient maternal vitamin A status is associated with babies of low birthweight and with a greater incidence of morbidity and mortality (1-3). Because maternal tissue is progressively depleted of vitamin A so as to supply fetal demands (4), pregnant women who initially possess marginal vitamin A reserves are at an increased risk of vitamin A inadequacy as pregnancy progresses. This situation may particularly develop in low-income households without sufficient funds to purchase fresh fruits and vegetables or to provide proper storage for dairy products. Much attention has been given to this problem in third-world countries, but only to a minor extent in the United States.

Marginal vitamin A status, which is defined as a condition of vitamin A inadequacy without the presence of clinical signs of deficiency, is best assessed biochemically by use of dose-response tests. The modified relative dose-response (MRDR) test, which was developed in this laboratory to assess marginal vitamin A status (5-8), is similar in principle to the relative dose-response (RDR) test (9). A dose of 3,4-didehydroretinyl acetate (DRA) administered orally elicits the release of accumulated apo-retinol-binding protein (apo-RBP) from the liver as holo-RBP (10). Five hours after dosage, both 3,4-didehydroretinol (DR) and retinol (R), which can readily be separated by high-pressure liquid chromatography (HPLC), are found in the plasma. Thus, the MRDR test requires only one blood sample, whereas the RDR test requires two. The ratio of DR to R at 5 h, above a selected cutoff value, is inversely related to vitamin A status.

In this study, we have investigated the response of low-income pregnant women in the third trimester of pregnancy to an oral dose of 8.8 μmol (2.5 mg) DRA. We have also

measured the concentrations of retinol, three carotenoids, α-tocopherol, and cholesterol in the serum of these subjects.

METHODS AND MATERIALS

Subjects

Low-income pregnant women were recruited from state and federally funded health-care programs in central Iowa. Field sites included the Broadlawns Hospital Family Clinic, Homes of Oakridge, the Women, Infants and Children (WIC) Clinic in Des Moines, and also the WIC clinics in Ames and Marshalltown, Iowa. All women involved in these clinics were required to be of low-income status, which is defined as a monthly income of $1,135 or less for a single mother and of $2,282 or less for a four-member family. Criteria for inclusion in the study included low-income status, general good health, adherence to a suggested diet preceding the test, and a willingness to have the test performed during the seventh month of pregnancy. Women who were not of low-income status, who were in poor health, or who were unwilling to undergo diet constraints or blood drawings were excluded. Consent forms were signed at the time of recruitment, and appointments were made for the subjects to return for the test during their seventh month of pregnancy.

Of approximately 170 pregnant women approached about the study, 92 expressed interest in participating. Thirty-five women failed to meet all inclusion criteria and were excluded. Of the 57 enrolled subjects, 45 were non-Hispanic White, 6 were Hispanic, 5 were Afro-American, and 1 was Asian. All were studied during the seventh month of pregnancy. Subjects ranged in age from 15 to 37 years. All subjects had access to prenatal multivitamins, which contained 4,000-10,000 IU of preformed vitamin A, 11-45 IU of vitamin E (as

dl-α-tocopheryl acetate), and 50-240 mg of vitamin C. None of the vitamin preparations contained provitamin A compounds, such as β-carotene. The subjects were asked not to ingest vitamin supplements on the day before or on the day of the test. A list containing approximately ten foods essentially free of vitamin A and carotenoids was provided to each participant. Subjects were also asked to avoid certain foods for the day before the test, such as milk and milk products, eggs, fortified cereals, and meats to avoid any interference with the test from a high intake of preformed vitamin A. On the day of testing, a 24-h recall for dietary intake was taken. Four subjects who ingested food products containing large amounts of preformed vitamin A that might have interfered with the test were excluded.

Methods

3,4-Didehydroretinyl acetate (DRA) was synthesized from retinoic acid (5,11) and then purified twice on a column of 8% water-deactivated alumina. Its purity, as judged by absorption at 350 nm, and determined HPLC, was 98.6%. No retinyl acetate (RAC), which separates well from DRA on HPLC, was detected (detection limit = 0.5 ng; or <0.01%). An oral dose of 8.8 μmol (2.5 mg) DRA dissolved in corn oil was pipetted directly into the mouth of each subject by using a Gilson positive-displacement pipette (Rainin Instruments, Woburn, MA). The dose was followed by an ice cream snack, essentially free of vitamin A, which contained approximately 10 g of fat to aid absorption of the retinoid. Five hours after the DRA was administered, venous blood samples were obtained by a licensed phlebotomist at the field site. Blood samples were collected into serum separation tubes, put in a cooler, and taken promptly back to the laboratory. Serum was collected after centrifugation at 5,500 rpm for 20 min, sealed under argon, and stored at -20°C for approximately four months. Thus, the concentrations of carotenoids, retiniol, and α-tocopherol may have been somewhat lower upon analysis. However, DR/R should not be affected by storage at these conditions.

The DR/R ratio was analyzed by standard procedures developed in our laboratory (5). In brief, 500 μl of serum from each volunteer was mixed with an equal volume of ethanol containing 50 μl (0.14 μg) RAC, which was used as an internal standard. Serum was extracted with 500 μl hexane (3 times). Hexane fractions were pooled and evaporated to dryness under a slow stream of argon gas. The residue was redissolved in 50 μl of methanol:dichloromethane (4:1 v/v) for analysis by HPLC. Each sample was applied to a 5 μm C_{18} Waters Resolve reversed-phase column by use of a WISP autoinjector (Waters, Milford, MA). A Waters 996 photo-diode array (PDA) detector monitored absorbance at 350 nm for DR and at 325 nm for R and RAC, while a Waters 510 pump was set at a flow rate of 1.0 ml/min. An isocratic solvent system (methanol:water, 90:10 v/v) was run for 13 min to separate the three analytes. Retention times for DR, R, and RAC were 7, 8, and 13 min, respectively. The Millennium 2010 software version 1.2, which was developed by Waters for use with their 996 PDA detector, performed data acquisition, processing, and management of chromatographic information. All data were acquired and stored in three-dimensional mode, which allowed detailed examination of selected spectra, integration of peak areas, and assessment of the purity of compounds. All analyzed data were printed by use of a Hewlett Packard Laser Jet II printer. The molar DR to R ratio was then calculated as previously described (5).

The analysis of carotenoids and tocopherols were conducted by standard procedures described in our laboratory (12). In brief, 100 μl of serum was mixed with 100 μl ethanol containing 50 μl (3.14 μg) all-*rac*-α-tocopheryl acetate, which was used as an internal standard. Serum was extracted by using 200 μl of hexane three times. Extracts were pooled and evaporated to dryness under a slow stream of argon gas. Residues were redissolved in 100 μl of methanol:methylene dichloride (75:25, v/v), and an aliquot (40 μl) was injected by use of a Waters WISP autoinjector onto a Waters Resolve 5 μm C_{18} 30-cm reversed-phase

HPLC column. The isocratic solvent system was composed of methanol, acetonitrile, ethylene dichloride, and octanol (50:50:15:0.12 by volumes) and contained 0.05% ammonium acetate. β-Carotene (t_r = 10.9 min), α-carotene (t_r = 10.2 min), and lycopene (t_r = 8.2 min) were monitored at 450 nm, α-tocopherol (t_r = 6.4 min) at 292 nm, and α-tocopheryl acetate (t_r = 7.0 min) at 285 nm. The HPLC setup was identical to that used for DR/R analysis, as already described.

Cholesterol analysis was performed using a Kodak Ektachem DT60II analyzer (Eastman Kodak Co., Rochester, NY). A 10 µl drop of serum from each subject was deposited on a Kodak Ektachem DT slide. In the analysis, cholesteryl esters are hydrolyzed to cholesterol, which is then oxidized by cholesterol oxidase to generate hydrogen peroxide. The hydrogen peroxide oxidizes a leuco dye in a peroxidase-catalyzed reaction to produce a colored compound. Colorimetric measurement by reflectance spectrophotometry provides the basis for the final cholesterol concentration value. Serum α-tocopherol concentrations are reported as µmol/L and as µmol/mmol cholesterol. All samples were analyzed in duplicate.

Statistics

Regression, univariate, and Pearson correlation coefficients were determined by use of the Statistical Analysis System, version 6.0, Cary, NC. Chi-square analysis of a contingency table was conducted by conventional procedures (13).

RESULTS

Characteristics of the whole study population

The age, MRDR ratios, and values for selected serum components are given in Table 1. The frequency distributions of serum concentrations of retinol, α-tocopherol, and cholesterol and of α-tocopherol/cholesterol ratios were approximately normal, whereas those

TABLE 1
Mean, Median, and Ranges of DR/R Ratios and of Serum Components in the Study Subjects

Variable	n	Mean ± SD	Median	Range
DR/R ratio (μmol/μmol)	57	0.026 ± 0.017	0.021	0.006 - 0.082
Retinol (μmol/L)	57	1.57 ± 0.51	1.55	0.82 - 3.12
β-Carotene (μmol/L)	56	0.16 ± 0.18	0.12	0.00 - 0.99
α-Carotene (μmol/L)	56	0.05 ± 0.04	0.056	0.00 - 0.16
Lycopene (μmol/L)	56	0.47 ± 0.40	0.36	0.04 - 2.14
α-Tocopherol (μmol/L)	56	32.6 ± 7.60	43.1	19.1 - 57.4
Cholesterol (mmol/L)	56	5.7 ± 1.39	5.5	3.23 - 9.05
α-Tocopherol/Cholesterol Ratio (μmol/mmol cholesterol)	56	6.0 ± 1.86	5.6	2.9 - 11.5
Age (years)	42	22.5 ± 5.0	21.5	15 - 37

of MRDR ratios and of the three carotenoid concentrations were skewed towards larger values (Fig. 1,2). By using suggested serum cutoff values of inadequacy for several nutrients (6, 14, 15) and of cardiovascular risk for serum cholesterol (16), the study population showed a state of inadequacy relative to vitamin A and of risk of atherosclerosis relative to cholesterol (Table 2).

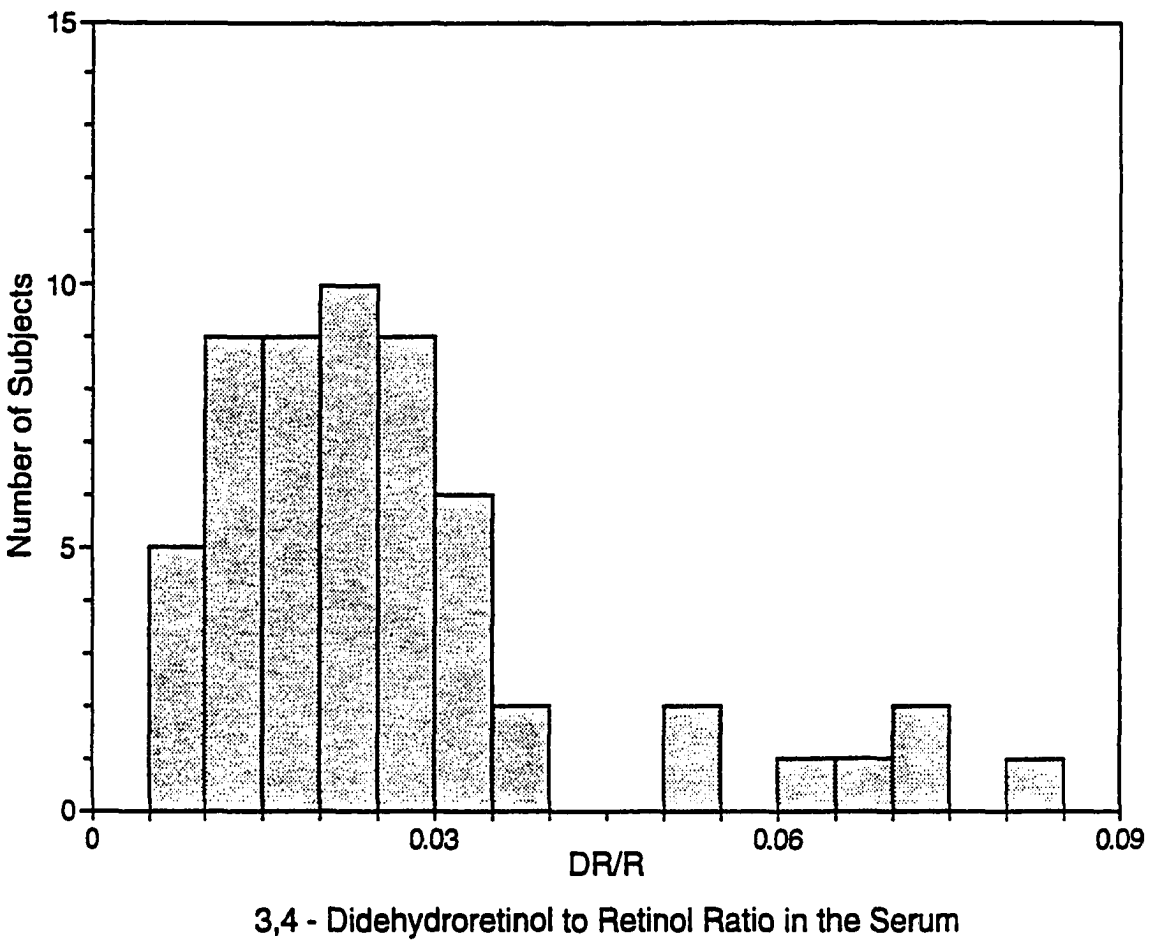

FIG 1. Frequency distribution of MRDR values for all pregnant women in the study.

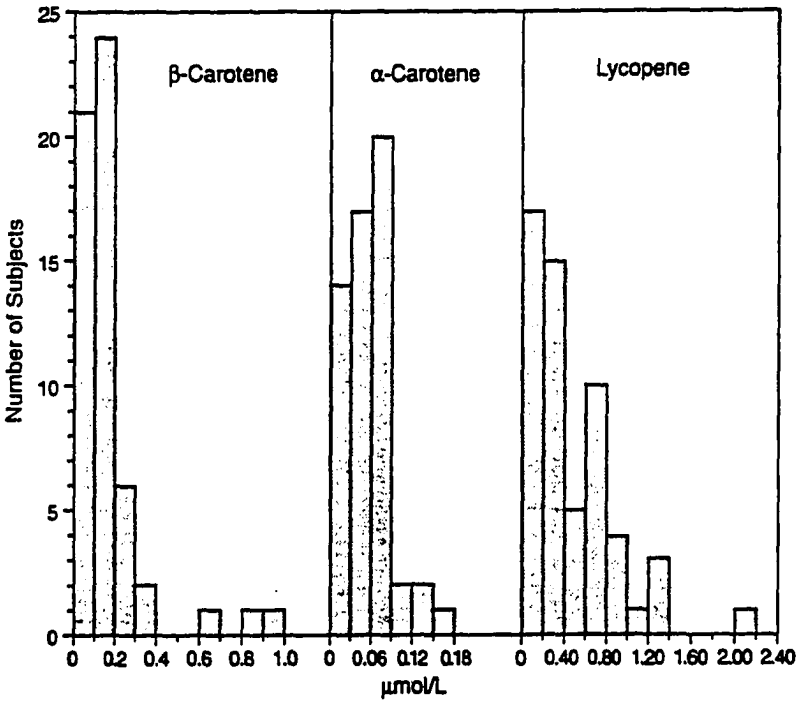

FIG 2. Frequency distribution of carotenoids for all pregnant women in the study.

TABLE 2
Percentages of Study Subjects at Nutritional Risk

Item	Cutoff value of inadequacy or risk	Percent at risk
MRDR ratio	≥0.03	26%
	≥0.06	9%
Retinol	<1.05 µmol/L	18%
	<0.70 µmol/L	0%
α-Tocopherol	<16 µmol/L	0%
α-Tocopherol/cholesterol	<2.2 µmole/mmole	0%
Cholesterol	>5.7 mmol/L	48%

Effects of ethnicity

Mean values for the MRDR ratios and for serum components as well as the percentages of various ethnic groups at risk are given in Table 3. Although vitamin A inadequacy was noted in all ethnic groups except for the single Asian studied, pregnant Hispanic and Afro-American women seemed to be more affected than the non-Hispanic White group. Probably because the former two groups were small, however, the differences by Chi-square analysis were not significant ($p > 0.10$) when a cutoff value of 0.03 was used and only approached significance ($p < 0.10$) when a cutoff value of 0.06 was employed.
Carotenoid patterns differed among the four groups. The sum of the three measured carotenoids in the Afro-American group was less than half that in the other groups. The Hispanic and Asian groups showed relatively larger amounts of α- plus β-carotene (50-62%) and smaller amounts of lycopene (38-50%), whereas the Afro-American and non-Hispanic White groups showed just the opposite, low α- and β-carotene (25-28%) and high lycopene (72-75%).

Correlations between measured parameters

The Pearson correlation coefficients and probability (P) values (two-tailed) of selected compared values are given in Table 4. MRDR values were inversely and significantly related to serum retinol concentrations, a correlation that is largely driven by MRDR ratios ≥0.03 (Fig. 3). In this regard, MRDR values <0.03 were not correlated ($r = -0.07$, $p = 0.65$) with serum retinol concentrations of subjects in this subgroup. Serum carotenoid concentrations were correlated with each other and, except for β-carotene, also with serum α-tocopherol levels. MRDR ratios did not correlate with any other measured parameter (Table 4), nor were any other two parameters correlated in any significant way.

TABLE 3
MRDR Ratios, Mean Values of Serum Components, and Percentages at Risk for Ethnic Groups among the Pregnant Women in the Study

Parameter	Non-Hispanic White (n = 45)	Hispanic (n = 6)	Afro-American (n = 5)	Asian (n = 1)
MRDR ratio	0.023 ± 0.023	0.040 ± 0.023	0.038 ± 0.030	0.013
% >0.03	22%	50%	40%	0
% >0.06	4%	33%	20%	0
Retinol (μmol/L)	1.6 ± 0.5	1.5 ± 0.6	1.2 ± 0.3	2.0
% <1.05 μmol/L	13%	17%	60%	0%
α-Tocopherol/cholesterol (μmol/mmol)	6.2 ± 1.9	6.2 ± 1.8	4.2 ± 1.0	5.9
% <2.2 μmol/mmol	0%	0%	0%	0%
Cholesterol (mmol/L)	5.7 ± 1.4	5.3 ± 0.9	6.4 ± 1.3	5.8
% >5.7 mmol/L	49%	40%	60%	
β-Carotene (nmol/L)	152 ± 165 (125)*	301 ± 285 (208)*	60 ± 27 (57)*	317
α-Carotene (nmol/L)	50 ± 35 (71)*	86 ± 8 (85)*	23 ± 34 (0)*	160
Lycopene (nmol/L)	513 ± 426 (384)*	391 ± 238 (470)*	250 ± 145 (286)*	289

*Median values are given in parentheses.

TABLE 4
Pearson Correlation Coefficients of Compared
Parameters in the Whole Study Group of Pregnant Women

Values compared	r	p
MRDR vs. retinol	-0.48	0.0002
β-Carotene vs. α-carotene	0.50	0.0001
β-Carotene vs. lycopene	0.31	0.02
α-Carotene vs. lycopene	0.49	0.0001
α-Tocopherol vs. α-carotene	0.35	0.007
α-Tocopherol vs. lycopene	0.42	0.001
α-Tocopherol vs β-carotene	0.05	0.74
MRDR vs β-carotene	-0.02	0.87
MRDR vs α-carotene	-0.12	0.37
MRDR vs lycopene	-0.16	0.22
MRDR vs α-tocopherol	-0.16	0.23
MRDR vs age	-0.15	0.34

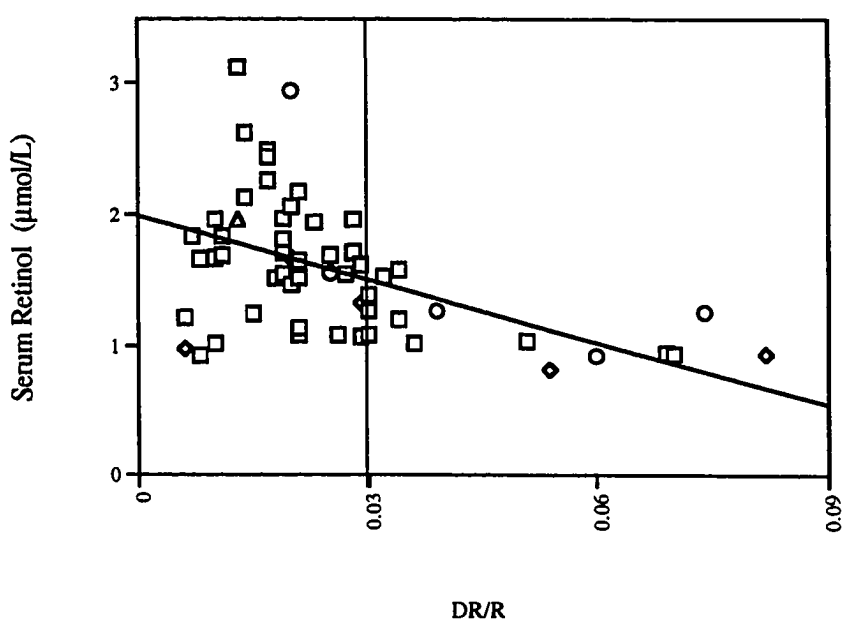

FIG 3. The relationship between the ratio of 3,4-didehydroretinol to retinol (DR/R) and serum retinol concentrations in all ethnic groups of pregnant women studied. Non-Hispanic White (□), Hispanic (O), Afro-American (◊) and Asian (Δ). The formula for the linear regression line is $y = -15.993x + 1.986$, $r = -0.48$, $p \leq 0.0002$.

DISCUSSION

Pregnant women are susceptible to vitamin A depletion because of increased demands during pregnancy and altered dietary habits. Although clinical vitamin A deficiency among presumably healthy persons, including pregnant women, is rarely observed in the United States, plasma vitamin A concentrations are reduced in women during gestation (17-21). A 25% reduction of serum retinol concentrations, due in part to hemodilution, is typical in women followed longitudinally during pregnancy (4). The National Health and Nutrition Examination Survey (NHANES 1) and the survey of Hispanic Americans living in the southwestern United States (14) both confirm this trend in pregnant females in the United States.

Both the RDR and MRDR tests have been successfully employed to assess marginal vitamin A status in humans (6-8, 22-26). As vitamin A is depleted in an individual, the MRDR value increases. A tentative cutoff point for individuals has been set at 0.03 in the United States (6) and at 0.06 in Indonesia (23, 24). Probable reasons for these geographic differences in cutoff values have been discussed (23, 24). While these cutoff values may seem arbitrary, they are based on the following observations: 1) MRDR ratios of healthy children and adults in the U.S. are <0.03; 2) MRDR ratios of subjects in the U.S. that are >0.03 fall to values <0.03 in all instances following a dose of vitamin A; 3) MRDR ratios in healthy Indonesian children are usually <0.06; and 4) MRDR ratios in Indonesia that are >0.06 fall to values <0.06 following a dose of vitamin A (23). Thus, DR/R ratios <0.03 imply adequate total body reserves of vitamin A, ratios in the 0.03-0.06 range currently are considered as a zone of probable inadequacy, and ratios ≥0.06 clearly indicate inadequate reserves.

In our sample of socioeconomically disadvantaged pregnant women, 26% and 9% were in a marginal status at the 0.03 and 0.06 cutoff ratios, respectively. This observation surprised us, inasmuch as the average intake of vitamin A in pregnant women in the United States is approximately 1000 μg retinol equivalents (27, 28). On the other hand, the median intake is only 540 retinol equivalents (28), primarily because the pattern of vitamin A and carotenoid intakes is highly skewed. In general, the nutritional status of lower socioeconomic groups tends to be poorer than that of wealthier, better-educated persons. Although all the women in this study were eligible for WIC, which provides nutritional advice and multivitamin preparations, advice need not be followed nor vitamin preparations regularly taken. If supplements had been ingested, the authors believe that all MRDR values would have been ≤0.03. Most subjects stated they took the vitamin, although validation of their claims was not possible. Thus, we conclude that at least one quarter, and probably more, of the subjects did not take the supplements on a regular basis, if at all.

Inadequate maternal vitamin A stores, particularly late in pregnancy, may not provide sufficient vitamin A needed by the rapidly growing fetus or, thereafter, satisfactory breast milk concentrations. Low birthweight, as a consequence of intrauterine growth retardation, and premature birth are both correlated with a low vitamin A status of the mother (3,29). Hemodilution of pregnancy is responsible for some reduction of serum vitamin A values. However, since the amount of DR-RBP released from the liver is also diluted, the DR/R ratio is only minimally affected by hemodilution.

The Hispanic and Afro-American groups seemed to be most at risk, with 50% and 40% (respectively) of their MRDR values ≥0.03. Unfortunately, great difficulty was encountered in recruiting pregnant women from these two racial groups. Hence, the sample size is quite small, which reduces the probability of finding statistical differences between groups. However, MRDR values ≥0.06 are quite prevalent in these two groups (33% for

Hispanic, 20% for Afro-American) with relatively few present in the non-Hispanic White group (4%) (Table 3). Thus, vitamin A inadequacy may not only be more prevalent among these racial groups, but also may be more severe. In this regard, pregnant Afro-American women have less availability to, and are less likely to receive, timely prenatal care than White women (30). Indeed, while most subjects in our study took advantage of the WIC program, the Afro-American subjects did not, primarily due to lack of both interest and transportation to the clinic.

Mean serum β-carotene, α-carotene, and lycopene values in our study population are approximately 50%, 50%, and 70%, respectively, of other reported values for healthy non-pregnant adult females (31, 32). The hemodilution of pregnancy may account for some of this difference. Because serum carotenoid values are positively correlated with dietary carotenoid intake (33), however, a reduced intake of carotenoids in our study population is likely. Foods containing high carotenoid levels, such as fresh fruits and vegetables, are often prohibitive in cost for low-income households. Food habits must also play an important role. Serum carotenoid levels for the Afro-American group, for example, are extremely low (Table 3), while those of the non-Hispanic White, Hispanic and Asian groups of similar socioeconomic status are just slightly below levels reported for healthy American women (31, 32). These differences between ethnic groups probably are due to dietary variations, but one might speculate that genetic differences in carotenoid metabolism may also occur, although no supporting data are available.

The reported mean ratio of serum α-tocopherol to cholesterol among healthy U.S. adults is 4.08 ± 0.14 mg/g cholesterol (3.66 μmole/mmole) (34). The mean ratio for each ethnic group in the study population falls above this level, which indicates satisfactory vitamin E nutriture. Circulating tocopherol levels increase during pregnancy, along with increased lipid levels (35).

Mean serum cholesterol levels were elevated 24% for the study population relative to mean values reported for non-pregnant adult women (5.7 mmol/L vs. 4.6 mmol/L) (16). A 16% increase in cholesterol concentrations during late pregnancy has been reported (36). This increase probably is due to an increase in apolipoprotein A-I and HDL cholesterol concentrations. Serum cholesterol values return to normal concentrations postpartum (35). When pregnancy is considered, therefore, serum cholesterol levels in our study population do not differ markedly from those of women in the general population.

In summary, a significant number of pregnant women in our study population showed an inadequate vitamin A status by application of the sensitive MRDR test. In the only other comparable study, conducted among pregnant Indonesian women, the percentages with DR/R ratios $\geq$0.03 and $\geq$0.06 were 52% and 17%, respectively (S. Tanumihardjo and J.A. Olson, unpublished observations), just twofold greater than those found here. Thus, vitamin A inadequacy among a high-risk group clearly is present in the American population. A preliminary report of a portion of this study has been published (37).

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5. GENERAL SUMMARY

Adequate vitamin A nutriture is essential for reproduction, immune function, vision, and maintenance of cellular differentiation in mammals. During gestation, the supply of vitamin A must be strictly regulated, ensuring that adequate, but not teratogenic, levels are supplied to the developing offspring. As gestation progresses, increased levels of vitamin A are needed to meet the demands of increased cell proliferation, and to maintain maternal supplies for adequate breast-milk concentrations.

The use of 3,4-didehydroretinyl acetate in the MRDR assay has proven to be useful in assessing marginal vitamin A status. Twenty six percent of the pregnant women in the lower socioeconomic population studied were found to be in less than adequate vitamin A status, with MRDR values ≥0.03, while 9% had values ≥0.06. Among the ethnic groups tested, Hispanic and Afro-American groups were most at risk for vitamin A deficiency, with 50% and 40% (respectively) of their MRDR values ≥0.03. MRDR values ≥0.06 were also quite prevalent for these two ethnic groups (33% for Hispanic, 20% for Afro-American). Very few subjects (4%) in the non-Hispanic White group exhibited MRDR values ≥0.06. Serum carotenoid concentrations for the entire study population were also below average values for healthy adult females. Values for the Afro-American group were extremely low, while levels for the other ethnic groups were only moderately low. The significant number of subjects in our study with inadequate vitamin A status indicates that a portion of socioeconomically disadvantaged pregnant women in America are at high-risk for vitamin A deficiency, especially among Hispanic and Afro-American groups.

The use of vitamin A2 in the MRDR assay has been quite useful. Significant toxicity and teratogenicity of A2 seemed unlikely, given that A2 has been shown to be only 30-40% as biologically active as A1 in promoting growth. Also, high teratogenic activity of naturally occurring retinoids has correlated with the presence of an unsubstituted carboxylic acid group at the end of the tetraene chain, which is not present on A2. Thus, the finding that A2 as more toxic and teratogenic than A1 by several orders of magnitude was somewhat of a surprise. While A2 did not possess the teratogenic potency of RA in our studies, the no observable effect level (NOEL) determined for these two compounds in regards to embryolethality was the same (3.5 μmol/kg BW). The biological response to, and metabolism of, an increased dose of A2 was also unique compared to other retinoids. An A2 dose tended to increase concentrations of RA in embryonic and maternal liver tissues, correlating with increased teratogenic effects.

The dose response curve for A2 was also peculiar among retinoids. Teratogenic response to retinoids has generally occurred in a dose-dependent manner. However, the embryotoxic potency of A2 tended to peak at a mid-range dose. Likewise, a mid-range A2 dose produced profound behavioral changes in six week old rats dosed prenatally, while both higher and lower doses did not produce changes. Thus, it appears that A2 is metabolized and transported in a manner unique among naturally occurring retinoids. Whether this difference is mediated by differences in catabolism, differences in binding to nuclear retinoids receptors, or both is unclear.

Extrapolation of these data to determine human risk from a single dose of A2 can be made based on the NOEL for A2 in regards to embryolethality, which was determined to be 3.5 μmol/kg BW. This amount is 25 fold greater than the amount given to a pregnant woman in the third trimester of pregnancy, which is 0.14 μmol/kg BW. Behavioral effects were seen in the female offspring of pregnant rats dosed with A2 at 1 μmol/kg BW. This is a seven fold greater amount than that used in the MRDR for pregnant women. While amounts of A2 given to pregnant women are lower than those inducing effects in laboratory animals, a wider margin of safety is preferred. Generally, a 100 fold margin of safety is required for compounds to be approved for human use by the FDA. While no toxic effects have been reported from use of A2 in human populations, our animal studies indicate that using the MRDR to assess vitamin A status in pregnant women may be putting the offspring at risk. Further investigation into the pharmacokinetics of A2 between the dam and fetal compartments is needed to clarify this issue. Studies are also needed to investigate the risk of using the MRDR assay in lactating women and small children, since A2 crosses readily into breast milk, and the CNS is not fully developed in young humans until approximately six months of age.

Thus vitamin A2 has proven to be quite useful in assessing vitamin A status in a population of pregnant women. However, risks may be associated with its use in pregnant women, as indicated by the outcome of our animal studies.

6. GENERAL CONCLUSIONS

- A2 is more toxic and teratogenic than A1.

- Resorption and terata are not necessarily related even with a similar class of compounds such as RA, A1 and A2.

- The biological activity of a compound in growth is not necessarily related to its toxicity.

- The A2 curve for resorption is not dose dependent.

- A2 produces significant behavioral changes in rats when given prenatally in a manner that is not dose-dependent.

- Socioeconomically disadvantaged pregnant women in Iowa, particularly from Hispanic and Afro-American ethnic groups, tend to be at risk of vitamin A deficiency.