7. Other Toxic Effects

7.1 Adverse effects

7.1.1 Human studies

Toxic effects due to hypervitaminosis A occur in the skin, the circulation (e.g., plasma proteins), internal organs (e.g., liver), the nervous system and the musculo-skeletal system. The manifestations of acute and chronic toxicity vary with dose and body mass, age (paediatric, adult, and elderly), sex and reproductive status (pregnant, lactating), disease conditions (e.g., liver or renal impairment; nutritional status) and concurrent drug administration or environmental chemical exposures. Toxicities associated with acute and chronic excess of vitamin A intake have been extensively reviewed (National Research Council, 1989; US Food and Drug Administration, 1976; Underwood, 1986; Olson, 1987; Hathcock *et al.*, 1990).

Approximately 10–15 cases of toxic reactions to vitamin A are reported per year in the United States, usually due to doses of over 100 000 IU retinol per day (Meyers *et al.*, 1996). Reported cases of overt signs of hypervitaminosis A resulting from food intake and/or supplements have been summarized graphically by Bendich and Langseth (1989) in time blocks from 1850 through 1987. Except for the periods 1952–55 and 1970–72, when infant supplements and treatments for dermatological disorders were introduced, the numbers of cases per year appear consistent with that reported by Meyers *et al.* (1996). In clinical trials, particularly those in which adults are enrolled for cancer preventive studies, eligibility criteria and frequent clinical evaluations tend to limit the occurrence of the more severe side-effects of hypervitaminosis A and the less severe effects are rapidly reversible.

Acute toxicity from large doses, relative to body mass, is more common in young children than in adults and is frequently associated with erroneous or over-zealous administration of supplements. In controlled settings, the frequency of acute toxicity has been evaluated in infants and young children to whom vitamin A (≥25 000 IU) was being administered in an effort to reduce childhood morbidity and mortality associated with vitamin A deficiency

(Florentino *et al.*, 1990; Stansfield *et al.*, 1993; Rahman *et al.*, 1995). Retinol distributes to maternal milk and doses of vitamin A given to lactating women with an inadequate vitamin A status improve that of nursing infants. However, 2000 IU of vitamin A has been safely and effectively administered, with plasma monitoring, to premature infants at risk for bronchopulmonary dysplasia (Robbins & Fletcher, 1993). Acute toxicity, in the context of organogenesis (teratogenesis), is well established through animal studies and case reports of fetal malformations (central nervous system, cardiovascular, palate and ear) following ingestion of ≥25 000 IU/day (≥0.4 μmol/kg bw/day) during pregnancy in humans (Schardein, 1993) (see also Section 7.2.2). Although a teratogenic threshold for vitamin A supplements of 10 000 IU/day has been suggested (Rothman *et al.*, 1996), this is not consistent with other data on teratogenicity (Mills *et al.*, 1997) and the study has been criticized on several scientific grounds (see Section 7.2.1) (Brent *et al.*, 1996; Khoury *et al.*, 1996; Watkins *et al.*, 1996; Werler *et al.*, 1996).

Information concerning the common toxicities associated with chronic administration of low doses of vitamin A to healthy adults is available from the Beta-Carotene and Retinol Efficacy Trial (CARET), a primary cancer prevention study (see Section 4.1.2.1(*b*)). No effect on liver function was observed after more than three years' administration of 25 000 IU retinol per day (*c.* 0.4 μmol/kg bw/day) with or without β-carotene (30 mg) in the pilot studies preceding CARET (Goodman *et al.*, 1993; Omenn *et al.*, 1993a). Only a negligible increase in serum triglyceride levels was observed in the CARET vanguard cohort of 1845 heavy smokers and asbestos-exposed workers, representing 10 184 person-years of intervention (Omenn *et al.*, 1994a). No other common toxicities were cited.

In the context of secondary cancer prevention, daily doses of 300 000 to 600 000 IU vitamin A as retinol for several months have usually been required to produce signs of hypervitaminosis A, although intake as low as 50 000 IU/day has been reported to be toxic after >18 months (Gossel & Bricker, 1994). Interestingly, 300 000 IU retinyl palmitate in an emulsion formulation (A-mulsin®, Mucos Pharma, Munich, Germany) given daily for twelve months to patients with resected stage I non-small-cell lung cancer did not produce major hepatic toxicity in the completed Italian adjuvant trial (Pastorino *et al.*, 1993). Serum levels of γ-glutamyltranspeptidase (GGT) rose during treatment, but were significantly higher only after two years (149 versus 57 IU/L). Serum triglyceride levels increased 63% over the first year of treatment and were significantly higher than those of controls at 8 and 12 months (Pastorino *et al.*, 1991; Infante *et al.*, 1991). The majority of adverse events were dermatological (dryness, desquamation, itching).

7.1.1.1 Mucocutaneous toxicity

Vitamin A is important in the differentiation of the skin, hair and mucous membranes and has been used in dermatology for many years (Peck & Di Giovanna, 1994). Side-effects of systemic vitamin A administration are common, as observed in the adjuvant trial above, but are reversible: dry and scaly skin (desquamation), mouth or lip fissures or chapping (cheilitis), dryness of mucous membranes (including conjunctivitis), brittle nails, skin rashes and erythema, and hair loss (alopecia) (Bendich & Langseth, 1989; Hathcock *et al.*, 1990). Emollients can reduce the discomfort of some of these symptoms.

Topical application causes similar focal reactions but typically not systemic reactions. Both retinyl acetate and retinoic acid have been evaluated as topical treatments for chemoprevention and they produce similar toxicities. After treatment of skin with retinoic acid (0.001–0.1% [unknown volume]) for up to 22 months, the most common adverse events were peeling, erythema and a burning sensation (Thorne, 1992; Mitchell *et al.*, 1995). Both retinoids have been further investigated as intravaginal treatments for cervical intraepithelial neoplasia (CIN) (Mitchell *et al.*, 1995). In a phase I/II trial, CIN I/II patients applied 3, 6, 9 or 18 mg retinyl acetate or placebo intravaginally for seven days beginning on day 5 of three sequential menstrual cycles (Romney *et al.*, 1985). Frequent severe adverse events at the highest dose were vulvar irritation and itching

and 14% of all treated patients had vaginal burning during the trial. The most common general complaints were fatigue and irritability. Phase I/II trials evaluated the safety of retinoic acid applied to a sponge and inserted in a cervical cap or diaphragm. Doses of 0.05–0.2% (5 mL/day) for four days commonly produced vaginal irritation, ulceration and discharge, but no evidence of systemic toxicity (Surwit *et al.*, 1982). In a second study, the same group evaluated a range of doses from 0.05% (1 mL, or approx. 0.002 μmol/day) for four days; the maximum tolerated dose was 0.372% (1 mL/day) (Meyskens *et al.*, 1983). A subsequent phase II study using this dose for induction and maintenance (2 days during months 3, 6, and 9) found mild local effects (cervical inflammation, vaginal discharge and itching) more frequent during induction (Graham *et al.*, 1986). Mild systemic effects (dry skin, chapped lips, mood change, headache and fever) were also noted, although a previous pharmacokinetic study failed to detect retinoic acid in serum up to 24 h after a one-day insertion at the same dose (Peng *et al.*, 1986).

7.1.1.2 Circulatory toxicity

Vitamin A affects lipid metabolism. Hypervitaminosis A may cause hypertriglyceridaemia, possibly due to increases in VLDL. Patients given 300 000 IU retinyl palmitate for 12 months had a mean serum triglyceride level of 283 mg/dL (n = 138) versus 179 mg/dL in 145 control patients (Pastorino *et al.*, 1991) Increases in cholesterol and apoprotein B and decreases in HDL cholesterol are also common (Marsden, 1989). Although the effects are reversible, they have implications for acute pancreatitis and atherosclerotic cardiovascular disease (Armstrong *et al.*, 1994). In the CARET vanguard cohort of 1845 participants, retinyl palmitate (25 000 IU) produced only negligible increases in serum triglyceride levels during five years (Omenn *et al.*, 1994b).

Due to effects on other organs, there may also be other changes in measures of physiological chemistry and haematology. Increase in serum alkaline phosphatase and hypercalcaemia may reflect alterations in bone cell biology. Approximately three-fold increases in GGT have also been reported (Infante *et al.*, 1991). Petechia and haemorrhage, such as bleeding nose or gums, have been reported as symptoms (Bendich & Langseth, 1989). Hypothrombinaemia related to vitamin K deficiency may be due to competitive intestinal absorption (McCarthy *et al.*, 1989).

7.1.1.3 Internal organ toxicity

(a) Liver

About 90% of the total vitamin A is stored in the liver, the majority (= 75%) as retinyl esters in lipid droplets of stellate cells (see Section 3.2) (Blaner & Olson, 1994; Hathcock *et al.*, 1990). Excess storage of vitamin A may result in fatty liver. Hepatomegaly and palpable or tender liver were mentioned in 16% of case reports of hypervitaminosis A (Bendich & Langseth, 1989). Viral hepatitis may precipitate symptoms of hypervitaminosis A, possibly due to low levels of retinol-binding protein. Protein malnutrition, particularly in children and the elderly, may predispose to hypervitaminosis A, also due to low levels of retinol-binding protein. In a study of 41 patients with vitamin A hepatoxicity (Geubel *et al.*, 1991), there were 17 cases of cirrhosis, 10 of mild chronic hepatitis, five of noncirrhotic portal hypertension and nine of increased storage. The authors concluded that vitamin A consumption in the low therapeutic range might be an appreciable cause of liver disease. Pre-existing liver disease may predispose individuals to vitamin A toxicity. Additional case reports indicate that in countries where vitamin A is available as a supplement, hypervitaminosis A should be investigated in cases of liver dysfunction, such as portal hypertension of unknown origin (Dubois *et al.*, 1991; Kowalski *et al.*, 1994).

(b) Gastrointestinal tract

Nausea and vomiting were the most commonly reported symptoms of hypervitaminosis A, occurring in 34% of cases (Bendich & Langseth, 1989). Anorexia, diarrhoea and weight loss may also occur. Adverse events of these types are rapidly reversible upon cessation of vitamin A administration. Symptoms of fatigue, malaise, lethargy, somnolence, weakness and irritability were also commonly reported (Bendich & Langseth, 1989).

7.1.1.4 Neurological toxicity

Headache is a common symptom associated with hypervitaminosis A and may occur transiently with supplement doses. More severe reactions include elevated pressure of the cerebrospinal fluid, cranial hypertension, altered vision, and papilloedema (Bendich & Langseth, 1989; Hathcock *et al.*, 1990). Vitamin A toxicity should also be considered with the diagnosis of pseudotumor cerebri (Benrabah *et al.*, 1995; Moskowitz *et al.*, 1993; Sharieff & Hanten, 1996; Hathcock *et al.*, 1990). There are case reports of drug interactions: headache, visual disturbance, optic disc oedema, and intracranial hypertension in patients combining vitamin A with minocyclin (a synthetic tetracycline) (Benrabah *et al.*, 1995; Moskowitz *et al.*, 1993; Sharieff & Hanten, 1996; Hathcock *et al.*, 1990). In prophylactic treatment of vitamin A deficiency, among 100 infants who received 25 000 IU vitamin A and 98 who received placebo concurrently with three sequential doses of diphtheria/pertussis/tetanus (DTP)/oral polio vaccination, there were eight versus one transient episodes of bulging fontanelles without associated symptoms and without significant increases in serum retinol levels (Rahman *et al.*, 1995). Among 2471 Haitian children, 1–6 years old, randomized to receive placebo or 100 000 IU (30 mg) or 200 000 IU (60 mg) of vitamin A, symptoms of headache, nausea and/or vomiting were dose-related and occurred in 12–24 h (Florentino *et al.*, 1990).

7.1.1.5 Musculo-skeletal toxicity

Hypervitaminosis A is known to cause demineralization of bone, periosteal calcification and hypercalcaemia in all age groups (Peck & Di Giovanna, 1994; Frame, 1974). Retinoid metabolites used in dermatology have caused altered bone remodelling, demineralization and closure of epiphyses. Disseminated idiopathic skeletal hyperostosis, including calcification of tendons and ligaments in the spine and elsewhere, can also occur (Peck & Di Giovanna, 1994). Vitamin A may interact with vitamin D and parathyroid hormone to stimulate osteoclastic activity.

Bone and joint pain and myalgia are listed as commonly associated symptoms of hypervitaminosis A (Bendich & Langseth, 1989; Hathcock *et al.*, 1990). However, only two case reports in the recent literature have described these symptoms, in a 59-year old woman and in an eight-year old boy with renal failure (Doireau *et al.*, 1996; Romero *et al.*, 1996). Mechanistic research is being conducted in animal models (see below).

7.1.2 Experimental studies

The single-dose acute toxicity of vitamin A and its analogues in laboratory rodents is summarized in Table 29 (Kamm *et al.*, 1984; Kelloff *et al.*, 1996). In young monkeys, the LD_{50} for retinyl acetate was 168 mg (0.56 x 10^6 IU) retinol/kg bw. No animals receiving the equivalent of 100 mg (0.33 x 10^6 IU) retinol/kg bw died (Hathcock *et al.*, 1990).

As in humans, vitamin A can be toxic to laboratory animals due to bioaccumulation when it is administered chronically at low daily doses. In rats, symptoms of hypervitaminosis A have been produced by retinol doses as low as 3 mg/day (10 000 IU/day) after a few days to several weeks (Hathcock *et al.*, 1990). The toxicities generally reflected those seen in humans and occurred in the skin, circulation (e.g., plasma proteins), internal organs (e.g., liver) and musculo-skeletal system. No studies in animals have modelled the human neurological toxicity. Efficacy and toxicity at low doses are mediated by metabolism of vitamin A to retinoic acid metabolites that bind to the RAR and RXR hormone receptor classes (see Section 3.1) and activate gene expression. Receptor-selective agonists/antagonists are being developed in an attempt to separate efficacy and toxicity. Homozygous RAR-γ mutant mice have increased resistance to toxicity (Look *et al.*, 1995). Toxicity at high doses may also be due to overloading of the capacity of transport molecules, with the result that free vitamin A

Table 29. Oral LD_{50} values for retinol and retinyl esters in mice and rats

Retinoid	Species	LD_{50} (mg/kg bw)
Retinol	Mouse	2570
Retinyl acetate	Mouse	4100
Retinyl palmitate	Mouse	6060
	Rat	7910

exerts a cytotoxic, detergent-like effect on membranes.

The LD_{50} rank-order of the vitamin A esters and metabolites is reflected in their long-term toxicity. Retinyl palmitate, at 250 times the human recommended dietary allowance (RDA), produced no adverse effects in rats given up to 27.5 mg/kg bw/day (50 000 IU/kg bw/day) or dogs given up to 13.8 mg/kg bw/day (25 100 IU/kg bw/day) for 10 months. However, retinoic acid administered to rats and dogs at doses of 5 and 50 mg/kg bw/day for 13 weeks showed toxicity (Kamm *et al.*, 1984). Rats in the low-dose group displayed hair loss, dermal and mucosal alterations, inhibition of spermatogenesis and weight loss. At the high dose, serum transaminase and alkaline phosphatase activities were elevated, total protein declined and 20% of the animals died. Similar signs were seen in the dogs; however, mortality at the high dose was 50%. In mice, doses of 150–250 mg/kg bw/day caused alopecia, weight loss and skin and membrane changes after five days.

7.1.2.1 Mucocutaneous toxicity

The commonly observed effects of retinoids on the skin of laboratory animals include erythema, epidermal thickening, scaling, loosening of the stratum corneum, increases in transepidermal water loss and conjunctivitis (Armstrong *et al.*, 1994). The effect is more pronounced on smooth and bare skin. Alopecia is also seen. Ophthalmological changes are more common with the retinoid metabolites. Cellular proliferation is increased, resulting in hyperplasia, particularly in the stratum spinosum (acanthosis), and hypergranulosis occurs. Vitamin A causes a broad spectrum of biological effects in the epidermis, including decreased numbers of tono-filaments and desmosomal attachments, increased gap junction proliferation, suppressed expression of epidermal transglutaminase and cornification, altered pattern of keratin expression towards fetal K19 and K13 forms and decreased total keratin content, and altered cell surface proteins and receptors (Peck & Di Giovanna, 1994). The effects are dose- and substance-specific, so that the histology of epidermal hyperplasia on the dorsal skin of hairless mice can be used to rank-order natural and synthetic retinoids by potency (Connor *et al.*, 1986). Retinol and retinyl palmitate have been extensively evaluated for toxicity in mice and rats, as well as clinically, and are considered to be safe as cosmetic ingredients in products containing 0.1–1.0% (Cosmetic Ingredient Review Expert Panel of the American College of Toxicology, 1987).

7.1.2.2 Circulatory toxicity

Elevations of serum levels of triglycerides, cholesterol and alkaline phosphatase and decreases in blood erythrocyte count and haemoglobin concentration have been reported, but vary with the form of vitamin A (Armstrong *et al.*, 1994). Hypertriglyceridaemia was induced in rats by doses of 33 mg retinol per day (110 000 IU/day) and by 550–1100 mg retinyl palmitate per day (1–2 x 10^6 IU/day), but in another study was not produced by a dose of 185 mg (336 000 IU) retinyl palmitate/100 g body weight/day (Hathcock *et al.*, 1990). The increase in cholesterol is less consistent and varies with the form of vitamin A. Alkaline phosphatase increases are also variable and may represent activity from bone osteoblasts and/or from the liver. Other reported serum enzyme changes (e.g., alanine aminotransferase (ALAT), aspartate aminotransferase, GGT) are associated with liver function. With respect to haematological changes, retinyl acetate at a dose of 0.5% in the diet reduced prothrombin to 65% and kaolin-activated partial thromboplastin times to 28% of control values; this dose was considered to have relatively weak haemorrhagic effects in the rat (Takahashi, 1995). The haemorrhagic action of retinyl acetate and other vitamin A compounds was prevented by dietary vitamin K supplementation (McCarthy *et al.*, 1989).

7.1.2.3 Internal organ toxicity

(a) Liver

In rats, after six months of dietary supplementation with 328 mg retinyl palmitate/kg, hepatic levels of the ester were increased ten-fold compared with vehicle-control animals (Grubbs *et al.*, 1990). Hypervitaminosis A causes fatty infiltration of rodent liver. Tissues such as adipose, kidney, testes, lung and bone marrow may also take up significant amounts of lipid and undergo histological changes.

In Kupffer cells isolated from male rats treated for 3–7 days with 75 mg/kg bw/day of retinol by oral gavage once daily, respiratory and phagocytic activity and the release of reactive oxygen species, tumour necrosis factor alpha and prostaglandin E_2 were elevated (Hoglen *et al.*, 1997). Also in rats, excessive intake of vitamin A for seven days caused activation of Kupffer cells and induced accumulation of lipid drop-lets in fat-storing cells, as well as proliferation of these cells (Lettinga *et al.*, 1996). Increased alkaline phosphatase pointed to activation of retinyl ester transport across bile canalicular membranes, while changes in metabolic enzyme markers suggested decreases in purine breakdown, antioxidant capacity, phagocytotic capacity and ammonia regulation. However, no cell damage was apparent.

With regard to drug interactions, the effect of vitamin A on ethanol-induced liver toxicity has been investigated in efforts to develop a model of human alcoholic liver disease. However, the role of vitamin A as a major risk factor in the pathogenesis of alcoholic liver fibrosis has not been established. Liver fibrosis, characterized by perivenular fibrosis and central vein thickening, was elicited in Sprague-Dawley rats when ethanol and retinyl acetate (up to 29 000 IU per litre of liquid diet) were incorporated into the diet for nine months (Leo & Lieber, 1983). Other investigators, using a similar experimental design, failed to produce fibrosis, as measured histologically or as increased collagen, after 16 months of treatment. They tested two different strains of rats (BN/BiRij and WAG/Rij), which develop fibrosis after carbon tetrachloride treatment (Bosma *et al.*, 1991; Seifert *et al.*, 1991). There was evidence of steatosis (fatty degeneration), round cell inflammatory infiltration and elevations in liver enzymes and serum lipids. However, neither group observed the characteristic histological picture of human alcoholic hepatitis in connection with hypervitaminosis A.

Pretreatment of rats with a single high dose of vitamin A (250 000 IU) led to increased hepatoxicity of vinylidene chloride (Wijeweera *et al.*, 1996). Vitamin A treatment activated Kupffer cells and induced CYP2E1 which, in turn, potentiated vinylidene chloride metabolism and induced an increase in plasma ALAT *in vivo*, an increase in potassium ion leakage from liver slices *in vitro* and histological evidence of centrilobular necrosis. Similarly, vitamin A pretreatment for seven days potentiated the hepatotoxicity of carbon tetrachloride (ElSisi *et al.*, 1993a,b,c). Other investigators found that when vitamin A (12 500 IU retinyl palmitate twice a week for four weeks) was given during carbon tetrachloride treatment of rats, parenchymal cell damage and fibrosis were enhanced, whereas vitamin A post-treatment after carbon tetrachloride strongly reduced fibrosis (Knook *et al.*, 1995). Conversely, in mice, retinol pretreatment at 75 mg/kg bw/day for seven days protected against increases in ALAT and histopathological necrosis induced by carbon tetrachloride and phalloidin, but potentiated the toxicity of allyl alcohol, acetaminophen and D-galactosamine (Rosengren *et al.*, 1995). The results of drug interaction studies in rats and mice indicate that there are species differences in the effect of vitamin A on the liver and that the timing of vitamin A administration may also affect the hepatotoxicity of the drug or chemical.

(b) Gastrointestinal tract

In rat intestine, vitamin A doses up to ten times the RDA for rats (4 μmol/kg) for seven days did not cause disturbance in absorptive cell processes (Suzuki *et al.*, 1995). However, at higher doses (100 times the RDA), the unesterified retinol/cellular retinol binding protein ratio (type II) was > 3, while at 1000 times the RDA the ratio was > 19 and lecithin:retinol acyltransferase activity was significantly elevated. In hamster, retinyl acetate, administered as 250 IU/g of diet, produced gallstones and elevations of serum ALAT (Cardenas *et al.*, 1991). Systemic effects of hypervitamosis A on the upper gastrointestinal epithelium have also been described. When rats were given daily intraperitoneal injections of 150 IU vitamin A per gram body weight for 10 days, there was histological evidence of hyperplasia and hypertrophy of the oesophageal epithelium, with increased mitoses and numbers of immature cells (Oliveira *et al.*, 1990). The alterations were more evident in the lower third of the oesophagus.

(c) Other organs

Extensive foci of degenerative myocardial fibres, associated with electrocardiogram changes, were reported in rats treated with vitamin A equivalent to 3 or 6 mg (10 000 or 20 000 IU) retinol/kg bw/day for three months (Hathcock *et al.*, 1990). Fatty changes and haemosiderosis in the spleen, glomerulonephritis and necrotizing nephrosis in the kidney, and testicular atrophy in adults and degenerative testicular changes in weanlings have been described (Kamm *et al.*, 1984).

7.1.2.4 Musculo-skeletal toxicity

Demineralization, thinning of the long bones, cortical hyperostosis, periostosis and premature closure of the epiphyses have been described in animals treated with high doses of vitamin A. In adult cats, confluent exostoses in the cervical spine were observed (Peck & Di Giovanna, 1994). Retinyl acetate fed to rats at doses of 8.5–13.6 mg retinol/day (28 300–45 300 IU) caused a limping gait and fractures. Bone mineralization, determined as bone-ash, was normal, so the toxicity was associated with alterations in the bone matrix (Hathcock *et al.*, 1990). Mice fed retinyl acetate (75–300 µg daily) in the diet for 3–16 months developed radiographic and histological evidence of arthritis and periarticular bone formation similar to diffuse idiopathic skeletal hyperostosis (Boden *et al.*, 1989). Retinyl palmitate fed at 60, 200 or 350 IU/g diet for 23 weeks caused dose-related increases in bone fractures, osteoporotic lesions, metaphyseal flares and bone deformities in mice treated with DMBA and TPA (Forsyth *et al.*, 1989). The bone toxicity of hypervitaminosis A has also been demonstrated in dogs, cats, calves and hogs (Kamm *et al.*, 1984).

7.2 Reproductive and developmental effects

7.2.1 Human studies

There are case reports of malformations following very high intakes of vitamin A early in pregnancy (Rosa *et al.*, 1986). The estimated doses vary from 25 000 IU to 500 000 IU, and the adverse outcomes include a range of abnormalities of the central nervous system, kidneys and urinary system and adrenal gland, some of which are similar to abnormalities seen in offspring of animals dosed with vitamin A (Monga, 1997). On their own, these case reports do not provide strong evidence of cause and effect, as the doses, patterns of ingestion and constellation of abnormalities vary considerably. However, suspicion that there is an effect is raised by laboratory studies showing teratogenic effects of vitamin A in all species tested, although the doses required vary considerably between species. Moreover, it is well known that synthetic chemicals with vitamin A activity are powerful human teratogens. For example, isotretinoin, a derivative of retinoic acid, introduced in the early 1980s for oral treatment of acne, was associated with an almost ten-fold increase in the occurrence of major malformations when mothers took the drug in the first month of pregnancy (Lammer *et al.*, 1985).

Three case–control studies have investigated the role of vitamin A as a human teratogen. A Spanish study compared 11 293 cases of congenital malformation (excluding chromosomal abnormalities) with 11 193 controls, matched for sex, hospital and day of birth (Martínez-Frías & Salvador, 1990). Maternal intake of vitamin A from diet and supplements during the pregnancy was assessed by questionnaire. Few women (16 with birth defects, 14 controls) had intakes above 10 000 IU per day, and no increased risk was observed in this group (RR, 1.1; $p = 0.4$). The risk was increased in the highest-dose group (above 40 000 IU; 11 cases, 4 controls); this finding bordered on significance (RR, 2.7; $p = 0.06$) and was stronger for women exposed in the first two months of pregnancy (RR, 4.0; $p = 0.19$; 4 cases, 1 control) than for those exposed in the last five months of gestation (RR, 2.0; $p = 0.35$; 4 cases, 2 controls).

A study in the United States included 2658 infants with malformations of structures derived from cranial neural crest cells (Werler *et al.*, 1990), as animal experiments have indicated that this tissue is particularly sensitive to effects of retinoids *in utero*. The control group included a similar number of infants with other malformations. Vitamin A supplementation was defined as daily use, for at least seven days, of retinol with or without vitamin D, or of fish oils. Relative risk estimates were 2.5 (95% CI, 1.0–6.2) for use in the first month of pregnancy, 2.3 (95% CI, 0.9–5.8) in month 2, and 1.6 (95% CI, 0.6–4.5) in month 3. There was

a slight and statistically non-significant increase in risk associated with use of multivitamins containing vitamin A. No information was obtained on dose provided by these supplements nor on vitamin A intake from diet.

In a study of all pregnancies in California and in Illinois, United States, between 1985 and 1987, infants with neural tube defects (n = 548) were compared with infants with other major malformations (n = 387) and normal controls (n = 573) (Mills *et al.*, 1997). There was no evidence of an increased risk of neural tube defects or other major malformations associated with high doses of vitamin A (greater than 10 000 IU) (RR for major malformations, 0.7; 95% CI, 0.3–2.0). Similar results were obtained when the cases were restricted to cranial neural crest defects. The odds ratios were slightly greater when intake from supplements only was considered rather than supplements plus dietary sources, but the differences were small, all risk estimates being close to unity, and were statistically non-significant. High intakes were uncommon: fewer than 1% of participants had vitamin A exposures estimated to be greater than 15 000 IU per day.

Data from a population-based case–control study of major birth defects in the 1980s were subsequently re-analysed to search for an association between vitamin A intake and occurrence of cranial neural crest defects (Khoury *et al.*, 1996). Exposure was defined as use of vitamin A, alone or as a component of multivitamin supplements, for three days per week or more frequently, in the period from one month before conception to the end of the first trimester of pregnancy. There was no evidence of an increased risk associated with use of vitamin A supplements (RR, 0.5; 95% CI, 0.2–1.3). It was estimated that the women taking these supplements seldom received doses above 10 000 IU per day. The relative risk in women who took only vitamin A was 0.6 (95% CI, 0.2–1.4).

The findings of Rothman *et al.* (1995) prompted a review of data from the Californian Birth Defects Monitoring Program, to compare use of vitamin A supplements by mothers of children with specific defects (orofacial clefts and conotruncal heart defects) with that of control women (Shaw *et al.*, 1996). The data were collected from a number of case–control studies conducted in the late 1980s, at which time it is thought that vitamin A supplements contained 10 000–15 000 IU retinol. There was no evidence of increased exposure among the cases compared with the controls in this analysis (for orofacial clefts, RR, 0.6, 95% CI 0.2–1.6; for conotruncal heart defects, none of the 207 case mothers took supplements).

In a cohort study, 22 748 women in the United States were recruited in the second trimester of pregnancy and information was obtained by telephone interview on diet and use of vitamin supplements (Rothman *et al.*, 1995). Information on the outcome of pregnancy was obtained from questionnaires mailed to obstetricians, or if the obstetricians did not reply, from the women themselves. It was estimated that about 2% (n = 455) of the women had total daily intake of vitamin A above 10 000 IU. The prevalence of all birth defects was twice as great in women taking 15 000 IU or more per day compared with those in the lowest-intake category (< 5000 IU per day) (prevalence ratio 2.2, 95% CI, 1.3–3.8). There was a more marked effect for neural crest defects (excluding neural tube anomalies), particularly among women whose excess vitamin A intake came from supplements: for intakes above 10 000 IU compared with less than 5000 IU, the prevalence ratio was 4.8 (95% CI, 2.2–10.5). This is consistent with data from studies of human volunteers, showing that exposure to retinoic acid was markedly lower after consumption of liver than after taking supplements of the same retinol equivalent dose (Buss *et al.*, 1994), presumably reflecting differences in the extent of oxidation of retinol to retinoic acid depending on the form in which the retinol is presented. The increased prevalence of abnormalities was apparent only among women with relatively high vitamin A intake around the time of conception or in the first six weeks of pregnancy; no effect was associated with high intake confined to later stages of pregnancy.

It has been pointed out that the study by Rothman *et al.* relied on birth defect data reported by physicians and mothers, that the category of 'neural crest defects' was very broad

and that the prevalence of major defects in the cohort was less than would be expected (Brent *et al.*, 1996). Unless there was differential misclassification with respect to vitamin A intake, these sources of error would not cause an apparent effect to be reported when one did not exist. However, the findings in the cohort study were based on relatively small numbers (for example, a total of 10 defects among women taking > 10 000 IU/day) and have not been replicated.

The study by Rothman *et al.* (1995), despite its limitations, provides the strongest evidence available that retinol may act as a teratogen in human populations. If it does, the critical question is then: at what level of intake does the effect occur? It has been argued on the basis of the non-human primate data and the case–control findings in human populations that teratogenic effects are unlikely to occur below about 30 000 IU per day (Miller *et al.*, 1998). However, Rothman *et al.* (1996) have stated that further analysis of the cohort data provides evidence for an effect in the range of 10 000–20 000 IU per day, with an approximately four-fold increase in prevalence of defects compared with the lowest-dose category. It should be noted also that a number of the case–control studies used as controls infants with malformations other than neural crest-derived lesions. Therefore, if there is a relationship between vitamin A and a wide range of malformations, as suggested by the cohort study, these case–control investigations may have underestimated the effect of vitamin A.

Evidence of safety of intakes below 10 000 IU, at least when given in conjunction with folic acid, is provided by a randomized controlled trial in Hungary of vitamin supplementation in early pregnancy (Czeizel & Dudás, 1992). In this study, the active agent contained vitamin A (4000 or 6000 IU) and folic acid; the observed rates of both neural tube defects and of other major malformations were lower in the vitamin user group than in subjects taking a control capsule. [The Working Group noted that since folic acid deficiency may be an important cause of certain malformations, the safety of vitamin A alone is difficult to ascertain on the basis of this study.]

7.2.2 Experimental studies

7.2.2.1 Effects on embryonic development

(a) Vitamin A deficiency

The importance of vitamin A for embryonic development was first demonstrated in the 1930s, when pregnant pigs fed a diet deficient in vitamin A were found to give birth to offspring that were blind and had severe malformations, such as cleft palate and cleft lip (Hale, 1933; Kalter & Warkany, 1959). In rats, vitamin A deficiency before and during gestation leads to increased embryolethality and a wide variety of abnormalities in the live offspring, including malformations of the eyes, urogenital system, respiratory tract, heart and great vessels. Administration of retinol to vitamin A-deficient rats during midgestation is essential for prevention of late fetal death as well as for delivery and neonatal survival, and retinoic acid cannot replace retinol in these functions (Wilson *et al.*, 1953). These results suggest that retinol itself or one of its metabolites, other than all-*trans*-retinoic acid, may be responsible for late developmental events in gestation (Thompson *et al.*, 1964; Takahashi *et al.*, 1975; Wellik & DeLuca, 1995; Wellik *et al.*, 1997).

(b) Retinoid excess

The first report on the teratogenicity of excess vitamin A appeared in the early 1950s. Oral doses of about 60 mg retinol/kg bw administered to pregnant rats daily during gestational days 2 to 16 resulted in high rates of embryolethality and severe fetal malformations, such as exencephaly, cleft lip and palate and eye defects (Cohlan, 1953). Subsequent studies in many animal species have consistently shown embryotoxic effects of excess of vitamin A or retinoic acid at doses that do not cause overt signs of maternal toxicity (Geelen, 1979; Agnish & Kochhar, 1993). Furthermore, the embryotoxic effects of retinoids are not restricted to mammals, but can also be induced in other vertebrate classes, such as birds (Tickle *et al.*, 1982), amphibians (Durston *et al.*, 1989) and fishes (Holder & Hill, 1991).

The teratogenic effects of retinoids cover a wide spectrum and show marked stage-specificity. For example, exposure of the early postimplantation embryo can result in cranio-

facial, central nervous system, cardiovascular and thymus defects, whereas exposure at later stages of development is associated with defects of the limbs, palate and genitourinary tract (Kalter & Warkany, 1961; Shenefelt, 1972; Kistler, 1981; Sulik *et al.*, 1995).

Studies with synthetic retinoids in rodents have revealed interesting structure–activity relationships. For example, the presence of an acidic terminus group and a polyene side-chain of more than five carbon atoms or an aromatic system are important structural requirements for the teratogenic activity of retinoids. Modifications of the β-ionone ring of retinoids, such as hydroxylation or oxygenation at C-4, affect the teratogenic activity of retinoic acid to only a small extent. Finally, the *cis–trans* configuration of the side-chain can be of great significance; thus 13-*cis*-retinoic acid is only marginally active, although all-*trans*-retinoic acid is highly active in rodent species (Kraft *et al.*, 1987; Wilhite *et al.*, 1989).

(c) Molecular basis for the role of retinoids in embryonic development

Significant progress has been made in the last decade towards elucidating the role of retinoids in normal and abnormal embryonic development. The many investigations in this field can be roughly divided into the following categories (presented in part in Tzimas, 1996).

(i) Endogenous retinoids: The retinoids that occur naturally in the developing embryo (Table 30) have been analysed by methods such as HPLC or either HPLC alone or gas chromatography combined with mass spectrometry. Endogenous retinoic acid and retinol have been identified in the embryo of all species examined to date, including birds (Thaller & Eichele, 1987; Scott *et al.*, 1994; Dong & Zile, 1995), rodents (Collins *et al.*, 1994; Tzimas *et al.*, 1995; Satre & Kochhar, 1989), rabbits (Tzimas *et al.*, 1996a), primates, including humans (Hummler *et al.*, 1994; Kraft *et al.*, 1993), *Xenopus* (Durston *et al.*, 1989) and zebrafish (Costaridis *et al.*, 1996). Notably, there is a concentration gradient of retinoic acid across the anterior–posterior axis of the developing chick limb bud, with highest concentrations posteriorly (Thaller & Eichele, 1987; Scott *et al.*, 1994). A similar gradient from the forebrain (lowest) to the spinal cord (highest) of the early mouse embryo has also been reported (Horton & Maden, 1995). Using an alternative approach, Hogan *et al.* (1992) examined the capacity of embryonic tissues to convert radiolabelled retinol to retinoic acid *in vitro* and found that the Hensen's node and the primitive streak of the early mouse embryo are more important sites of all-*trans*-retinoic acid synthesis than more anterior tissues (Costaridis *et al.*, 1996).

In addition, 3,4-didehydroretinol and 3,4-didehydroretinoic acid are endogenously found in avian (Scott *et al.*, 1994) and rabbit embryos (Tzimas *et al.*, 1996a), and 3,4-didehydroretinol is also present in the embryo of monkeys (Tzimas, 1996) and humans (Sass, 1994). Surprisingly, none of these retinoids occurs physiologically in the embryos of mice or rats (Collins *et al.*, 1994; Tzimas *et al.*, 1995).

Table 30. Endogenous concentrations of plasma retinoids in several species[a]

Retinoid	Rat[b]	Cynomolgus monkey[c]	Rabbit[d]	Human[e]
All-*trans*-retinoic acid	0.4 ± 0.03	0.5 ± 0.3	1.4 ± 0.3	1.3 ± 0.6
13-*cis*-retinoic acid	n.d.	n.d.	1.7 ± 1.1	0.9 ± 0.2
Retinol	100 ± 42.4	219 ± 12.6	826 ± 140	477 ± 71.0
Retinyl palmitate	102 ± 19.5	104 ± 46.4	35.6 ± 13.3	10.5 ± 7.1

[a] Concentrations are expressed in ng/mL (means ± SD); measured by HPLC
[b] Pregnant Wistar rats on gestational day 12. Data from Collins *et al.* (1994)
[c] Pregnant cynomolgus monkeys on gestational day 31. Data from Tzimas (1996)
[d] Pregnant Swiss hare rabbits on gestational day 12. Data from Tzimas *et al.* (1996a)
[e] Nonpregnant female volunteers. Data from Peiker *et al.* (1991)
n.d., not determined

Other endogenous embryonic retinoids include all-*trans*-4-oxoretinoic acid and all-*trans*-4-oxoretinal in *Xenopus* embryos (Pijnappel *et al.*, 1993; Blumberg *et al.*, 1996) and all-*trans*-retinal in *Xenopus* (Durston *et al.*, 1989) and zebrafish (Satre & Kochhar, 1989) embryos. 9-*cis*-Retinoic acid has been tentatively identified in *Xenopus* embryos (Kraft *et al.*, 1994), but this finding has been questioned (Blumberg *et al.*, 1996). None of these retinoids was detected in the embryo of mammalian or avian species, suggesting that either they do not play a significant physiological role, at least at the developmental stages examined, or they elicit biological effects at concentrations which are lower than the detection limits of the analytical methods used.

To allow retinoid determination in small tissue segments of early embryos, the more sensitive reporter gene assays have been employed; these assays make use of transgenic mice carrying a reporter gene which is under the transcriptional control of a retinoic acid response element (RARE) (Rossant *et al.*, 1991; Mendelsohn *et al.*, 1991; Balkan *et al.*, 1992) or cell lines transfected with a retinoic acid-driven reporter gene (Wagner *et al.*, 1992; Chen *et al.*, 1992a, 1994). The response of the reporter systems is assumed to reflect the presence of all-*trans*-retinoic acid or other retinoids that induce the expression of the reporter gene, but cannot be considered conclusive with respect to the chemical identity of the retinoids. Despite this caveat, studies using such methods have clearly demonstrated the existence of some 'hot spots' of embryonic retinoid concentrations, such as the Hensen's node of the chicken embryo (Chen *et al.*, 1992b); furthermore, anterior–posterior gradients of retinoid concentrations were found in early neurula *Xenopus* embryos (Chen *et al.*, 1994) and the central nervous system tissue of rat embryos (Wagner *et al.*, 1992), with highest levels posteriorly rather than anteriorly. It will be important to complement these reporter gene studies with direct chemical identification and quantitation of endogenous retinoids.

(ii) Cellular retinoid-binding proteins and retinoid receptors: The cellular retinoid-binding proteins and the retinoid receptors (see Section 3.1) display characteristic spatial and temporal patterns of expression during mouse and chicken embryogenesis (Giguere, 1994; Mendelsohn *et al.*, 1992). CRABP-I is abundantly expressed in the mesenchyme of the hindbrain, the cranial neural crest cells and the limb buds, reflecting the structures of the embryo that are susceptible to retinoic acid-induced teratogenicity, whereas CRBP is found in the epithelium of the same regions. In contrast, the expression of CRABP-II is more widespread and not restricted to the retinoid-sensitive embryonic regions (Ruberte *et al.*, 1992; Gustafson *et al.*, 1993; Lyn & Giguere, 1994; Vaessen *et al.*, 1990; Maden, 1994). RAR-α and RXR-β are widely distributed, whereas the other receptors are temporally and spatially restricted (Giguere, 1994; Mangelsdorf *et al.*, 1994).

The role of CRABP-I and -II during embryonic development has been subject of controversy. One hypothesis is that CRABP-I (and perhaps CRABP-II as well) regulates the cytoplasmic levels of retinoic acid by binding the majority of this acid or enhancing the rate of its catabolism, thus allowing only small amounts of free ligand to enter the nucleus and activate the retinoid receptors (Napoli, 1994; Ruberte *et al.*, 1992). This would be consistent with data from studies with cell lines, which showed that overexpression of CRABP-I reduced the ability of retinoic acid to stimulate RAR-mediated gene transcription (Boylan & Gudas, 1991). Alternatively, CRABP-I may shuttle its ligand to the nucleus (Takase *et al.*, 1986); this hypothesis has recently been supported by the demonstration of nuclear localization of CRABP-I in mouse embryonic cells (Gustafson *et al.*, 1996). However, other studies have shown that mutant mice deficient in either CRABP-I or CRABP-II, or both, are essentially normal, both during development and in adult life, and are as susceptible to the teratogenic effects of retinoic acid as their wild-type littermates (Gorry *et al.*, 1994; de Bruijn *et al.*, 1994; Fawcett *et al.*, 1995; Lampron *et al.*, 1995). However, the double-knockout mice exhibit increased mortality by six weeks of age compared with wild-type controls. It remains to be determined whether the absence of CRABPs affects susceptibility to vitamin A deficiency (Li & Norris, 1996).

The physiological role of RARs in embryonic development has been studied by targeted modification of certain members of the RAR family (Lohnes *et al.*, 1995). Notably, most congenital defects of the fetal vitamin A deficiency syndrome were observed after combined disruption of various genes within the RAR family, but not in RAR single mutants, suggesting functional redundancy between the members of the RAR family. Other studies have shown the significance of RXR-α for heart and eye morphogenesis (Kastner *et al.*, 1994; Sucov *et al.*, 1994) and of RXR-β for normal spermatogenesis (Kastner *et al.*, 1996). In addition, combined disruption of RXR-α and RAR isoforms resulted in even more severe phenotypes, indicating that RAR/RXR-α heterodimers mediate retinoid signalling *in vivo* (Kastner *et al.*, 1994, 1997).

(iii) Regulation of morphogenesis and gene expression by retinoids: The fundamental role of retinoids in vertebrate embryonic development has been further emphasized by studies that examined the effects of retinoids on pattern formation of structures of the embryonic body such as the central nervous system, the vertebral column, and limbs (Hofmann & Eichele, 1994). For example, retinoic acid released from a bead implanted at the anterior margin of a chick limb bud can alter the normal digit pattern and even cause pattern duplications (Tickle *et al.*, 1982). Thus, retinoic acid mimics the zone of polarizing activity, which is a group of cells located at the posterior region of the limb bud that is well known to evoke the development of additional digits when grafted at the anterior edge of another limb bud (Tickle *et al.*, 1975). The polarizing activity of retinoic acid in this system and the demonstration of its physiological occurrence in limb tissue (Thaller & Eichele, 1987) have led to the speculation that retinoic acid is the endogenous morphogen of limb development. Morphogens are thought to be distributed in gradients and determine the fate of cells in a concentration-dependent manner.

The results of more recent studies have, however, challenged the proposed existence of a concentration gradient for morphogenetically active retinoids. First, the endogenous concentrations of 3,4-didehydroretinoic acid do not form a gradient across the anterior–posterior axis of the chicken limb bud (Scott *et al.*, 1994), although this retinoid is as potent as all-*trans*-retinoic acid in inducing pattern duplications after local application to the anterior margin of the limb bud (Thaller & Eichele, 1990). Second, in contrast to the situation in the chick limb bud, retinoic acid does not display an anterior–posterior concentration gradient in the mouse limb bud (Scott *et al.*, 1994; Ang *et al.*, 1996). Third, immunohistochemical analysis of a monoclonal antibody raised against retinoic acid revealed that this acid is distributed in the peripheral rather than the core mesenchyme of the developing chick limb bud (Tamura *et al.*, 1990). Thus, the small posterior tissue segment (one-fourth of the limb) assayed by Thaller and Eichele (1987) and Scott *et al.* (1994) would contain less core mesenchyme with a low retinoic acid concentration than the larger anterior segment, and the anterior–posterior gradient of retinoic acid (Thaller & Eichele, 1987; Scott *et al.*, 1994) may therefore be an artefact.

Other studies have also challenged the validity of retinoic acid being the proximate morphogen of the zone of polarizing activity, based on differential effects of retinoic acid and the zone of the polarizing activity at the molecular level (Noji *et al.*, 1991), as well as the discovery of several signalling molecules, such as the sonic hedgehog protein (shh), which act downstream of retinoic acid (Riddle *et al.*, 1993; Ogura *et al.*, 1996). Therefore, retinoic acid seems to be an important but intermediate signal of limb morphogenesis.

Recent investigations have focused on the effects of all-*trans*-retinoic acid on the expression of growth factors within the developing embryo, such as homeotic genes (*hox* genes), the *shh* gene and members of the fibroblast growth factor (FGF) and TGFβ families. The products of these genes are believed to play important roles in determining the developmental fate of many embryonic structures, such as the rhombomeres of the hindbrain and the limb bud (Gudas, 1994; Means & Gudas, 1995). Retinoic acid activates the expression and alters the spatial pattern of expression of many of these genes, as shown in cell culture systems and in developing embryos. This up-regulation may result from transcriptional

activation via the RAR/RXR pathway, as the identification of RAREs in several *hox* genes suggests, or, indirectly, as a result of induction by *shh*. Furthermore, null mutations of some *hox* genes in mice induce phenotypes similar to the teratogenic phenotypes induced by retinoids. These findings suggest that alteration of the expression of *hox* genes is part of the mechanism of retinoid teratogenicity (Gudas, 1994; Means & Gudas, 1995).

(d) Interspecies variations in retinoid teratogenesis

The relative teratogenic potencies of retinoids used in human therapy have been investigated in numerous animal species. In general, retinoids induce qualitatively similar patterns of abnormalities in every species tested, depending on the dose and the developmental stage at the time of exposure to the drug. Unlike the spectrum of defects, the doses of retinoids required to induce teratogenic effects often show pronounced interspecies variation. Table 31 lists the lowest reported teratogenic doses of retinol in various species. This information has been obtained mainly from studies in which vitamin A was administered to animals by gavage daily during the period of organogenesis, although in some studies with monkeys, the treatment began before the onset of organogenesis, and the drug was administered twice daily on the last two to four days of the dosing period.

Table 31. Lowest reported teratogenic doses of vitamin A following oral administration

Species	Retinol dose[a]	
	mg/kg	IU/kg
Mouse[b]	25	83 000
Rat[b]	50	167 000
Rabbit[b]	5.5	18 000
Cynomolgus monkey[c]	6	20 000
Human[d]	(0.2–1.5)[e]	700–5000

[a] Doses of retinyl palmitate are expressed as retinol
[b] From experiments with daily dosing throughout organogenesis (Tzimas *et al.*, 1994; Collins *et al.*, 1994; Agnish & Kochhar, 1993; Agnish *et al.*, 1990)
[c] Daily dosing with 13-*cis*-retinoic acid and all-*trans*-retinoic acid began before the onset of organogenesis, followed by dosing twice daily on the last 2–4 days of the dosing period (Hummler et al., 1990; Hendrickx & Hummler, 1992). Retinol was dosed daily from gestational day 16 to 27 (Hendrickx *et al.*, 1997)
[d] The numbers indicated refer to the therapeutic dose of 13-*cis*-retinoic acid (Lammer *et al.*, 1985) or the estimated doses of retinol obtained via supplementation and food
According to case reports (Rosa, 1993)

The lowest teratogenic doses of retinol in mice and rats are at least one order of magnitude higher than in rabbits, cynomolgus monkeys and humans.

Much effort has been expended to determine the reasons for the marked interspecies variations. It is generally accepted that a direct embryotoxic effect of a drug is determined by pharmacodynamic factors, such as the inherent activity of the drug and the intrinsic sensitivity of the embryo, as well as pharmacokinetic factors that determine the extent of embryonic exposure to the drug and its active metabolites (Wilson, 1977; Nau, 1986).

(e) Influence of placenta type on transplacental distribution of retinoids

In rodents, the type of placenta changes with gestational age. In the rat, for instance, the choriovitelline (yolk-sac) placenta is formed after angiogenesis of the chorionic placenta. Another type of placenta, the chorioallantoic placenta, differentiates from gestational day 11.5 and gradually becomes functional from day 12 onward (Beck, 1976; Garbis-Berkvens & Peters, 1987; Jollie, 1990). Most information on the transplacental distribution of retinoids in rodents is derived from studies performed at mid-organogenesis stages, such as gestational day 11 in mice and day 12 in rats, before full development of the chorioallantoic placenta. In contrast, development of the chorioallantoic placenta in the monkey takes place much earlier during gestation and is already complete at the time when measurements of embryonic retinoid concentrations can be performed (Beck, 1976). Therefore, it was previously hypothesized that the relatively extensive placental transfer of 13-*cis*-retinoic acid to the monkey embryo, as compared to rodent embryos at midgestation, was related to the type of placenta (Hummler *et al.*, 1994). A recent study further addressed this question by comparing the placental transfer of 13-*cis*-retinoic acid in mice and rats at gestational ages at which the chorioallantoic placenta is either starting to differentiate (day 11 for mice and day 12 for rats) or well established

(day 14 for mice and day 16 for rats) and found more efficient transfer at the later than earlier gestational ages (Tzimas *et al.*, 1995).

(f) Duration of retinoid exposure is a major determinant of teratogenic outcome

Interspecies comparison of the transplacental pharmacokinetics of all-*trans*-retinoic acid and 13-*cis*-retinoic acid has not only helped to explain the marked interspecies variations of the lowest teratogenic doses of 13-*cis*-retinoic acid, but has also shown that embryonic AUC values of the corresponding retinoic acid isomer are similar across species after equipotent dosing regimens. In contrast, this was not the case for the C_{max} values. These results point out the importance of prolonged exposure to retinoids for induction of teratogenicity, rather than short-lived exposure reflected by relatively high C_{max} and rapid elimination. Other investigations have also addressed this issue by various approaches, as described later in this section.

The aforementioned postulate is in harmony with results of other studies, which have shown that the elevation of RAR-β2 expression above physiological levels in retinoid-sensitive regions of the mouse embryo is a specific marker of retinoid-induced teratogenicity (Harnish *et al.*, 1990). Thus, the elevation in RAR-β2 expression in mouse embryos must persist for at least 6 to 9 h after administration of teratogenic doses of all-*trans*- or 13-*cis*-retinoic acid to result in dysmorphogenesis, whereas the proportional extent of the increase in RAR-β2 expression did not correlate with the teratogenic outcome (Soprano *et al.*, 1994). Thus, the hypothesis that prolonged embryonic exposure to active retinoids — even if obtained by relatively low concentrations — is the decisive determinant of the teratogenic potency of retinoids has been confirmed by two different approaches, one based on pharmacokinetics, and one based on the effects of retinoids at the molecular level.

7.2.2.2 Interspecies comparison of the metabolism and bioactivation of vitamin A following teratogenic doses

(a) Mouse

The metabolism of vitamin A in mice following doses of different teratogenic activity has been examined in four studies (Kochhar *et al.*, 1988; Eckhoff *et al.*, 1989; Collins *et al.*, 1992; Tzimas *et al.*, 1996a,b).

Kochhar *et al.* (1988) reported plasma and embryonic pharmacokinetic data for retinoids following a highly teratogenic oral dose of 200 mg retinol/kg bw or a nonteratogenic oral dose of 100 mg/kg to ICR mice on gestational day 11. Notably, substantial concentrations of all-*trans*-retinoic acid, all-*trans*-4-oxoretinoic acid and 13-*cis*-retinoic acid were measured in maternal plasma and embryos after the teratogenic dose, whereas little if any acidic retinoids were detected following the nonteratogenic dose. Furthermore, a marked increase in retinol concentrations in maternal plasma and embryos was observed (up to 36-fold and 8-fold, respectively). Retinyl esters were not determined in this study due to limitations of the analytical method.

Eckhoff *et al.* (1989) reported transplacental pharmacokinetics of retinoids following administration of a highly teratogenic oral dose of 100 mg retinol/kg bw or a nonteratogenic dose of 10 mg/kg to NMRI mice on gestational day 11. High concentrations of all-*trans*-retinoic acid (C_{max} values of 593 ng/mL plasma and 327 ng/g embryo) and all-*trans*-4-oxo-retinoic acid (C_{max} values of 174 ng/mL plasma and 143 ng/g embryo) were determined after the teratogenic dose. However, in contrast to the results of Kochhar *et al.* (1988), the plasma concentrations of 13-*cis*-retinoic acid were much lower (< 10% of those of all-*trans*-retinoic acid) and no 13-*cis*-retinoic acid was found in the embryo, findings which were reproduced in subsequent experiments with this strain of mice (Collins *et al.*, 1992; Tzimas *et al.*, 1996b). The lower concentrations of 13-*cis*-retinoic acid found in this study as compared to that of Kochhar *et al.* (1988) may be due to a strain difference. It should also be noted that after doses of 100 mg/kg, the C_{max} of retinol was 40-fold (plasma) and 13-fold (embryo) higher than physiological concentrations. In addition, levels of retinyl esters in both plasma and embryos were markedly increased after the 100 mg/kg dose. In contrast, the increase in plasma and embryonic concentrations of retinol and retinyl esters

was much less after administration of the 10 mg/kg dose. The formation of all-*trans*-retinoic acid and all-*trans*-4-oxoretinoic acid was very limited after the nonteratogenic dose, and the concentrations of these retinoids were much less than 10% of those obtained after the ten-fold higher dose (Eckhoff *et al.*, 1989).

Furthermore, 14-hydroxy-4,14-retro-retinol (14-HRR) was identified by Tzimas *et al.* (1996b) as a major metabolite of vitamin A in plasma, maternal and embryonic tissues of pregnant NMRI mice following administration of a teratogenic dose of retinol on gestational day 11. Concentrations of 14-HRR increased relatively slowly to their maximum and decreased slowly afterwards, in contrast to the rapid increase and subsequent elimination of all-*trans*-retinoic acid and all-*trans*-4-oxo-retinoic acid. Embryonic concentrations of 14-HRR were two- to five-fold higher than those in plasma, but it remains to be elucidated whether this is due to extensive transplacental passage of 14-HRR and/or to synthesis from retinol occurring *in situ* in the embryo.

The major question which arises from the results of these studies is whether retinol itself or some of its metabolites are responsible for the teratogenic effects observed after retinol administration. The discovery of RARs and their participation in the mediation of retinoid action in a wide variety of biological systems make plausible the assumption that RAR-binding of all-*trans*-retinoic acid and all-*trans*-4-oxo-retinoic acid mediates retinol-induced teratogenicity, at least in part.

Two lines of evidence suggest that exposure solely to all-*trans*-retinoic acid or its 4-oxo metabolite cannot explain the teratogenic effects of retinol treatment:

First, when NMRI mice were treated with teratogenic doses of either all-*trans*-retinoic acid (Kraft *et al.*, 1989) or retinol (Eckhoff *et al.*, 1989), embryonic exposure to all-*trans*-retinoic acid was substantially higher following all-*trans*-retinoic acid administration than after treatment with retinol, although the dosing regimen with all-*trans*-retinoic acid was less teratogenic than that with retinol.

Table 32. Area under the curve (AUC) values of retinoids in plasma and embryos of mice and rates of skeletal anomalies following administration of a teratogenic dose of retinol with or without pretreatment with 4-methylpyrazole[a]

Parameter	Retinol[b]	Retinol + 4-methylpyrazole[c]
AUC values in plasma[d]		
Retinol	9.48	8.50
All-*trans*-retinoic acid	3.75	0.156
All-*trans*-4-oxoretinoic acid	1.56	0.112
AUC values in embryos[d]		
Retinol	9.08	7.69
All-*trans*-retinoic acid	3.43	0.547
All-*trans*-4-oxoretinoic acid	1.50	0.310
Teratogenicity[e]		
Forelimb anomalies	55.6%	31.3%[f]
Hindlimb anomalies	43.9%	24.0%[f]
Craniofacial anomalies	56.0%	31.3%[f]

[a] From Collins *et al.* (1992)

[b] Following a single oral administration of 50 mg retinol/kg bw to pregnant mice on gestation day 11

[c] Following a single oral administration of 50 mg retinol/kg bw and pretreatment with an intraperitoneal dose of 100 mg 4-methylpyrazole/kg before retinol administration to pregnant mice on gestation day 11

[d] AUC values are expresssed in mg x h/g and were calculated for the time interval 0–8 h post-treatment.

[e] Percentage of fetuses affected

[f] Significantly lower than in the group receiving retinol alone (χ^2, $p < 0.01$)

Second, in a further study, the oxidative metabolism of retinol was blocked by pretreatment of pregnant NMRI mice with 4-methylpyrazole before retinol administration (Collins *et al.*, 1992). This led to a drastic reduction in the generation of all-*trans*-retinoic acid and all-*trans*-4-oxoretinoic acid and in the total embryonic exposure to these retinoids. Teratogenic effects, however, were reduced only partially (Table 32). However, the total embryonic exposure to retinol and 14-HRR was not affected by 4-methylpyrazole.

These results show that all-*trans*-retinoic acid is only in part responsible for the teratogenic effects induced by retinol treatment in mice, as proposed earlier (Kochhar *et al.*, 1988; Eckhoff *et al.*, 1989) and suggest that retinol may participate in eliciting teratogenicity.

(b) Rat

Retinoid profiles in maternal plasma and embryos of Wistar rats at gestational day 12 were determined after a teratogenic dosing regimen, consisting of six daily oral administrations of 90 mg retinyl palmitate/kg bw on days 7–12 (Collins *et al.*, 1994). In addition, retinoid pharmacokinetics have been examined after a single oral administration of 90 mg/kg on gestational day 12 (Tzimas, 1996); this dose is not expected to be teratogenic when given only once (Piersma *et al.*, 1996). The plasma concentrations of all-*trans*-retinoic acid and 13-*cis*-retinoic acid were in the low ng/mL range and increased over endogenous levels only sporadically following either single or multiple daily dosing with retinyl palmitate. All-*trans*-4-oxoretinoic acid and 13-*cis*-4-oxoretinoic acid were found in rat plasma at higher concentrations than the retinoic acid isomers, but only after multiple dosing. Retinyl β-glucuronide and 14-HRR were also identified in rat plasma, and exposure to these retinoids seemed to increase during long-term administration of retinyl palmitate.

As observed in the maternal plasma, embryonic concentrations of all-*trans*-retinoic acid were not significantly elevated over endogenous levels after either the single-dose or the multiple-dose regimen. Thus, the AUC of all-*trans*-retinoic acid in the embryo in the multiple-dose experiment was only 21% above the 'endogenous' AUC value obtained by extrapolation of the endogenous concentration over a 24-h period. Embryonic exposure to retinyl β-glucuronide, 13-*cis*-retinoic acid and the 4-oxo isomers was also relatively low or negligible, in contrast to the relatively high embryonic exposure to 14-HRR, retinol and retinyl esters.

These results clearly show that embryonic exposure to metabolically generated all-*trans*-retinoic acid is negligible following a single dose or during long-term administration of retinyl palmitate. Thus, the pattern of embryonic retinoids in the rat is very different following administration of the lowest teratogenic doses of either all-*trans*-retinoic acid or retinyl palmitate. Following treatments with retinyl palmitate, rat embryos were highly exposed to retinol and 14-HRR, and to a very small extent to all-*trans*-4-oxoretinoic acid and 13-*cis*-retinoic acid. On the other hand, after all-*trans*-retinoic acid administrations, rat embryos were exposed mainly to all-*trans*-retinoic acid and to a minor degree to all-*trans*-4-oxoretinoic acid and 13-*cis*-retinoic acid. This divergence between two dosing regimens with approximately equal teratogenic potency suggests that retinoids other than all-*trans*-retinoic acid are responsible for the teratogenic potency of retinyl palmitate.

Tembe *et al.* (1996) reached a similar conclusion about the role of the acidic retinoids in retinol-induced teratogenicity, after comparing the profiles of retinoids in maternal plasma of rats at gestational day 10 following different doses of either all-*trans*-retinoic acid or retinol. Doses of 500 mg retinol/kg bw induced gross structural malformations in all fetuses, and plasma concentrations of all-*trans*-retinoic acid and all-*trans*-4-oxo-retinoic acid reached 250 and 50 ng/mL, respectively. However, both the concentrations and the AUC values of the acidic retinoids were < 10% of the corresponding values seen after administration of an equipotent dose of all-*trans*-retinoic acid (50 mg/kg). It should be noted that the doses used were in the upper range of the dose–response curve, in contrast to the lowest teratogenic doses of all-*trans*-retinoic acid and retinyl palmitate used in the study of Collins *et al.* (1994).

(c) Rabbit

A single study has been reported dealing with the transplacental pharmacokinetics of retinoids in pregnant rabbits following administration of vitamin A (Tzimas *et al.*, 1996a). Retinyl palmitate was given orally to pregnant Swiss hare rabbits at a dose level of 10 mg/kg bw once daily on gestational days 7–12, and retinoid profiles in plasma and embryos were determined after the last dosing on day 12. This dosing regimen was within the teratogenic dose range and was clearly embryotoxic, based on the high resorption rate determined on gestational day 12 during sample collection.

Retinol and several of its esters were the predominant retinoids in both plasma and embryos. The major polar metabolite of retinol in plasma was 9,13-di-*cis*-retinoic acid, but its embryonic concentrations were about 6% of those in plasma. Other major plasma retinoids were retinyl β-glucuronide and 13-*cis*-4-oxo-retinoic acid, neither of which was detected in the embryo due to their limited placental transfer. 9-*cis*- and 13-*cis*-retinoic acid and 14-HRR were found in plasma at trace amounts; among those minor metabolites, only 14-HRR was found in measurable amounts in the embryo (C_{max} 37.4 ng/g). Finally, levels of all-*trans*-retinoic acid were very low in maternal plasma, and this was reflected by its AUC, which was only 2.4% of that of 9,13-di-*cis*-retinoic acid. In contrast, embryonic concentrations of all-*trans*-retinoic acid were about two-fold higher than endogenous levels.

The embryonic exposure to retinoids following the teratogenic dosing regimen with retinyl palmitate was compared with that observed after a teratogenic dosing regimen with all-*trans*-retinoic acid (Table 33). The similarity of the potency of the two dosing regimens is consistent with the fact that the AUC values of both all-*trans*-retinoic acid and all-*trans*-4-oxo-retinoic acid in the embryo were virtually identical in the two studies. It has therefore been suggested that the embryonic exposure to all-*trans*-retinoic acid and all-*trans*-4-oxo-retinoic acid is sufficient to explain the embryotoxic effects of dosing with retinyl palmitate in the rabbit (Tzimas *et al.*, 1996a). Rabbit embryos were also considerably exposed to retinol, but in view of the identical levels of exposure to all-*trans*-retinoic acid and all-*trans*-4-oxoretinoic acid after the two dosing regimens, retinol does not seem to play a significant role in the teratogenicity of retinyl palmitate in this species. Finally, the relatively low exposure to 14-HRR after dosing with retinyl palmitate suggests that this retinoid makes little contribution to the mediation of the embryotoxic effects of the applied dosing regimen in the rabbit.

Table 33. Area under the curve (AUC) values of retinoids in embryos of rabbits following roughly equipotent dosing regimens with all-*trans*-retinoic acid or retinyl palmitate[a]

Retinoid	All-*trans*-retinoic acid (6 mg/kg body wt per day)	Retinyl palmitate (10 mg/kg body wt per day)
All-*trans*-retinoic acid	954	929
	(404)[b]	(379)[b]
All-*trans*-4-oxo-retinoic acid	276	217
13-*cis*-Retinoic acid	83	–[c]
9,13-Di-*cis*-retinoic acid	–[c]	136
14-Hydroxy-4,14-retro-retinol	–[c]	271
Retinol	6720	9051
	(1104)[b]	(3435)[b]

[a] AUC values are expressed in ng x h/g and were calculated for the time interval 0–24 h after the last of six daily oral administrations of 6 mg all-*trans*-retinoic acid/kg bw or 10 mg retinyl palmitate/kg to rats from gestation day 7 to 12 (Tzimas *et al.*, 1994, 1996)

[b] The values in brackets represent only the excess of all-*trans*-retinoic acid or retinol over the endogenous concentrations

[c] Below limit of detection

(d) Monkey

The few data on retinoid metabolism following vitamin A administration to cynomolgus monkeys (Eckhoff *et al.*, 1990, 1991b; Eckhoff, 1991) were published before the determination of the lowest teratogenic dose of vitamin A in this species (Hendrickx *et al.*, 1997).

Eckhoff *et al.* (1990, 1991b) examined the plasma profiles of retinoids in nonpregnant cynomolgus monkeys following increasing oral doses of vitamin A (2, 10 and 50 mg retinol/kg bw), given in either an oil-based vehicle or a detergent-based vehicle. A wide variety of polar retinoids, including all-*trans*- and 13-*cis*-retinoic acid, all-*trans*- and 13-*cis*-4-oxoretinoic acid, all-*trans*-retinoyl-β-glucuronide and retinyl β-glucuronide, were identified as plasma metabolites of retinol. It remains to be elucidated whether and, if so, to what extent, retinol is metabolized to 14-HRR and 9,13-di-*cis*-retinoic acid in cynomolgus monkeys. The relative abundance of some of the retinoid meta-bolites was shown to be dose- and vehicle-dependent. For example, exposure to the acidic retinoids (all-*trans*- and 13-*cis*-retinoic acid, all-*trans*- and 13-*cis*-4-oxoretinoic acid) was increased overproportionally to the dose between 2 and 10 mg/kg (with the detergent-based vitamin A preparation) and between 10 and 50 mg/kg (with the oil-based preparation). Concentrations of retinol, retinyl esters and the polar metabolites tended to be higher after dosing with the detergent-based vehicle (Eckhoff *et al.*, 1991b).

The same investigators further compared plasma retinoid profiles in pregnant and nonpregnant female monkeys after a single oral dose of 5 mg retinol/kg bw dissolved in a detergent-based vehicle (Eckhoff, 1991). Surprisingly, the C_{max} values of most of the polar metabolites of retinol were much lower in pregnant than nonpregnant animals. It was hypothesized that the pregnant monkey has a more limited capacity to metabolize retinol, compared with nonpregnant animals, although the possibility of decreased absorption of retinol during pregnancy cannot be ruled out (Eckhoff, 1991).

Finally, in a further experiment, embryonic levels of retinoids were determined in pregnant monkeys following a single oral dose of 5 mg retinol/kg bw ($n = 1$) or 25 mg/kg ($n = 2$) during midgestation (Eckhoff, 1991). The results provide a very limited basis for estimation of embryonic retinoid exposure after dosing with vitamin A, but suggest that substantial embryonic exposure to retinoids may have occurred only after administration of the 25 mg/kg dose, and that, with the exception of the glucuronides, all metabolites which were measured in the plasma were also present in the embryo. In addition, embryonic concentrations of retinol and retinyl esters were highly elevated above endogenous levels (Eckhoff, 1991).

The teratogenic activity of vitamin A in cynomolgus monkeys has only recently been reported (Hendrickx *et al.*, 1997). Oral administration of daily doses of retinol (6 to 24 mg/kg bw) to the dams on gestational days 16–27 resulted in manifestation of embryolethality and retinoid-typical fetal anomalies in the craniofacial region, heart and thymus. The lowest teratogenic dose was 6 mg/kg bw; a dose of 2.25 mg/kg did not induce any observable fetal anomalies. It would therefore be valuable to examine plasma and embryonic retinoid profiles following these dosing regimens, in order to identify the retinoids which may be responsible for the observed teratogenicity.

(e) Human

The metabolism of vitamin A in humans *in vivo* has been examined following vitamin A (retinyl palmitate) supplementation (Eckhoff *et al.*, 1991a; Tang & Russell, 1991; Buss *et al.*, 1994; Peiker *et al.*, 1991; Chen *et al.*, 1996b) as well as consumption of fried liver (Buss *et al.*, 1994; Arnhold *et al.*, 1996) or other meals rich in vitamin A and provitamin A. In these studies, concentration profiles of retinoids were monitored in the plasma of volunteers following a single or long-term administration of vitamin A.

All-*trans*-retinoic acid, 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid are physiological constituents of human plasma (Eckhoff & Nau, 1990a; Tang & Russell, 1990). Furthermore, these metabolites, together with all-*trans*-4-oxoretinoic acid, have been identified as plasma metabolites of exogenously administered vitamin A (Eckhoff *et al.*, 1991a; Tang & Russell, 1991; Buss *et al.*, 1994). 9-*cis*- and

9,13-di-*cis*-retinoic acid and 14-HRR have been detected in human plasma after liver consumption (Arnhold *et al.*, 1996); these retinoids were not searched for in previous studies (Eckhoff *et al.*, 1991a; Tang & Russell, 1991; Peiker *et al.*, 1991). Neither retinyl β-glucuronide nor any isomer of retinoyl β-glucuronide or 4-oxoretinoyl β-glucuronide was detected in human plasma after vitamin A intake (Eckhoff *et al.*, 1991a; Arnhold *et al.*, 1996), and it is not known whether these glucuronides are excreted in the urine.

With respect to the quantitative pattern of plasma retinoids following vitamin A intake, the results of the human studies can be summarized as follows:

(i) Plasma levels of retinyl esters increase substantially over baseline values following vitamin A supplementation or liver consumption (Eckhoff *et al.*, 1991a; Buss *et al.*, 1994). In contrast, a relatively low increase of retinol concentrations over endogenous values was seen only after vitamin A intake at doses corresponding to 0.8 mg retinol/kg bw or higher (Buss *et al.*, 1994; Arnhold *et al.*, 1996).

(ii) Plasma concentrations of 13-*cis*- and 9,13-di-*cis*-retinoic acid and 13-*cis*-4-oxo-retinoic acid were elevated to a much higher degree than those of all-*trans*- and 9-*cis*-retinoic acid, all-*trans*-4-oxoretinoic acid and 14-HRR after vitamin A intake (Eckhoff *et al.*, 1991a; Tang & Russell, 1991; Buss *et al.*, 1994; Arnhold *et al.*, 1996). Concentrations of retinoic acid isomers reached their peak about 1–3 h earlier than retinyl esters in some studies (Eckhoff *et al.*, 1991a; Buss *et al.*, 1994); this was proposed to result from oxidation of free retinol which escaped esterification in the intestinal mucosa cells after absorption and reached the liver via the portal blood.

(iii) A steady-state plasma concentration of 13-*cis*-4-oxoretinoic acid was reached upon long-term administration of vitamin A (Eckhoff *et al.*, 1991a; Chen *et al.*, 1996b), whereas a steady-state for 13-*cis*-retinoic acid was found only in one study (Chen *et al.*, 1996b). All other retinoids were rapidly eliminated from the blood during the period between daily doses.

(iv) Controversy exists about the extent of the increase of plasma concentrations of all-*trans*-retinoic acid following vitamin A intake. For instance, the ratios of the C_{max} versus the endogenous concentration (C_{end}) of all-*trans*-retinoic acid were 35 and 62 after pharmacological doses of 0.8 and 2.3 mg retinol/kg, respectively, in one study (Buss *et al.*, 1994), but only 3.9 after a dose of 2.25 mg retinol/kg in another (Tang & Russell, 1991). Following vitamin A supplementation with less than 0.8 mg retinol/kg bw as well as after liver consumption, the C_{max} of all-*trans*-retinoic acid was only 1.3- to 3-fold higher than the C_{end} (Eckhoff *et al.*, 1991a; Buss *et al.*, 1994; Arnhold *et al.*, 1996). In contrast, no elevation of all-*trans*-retinoic acid levels over the C_{end} was observed after consumption of meals rich in vitamin A and provitamin A, corresponding to an intake up to 170 000 IU (Chen *et al.*, 1996b). A possible explanation is the inclusion of plant sources of carotenoids in the composition of the menu; however, the conversion of carotenoids to all-*trans*-retinoic acid may be much more limited than the generation of the latter from retinol.

(v) Exposure to all-*trans*-retinoic acid was markedly lower after liver consumption than after supplementation with the same dose (Buss *et al.*, 1994). Thus, the plasma AUC of all-*trans*-retinoic acid after liver consumption (corresponding to a dose of 2.3 mg retinol/kg bw) was about 13% of that after administration of the same dose as a supplement. This phenomenon was not observed, at least to this extent, for the other polar retinoids. The reason for this discrepancy is not clear; differences in the milieu of the intestinal lumen depending on the material ingested (liver versus galenic formulation) may perhaps affect the extent to which retinol becomes available for oxidation to all-*trans*-retinoic acid.

(f) Interspecies comparison of vitamin A metabolism and risk of vitamin A exposure in humans

(i) Interspecies comparison of vitamin A metabolism: The metabolism of exogenously administered retinol and retinyl esters is qualitatively similar across species and comprises esterification of retinol with fatty acyl moieties, oxidation to retinoic acid isomers and their 4-oxo metabolites,

hydroxylation to 14-HRR (not yet shown in monkeys) and β-glucuronidation of retinol and all-*trans*-retinoic acid (not yet shown in mice and humans) (see also Section 3.2).

However, there are pronounced interspecies differences in terms of the relative exposure to individual retinoids. In Table 34, the polar metabolites of retinol in five species are categorized as major and minor ones, depending on the relative contributions of their plasma AUC values to the total AUC of all polar retinoids. This evaluation was based on pharmacokinetic data derived after teratogenic dosing regimens (for mice, rats and rabbits) (Eckhoff *et al.*, 1989; Tzimas *et al.*, 1994, 1996a; Collins *et al.*, 1994) or dosing regimens suspected to be teratogenic (for monkeys and humans) (Eckhoff *et al.*, 1991a,b; Buss *et al.*, 1994; Arnhold *et al.*, 1996). The following conclusions can be drawn:

(1) The conversion of retinol to all-*trans*-retinoic acid and the further oxidation to all-*trans*-4-oxoretinoic acid are much more extensive in mice and monkeys than in rats, rabbits and humans. This conclusion is, however, based on plasma data; the total body burden of the all-*trans*-retinoids may be higher in rats, rabbits and humans, because of possible interspecies differences in the volume of distribution.

(2) 9,13-Di-*cis*-retinoic acid is the predominant polar metabolite of vitamin A in rabbits and a major one in humans, but it is not present in rats, mice, or monkeys.

(3) The formation of 14-HRR is more extensive in mice and rats than in rabbits and humans (no information about this metabolic pathway in monkeys is available).

(4) Two of the three major plasma metabolites of vitamin A in humans are major retinoids also in rabbits (9,13-di-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid) and monkeys (13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid), whereas only 13-*cis*-4-oxoretinoic acid is a major plasma retinoid in rats. In contrast, concentrations of 13-*cis*-retinoic acid in mouse plasma were very low, and the other two retinoids were not formed at all. Notably, the sum of the plasma AUC values of 9,13-di-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid in

Table 34. Interspecies comparison of vitamin A metabolism based on plasma area under the curve (AUC) values of retinoid metabolites following dosing with vitamin A

Category[a]	Mouse	Rat	Rabbit	Monkey	Human
Major metabolites	All-*trans*-retinoic acid All-*trans*-4-oxo-retinoic acid 14-Hydroxy-retro-retinol	13-*cis*-4-Oxoretinoic acid 14-Hydroxy-retro-retinol Retinol glucuronide All-*trans*-4-oxo-retinoic acid	9,13-Di-*cis*-retinoic acid 13-*cis*-4-Oxoretinoic acid Retinyl glucuronide	All-*trans*-retinoic acid 13-*cis*-Retinoic acid All-*trans*-4-Oxo-retinoic acid 13-*cis*-4-oxoretinoic acid Retinoyl glucuronide Retinoyl glucuronide	13-*cis*-4-Oxoretinoic acid 13-*cis*-Retinoic acid 9,13-Di-*cis*-retinoic acid
Minor metabolites	13-*cis*-Retinoic acid Retinyl glucuronide Retinoyl glucuronide	All-*trans*-retinoic acid 13-*cis*-Retinoic acid 9-*cis*-Retinoic acid	13-*cis*-Retinoic acid All-*trans*-retinoic acid 9-*cis*-Retinoic acid All-*trans*-4-oxoretinoic acid 14-Hydroxy-retro-retinol	9-*cis*-retinoic acid all-*trans*-4-oxo-retinoic acid	All-*trans*-retinoic acid 9-*cis*-Retinoic acid All-*trans*-4-oxo-retinoic acid 14-Hydroxy-retro-retinol

[a] The categorization to major and minor metabolites is based on the ratio of the plasma AUC value of individual retinoids to the sum of the AUC values of all polar retinoids; ratios <0.1 led to characterization of a metabolite as 'minor'. For these calculations, AUC values were corrected for different molecular masses.

rabbits accounted for 83% of the sum of AUC values of all polar metabolites of retinol. This is very close to the relative contribution of 13-*cis*-4-oxoretinoic acid, 13-*cis*-retinoic acid, and 9,13-di-*cis*-retinoic acid in humans (87%), whereas the corresponding percentages were lower for 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid in monkeys (18–60%, depending on the dose of retinol administered) and for 13-*cis*-4-oxoretinoic acid in rats (33%).

Finally, great interspecies variations exist in the degree of the increase in plasma retinol levels above endogenous levels following teratogenic dosing regimens with vitamin A. Rabbits and monkeys appear to be more similar to humans than are mice or rats with respect to the relative increase of plasma retinol. However, since the monkey data were derived from experiments performed with nonpregnant monkeys, additional studies with pregnant monkeys are required to address the similarity of humans to monkeys.

From all these comparisons, it seems that among the species tested so far, the rabbit shares the most similarities to humans with respect to maternal metabolism of vitamin A.

(ii) The proximate retinoid teratogens of vitamin A dosing are different across species: Data on embryonic exposure to retinoids following teratogenic dosing regimens with vitamin A in various species strongly suggest that the proximate retinoid teratogens of vitamin A differ across species. Thus, retinol and 14-HRR are the most probable candidates in this regard in rats, whereas all-*trans*-retinoic acid (and to a smaller extent all-*trans*-4-oxoretinoic acid) seems to be the ultimate teratogen in rabbits (Table 33). The relative contributions of retinol and its polar metabolites to the teratogenicity of vitamin A could not be dissociated in the mouse and cynomolgus monkey (Table 32).

The different pathways of metabolic activation of retinol in rats compared with rabbits may well explain why the lowest teratogenic dose of retinol is about nine-fold higher in rats than in rabbits (Table 31). This may result from the limited, if any, embryonic exposure of the rat embryo to all-*trans*-retinoic acid (Collins *et al.*, 1994); in contrast, the rabbit embryo is substantially exposed to all-*trans*-retinoic acid (Tzimas *et al.*, 1996a). However, a threshold of embryonic exposure to retinol and 14-HRR, resulting in teratogenic effects, may exist, and this seems to be attained in rats after the teratogenic dose (Collins *et al.*, 1994).

(iii) Risk of vitamin A exposure in humans: Information on the teratogenicity, metabolism and bioactivation of vitamin A in various animal species may be utilized for a rational risk assessment of vitamin A exposure of humans. Due to the wide interspecies differences in the bioactivation of retinol, it is necessary to know which species is most appropriate for comparison of human and animal data. For this purpose, several parameters should be compared between animal species and humans, such as the lowest teratogenic doses of vitamin A, the endogenous pattern of retinoid metabolism, and the metabolism and pharmacokinetics of retinoids following administration of vitamin A.

First, the lowest teratogenic doses of vitamin A in rabbits and cynomolgus monkeys, rather than other species, are quite close to those suspected to be teratogenic in humans, namely 10 mg retinyl palmitate/kg bw (equivalent to 5.5 mg retinol/kg) in rabbits, 6 mg retinol/kg in monkeys, and 0.2–1.5 mg retinol/kg in humans (cf. Table 31). An even lower teratogenic threshold dose (10 000 IU/day) has been suggested in an epidemiological study (Rothman *et al.*, 1995), but the validity of the estimate has been questioned (see Section 7.2.1).

Second, the pattern of endogenous plasma retinoids in rabbits resembles that in humans with respect to the presence of all-*trans*-retinoic acid and 13-*cis*-retinoic acid at concentrations of 1–2.5 ng/mL as well as the high abundance of retinol and much lower concentrations of retinyl esters (Tzimas *et al.*, 1996a; Eckhoff *et al.*, 1991a). In comparison, much lower physiological concentrations of retinoic acid isomers and a higher ratio of esterified versus nonesterified retinol have been measured in the plasma of mice, rats and monkeys (Hummler *et al.*, 1994; Eckhoff *et al.*, 1991b; Collins *et al.*, 1994; Tzimas *et al.*, 1995).

Third, among all species examined, the available data suggest that rabbits share the

most similarities to humans with respect to maternal retinoid metabolism following dosing with vitamin A. The close similarity between rabbits and humans comprises the following points:

(i) The fairly low relative increase in plasma retinol concentrations after dosing with vitamin A.

(ii) The predominant plasma retinoic acid isomer is not the all-*trans* form; instead, 9,13-di-*cis*-retinoic acid is the main retinoic acid isomer in rabbit plasma, while both 13-*cis*-retinoic acid and 9,13-di-*cis*-retinoic acid are major retinoids in human plasma, at least following liver consumption (Tzimas *et al.*, 1996a; Eckhoff *et al.*, 1991a; Buss *et al.*, 1994; Arnhold *et al.*, 1996).

(iii) 13-*cis*-4-Oxoretinoic acid is a major plasma retinoid in both rabbits and humans, with similar steady-state concentrations and systemic exposure levels (Tzimas *et al.*, 1996a; Eckhoff *et al.*, 1991a; Buss *et al.*, 1994; Arnhold *et al.*, 1996; Chen *et al.*, 1996b).

(iv) Plasma levels of 14-HRR are low in both species (Tzimas *et al.*, 1996b; Arnhold *et al.*, 1996).

(v) Plasma concentrations of all-*trans*-retinoic acid are marginally elevated in both species after dosing with vitamin A. For example, plasma concentrations of all-*trans*-retinoic acid in rabbits after the last vitamin A administration were in most cases 1.3- to 2.3-fold higher than endogenous levels (Tzimas *et al.*, 1996a). Similarly, the plasma C_{max} of all-*trans*-retinoic acid in humans after liver consumption was 1.6- to 2.9-fold higher than endogenous concentrations (Buss *et al.*, 1994; Arnhold *et al.*, 1996), whereas the relative increase in plasma all-*trans*-retinoic acid after supplementation was similar after doses up to 0.25 mg retinol/kg (Eckhoff *et al.*, 1991a).

Fourth, retinoid pharmacokinetic profiles are similar in both species. In particular, apparent steady-state concentrations were reached for all retinoids in rabbit plasma following repeated daily dosing with vitamin A. Similarly, steady-state concentrations of 13-*cis*-4-oxo-retinoic acid and 13-*cis*-retinoic acid were observed in human plasma during long-term administration of vitamin A supplements (Eckhoff *et al.*, 1991a; Chen *et al.*, 1996b).

Overall, therefore, it appears that the rabbit is the most appropriate species for extrapolation of embryonic exposure data for human risk assessment (Tzimas *et al.*, 1996a). Remarkably, despite the very low systemic exposure of rabbits to all-*trans*-retinoic acid, the embryonic exposure to this acid following dosing with vitamin A was substantial and probably sufficient to account for the embryotoxic effects of the dosing regimen used (Table 33). Two sources of all-*trans*-retinoic acid in the rabbit embryo after maternal dosing with vitamin A are possible. First, all-*trans*-retinoic acid can efficiently be transferred from the maternal circulation to the embryo, a process which may be mediated by embryonic CRABP-I or -II. Notably, specific binding of all-*trans*-retinoic acid to human embryonic proteins (probably CRABPs) has been reported (Nau, 1990), in agreement with findings in rodent and avian embryos (Scott *et al.*, 1994; Ruberte *et al.*, 1992; Gustafson *et al.*, 1993; Lyn & Giguere, 1994; Vaessen *et al.*, 1990; Maden, 1994). Second, and perhaps more relevant in the rabbit, all-*trans*-retinoic acid can be produced locally from retinol in the embryo (Tzimas *et al.*, 1996a). Several enzymes have been proposed to catalyse this process, but their expression in rabbit and human embryonic tissues has not been investigated. The available data for the rabbit, together with numerous similarities between rabbits and humans, do not rule out the possibility of substantial exposure of the human embryo to all-*trans*-retinoic acid, in spite of the marginal increases in plasma concentrations of all-*trans*-retinoic acid in vitamin A-exposed pregnant women.

The human risk for embryotoxic effects induced by vitamin A dosing may be further enhanced due to exposure to 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid. Only limited amounts of these retinoids were found in the rabbit embryo following administration of retinyl palmitate (Tzimas *et al.*, 1996a). However, in humans, higher embryonic exposure might occur, especially if these retinoids are transferred across the human placenta more readily than across the rabbit placenta. This hypothesis is based on the more extensive transfer of the two 13-*cis*-retinoids across the monkey placenta than the rodent placenta and the close similarities of the placentas of humans and monkeys (Tzimas *et al.*, 1996a; Beck, 1976).

7.2.2.3 Role of vitamin A in reproduction, in particular spermatogenesis

Animals maintained on a vitamin A-deficient diet can be rescued by treatment with all-*trans*-retinoic acid and most of the symptoms of vitamin A deficiency are relieved except for those related to vision and reproduction. Retinoic acid cannot be reduced to retinal and therefore the cofactor of rhodopsin cannot be produced, resulting in eye defects. Vitamin A-deficient rats supplemented regularly with retinoic acid fail to reproduce (Thompson *et al.*, 1964); they continue their normal estrous cycles, mate with healthy males, and become pregnant, but invariably resorb their fetuses around gestational day 15. Retinol must be administered before day 10 to rescue the fetuses (Wellik & DeLuca, 1996). Retinoic acid cannot substitute for retinol in spermatogenesis, probably because retinoic acid cannot cross the blood–testis barrier (made up mostly by Sertoli cells), and little uptake of radiolabelled retinoic acid by the rat testes occurs (Blaner & Olson, 1994). The testis appears to be quite an exceptional organ in this regard, since all-*trans*-retinoic acid transfers well into most other organs, in particular the well perfused liver and brain. It is thought that all-*trans*-retinoic acid is the active retinoid metabolite in spermatogenesis, but because of its poor uptake, the testis is dependent on vitamin A as a precursor for retinoic acid. This view is supported by the finding that injection of large doses of retinoic acid into the testes can restore spermatogenesis (van Pelt & de Rooij, 1991).

Although CRABP-I and -II as well as CRBP-I are present in the testis in a very specific distribution, the function of these proteins remains unclear and knock-out of these proteins does not effect development or adult life, including fertility (Gorry *et al.*, 1994; Lampron *et al.*, 1995). This is also true for transthyretin-deficient mice, although they do have very low retinol serum levels. The roles of RBP and CRBP remain speculative, but may reside in transfer, metabolism and storage of retinol.

Male null mutant mice in which either the RAR-α, the RAR-β or the RXR-β gene was deleted are sterile. Deletion of the RAR-α gene resulted in degeneration of seminiferous epithelium (Lufkin *et al.*, 1993), and mutation of the RAR-γ gene in squamous metaplasia of the glandular epithelia of the seminal vesicles (Lohnes *et al.*, 1993). Mutation of the RXR-β gene resulted in failure of spermatid release within the seminiferous epithelia; female mice were fertile (Kastner *et al.*, 1996). Lipid accumulation was found in the Sertoli cells, perhaps due to heterodimerization of the RXR-β with one of the PPARs. Deletions of the RAR-β, RXR-α and RXR-γ genes did not result in infertility or effects on spermatogenesis. It thus appears that RAR-β and RXR-β are the primary signalling receptors in the germ cells and Sertoli cells, respectively. The importance of RXR-β also suggests that 9-*cis*-retinoic acid may be essential in spermatogenesis and this isomer was indeed identified in epididymal fluids. Recently, 9-*cis*-retinol dehydrogenase was found in the human testis (Mertz *et al.*, 1997) and this could possibly play a role in the generation of 9-*cis*-retinoic acid from 9-*cis*-retinol. Further-more, an extracellular binding protein for all-*trans*-retinoic acid was identified in the epididymis. This indicates a very special role for all-*trans*-retinoic acid and possibly its 9-*cis* isomer in sperm maturation, because in all other tissues or fluids only intracellular, but not extracellular binding proteins for retinoic acid were identified.

7.2.2.4 Possible metabolic basis of retinoid-induced teratogenicity

The teratogenicity induced by vitamin A is not associated with embryonic exposure to all-*trans*-retinoic acid in all cases. Teratogenic activity of vitamin A in mice and rats cannot be explained by embryonic exposure to all-*trans*-retinoic acid only; retinol and 14-HRR may be involved in these species. The results implicate 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid as well as retinol and 14-HRR as possible proximate teratogens.

It is currently believed that retinoid toxicity (including teratogenicity) results from interaction of active retinoids with RARs and RXRs (Armstrong *et al.*, 1994). Contradictory information exists on the binding of retinol to RARs; no appreciable binding was reported in two studies (Crettaz *et al.*, 1990; Keidel *et al.*, 1992), whereas Repa *et al.* (1993) showed that retinol is one order of magnitude less potent than

all-*trans*-retinoic acid in binding to RARs, and the binding observed was not the result of metabolism to all-*trans*-retinoic acid during the binding experiment. Finally, 14-HRR does not bind to any of the known retinoid receptors (Ross & Hämmerling, 1994).

The apparent differences in the lowest teratogenic doses of vitamin A in rats and rabbits probably result from interspecies differences in the bioactivation of retinol. Thus, teratogenic dosing regimens with vitamin A led to significant embryonic exposure to all-*trans*-retinoic acid in the rabbit, at levels similar to those obtained after dosing with all-*trans*-retinoic acid. In contrast, rat embryos were appreciably exposed to retinol and 14-HRR, but not to all-*trans*-retinoic acid, following vitamin A administration. Therefore, different retinoids may be responsible for vitamin A-induced teratogenicity in different species. The relative contributions of retinol and its metabolites to the teratogenicity induced by excess vitamin A in mice, monkeys and humans cannot yet be fully evaluated.

Among all the species examined to date, the rabbit appears the most similar to the human with respect to the endogenous retinoid profile and the pattern of plasma retinoids following vitamin A intake. Therefore, the marginal systemic exposure to all-*trans*-retinoic acid observed in humans after vitamin A supplementation or liver consumption does not rule out the possibility of high embryonic exposure to this retinoid, as is the case in rabbits. The potential involvement of 13-*cis*-retinoic acid and 13-*cis*-4-oxoretinoic acid, both of which are major retinoids in human plasma after vitamin A intake, in the induction of teratogenic effects should also be considered. Therefore, a teratogenic risk of high vitamin A intake in humans cannot at present be excluded.

7.3 Genetic and related effects

7.3.1 Human studies

As reported in Section 4.1.3, phase II chemoprevention studies have shown the ability of vitamin A, either alone or in combination with other agents, to decrease the frequency of micronuclei in cells of individuals exposed to genotoxic agents. Moreover, molecular epidemiology studies have provided evidence for an inverse relationship between either the dietary intake or serum/plasma levels of vitamin A and certain intermediate biomarkers, such as levels of aromatic DNA adducts in cells of smokers (see Section 4.1.3).

One study evaluated the detection rate of the major aflatoxin B_1–DNA adduct, i.e., aflatoxin B_1–N^7-guanine, in the urine of healthy males in Taiwan. After adjusting for chronic hepatitis B surface antigen (HBsAg) carrier status and other potential confounders, no association was found between adduct levels and the plasma levels of retinol. In the same study, this biomarker was positively associated with plasma levels of α-carotene and β-carotene and inversely correlated with plasma levels of lycopene (Yu *et al.*, 1997).

7.3.2 Experimental studies

Published data concerning the assessment of genetic and related effects of retinol, retinal, retinoic acid, retinyl acetate and retinyl palmitate *in vitro* and/or *in vivo* are summarized in Table 35 and represented diagrammatically in Appendix 4. Most data were generated in studies evaluating the ability of these compounds to modulate genetic and related effects produced by genotoxic agents (see Sections 4.2.2 and 4.2.3.2).

Retinol failed to revert the *Salmonella typhimurium his*⁻ strains TA1538, in the absence of S9 mix, and, irrespective of the addition of the exogenous metabolic system, TA1535, TA98, TA100 and TA102 (Baird & Birnbaum, 1979; White & Rock, 1981; Qin & Huang, 1985; Wilmer & Spit, 1986). In one study, however, retinol was reported to be mutagenic in strain TA104, in the presence of S9 mix (Han, 1992) [The Working Group noted that this result contrasts with the lack of mutagenicity of retinyl palmitate and retinoic acid in the same strain (see below)]. Retinol induced the mitochondrial mutation to respiratory deficiency (*petite*) mutation in *Saccharomyces cerevisiae* strain 6-81, but was not mutagenic to nuclear genes, as shown by the lack of reversion to methionine prototrophy (Cheng & Wilkie, 1991). Retinol did not affect a variety of endpoints in cultured mammalian cells, including DNA single-strand breaks (Alaoui-Jamali *et al.*,

1991a) and unscheduled DNA synthesis in primary rat hepatocytes (Budroe *et al.*, 1987), hypoxanthine phosphoribosyl transferase (HGPRT) mutation in Chinese hamster ovary (CHO) cells (Budroe *et al.*, 1988), 6-TG^R mutation (Ferrari *et al.*, 1989; Kuroda, 1990), sister chromatid exchanges (Sirianni *et al.*, 1981; Huang *et al.*, 1982; Qin & Huang, 1985; Alaoui-Jamali *et al.*, 1991a) and chromosomal aberrations (Qin & Huang, 1985) in Chinese hamster V79 cells, sister chromatid exchanges in Chinese hamster epithelial liver (CHEL) cells (Cozzi *et al.*, 1990) and morphological differentiation in mouse melanoma B-16 cells (Hazuka *et al.*, 1990). Retinol significantly decreased the 'spontaneous' chromosome instability, measured in terms of frequency of chromatid bridges and fragments at anaphase and telophase, in mouse C127 cells transformed by bovine papillomavirus DNA (Stich *et al.*, 1990). In contrast to these negative results, retinol produced a statistically significant increase in sister chromatid exchanges in cultured human lymphocytes (Dozi-Vassiliades *et al.*, 1985) [The Working Group noted the quite small difference in mean SCE/cells ± SE in controls (9.9 ± 0.3) and retinol-treated cells (11.7 ± 0.3)].

The genotoxicity of retinal was investigated in a single study which showed its lack of influence on the frequency of sister chromatid exchanges in Chinese hamster V79 cells (Sirianni *et al.*, 1981).

Retinoic acid failed to revert *S. typhimurium* strains TA1535, TA98, TA100 and TA102, irrespective of the presence of S9 mix (Qin & Huang, 1985; Wilmer & Spit, 1986) and TA104, in the absence of an exogenous metabolic system (Han, 1992; De Flora *et al.*, 1994). Retinoic acid did not induce unscheduled DNA synthesis in primary rat hepatocytes (Budroe *et al.*, 1987), HGPRT mutation in CHO cells (Budroe *et al.*, 1988), sister chromatid exchanges in Chinese hamster V79 cells (Sirianni *et al.*, 1981) or CHEL cells (Cozzi *et al.*, 1990). Retinoic acid displayed the same potency as retinol in attenuating the intrinsic chromosome instability in mouse C127 cells transformed by bovine papilloma-virus DNA (Stich *et al.*, 1990).

Irrespective of the presence of S9 mix, retinyl acetate did not affect the 'spontaneous' mutation frequency in *S. typhimurium* strains TA1535, TA98, TA100 and TA102 (Qin & Huang, 1985; Wilmer & Spit, 1986). It did not induce mutations in human heteroploid epithelial-like EUE cells (Ferreri *et al.*, 1986) or sister chromatid exchanges in Chinese hamster V79 cells (Sirianni *et al.*, 1981). Administration of retinyl acetate to C57BL/6J mice with the diet (20 mg/kg diet) for 10 weeks did not affect the frequency of sister chromatid exchanges in bone marrow cells (Qin & Huang, 1986).

Retinyl palmitate did not revert *S. typhimurium* strain TA104 (without S9 mix) (De Flora *et al.*, 1994). It was mutagenic, as measured by selection against diphtheria toxin, in human heteroploid epithelial-like EUE cells (Ferreri *et al.*, 1986) and it failed to induce DNA single-strand breaks, as evaluated by alkaline elution assay, when given for eight weeks in the diet (500 IU/g diet) of Sprague–Dawley rats. When given by gavage (32 mg/kg bw) twice a week for seven weeks, it did not affect the frequency of micronuclei in bone marrow cells of NMRI mice (Busk *et al.*, 1984) or in bone marrow cells of Swiss albino mice after a single oral administration (150 IU) of vitamin A [unspecified] (Rao *et al.*, 1986). Studies performed in a single laboratory showed the ability of retinyl palmitate to induce chromosomal aberrations in mouse cells *in vivo*. In particular, after treatment of Swiss albino mice with 132 IU/kg bw/day for 14 days, retinyl palmitate increased total chromosomal aberrations [1.6 times] (not significant) in bone marrow cells and [2.0 times] in spermatocytes (Kumari & Sinha, 1994). Under the same conditions but extending the treatment period to 14 weeks, retinyl palmitate increased the frequency of micronuclei [2.1 times] (not significant) in polychromatic and normochromatic erythrocytes from bone marrow, and total chromosomal aberrations [2.9 times] ($p < 0.002$) in bone marrow cells and [2.6 times] ($p < 0.002$) in spermatocytes (Sinha & Kumari, 1994). As reported in the same studies, the frequency of spermatozoa showing abnormal head morphology was unchanged after 14 days but was significantly enhanced ($p < 0.002$) after 14 weeks of treatment with retinyl palmitate.

Table 35. Genetic and related effects of retinol, retinal, retinoic acid and retinyl esters in *in vitro* and *in vivo* test systems

End-point*	Code*	Test system	Result[a]		Dose[b] (LED or HID)	Reference
			Without exogenous metabolic system	With exogenous metabolic system		
Retinol						
G	SA0	*Salmonella typhimurium* TA100, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA2	*Salmonella typhimurium* TA102, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA5	*Salmonella typhimurium* TA1535, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA8	*Salmonella typhimurium* TA1538, reverse mutation	–	0	100 µM	White & Rock (1981)
G	SA9	*Salmonella typhimurium* TA98, reverse mutation	0	–	200 µg/plate	Baird & Birnbaum (1979)
G	SA9	*Salmonella typhimurium* TA98, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA9	*Salmonella typhimurium* TA98, reverse mutation	0	–	0.5 µmol/plate	Wilmer & Spit (1986)
G	SA4	*Salmonella typhimurium* TA104, reverse mutation	+	0	1 µmol/plate	Han (1992)
G	SCF	*Saccharomyces cerevisiae* 6–81, mitochondrial (petite) mutation	+	0	4 mg/mL	Cheng & Wilkie (1991)
G	SCR	*Saccharomyces cerevisiae* 6–81, reverse mutation	–	0	4 mg/mL	Cheng & Wilkie (1991)
D	DIA	DNA single-strand breaks in primary rat hepatocytes	–	0	139.6 mM	Alaoui-Jamali *et al.* (1991a)
D	URP	Unscheduled DNA synthesis in primary rat heptocytes	–	0	50 µM	Budroe *et al.* (1987)
G	GCO	HGPRT mutation in Chinese hamster ovary (CHO cells)	–	–	50 µM	Budroe *et al.* (1988)
G	G9H	6-TG^R mutation in Chinese hamster V79 cells	–	–	50 µM	Ferrari *et al.* (1989)
G	G9H	6-TG^R mutation in Chinese hamster V79 cells	–	0	100 µg/mL	Kuroda (1990)
S	SIC	Sister chromatid exchanges in Chinese hamster V79 cells	–	0	4 µg/mL	Sirianni *et al.* (1981)
S	SIC	Sister chromatid exchanges in Chinese hamster V79 cells	–	–	32 µg/mL	Huang *et al.* (1982)
S	SIC	Sister chromatid exchanges in Chinese hamster V79 cells	–	–	16 µg/mL	Qin *et al.* (1985)
S	SIC	Sister chromatid exchanges in Chinese hamster V79 cells	0	–	139.6 mM	Alaoui-Jamali *et al.* (1991a)
S	SIC	Sister chromatid exchanges in Chinese hamster epithelial liver (CHEL) cells	–	0	25 µM	Cozzi *et al.* (1990)
C	CIC	Chromosomal aberrations in Chinese hamster V79 cells transformed by bovine papillomavirus DNA	–	–	32 µg/mL	Qin *et al.* (1985)
–	–	Chromosome instability in mouse C127 cells	*	0	0.5 µM(ID50)	Stich *et al.* (1990)
–	–	Morphological differentiation, mouse B-16 melanoma cells	–	0	18.6 µM	Hazuka *et al.* (1990)
S	SHL	Sister chromatid exchanges in cultured human lymphocytes	(+)	0	4 µg/mL	Dozi-Vassiliades *et al.* (1985)

Table 35 (Contd)

End-point*	Code*	Test system	Result[a]		Dose[b] (LED or HID)	Reference
			Without exogenous metabolic system	With exogenous metabolic system		
Retinal						
S	SIC	Sister chromatid exchanges in Chinese hamster V79 cells	–	0	1 µg/mL	Sirianni *et al.* (1981)
Retinoic acid						
G	SA0	*Salmonella typhimurium* TA100, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA2	*Salmonella typhimurium* TA102, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA5	*Salmonella typhimurium* TA1535, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA9	*Salmonella typhimurium* TA98, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA9	*Salmonella typhimurium* TA98, reverse mutation	0	–	0.5 µmol/plate	Wilmer & Spit (1986)
G	SAS	*Salmonella typhimurium* TA104, reverse mutation	–	0	10 µmol/plate	Han (1992)
G	SAS	*Salmonella typhimurium* TA104, reverse mutation	–	0	4 µmol/plate	De Flora *et al.* (1994)
D	URP	Unscheduled DNA synthesis in primary rat hepatocytes	–	0	50 µM	Budroe *et al.* (1987)
G	GCO	HGPRT mutation in Chinese hamster ovary (CHO) cells	–	–	50 µM	Budroe *et al.* (1988)
S	SIC	Sister chromatid exchanges in Chinese hamster V79 cells	–	0	4 µg/mL	Sirianni *et al.* (1981)
S	SIC	Sister chromatid exchanges in Chinese hamster epithelial liver (CHEL) cells	–	0	50 µM	Cozzi *et al.* (1990)
–	–	Chromosome instability in mouse C127 cells transformed by bovine papillomavirus DNA	*	0	0.5 µM(ID50)	Stich *et al.* (1990)
Retinyl acetate						
G	SA0	*Salmonella typhimurium* TA100, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA2	*Salmonella typhimurium* TA102, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA5	*Salmonella typhimurium* TA1535, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA9	*Salmonella typhimurium* TA98, reverse mutation	–	–	40 µg/plate	Qin & Huang (1985)
G	SA9	*Salmonella typhimurium* TA98, reverse mutation	0	–	0.5 µmol/plate	Wilmer & Spit (1986)
G	GIH	Diphtheria toxin mutation in human heteroploid epithelial-like (EUE) cells	–	0	1 µM	Ferreri *et al.* (1986)
S	SIC	Sister chromatid exchanges in Chinese hamster V79 cells	–	0	10 µg/mL	Sirianni *et al.* (1981)
S	SVA	Sister chromatid exchanges in mouse bone marrow cells	–	NA	20 mg/kg diet for 10 weeks	Qin & Huang (1986)

Table 35. (Contd)

End-point*	Code*	Test system	Result[a]		Dose[b] (LED or HID)	Reference
			Without exogenous metabolic system	With exogenous metabolic system		
Retinyl palmitate						
G	SAS	*Salmonella typhimurium* TA104, reverse mutation	–	0	4 µmol/plate	De Flora *et al.* (1994)
D	DVA	Single-strand breaks in rat hepatocytes	NA	–	500 IU/g diet for 8 weeks	Decoudu *et al.* (1992)
G	GIH	Diphtheria toxin mutation in human heteroploid epithelial-like (EUE) cells	–	0	10 µM	Ferreri *et al.* (1986)
M	MVM	Micronuclei in mouse peripheral blood erythrocytes	–	NA	132 IU/kg	Sinha & Kumar (1994
M	MVM	Micronuclei in mouse bone marrow cells	–	NA	bw/day for 14 weeks 150 IU (single administration)	Rao *et al.* (1986)
M	MVM	Micronuclei in mouse bone marrow cells	–	NA	32 mg/kg bw by gavage twice a week for 7weeks	Busk *et al.* (1984)
C	CBA	Chromosomal aberrations in mouse bone marrow cells	–	NA	132 IU/kg bw/ day for 14 days	Kumari & Sinha (1994)
C	CBA	Chromosomal aberrations in mouse bone marrow cells	+	NA	132 IU/kg bw/ dayfor 14 weeks	Sinha & Kumari (1994)
C	CCC	Chromosomal aberrations in mouse spermatocytes	(+)	NA	132 IU/kg bw/ day for 14 days	Kumari & Sinha (1994)
C	CCC	Chromosomal aberrations in mouse spermatocytes	(+)	NA	132 IU/kg bw/day for 14 weeks	Sinha & Kumari (1994)

[a] Result: +, positive; (+), weak positive; –, negative; 0, not tested; NA, not applicable (in vivo assay); *, inhibition of the investigated end-point

[b] LED, lowest effective dose; HID, highest ineffective dose; ID50, dose inhibiting the 50% of the investigated effect. The units are as reported by the authors.

*See Appendix 2 for codes; – test or end-point is not defined and is not shown in the activity profile.