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EARLY CARDIOVASCULAR DEVELOPMENT
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IN RESPONSE TO RETINOIDS

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Helen Dersch

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EARLY CARDIOVASCULAR DEVELOPMENT OF VITAMIN A-DEFICIENT JAPANESE QUAIL EMBRYOS IN RESPONSE TO RETINOIDS

By

Helen Dersch

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ABSTRACT

EARLY CARDIOVASCULAR DEVELOPMENT OF VITAMIN A-DEFICIENT JAPANESE QUAIL EMBRYOS IN RESPONSE TO RETINOIDS

By

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It has previously been demonstrated that vitamin A-deficiency causes specific abnormalities of cardiogenesis in Japanese quail embryos. Administration of various retinoids to vitamin A-deficient avian embryos induces normal cardiovascular development. The active form of vitamin A is thought to be retinoic acid, but this has not been directly confirmed. In the present study, all-trans-retinoic acid (0.025-1.5 μg) was administered to vitamin A-deficient embryos via injection into the egg air sac. Normal cardiovascular development was induced in embryos treated with retinoic acid within the time period of 0-30 hours of incubation. Administration of 0.1 μg of all-trans-retinoic acid at 24 hours of incubation resulted in the highest percentage (34%) of embryos with normal cardiovascular development. 3,4-di-dehydro- and 13-cis-retinoic acids were less active than all-trans-retinoic acid. Retinol and di-dehydroretinol were most active when administered at the beginning of incubation; the activity of these retinoids decreased when administered at 24 hours of incubation. A sharp decline in activity was observed with all retinoids tested when administered at 36 hours of incubation or later. Retinol was the most active of the retinoids tested, inducing normal cardiogenesis in 97% of

vitamin A-deficient embryos when administered at the beginning of incubation at its optimal dose (1.0 μg). However, when retinol and retinoic acid were each administered at the 0.1 μg dose and at 24 hours of incubation when retinoic acid was maximally effective, the activity of retinoic acid was greater than that of retinol.

HPLC analysis of retinoids following a pulse dose of radiolabeled retinol indicated that di-dehydroretinol was the major metabolite in the tissues examined. Although most of the unchanged retinol was found in the extraembryonic membranes, the ratio of di-dehydroretinol to retinol was highest within the embryo itself. Retinoic acid was not conclusively demonstrated as a metabolite of retinol.

These experiments suggest that all-trans-retinoic acid is the active form of vitamin A for cardiovascular development of the quail embryo, but the synthesis of retinoic acid within the embryo remains to be elucidated.

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INTRODUCTION

Historical perspective of vitamin A

Vitamin A was the first "vitamin" to be discovered. It was first described by McCollum and Davis (1913) who isolated a factor with growth-promoting activity from animal fat and fish oils which they named "fat-soluble A"; Drummond (1920) suggested the name "vitamin A". In the following years, a deficiency of vitamin A was found to cause a number of pathological conditions, including xerophthalmia (McCollum and Simmonds, 1917) and night blindness (Fridericia and Holm, 1925). Wolbach and Howe (1925) gave detailed descriptions of histopathological changes in epithelia associated with vitamin A deficiency, and Green and Mellanby (1928) recognized the association of vitamin A-deficiency with infectious disease. The identification of vitamin A-deficiency diseases led to investigations aimed at determining the requirements for vitamin A in humans, other mammals, and birds (Baumann, 1953; Moore, 1957), and to the development of vitamin A-deficient diets to facilitate the study of vitamin A functions (Moore, 1957). Drummond and Crawford (1920) noted the difficulty in accurately assessing the effects of

vitamin A in *in vivo* studies which relied upon a vitamin A -deficient diet.

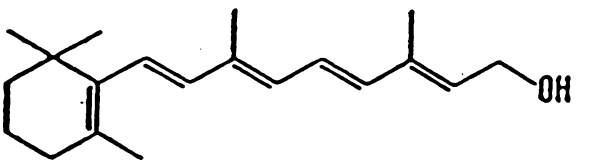
Continued research of vitamin A led to the discovery of the structure of various vitamin A forms and to their synthesis. Moore (1930) demonstrated that β-carotene from plant sources had provitamin A activity. Karrer *et al.* (1931a, 1931b) proposed the exact structural formulas for vitamin A as well as for β-carotene and β-di-hydrocarotene (Karrer *et al.*, 1930; Karrer and Morf, 1931). Vitamin A-2 was isolated from fish liver oils in 1937 and identified as the di-dehydro form of retinol (Sebrell, 1967). The aldehyde form of vitamin A, retinene or retinal, was synthesized by Morton and Goodwin (1944).

Between 1935 and 1953 Wald and other researchers discovered the link between vitamin A and the visual process (Wald, 1935-36a, 1935-36b), identified rhodopsin as containing vitamin A aldehyde (Morton, 1944), and elucidated the visual cycle (Wald, 1953), work for which Wald received the Nobel Prize in 1967. Another active vitamin A compound, retinoic acid, was synthesized in 1946 and its biological activity described (Arens and van Dorp, 1946; van Dorp and Arens, 1946). Retinoic acid was found to be active in growth and in epithelial cell differentiation but not in vision (Dowling and Wald, 1960) and also could not support mammalian reproduction (Thompson JN *et al.*, 1964). The work of these pioneering researchers demonstrated the multiple functions of vitamin

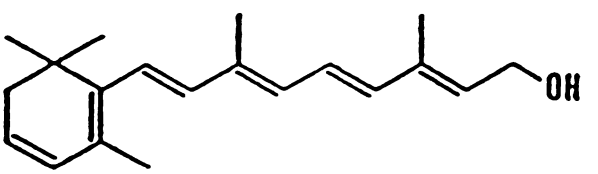
A in many tissues and established that several compounds had vitamin A activity. Vitamin A was shown to play a role in growth, maintenance of bone and epithelial tissues, reproduction, and embryonic development, reviewed by Sporn and Roberts (1984). Further research on metabolism of the various forms of vitamin A led to the discovery and isolation of a plasma transport binding protein for retinol, RBP, (Kanai *et al.*, 1968), and of separate cellular binding proteins for retinol, CRBP (Bashor *et al.*, 1973) and CRBP II, (Levin *et al.*, 1987), and for retinoic acid, CRABP, (Ong and Chytil, 1978). Most recently, nuclear receptors for retinoic acid, RARs, have been discovered (Giguere *et al.*, 1987; Petkovich *et al.*, 1987); much of the current vitamin A research is focusing on the regulation of gene expression by retinoic acid.

Major forms of vitamin A

The naturally occurring active vitamin A forms share a similar structure. Of interest in the present study are two forms of retinol, all-trans retinol and 3,4-didehydroretinol, and three forms of retinoic acid, all-trans-retinoic acid, the 13-cis isomer and the 3,4-didehydro derivative (Figure 1, 2). Retinol can be converted to retinoic acid in a 2-step process with the aldehyde, retinal, as an intermediate. This conversion is shown in Figure 3. The reaction from retinol to retinal is reversible, but the oxidation to retinoic acid is

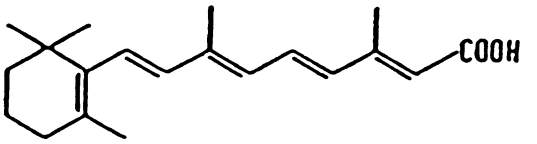


Retinol (Vitamin A_1)

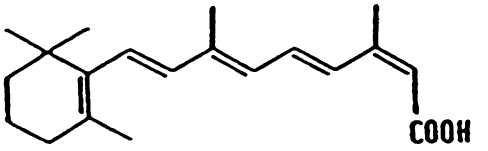


Di-dehydroretinol (Vitamin A_2)

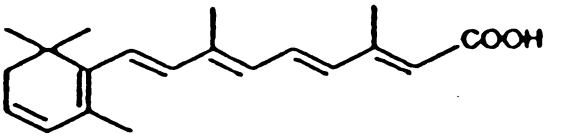
Figure 1: Structures of retinols



All-trans-retinoic acid



13-cis-retinoic acid



3,4-di-dehydro-retinoic acid

Figure 2: Structures of retinoic acids

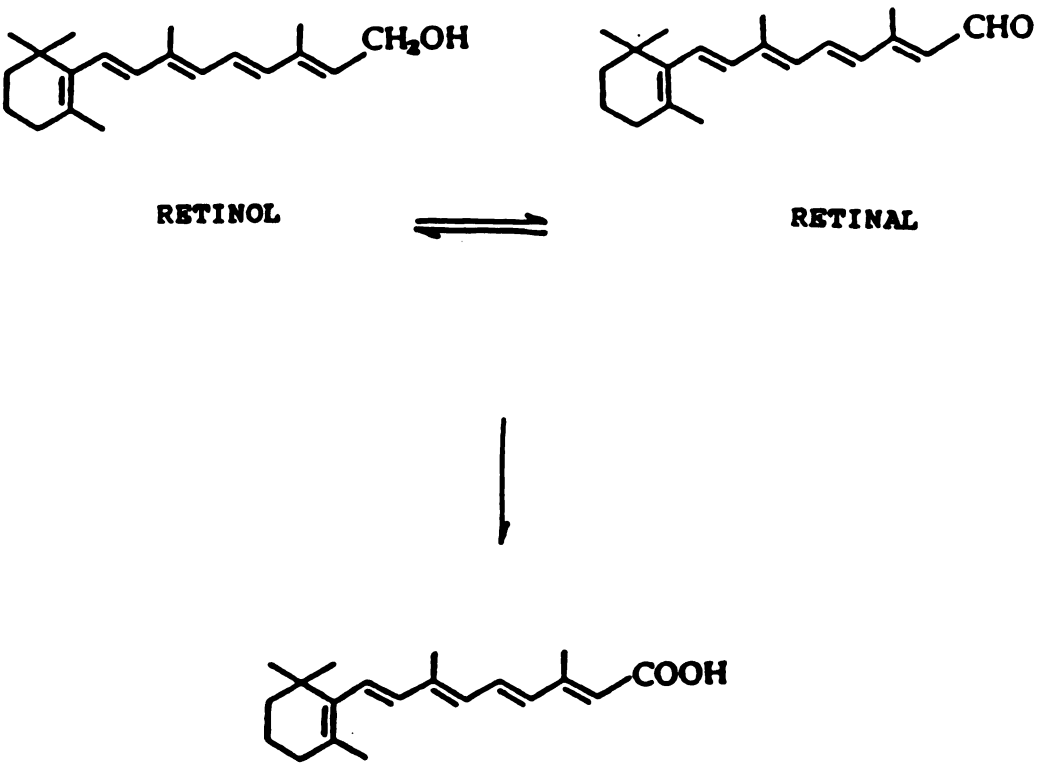


Figure 3: Conversion of retinol to retinoic acid

irreversible. Retinoic acid is further metabolized to less active or inactive forms by oxidation or conjugation. All-trans-retinol can also be converted to 3,4-di-dehydro-retinol which, in turn, can be oxidized to di-dehydro-retinoic acid.

In addition to the naturally occurring forms of vitamin A and their metabolites, several related synthetic compounds have been developed. The term "retinoid" has been adopted as a general term which includes both natural forms of vitamin A and synthetic analogs. Some authors have based usage of the term on the biological activity of the compound (Sporn and Roberts, 1985), while others have used a broader definition to include compounds with chemical structures which are similar to those of the biologically active forms, but including related inactive compounds, since there is a wide range of biological activity (Goodman, 1981).

Metabolism of vitamin A

The liver is a primary tissue in the metabolism and regulation of vitamin A homeostasis because of its roles in storage and in release of vitamin A into the blood as needed by tissues. Retinol binding protein (RBP) is a small protein, (20,000 *D*), synthesized primarily by the liver, which transports a single molecule of retinol (ROL) in circulation (Kanai *et al.*, 1968). In plasma, the RBP-ROL molecule combines with transthyretin (TTR) in a ratio of 1:1.

Transthyretin is also the carrier protein for thyroxine. This large transport complex is thought to protect retinol from reaction with other substances and from filtration by the kidney. The plasma level of ROL is quite tightly regulated and constant. Only in vitamin A-deficiency, when the liver stores of vitamin A become very low, does the plasma level of ROL decrease substantially.

It is not clear how retinol is taken up by peripheral cells, but it is presumed that there is some recognition of the TTR-RBP-ROL complex. The RBP-ROL is released from this complex, and only retinol crosses the cell membrane (reviewed by Wolf, 1984). Apo-RBP remains in the circulaticn where it may be a signal for the liver to release additional RBP-ROL into the circulation (Gerlach and Zile, 1991). The kidney filters and degrades the apo-RBP. Retinoic acid does not have a specific plasma binding protein but binds to albumin (Smith *et al.*, 1973). It is assumed that there is no specific transport mechanism of retinoic acid for uptake into the cells (Goodman, 1981).

Within cells there are specific vitamin A binding proteins, cellular retinol binding protein (CRBP) (Bashor *et al.*, 1973), and cellular retinoic acid binding protein (CRABP) (Ong and Chytil, 1978), respectively. Expression of these binding proteins is regulated in a highly specific way, both anatomically and temporally. A second CRBP, CRBP-II, transcribed by a separate gene, is found in intestinal mucosa (Levin *et al.*, 1987); its proposed role is the uptake and

esterification of dietary retinol and, perhaps, of carotene.

Retinoid binding proteins have been found in many tissues but the tissue distributions and concentrations of CRBP and CRABP are different from each other (Ong *et al.*, 1982; Porter *et al.*, 1985). This suggests multiple actions of retinoids with local and specific regulation of vitamin A functions (Ross, 1991). Binding of retinol (ROL) to CRBP-II in the intestine may be important in esterification of retinol by membrane-bound enzymes of the intestinal mucosa (Ong *et al.*, 1987); CRBP may play a similar role in liver as the ROL-CRBP I complex (Yost *et al.*, 1988).

A potentially important function of the retinoid binding proteins may be to solubilize the ligands and provide specificity for recognition of the bound ligands by various enzymes. In this role, the cytosolic retinoic acid-binding protein, CRABP, may function to control the movement of retinoic acid (RA) into the nucleus and to regulate the nuclear concentration of this ligand. CRABP (and possibly other retinoid binding proteins) could serve regulatory roles which could include binding retinoic acid at its site of synthesis in the cytoplasm and then controlling its delivery to the nucleus for association with a receptor (RAR). CRABP could be important in establishing equilibria between the cytoplasmic and nuclear concentrations of retinoic acid and between CRABP-bound and RAR-bound retinoic acid in the nucleus (Ross, 1991). Positive correlations have been found between retinoid activity and the affinity of retinoids for CRABP

(Jetten and Jetten, 1979; Lotan, 1980), which is consistent with such a regulatory role of the binding proteins.

The varied functions of the different forms of vitamin A are well established (reviewed by Wolf, 1984). Regulation of inter-conversions between the vitamin A forms is likely to be critical for maintaining appropriate tissue concentrations of each of the metabolites (reviewed by Ross, 1991). Retinol is the major transport form of vitamin A and is in higher concentration in plasma than other forms of vitamin A. When esterified with long-chain fatty acids, it becomes the major storage form; the esterified form as palmitate is the form of vitamin A in high concentration in the liver. Retinal is produced by enzymatic oxidation of retinol or is made directly from cleavage of β-carotene in the intestine and the liver. Retinal is the chromophore of rhodopsin in the visual cycle; it is also an obligatory intermediate in conversion of retinol to retinoic acid. Oxidation of retinol to retinal is reversible, but further oxidation of retinal to retinoic acid is irreversible. In most tissues other than the retina of the eye, retinal is thought to be only an intermediate in the rapid conversion of retinol to retinoic acid. In vision, the aldehyde form of vitamin A is the active form, and the visual cycle involves the isomerization of two forms of retinal (Wald, 1953, 1968).

Retinoic acid is the form of vitamin A which has been the center of interest in recent years. In the mid-1980's, retinoic acid was identified as the active form of vitamin A

in most differentiation systems (reviewed by Sporn *et al.*, 1984; Williams and Napoli, 1985). Cellular retinoic acid comes from synthesis from retinol (Roberts and DeLuca, 1967). The conversion of retinol to retinoic acid is thought to take place in two steps: oxidation of retinol to retinal catalyzed by alcohol dehydrogenase and the likely rate-limiting step, and then oxidation of retinal to retinoic acid catalyzed by aldehyde dehydrogenase (Bhat *et al.*, 1988; Posch *et al.*, 1989). Only a small amount of available retinol is converted to retinoic acid (2% in kidney, the most active tissue in this conversion) (Bhat *et al.*, 1988), and then the retinoic acid is rapidly metabolized (Roberts and DeLuca, 1967). The ability of cells to convert retinol to retinoic acid is not related to the presence or the concentrations of the cellular binding proteins for these two retinoids (Napoli and Race, 1987).

Citral is a competitive analogue of retinal and can act as a substrate for the enzymes in both of the above steps (Connor and Smit, 1987; Connor, 1988). Citral has been found to inhibit the conversion of retinol to retinoic acid in several tissues including mouse epidermis (Connor and Smit, 1987; Connor, 1988) and chick epithelium (Aydelotte, 1963a, 1963b, 1963c). The ability of citral to inhibit the biological effects of retinol indicates that retinoic acid formation is obligatory for at least some functions of vitamin A in the tissues examined (Connor and Smit, 1987; Connor, 1988). While much of the study of the biosynthesis of retinoic acid has focused on cellular conversion of retinol to

retinoic acid, serum retinoic acid may also be a source of retinoic acid for target tissues, particularly following ingestion of dietary retinyl esters which may be metabolized to retinoic acid within the intestine and subsequently released into the serum (Tang and Russell, 1991). In human subjects after ingestion of all-trans-retinyl esters, levels of both 13-cis- and all-trans-retinoic acids increased significantly, while serum retinol, known to be closely regulated, did not change (Tang and Russell, 1991).

Recent research has identified specific nuclear receptors (RARs) for retinoic acid (Giguere *et al.*, 1987; Petkovich *et al.*, 1987). These receptors are members of a receptor "superfamily" consisting of the steroid hormone receptors (glucocorticoid, mineral-corticoid, estrogen, progesterone), thyroid receptors T_3R (alpha & beta), and the vitamin D receptor, all regulated by a related family of genes. Each of these receptors is about 50,000 daltons in size and has two major regions, the ligand binding region, and the nuclear binding region containing zinc fingers. The DNA-binding zinc fingers are very homologous between the receptors for the steroid hormones, thyroxine, vitamin D, and retinoic acid. The RARs are especially closely related to T3Rs (Umesono *et al.*, 1988).

The most likely mechanism of retinoid action for differentiation and morphogenesis is thought to be similar to the action of steroid and thyroid hormones. This model of retinoid action involves a cellular binding protein, CRABP or

CRBP, which binds its ligand within the cytoplasm with high affinity and specificity and then transports the ligand into the cell nucleus where the retinoid binds to receptor sites which alters gene transcription (Takase *et al.*, 1986).

Two important concepts have emerged from the recent studies of retinoid metabolism: (1) retinoids bind to specific extracellular or cytoplasmic proteins which probably serve to transport retinoids, and possibly regulate their metabolism and activity by delivery to appropriate enzymes or receptors; and (2), retinoic acid directs the expression of retinoic acid-responsive genes through interaction with receptors in the cell nucleus (reviewed by Ross, 1991).

Vitamin A in reproduction and embryonic development

It is well established that vitamin A is required for normal mammalian reproduction, as demonstrated by complete or partial infertility of vitamin A-deficient animals (Moore, 1957; Thompson JN *et al.*, 1964). In studies of mammals including rats, mice and guinea pigs, atrophy of the testes and failure of spermatogenesis were found in males (Moore, 1957); vitamin A-deficient female rats had localized areas of infection and placental necrosis, as well as prolongation of pregnancy and difficult delivery with excessive uterine bleeding and sometimes death of the dam either before or during delivery (Moore, 1957). Vitamin A is also necessary for reproduction in birds; egg production ceased in chickens several weeks before they succumbed to the effects of the

vitamin deficiency, and eggs with low levels of vitamin A did not hatch (Moore, 1957).

Vitamin A is required for normal embryonic and fetal development (Wilson *et al.*, 1953; Thompson JN *et al.*, 1964). These early studies described anatomical abnormalities resulting from vitamin A-deficiency during embryogenesis. In rats and pigs, the earliest embryonic stages of development proceed normally, but continued lack of vitamin A resulted in placental necrosis and fetal resorption (Moore, 1957; Thompson JN *et al.*, 1964) or in malformations of many organs, including small or absent eyes, harelip and cleft palate, and horseshoe or fused kidneys (Warkany and Schraffenberger, 1946; Wilson and Barch, 1949; Wilson and Warkany, 1948). Malformations of the heart included failure of the interventricular septum to close, abnormalities of the aortic arch, and retarded myocardial development (Wilson and Warkany, 1949). The most common types of malformations were hypoplastic in nature, resulting from retardation or failure of differentiation of tissues or organs (Moore, 1957). Vitamin A supplementation to the pregnant female restored normal development in some cases, although irreversible abnormalities in organogenesis of the fetus may have occurred (Wilson *et al.*, 1953; Moore, 1957; Thompson JN *et al.*, 1964).

Effect of vitamin A on embryonic epithelial tissues

Retinoids are known to function in differentiation and maintenance of most epithelial tissues (Chytil and Sherman,

1987). Both normally occurring and experimentally induced epithelial differentiation has been the subject of extensive research. Fell and Mellanby (1953) first described the mucous metaplasia in chick embryo epidermis, which differentiated into a secretory epithelium *in vitro* when the culture medium contained higher than physiological levels of vitamin A; the epidermis returned to normal when placed in culture medium containing vitamin A at physiological levels. Fell (1957) concluded that the degree of vitamin A-induced mucous metaplasia was dependent upon the degree of differentiation or the age of the tissue at the time of exposure to vitamin A. Peck *et al.*, (1977) demonstrated that the vitamin A-induced mucous metaplasia in epithelium was not only reversible and stage specific, but dose-dependent as well. Stage specificity is a characteristic feature of many retinoid effects (Johnson and Scadding, 1991).

The metaplastic responses to vitamin A in mammalian tissues are similar to those seen in chick epidermis. Skin cultures from older mouse embryos grown in culture in the presence of retinoids were unable to undergo the complete metaplasia observed in cultures of skin from younger embryos exposed to retinoids (Sweeney and Hardy, 1976; Hardy *et al.*, 1978). Sweeney and Hardy (1976) interpreted these results to indicate the existence of a period of special sensitivity when the epidermis was capable of responding to the level of vitamin A. Dhouailly *et al.* (1980) demonstrated that by varying the time of retinoic acid administration, the

primordia for chick scales were sensitive to retinoic acid only during specific developmental periods; specific "morphological markers" appeared during these sensitive periods. While cells were responsive to retinoic acid during the sensitive period, administration of retinoic acid just prior to or after the appearance of these markers rarely induced any morphogenetic alteration (Dhouailly *et al.*, 1980).

The epithelial cells of mammalian trachea also show metaplastic changes when cultured with varying concentrations of vitamin A, differentiating into ciliated, secretory cells when physiological levels of vitamin A are present, or into squamous keratinizing cells in the absence of vitamin A (Jetten *et al.*, 1986). This *in vitro* keratinization mimics that seen in vitamin A deficient animals (Wolbach and Howe 1925; Chytil 1986a,b). Jetten and Smits (1985) demonstrated a positive correlation between the activity of various retinoids in inhibiting keratinization in tracheal cultures and the capacity of the retinoids to bind CRBP or CRABP.

These studies illustrate several factors regarding vitamin A function in embryonic development. The function of retinoids is dependent upon their presence during specific time periods when the sensitivity of target tissues is high. The concentration of the retinoid is important in determining the response during this sensitive period; atypical development may occur in response to either the absence of vitamin A or to higher than physiological levels depending on the type of tissue. The response may also be related to the

presence of additional substances, such as binding proteins. Recent research has identified RARs in embryonic tissues, providing indirect evidence that retinoic acid is the active vitamin A form during embryogenesis and providing another mechanism for regulation of the different effects of retinoids on various tissues. There are temporal and spatial (tissue specific) differences in expression of the RAR alpha, beta and gamma isoforms identified in mouse embryos: RAR-alpha expression is ubiquitous and constitutive; RAR-beta is inducible by retinoic acid and is highly expressed in developing neural tissue; and expression of RAR-gamma is restricted to particular tissues such as skin, bone and cartilage (Dolle *et al.*, 1990; Science, 1990).

Effect of vitamin A on amphibian limb development

Vitamin A induces an alteration of pattern in both regenerating (Maden, 1982; Maden, 1983a, 1983b) and developing limbs (Summerbell, 1983; Tickle *et al.*, 1985). A considerable amount of research has been undertaken with models of these two systems.

After amputation, amphibian limbs regenerate those structures distal to the site of amputation to form a complete limb (Sharma and Anton, 1986). The presence of exogenous retinoids caused limb regeneration not only of the structures which were amputated but also duplication of structures which were not amputated (Maden 1982, 1983a,

1983b). These duplications took place distal to the site of amputation even though the newly regenerated limb included structures normally proximal to the site of amputation (Maden, 1982). The proportion of the whole limb which could be duplicated was dependent upon the level of amputation and the concentration and time of retinoid exposure. The more distal the amputation plane, the lower the exposure of retinoid required to cause duplication of given structures (Maden, 1982). Increased concentration and/or time of retinoid exposure resulted in greater duplication, distal to the site of amputation, of structures which had not been amputated and were still present proximal to the site of amputation. It was thus possible to regenerate a complete limb extending from the original limb which had been amputated at some midpoint along the proximodistal axis (Maden, 1982). Maden (1984) identified the target cells of retinoid action of the regenerating limb to be those of the blastemal mesenchyme rather than the epidermis.

Keeble and Maden (1986) measured the levels of CRABP in amphibian tissues, including both untreated and retinoic acid-treated blastemas. The highest levels of CRABP were found in early stage blastemas. There was a rapid and progressive decrease in level of CRABP during regeneration, approaching the level of the mature whole limb at the last stages of regeneration. The level of CRABP thus correlated with the ability of the retinoids to induce duplication;

the early stages were more susceptible to the effects of retinoic acid. No difference was observed in CRABP levels between untreated and retinoic acid treated blastemas, indicating that retinoic acid did not regulate the levels of its own binding protein in these regenerating tissues (Keeble and Maden, 1986).

McCormick *et al.* (1988) measured the ratio of apo-CRABP (without ligand) to holo-CRABP (with bound ligand) in regenerating limbs and found that the apo- to holo-CRABP ratio was highest during the early blastema stage, the stage most sensitive to retinoids. As regeneration progressed to later (and less retinoid sensitive) stages, the ratio decreased until very little apo-CRABP was present relative to the amount of holo-CRABP in the tissue. The researchers suggested that during early stages of regeneration, endogenous retinoic acid levels were low, allowing exogenous retinoid to readily bind to the high levels of apo-CRABP and be transported to the cell nucleus. As regeneration proceeded, increasing amount of endogenous retinoic acid limited the availability of apo-CRABP, resulting in a progressive decrease in the effects of the exogenous retinoic acid, although the source of the increased endogenous retinoic acid was not known (McCormick *et al.*, 1988).

Evidence that retinoids act at the genome level in the regenerating limb model comes from studies using actinomycin D, an inhibitor of RNA transcription, and

cycloheximide, an inhibitor of protein synthesis, both of which completely extinguished retinoid ability to respecify positional patterning in the regenerating limbs (Scadding, 1988). Giguere *et al.* (1989) identified a nuclear retinoic acid receptor (RAR) in regenerating limb tissue in the blastemal cells shortly after amputation; RAR continued to be expressed in high concentrations until after the completion of limb differentiation and morphogenesis. The expression of the RAR correlated with the stages of limb regeneration when retinoic acid was maximally effective in altering pattern specification.

Effect of vitamin A on chick limb development

Mirror image digital duplications were induced by local application of retinoic acid in stage 20[1] chick limb buds in a dose-dependent manner, with formation of additional digits with increased retinoic acid concentration (Summerbell, 1983; Eichele *et al.*, 1985; Tickle *et al.*, 1985). In addition, a time period of sensitivity to the retinoic acid was observed (Eichele *et al.*, 1985). Limb buds of stage 20 embryos required the presence of retinoic acid for at least 12 hours before any effect could be induced; this was interpreted as a "priming phase" of retinoid exposure, necessary for maximum response (Eichele *et al.*, 1985). However, this time of exposure by

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[1] Stages of chick embryo development are those described by Hamburger and Hamilton (1951) (see List of References).

itself was not sufficient to induce duplication; an additional 3-6 hours period was required, during which time increased exposure to the retinoic acid resulted, 2 days later, in increased duplication of the digits along with the normal digits (Eichele *et al.*, 1985). This suggests that a minimum time of exposure to retinoic acid is required to initiate any effect. Once cells are initiated, the extent of the response is influenced by the length of the additional exposure. Such an initiation process could include induction of receptors, binding proteins, or enzymes necessary for cell response to retinoids.

Studies by Wilde *et al.* (1987) provided further evidence of a time period of retinoid sensitivity in limb digit formation. Application of retinoic acid to the limb bud of stage 10 embryos, when limb cell specification was first evident, required 29 hours of exposure to cause pattern duplication; the exposure to retinoic acid necessary to cause duplication decreased in embryos treated at slightly later developmental stages, such that 24 hours of exposure was required beginning at stage 13 but only 18 hours of retinoid exposure was required beginning at stage 14 (Wilde *et al.*, 1987). The results of these studies suggests a time "window" of sensitivity to the retinoids. The presence of retinoic acid is necessary both for a minimum length of time within that window and also at a specific time at the end of the window.

Measurement of[^{3}H]-retinol released from an retinol-impregnated bead inserted in the limb bud of stage 20 and stage 24 chick embryos showed a concentration gradient of retinoic acid at physiologically significant levels from one side of the limb bud to the other side during the period of limb pattern formation (Tickle *et al.*, 1985; Eichele and Thaller, 1987). These authors suggest that the differential amount of retinoic acid specifies the pattern of digit formation. However, some authors have suggested that the difference in retinoic acid concentration across the gradient was too small to cause such profound effects on limb development (Franceschi, 1992). CRABP was found to be present at high levels in the limb buds of chick during this period of limb bud pattern development (Maden and Summerbell, 1986) and formed a gradient of CRABP reciprocal to that described for retinoic acid (Maden *et al.*, 1988). The effect of such opposing concentration gradients of retinoic acid and its carrier protein could be to steepen the retinoic acid gradient and facilitate its recognition by the limb bud cells (Maden *et al.*, 1988). However, Tamura *et al.* (1990) localized retinoids in the limb buds of stage 18 and 20-24 chick embryos with an monoclonal antibody to retinoids and found a more even distribution along the most distal edges of the limb bud, without evidence of a concentration gradient.

Analysis of the vitamin A content of chick embryos showed that the amount of vitamin A within the embryo approximately doubled each day from day 3, the time of earliest measurement, to day 7, from 0.04 μg to 0.82 μg (Romanoff, 1967). During the same period, the vitamin A content of the chicken egg yolk decreased by 16 μg/day for days 1-3, and decreased 7 μg/day for days 4-6 (Romanoff, 1967). These data suggest that mobilization of vitamin A from the egg yolk takes place beginning in the first day of incubation and that accumulation of vitamin A within the embryo tissues represents a small (0.6-5%) fraction of the vitamin A mobilized on a daily basis during this period. Thus, a relatively large pool of available vitamin A is present compared to the amount found in the embryo. There are no embryonic tissues present at this time of development which are known to be vitamin A storage sites. It is therefore reasonable to assume that the small accumulation of vitamin A in the embryo may represent endogenous tissues levels, which range from 6.5 nM on day 3, to 4-4.5 nM during days 4-8, assuming the vitamin A is present as retinol (Romanoff, 1967).

Cardiovascular development in the avian embryo

Vitamin A is required for development of the cardiovascular system. Research with vitamin A-deficient chick and quail embryo models demonstrated that abnormal development of the heart and vascular system resulted in

death of the embryo (Thompson *et al.*, 1969; Heine *et al.*, 1985). Supplementation of certain forms of vitamin A to the hen before laying, or injection of vitamin A directly into the egg before incubation, resulted in normal development of the embryo (Thompson *et al.*, 1969; Heine *et al.*, 1985). These studies demonstrated a vitamin A requirement for normal heart and vascular development in chick and quail embryos.

The cardiovascular system is one of the first organ systems to form during embryogenesis (Risau, 1991). A functioning cardiovascular system is necessary for the embryo to grow beyond the earliest stages of development when its small mass can be supplied with nutrients by simple diffusion from the yolk, and when there is direct gaseous interchange though the porous shell (Patten, 1951).

The three components of the cardiovascular system, the heart, the vessels within the body of the embryo, and the extraembryonic network of vessels and red blood cells, develop simultaneously; blood circulation is established within the first two days of development (Patten, 1951).

The primordial cardiogenic cells of the mesoderm are formed in the embryo by 24 hours of incubation. During the next 12 hours of development, dramatic development of the heart occurs. From about 25-28 hours of incubation the lateral endocardial primordia form a pair of tubular structures, one cell in thickness. These paired tubes come together in the mid-region, and by 29 hours of

incubation have fused to form a single, nearly straight heart tube. By 33-36 hours of incubation the heart tube is dilated and displaced to the right of the mid-line, and regular ventricular contractions have begun. The open anterior end of the heart tube continues to grow cephalically to become the ventral aorta and the posterior end of the heart is open and bifurcated into the paired omphalomesenteric veins (Freeman, 1974; Patten, 1951; Viragh *et al.*, 1984).

At the same time that the heart is developing, the extraembryonic blood cells and vessels are also developing. By 24 hours of incubation the mesoderm has grown peripherally into the proximal region of the area opaca forming the area opaca vasculosa where the extra-embryonic vasculature will develop. Between 24 and 33 hours of incubation, this vascular area enlarges rapidly with the peripheral boundary of the area vasculosa marked by the precursor of the sinus terminalis. The blood islands first appear as irregular clusters of mesoderm cells. Differentiation of blood islands begins in the peripheral part of the area vasculosa and extends toward the embryo. At the stage of 3-5 somites, the blood islands form into single layers of peripheral cells which may be regarded as the endothelial wall of blood channels. Primitive red blood cells containing hemoglobin are formed from the central cells within these channels. Extension and anastomosing of the blood islands results in a network of

small communicating channels. The first channels are very small and freely anastomosing as they extend toward the embryo. Some channels later become confluent while others disappear, and definite main vessels gradually develop (Patten, 1951).

Blood vessels are also developing within the embryo. The ventral aorta extends from the cephalic end of the heart, branching into the ventral aortic roots which curve around and continue caudad as the dorsal aortae. The paired anterior cardinal veins return blood from cephalic region of embryo to the heart while the pair of posterior cardinal veins return blood from the caudal region. Anterior and posterior cardinal veins of the same side of the body become confluent near the heart, forming the common cardinal veins or ducts of Cuvier. By 38 hours of incubation the anterior cardinal veins and ducts of Cuvier are readily recognized, and the posterior cardinal veins are also established. The last links in the circulatory path are the arterial channels from the dorsal aortae. At 38-40 hours of incubation the omphalomesenteric arteries open to the extraembryonic network of channels and carry blood from the dorsal aortae to the vitelline plexus, completing the closed loop for blood circulation (Patten, 1951).

The mechanisms by which the vascular precursors segregate from non-angiogenic mesoderm cells and form cords of endothelial cells are not known. It has been suggested

that blood vessel and cardiac precursors which have segregated from lateral mesoderm migrate along specific pathways within the embryo and differentiate in response to local cues (Reiss and Noden, 1989). The abnormalities of heart and blood vessel development in vitamin A-deficient embryos suggest that vitamin A may play a role in providing such a cue in cardiovascular development.

Vitamin A in avian cardiovascular development

The first morphologically evident abnormality in the vitamin A-deficient avian embryo is the failure of the paired omphalomesenteric veins to develop; instead, the heart tube closes off at the caudal end, preventing establishment of the vitelline circulation (Heine *et al.*, 1985). There is little development of the embryo beyond that achieved by about 40 hours of incubation (stage 17) (Heine *et al.*, 1985), and, although the embryo may survive for an additional day or two, the lack of blood circulation is ultimately a fatal abnormality (Thompson JN *et al.*, 1969). Development of the main intraembryonic vessels proceeds in the vitamin A-deficient embryo even without blood circulation (Arey, 1963; Heine *et al.*, 1985), but the development of the extraembryonic vessels does not take place in the absence of vitamin A. In the extraembryonic tissues, the blood island cells develop and differentiate into the red blood cells and into preliminary blood

channels, but complete extraembryonic vessels do not form (Risau, 1991; Heine *et al.*, 1985).

The studies of retinoid functions in various embryonic tissues demonstrate the diversity of retinoid effects on differentiation and morphogenesis and reflect not only varying effects on different tissue types but also varying effects on the same tissue during different stages of development. The fact that retinoids induce a particular effect only during a brief period of development supports the concept that retinoids act as switches for differentiation, and that the nature of the response of retinoid-treated cells is determined within the cells themselves (Johnson and Scadding, 1991). The precise sequence of events in retinoid functions have not been completely elucidated.

Hypothesis and objectives of this study

Much of the current knowledge of the biological activity of retinoic acid in embryogenesis comes from the studies of alterations in normal limb bud development and regeneration by administration of exogenous retinoids, particularly retinoic acid. The results of these and similar studies have been interpreted to mean that retinoic acid is the active form of vitamin A and acts as a morphogen in the determination of developmental pattern of embryonic limb structures. However, the essentiality and the functions of any physiologically active molecule is

best assessed in a model system in which that molecule is absent but can be reintroduced. The vitamin A-deficient quail embryo model initially described by Thompson *et al.* (1969) in their work with chick embryos and subsequently adapted for Japanese quail by Heine *et al.* (1984) was chosen for the present study. This model has several important advantages compared to models involving normal embryos or mammalian animals:

1) vitamin A-deficiency in avian embryos causes specific fatal abnormalities in early cardiogenesis which can be prevented by vitamin A supplementation;
2) the egg provides an isolated, self-contained system where embryonic development takes place independently of maternal influences;
3) embryos are available without any surgical or invasive procedure to the hen;
4) avian heart development during the early period of organogenesis is similar to that of mammals, making the avian embryo an appropriate model for application to humans and other mammals (Thompson RP and Fitzharris TP, 1979a, 1979b, 1985; Thompson RP *et al.*, 1983; Thompson RP *et al.*, 1985);
5) it is possible to produce avian eggs which do not contain vitamin A in the yolk, thus providing a vitamin A-deficient environment for embryonic development.

The purpose of this study was to test the hypothesis that retinoic acid is active in preventing the vitamin A-deficiency-associated abnormality of the cardiovascular system in Japanese quail embryos. Specific objectives were: 1) to determine the time point of vitamin A requirement for normal embryonic cardiogenesis; 2) to compare the biological activity of different forms of retinoic acid and retinol; and 3) to determine whether retinol is converted to retinoic acid by the embryo during the period of cardiogenesis.

MATERIALS AND METHODS

Quail embryo model

Vitamin A-deficient (-A) eggs were obtained from Japanese quail hens fed a semi-purified diet (Teklad, Madison, WI) adequate in all nutrients but with 10 mg of methyl retinoate/kg diet as the only source of vitamin A (Appendix A). Embryos from eggs of hens fed this diet develop a characteristic abnormality in cardiovascular development described for chick embryos by Thompson *et al.* (1969) and for Japanese quail embryos by Heine *et al.* (1985). Control, vitamin A-sufficient (+A) eggs were obtained from hens fed a normal ration, Purina Gamebird Chow, Startena for growing chicks and Bird Breeder Layena for the adult birds (Purina Mills, Inc., St. Louis, MO).

An initial group of quail were hatched from normal eggs and were divided into 2 groups of 25-30 chicks each. The control group was fed Purina starter/grower diet until the hens began to lay eggs (6-7 weeks of age); the feed was then changed to Purina breeder diet. The vitamin A-deficient chicks were fed the vitamin A-deficient starter/grower diet (Teklad) beginning within the first week after hatching until the hens began to lay eggs; the feed was then changed to the vitamin A-deficient breeder

diet. At the time the hens began to lay, the ratio of females to males in each group was established at approximately 2:1 by removing extra male birds from the cage. The quail were housed at the Michigan State University poultry farm.

It is assumed that in this vitamin A-deficient model, the hen is unable to transfer vitamin A to the egg yolk because the only source of vitamin A in the diet is methyl retinoate which cannot be converted to retinol or esterified to retinyl esters, the forms of vitamin A normally found in the egg yolk. However, no verification of the absence of methyl retinoate or other vitamin A active compounds in the yolk was reported by the previous researchers using this model (Heine *et al.*, 1985; Thompson JN *et al.*, 1969). HPLC analysis of the egg yolk was conducted to establish the vitamin A content of the yolk.

Yolks from eggs layed by hens fed methyl retinoate as the only source of vitamin A were lyophilized and the retinoids extracted from samples (75 mg of yolk/sample) with 2 ml of chloroform and 1 ml of methanol, vortexed for 10 seconds, placed on a rotary shaker for 10 minutes, centrifuged for 30 minutes and the upper phase transferred to a separate tube. This extraction was repeated with two additional ml of chloroform. The upper phase from the second extraction was combined with that from the first extraction from each sample and evaporated with a stream of nitrogen. The residue was redissolved in 400 μl of acetone

and applied to a ODS-III analytical column (Waters, Milford, MA) and eluted with the following mobile phases by a step-gradient method using 75:25 methanol:water (v/v) with 0.1 M ammonium acetate, 90:10 methanol:water (v/v) and 85:15 methanol:chloroform; between each sample run the column was washed with 100% methanol and re-equilibrated in 75:25 methanol:water. Retinoids were detected at 340 nm.

Assessment of embryonic development

Embryonic development was assessed according to the developmental stages of chicken embryos described by Hamburger and Hamilton (1951). The designation of an embryo as normal or vitamin A-deficient involved assessment of the development of the heart, the embryonic and extra-embryonic vessels, and establishment of blood circulation. The criteria for an embryo to be designated as responding to the added retinoid and developing a normal cardiovascular system was: (1) the development of a normal heart shape, (2) the presence of a vascular connection extending from the heart, (3) the presence of a developing extraembryonic vascular network, and (4) circulation of blood. Embryos designated as being vitamin A-deficient were characterized by a specific pattern of abnormal development manifested by: (1) a closed heart, (2) absence of embryonic vascular connections extending into the area vasculosa, (3) absence of vessel development within the

area vasculosa, and (4) absence of blood circulation. These criteria are summarized in Table 1.

Table 1: Classification of embryos as normal or vitamin A-deficient

	Embryo development[1]	
	Normal	Vitamin A-deficient
Heart shape	normal	abnormal
Omphalomesenteric veins	present	absent
Extraembryonic vessels	present	absent
Blood circulation	present	absent

[1] Observation of embryos after 65-75 hours of incubation

The previous work with this vitamin A-deficient avian embryo model by Thompson JN *et al.* (1964) and Heine *et al.* (1985) has established that all embryos from eggs of hens fed the vitamin A-deficient diet supplemented with methyl retinoate have the characteristic abnormalities of the heart and vascular system described above; if vitamin A is added to the egg or to the embryo early in development, normal cardiogenesis can occur.

An additional classification, abnormal embryos, was used in the present study for all embryos which developed any abnormality other than the constellation of cardiovascular abnormalities characteristic of vitamin A-deficiency. These other abnormalities included 1) small size of the embryo with developmental stage characteristic of a shorter incubation time than the actual incubation time, 2) a disproportionate development of some regions of the embryo in relation to other regions, or 3) the absence or dysmorphogenesis of parts of the embryo. These embryos were classified as having abnormalities unrelated to vitamin A-deficiency. Embryos which were dead at the time of observation were excluded from the classification except for the experiments to determine toxicity of retinoic acid, in which case the dead embryos were included in an abnormal/dead classification. Infertile eggs were also excluded from any classification of embryonic development and were not included in the number of embryos for a replication of the experiments.

Administration of retinoids to the embryo

Eggs were collected daily and stored at 17°C until used. Although embryonic development is not affected by the storage of eggs for up to one week, eggs from different days were evenly mixed within each experimental group to minimize any differences due to length of storage . Eggs were placed in an incubator at 38°C with 99.5% humidity and with a two-hour rotation cycle. Retinoids were administered to the embryos

in ovo at various times during incubation. Retinoids were dissolved in acetone, mixed with a solution containing saline:homogenized whole -A egg, 95:5 (v/v), and the acetone evaporated with a stream of nitrogen. Eggs were cleaned with 70% ethanol and a hole made with a 18-gauge needle on top of each egg at the site of the air sac. Twenty μl of retinoid solution was injected into the air sac of the egg using a Hamilton syringe. Controls were injected with the vehicle only. The hole in the egg shell was sealed with tape and the eggs were returned to the incubator (38°C). At least three trials were conducted for each time point, dose, and retinoid.

Quantities of retinoids used in this study were calculated on a μg basis to be more directly comparable to the earlier studies with this vitamin A-deficient avian embryo model. The molecular weights, and therefore the quantities expressed in moles of these retinoids are nearly identical (mol. wt. 284-300). The equivalent quantities in moles and μg of retinoids used in this study are shown in Appendix B.

After a total of 65-75 hours of incubation, embryos were dissected from the eggs and examined under a Bausch & Lomb dissecting microscope. This length of incubation was chosen because normal development of the cardiovascular system is evident in the vitamin A-sufficient control embryos and also in those vitamin A-deficient embryos responding to the added retinoid. However, untreated vitamin A-deficient embryos

and vitamin A-deficient embryos not responding to the retinoid treatment exhibit the characteristic cardiovascular abnormality associated with lack of vitamin A during cardiogenesis described above.

Retinoids and chemicals

3,4-di-dehydro-retinoic acid was a gift from Hoffmann-LaRoche, Nutley, NJ; 3,4-di-dehydroretinol was a gift from Dr. B.J. Burri, (Western Human Nutrition Research Center, San Francisco, CA); [^{3}H]-retinol was purchased from New England Nuclear-DuPont, (Boston, MA); other retinoids and chemicals were reagent grade purchased from Sigma Chemical Company, (St. Louis, MO).

Statistical analysis

Differences in the response of embryos to various amounts of retinoids and time points of retinoid administration, and differences in quantities of radiolabeled retinoids within embryo tissue sections were determined by statistical analysis of data using 1-, 2- and 3-way analysis of variance with multiple range tests on the arc sine square root transformation with small sample size modification (Snedecor and Cochran, 1989) and on the natural log transformation of the retinol metabolism data using STATGRAPHICS Statistical Graphic Systems (STATGRAPHICS, 1985-1991). Significance was determined at $P \leq 0.05$.

Retinol: dose-response and time-response

In this study, the biological activity of retinol was tested by using single doses of 0.1, 0.5, 1.0 or 2.0 μg injected into the vitamin A-deficient quail egg at various times (0, 24, 36 hours) during early embryonic development. These doses and times were chosen because retinol had previously been shown to be active in preventing the vitamin A-deficiency-associated cardiovascular abnormality in chicken embryos with an optimal dose of 2 μg, with the highest percentage of normal embryos occurring when retinol was administered at the beginning of incubation and the percentage of normal embryos decreasing when retinol was injected later during the first 48 hours of incubation (Thompson *et al.*, 1969).

Inhibition of the biological activity of retinol by citral

The administration of retinol or retinol together with citral was tested in vitamin A-deficient eggs to determine whether or not the presence of citral would inhibit the activity of retinol. Citral was not soluble in the saline:homogenized whole egg solution used for the other retinoid injections; a solution of 90:10 saline:dimethyl sulfoxide (DMSO) was used. Two μg of retinol/20 μl of solution, or the same amount of retinol with 10-15 μmols of citral/20 μl were injected into vitamin A-deficient eggs at 0 or 24 hours of incubation in the same manner as described above. Two μg of retinol was chosen since it had already

been established to be a dose capable of preventing the vitamin A-deficiency related abnormalities; the amount of citral selected was comparable to the amount used by others relative to the 2 μg of retinol (Connor and Smit, 1987; Connor, 1988). The embryos were examined under the dissecting microscope after a total of 65-75 hours of incubation to assess the development of the heart and vascular system.

All-trans-retinoic acid: dose-response and time-response

Several doses of all-trans-retinoic acid were injected into both normal (+A) and vitamin A-deficient (-A) eggs to determine whether retinoic acid would prevent the vitamin A-deficiency-associated abnormality of the cardiovascular system of the quail embryo, and, if so, to determine the most effective dose of retinoic acid that would not result in toxicity-related abnormality or in embryonic death. A single dose of retinoic acid was injected at the beginning of incubation or after 24 hours of incubation. Doses of 0-0.2 μg of all-trans-retinoic acid were used in the present study based on the results of Thompson *et al.* (1969) who reported dose-dependent toxicity effects in 20% to 100% of embryos with doses of 0.1 to 5 μg of retinoic acid administered to normal chicken embryos.

To determine the time point when retinoic acid injection would result in the maximum percentage of embryos with normal cardiovascular development, a single dose of 0.1 μg of

retinoic acid was injected into vitamin A-deficient eggs at different times during the first 2 days of development.

Comparison of the biological activity of various natural retinoids

The biological activity of retinol in preventing the vitamin A-deficiency-associated cardiovascular abnormality was compared to that of 3,4-di-dehydroretinol by injecting single doses of 0.1 or 1.0 μg of this retinoid into the vitamin A-deficient eggs at 0, 24 or 36 hours of incubation. The biological activity of different forms of retinoic acids was compared by injecting single doses of 0.1 μg of all-trans-retinoic acid, 13-cis-retinoic acid, or 3,4-di-dehydro-retinoic acid into vitamin A-deficient eggs at 0, 24 or 36 hours of incubation.

Metabolism of [^{3}H]-retinol in the embryo

The hypothesis that the embryo converts retinol to retinoic acid was tested by administration of ^{3}H-retinol to embryos. Vitamin A-deficient eggs were incubated for 32 hours before transfer of the entire egg contents to a culture vessel made from a 50 ml culture tube cut to 15 ml size. Twenty μl of 90:10 saline:1% bovine serum albumin (BSA) (v/v) containing 0.5 μCi of [^{3}H]-retinol and 0.2 μg of unlabeled all-trans retinol was administered to the embryo by injecting the solution with a Hamilton syringe just into the albumen layer covering the embryo. The culture tube was covered with

Permafilm and incubated for 3 hours at 38°C. The embryo was then dissected from the egg contents, rinsed 3 times in ice cold phosphate buffered saline (PBS) buffer, pH 7.4, containing 5 mg/ml each of ethylenedinitrilo-tetraacetic acid disodium salt (EDTA) and ascorbic acid to remove adherent albumen or yolk. The embryos were then separated into 3 sections: 1) the embryo with the area pellucida, 2) the area vasculosa of the area opaca, and 3) the remaining membrane of the area opaca. For simplicity throughout this thesis, these sections will be referred to as 1) the embryo, 2) the area vasculosa, and 3) the area opaca, which is understood to be that portion remaining after the area vasculosa has been dissected away. Each of these sections was rinsed 3 additional times in the PBS/ascorbic acid/EDTA buffer and then stored frozen (-20°C) in 50 µl of the buffer until used for extraction and HPLC analysis. Some samples were pooled in numbers of 3, 5, 6, 8 or 10 embryo sections before freezing. When extracted, samples were thawed before addition of the extraction solution containing an additional 50 µl of the PBS/ascorbic acid/EDTA buffer, 200 µl of methanol and 1 ml of hexane. Each sample was sonicated for 10 minutes, vortexed for 5 minutes, centrifuged for 1 minute in a microcentrifuge, and the hexane layer transferred to a separate tube. An additional ml of hexane was added to each original sample, vortexed, centrifuged, and transferred to the same tube as the first hexane portion; this process was repeated again so that each sample was extracted with a total of 3 ml of hexane.

The hexane from the 3 extractions of each sample was combined and evaporated to dryness under a stream of N_2. The residue was redissolved in 150 μl of methanol of which 100 μl was applied to an ODS-II column (Waters, Milford, MA) to be chromatographed using as mobile phases 65:35 methanol:water (v/v) containing 0.1 M ammonium acetate, 90:10 methanol:water and finally 85:15 methanol:chloroform; between each sample run the column was washed with 100% methanol and re-equilibrated in 65:35 methanol:water. Retinoids were detected by absorbance at 340 nm. Fractions were collected from which an 1.0 ml aliquot was mixed with 3.5 ml of scintillation cocktail and analyzed for 5 minutes in a Tri-Carb 4000 scintillation counter, with the radioactivity quantitated as dpm. Radioactivity was calculated as dpm/sample and as dpm/μg of protein determined by the modified method of Lowry (Markwell, 1981).

RESULTS

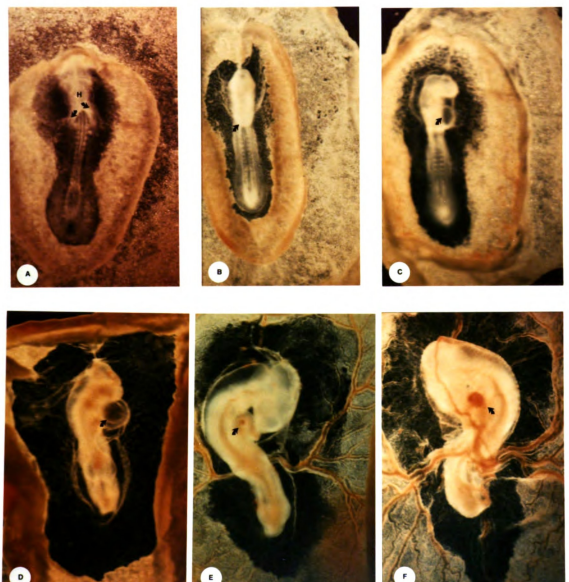
Determination of reference parameters for the quail embryo model

Figure 4 illustrates the appearance of the Japanese quail embryos during the time of cardiovascular development. Photomicrograph A illustrates the central heart with openings of the omphalomesenteric veins (arrows) characteristic of normal embryos or vitamin A-deficient embryos in which the heart has not yet become sealed; B-D illustrate the characteristic cardiovascular abnormalities of vitamin A-deficient embryos with a closed heart and the absence of omphalomesenteric veins and other blood vessels; E-F show normal cardiovascular development, present in the normal control embryos and also in those vitamin A-deficient embryos which responded to added retinoids.

Normal control eggs and vitamin A-deficient eggs were injected with the vehicle alone to determine its effect on embryonic development. Of 205 control embryos, 95.5% were normal and 4.9% were abnormal in appearance unrelated to the specific vitamin A-deficiency-associated abnormalities, as described in **Materials and Methods.** The same percentage (95.1%) of 285 vitamin A-deficient embryos showed the characteristic vitamin A-deficiency-associated

Figure 4: Photomicrographs of quail embryos during the time of cardiovascular development

A: embryo (28 h incubation) showing a central heart (H) and the openings of the omphalomesenteric veins (arrows);
B-C: vitamin A-deficient embryo (60 h) with a closed heart (arrows) and absence of omphalomesenteric veins and other vessels;
D: vitamin A-deficient embryo (72 h) with enlarged, ballooned heart (arrows);
E-F: vitamin A-deficient embryo (72 h) with normal heart (arrows) and cardiovascular development after administration of retinoids;


A
B
C
D
E
F

cardiovascular abnormality, described in **Materials and Methods** and summarized in Table 1; 4.9% of the vitamin A-deficient embryos had other abnormalities unrelated to vitamin A-deficiency.

Analysis of the yolks from eggs of hens fed the methyl retinoate diet showed the presence of methyl retinoate, ranging from 5-15 μg/yolk. However, embryos from these eggs did not develop normally unless vitamin A-active retinoids were administered to the eggs.

Effect of retinol on cardiovascular development of vitamin A-deficient quail embryos

The effect of the administration of different single-dose amounts of retinol on the cardiovascular development in vitamin A-deficient quail embryos is shown in Table 2. When 1.0 or 2.0 μg of retinol was administered at the beginning of the incubation period, normal cardiovascular development was observed in almost all (97%) of the embryos; when 1.0 or 2.0 μg of retinol was administered at 24 hours of incubation, a significant ($P \leq 0.05$) decrease in the percentage of embryos with normal cardiovascular development was observed (70% and 54% of the embryos, respectively). In addition, the percentage of embryos with normal development was significantly lower (22%) with the 2.0 μg dose compared to the 1.0 μg dose when administered at 24 hours of incubation. The administration of 0.1-0.5 μg of retinol at the beginning of incubation or at 24 hours of incubation was less effective

Table 2: Effect of retinol on cardiovascular development of vitamin A-deficient quail embryos

Time of treatment (hours of incubation)	µg of retinol injected	n[2]	Embryonic development at 65-75 h of incubation: % of embryos classified as:[1] Normal	Vitamin A-deficient	Abnormal[3]
0	0.1	3	47.1[4] (+8.7,-8.4)[5]	52.9 (+13.5,-13.9)	0
	0.5	3	75.8 (+8.3,-6.0)	21.2 (+8.9,-12.7)	3.0 (+4.4,-0.1)
	1.0	3	97.0 (+7.4,-5.5)	3.0 (+6.1,-3.5)	0
	2.0	3	96.9 (+1.4,-5.8)	3.1 (+5.0,-1.0)	0
24	0.1	3	21.4 (+6.5,-7.4)	78.6 (+6.3,-5.0)	0
	0.5	3	46.2 (+9.7,-7.5)	53.8 (+8.3,-10.7)	0
	1.0	3	70.0 (+8.9,-6.6)	30.0 (+10.0,-11.6)	0
	2.0	3	54.5 (+9.5,-7.9)	45.5 (+9.0,-10.9)	0
36	0.1	4	0	100	0
	0.5	4	6.7 (+6.9,-1.5)	93.3 (+2.3,-8.1)	0
	1.0	4	6.9 (+5.9,-2.9)	89.7 (+4.5,-6.9)	3.4 (+6.6,-0.3)
	2.0	4	4.3 (+6.6,-0.1	95.7 (+0.1,-6.2)	0

[1] Experimental details and classification of embryonic development are described in **Materials and Methods**
[2] Number of replications; mean of 8.7 embryos/replication
[3] Unrelated to vitamin A-deficiency
[4] Mean ± SE; statistical analysis was done on arc sine square root transformed data and,therefore, the SE is asymmetrical around the untransformed mean
[5] Significant differences ($P \leq 0.05$) are as follows:

Single dose level compared by time-pair: (0 & 24; 0 & 36; 24 & 36): each dose is significantly different within its time-pair combinations;

Different dose levels within the same treatment time:
- 0 h: all doses are different from the others except for 1.0µg compared to 2.0µg
- 24 h: 0.1µg is different from every other dose; 0.5µg, 1.0µg, 2.0µg are each only different from 0.1µg;
- 36 h: there are no significant differences between doses

than the administration of higher amounts (1.0 or 2.0 μg) of retinol in establishing normal cardiovascular development in vitamin A-deficient quail embryos. When administered at 36 hours of incubation or later, retinol had low activity (< 10% normal embryos) in inducing normal cardiovascular development; embryos treated with retinol later than 36 hours of incubation exhibited cardiovascular abnormalities typical of vitamin A-deficient embryos (Appendix C).

Inhibition of the biological activity of retinol by citral

Table 3 shows the results of the experiments to determine whether or not citral would inhibit the activity of retinol; 2 μg of retinol, alone or together with citral (10-15 μmols), were injected into the egg at 0 and 24 hours of incubation. Administration of citral together with retinol at the beginning of incubation, when retinol is most active in inducing normal cardiovascular development, decreased the biological activity of retinol by 15%; there was a slightly greater decrease (22%) in activity of retinol when it was administered with citral at 24 hours of incubation. However, there were no statistically significant differences in the outcomes from treatment at of the embryos with retinol alone or with retinol and citral together.

Table 3: Cardiovascular development of vitamin A-deficient embryos treated with retinol or retinol and citral

Time of treatment (h)	Treatment	n[2]	Embryonic development after 65-75 h of incubation: % of embryos classified as:[1] Normal	Vitamin A-deficient	Abnormal[3]
0	Retinol[4]	4	60.0[5] (+12.9,-9.8)	34.3 (+11.2,-17.2)	5.7 (+4.3,-1.5)
	Retinol and Citral[6]	3	51.9 (+8.8,-18.1)	40.7 (+18.0,-10.1)	7.4 (+8.1,-4.6)
24	Retinol	3	33.3 (+13.1,-12.2)	63.3 (+13.0,-12.2)	3.3 (+3.2,-0.4)
	Retinol and Citral	3	25.8 (+11.4,-3.8)	58.1 (+11.0,-10.5)	16.1 (±3.7)

[1] Experimental details and classification of embryonic development are described in **Materials and Methods**
[2] Number of replications; mean = 9.5 embryos/replication
[3] Unrelated to vitamin A-deficiency
[4] 2 µg of retinol
[5] 2 µg of retinol and 10-15 µmols of citral
[6] Mean ± SE; statistical analysis was done on arc sine square root transformed data and, therefore, the SE is asymmetrical around the untransformed mean; there are no significant differences between treatment with retinol or with retinol together with citral

Effect of di-dehydroretinol on cardiovascular development of vitamin A-deficient quail embryos

Two dose levels of di-dehydroretinol were used, 0.1 and 1.0 μg, and each was tested at 0, 24 and 36 hours of incubation. Results are shown in Table 4. The percentage of normal embryos after treatment with the 0.1 μg dose was very low regardless of time of treatment. The 1.0 μg dose prevented the vitamin A-deficiency-related cardiovascular abnormality in 62% and 50% of vitamin A-deficient embryos when given at 0 hours and 24 hours of incubation, respectively; the percentage of normal embryos decreased to 13% when the retinoid was administered at 36 hours of incubation. Di-dehydroretinol was less active in inducing normal development than retinol (see Table 2). At 1.0 μg, di-dehydroretinol was significantly less active than retinol when given at either 0 or 24 hours of incubation (35% and 28% less, respectively); at 0.1 μg, di-dehydroretinol was significantly less active than retinol when given at 0 hours of incubation ($P \leq 0.05$).

Embryotoxicity of retinoic acid

The response of normal quail embryos to various amounts of injected all-trans-retinoic acid is shown in Table 5. Retinoic acid doses of 0.05-0.1 μg administered in a single dose either at 0 hours or at 24 hours of incubation did not result in a greater percentage of embryos classified as having abnormalities (described in

Table 4: Effect of di-dehydroretinol on cardiovascular development of vitamin A-deficient quail embryos

Embryonic development at 65-75 h of incubation				
Hours of incubation at time of treatment	n[2]	% of embryos classified as:[1] Normal	Vitamin A-deficient	Abnormal[3]
		0.1 μg di-dehydroretinol		
0	3	2.6[4,5] (+6.0,-1.7)	97.4 (+1.7,-6.0)	0
24	3	2.6 (+6.4,-1.6)	94.8 (+0.05,-9.0)	2.6 (+8.9, -0.1)
36	3	2.7 (+5.7,-1.8)	97.3 (+1.1,-3.7)	0
		1.0 μg di-dehydroretinol		
0	3	62.5 (±9.8)	37.5 (±9.8)	0
24	3	50.0 (+14.5,-5.7)	50.0 (+5.7,-14.5)	0
36	3	13.3 (+5.8,-7.3)	84.5 (+7.3,-7.1)	2.2 (+5.3, -1.5)

[1] Experimental details and classification of embryonic development are described in Materials and Methods

[2] Number of replications at each treatment time; mean number of embryos/replication = 13.6

[3] Unrelated to vitamin A-deficiency

[4] Mean ± SE; statistical analysis was done on arc sine square root transformed data and, therefore, the SE is asymmetrical around the untransformed mean

[5] Significant differences ($P \leq 0.05$) are as follows:

The same dose compared at different times of treatment:
0.1 μg: no significant differences between any treatment times;
1.0 μg: significant differences between 0 and 24 h, and between 24 and 36 h;
Different dose compared at the same time of treatment:
The two doses are significantly different when given at 0 or at 24 hours of incubation, but are not different when given at 36 hours.

Table 5: Effect of retinoic acid administered to normal quail embryos

µg of retinoic acid	Embryonic development at 65-75 h of incubation: retinoic acid administered at 0 h			retinoic acid administered at 24 h		
	% of embryos classified as:[1]					
	n[2]	Normal	Abnormal[3] or dead	n	Normal	Abnormal or dead
0	24	96	4	16	100	0
0.025	--	--	--	4	100	0
0.050	16	100	0	17	94	6
0.100	8	100	0	9	100	0
0.200	5	100	0	9	78	22

[1] Experimental details and classification of embryonic development are described in **Materials and Methods**
[2] Number of embryos
[3] Unrelated to vitamin A-deficiency

Materials and Methods) compared to the percentage with such abnormalities when the vehicle alone was administered, and, therefore, these doses of retinoic acid (0.05 - 0.1 μg) were not considered toxic to the embryos. Although 0.2 μg of retinoic acid injected at the beginning of incubation did not cause abnormalities in the treated embryos, when this same dose was administered at 24 hours of incubation, abnormalities were observed in 22% of treated embryos. Single doses of 1.1 μg and 1.5 μg of retinoic acid caused abnormalities in 33%-100% of embryos when they were administered at 0 or 24 hours of incubation (Appendix D).

Effect of retinoic acid on cardiovascular development of vitamin A-deficient quail embryos

Retinoic acid in varying doses was administered to vitamin A-deficient embryos to determine the optimal amount for a single dose of all-trans-retinoic acid which would induce normal cardiovascular development with a minimum of retinoic acid-associated embryotoxicity. Results are shown in Table 6. A single dose of 0.2 μg of all-trans-retinoic acid resulted in normal cardiovascular development in 50% of vitamin A-deficient embryos when the retinoid was administered at the beginning of the incubation period. When the same amount of all-trans-retinoic acid was administered at the 24 hour time point, normal cardiovascular development was observed in only 14% of vitamin A-deficient embryos. At this dose level

Table 6: **Effect of all-trans-retinoic acid on cardiovascular development of vitamin A-deficient quail embryos**

	Embryonic development at 65-75 h of incubation							
	Retinoic acid injected at 0 h				Retinoic acid injected at 24 h			
	% of embryos classified as:[1]							
µg of retinoic acid	n	Normal	Vitamin A-deficient	Abnormal[2] or dead	n	Normal	Vitamin A-deficient	Abnormal or dead
0[3]	46	0	100	0	28	0	100	0
0.025	--	--	--	--	18	11	89	0
0.050	40	7.5	87.5	5	58	25.9	72.4	1.7
0.100	22	13.6	68.2	18.2	34	26.5	67.6	5.9
0.200	12	50	50	0	35	14.3	74.3	11.4

[1] Experimental details and classification of embryonic development are described in Materials and Methods
[2] Unrelated to vitamin A-deficiency
[3] Injection of vehicle only

abnormalities unrelated to vitamin A-deficiency were observed in 0-11% of the embryos while the remaining embryos exhibited the characteristic cardiovascular abnormalities associated with vitamin A-deficiency. Single doses of 0.05 - 0.1 μg of all-trans-retinoic acid, while demonstrating relatively low biological activity when given at the beginning of the incubation period (7.5 - 13.6% normal development), induced normal cardiovascular development in 26% of the vitamin A-deficient embryos when administered at 24 hours of incubation. While 0.05 μg dose produced a relatively small percent of abnormal embryos (2-5%), the 0.1 μg dose produced abnormalities in as high as 18% of embryos. The lowest amount of retinoic acid tested was 0.025 μg; with this dose, the biological activity for inducing normal cardiovascular development in vitamin A-deficient quail embryos was 40% that of the 0.1 μg dose when given at 24 hours of incubation. Embryotoxicity was evident in 50% of embryos when 1.1-1.5 μg doses of retinoic acid were administered at 24 hours of incubation (Appendix D).

Effect of 0.1 μg of all-trans-retinoic acid: time-response

Table 7 illustrates the results of single injections of 0.1 μg of all-trans-retinoic acid to establish the time during embryonic development when all-trans-retinoic acid is necessary in the vitamin A-deficient quail embryo for normal cardiovascular development. A time window of

Table 7: Effect of 0.1 μg of all-trans-retinoic acid on cardiovascular development of vitamin A-deficient quail embryos

Time of injection (h of incubation)	n[1]	% of embryos with normal cardiovascular development[2]
0	5	20.4 (+7.5,-4.5)[3,4]
14 - 18	7	9.3 (+4.4,-3.7)
20 - 22	9	19.0 (+1.7,-5.5)
24	8	33.9 (+2.6,-7.2)
25 - 26	6	24.0 (+3.4,-4.7)
27 - 28	5	23.6 (+3.6,-5.8)
29 - 30	7	14.1 (+1.4,-4.0)
34 - 38	5	5.5 (+1.5,-2.4)
40 - 45	4	2.0 (+2.3,-0.3)
46 - 50	4	0

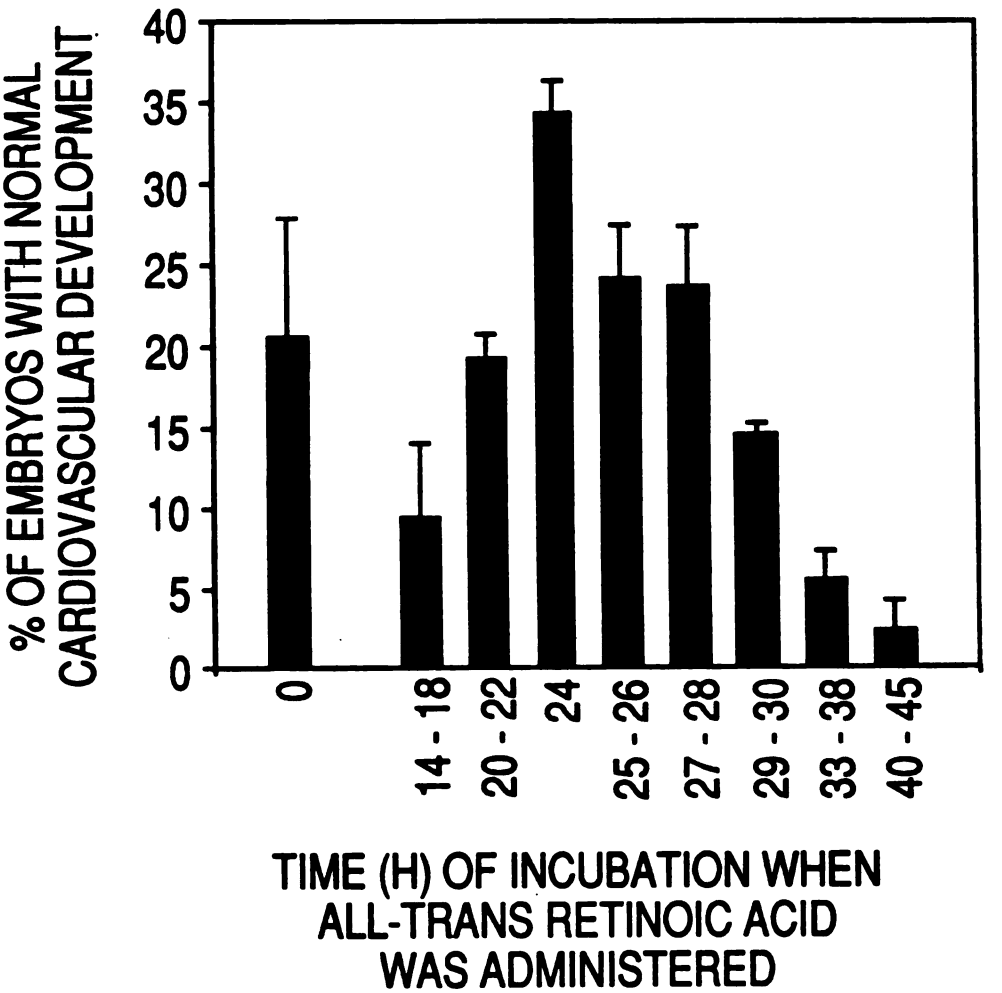
[1]Number of replications at each treatment time; mean number of embryos/replication =19.
[2]Embryos were examined and classified after 65-75 h of incubation as described in Materials and Methods.
[3]Mean ± SE; the statistical analysis was done on the arc sine square root transformed data and, therefore, the SE is asymmetrical around the untransformed mean;
[4]Statistically significant ($p \leq 0.05$) differences are:

Time:	compared to time:
0	14-18, 33-38, 40-45, 46-50;
14-18	0, 24, 25-26, 27-28, 46-50;
20-22	24, 33-38, 40-45, 46-50;
24	14-18, 20-22, 29-30, 33-38, 40-45, 46-50;
25-26	14-18, 33-38, 40-45, 46-50;
27-28	14-18, 33-38, 40-45, 46-50;
29-30	24, 40-45, 46-50;
33-38	0, 20-22, 24, 25-26, 27-28;
40-45	0, 20-22, 24, 25-26, 27-28, 29-30;
46-50	0, 14-18, 20-22, 24, 25-26, 27-28, 29-30.

8 hours (20-28 hours of incubation) was observed during which all-trans-retinoic acid resulted in normal cardiovascular development. The effect of all-trans-retinoic acid was maximal when it was administered at 24 hours of incubation; normal development was observed in a significantly higher percentage of embryos treated at 24 hours than when the treatment was given at the 14-18 and 20-22 hour points ($P \leq 0.05$). There was no significant difference in the percentage of normal embryos when the injections were given at 0 hours or within the period of 24-28 hours of incubation. However, when the injections were given at 14-18 hours or at 28 hours of incubation or later, the percentage of normal embryos was significantly lower than that observed with the 0 hour injection time point and also the 24-28 hour injection times. Injections given at 33-38 hours of incubation also resulted in a significantly lower percentage of normal embryos that when the injections were given at 0 or 20-28 time points. Injections at 40 hours of incubation or later resulted in essentially no biological activity and resulted in significantly fewer normal embryos compared to that obtained from other times of injection, except for the 14-18 hour treatment time. These data are presented in graphic form (Figure 5) for ease of observing the pattern of activity of retinoic acid in inducing normal cardiovascular development.

Figure 5: Cardiovascular development of vitamin A-deficient quail embryos after the administration of 0.1 µg of all-trans-retinoic acid

A single dose of retinoic acid was injected into the air sac of the egg. Embryos were examined after 65-75 hours of incubation. Data are from Table 7.


% OF EMBRYOS WITH NORMAL CARDIOVASCULAR DEVELOPMENT
40
35
30
25
20
15
10
5
0
0
14 - 18
20 - 22
24
25 - 26
27 - 28
29 - 30
33 - 38
40 - 45
TIME (H) OF INCUBATION WHEN ALL-TRANS RETINOIC ACID WAS ADMINISTERED

Comparison of the biological activities of various retinoids in inducing normal cardiovascular development of vitamin A-deficient quail embryos

The comparative effectiveness of a single dose of 0.1 μg of three different forms of retinoic acids for induction of normal cardiovascular development is shown in Table 8. All-trans-retinoic acid administered at 24 hours of incubation was 2- and 6-fold more effective than didehydro-retinoic acid or 13-cis-retinoic acid, respectively, in inducing normal cardiovascular development in vitamin A-deficient quail embryos. All-trans-retinoic acid was also the most active of these retinoids when they were administered at the beginning of incubation. 13-cis-retinoic acid was 2-fold more active when administered at the beginning of incubation as compared to administration at 24 hours of incubation.

Figure 6 illustrates the comparative effectiveness of the three forms of retinoic acid and two forms of retinol tested in this study, one (or more) of which might be the active form of vitamin A for normal cardiovascular development during quail embryogenesis. Comparison was made of the biological activities of 0.1 μg amounts of each of the retinoids, a dose that for all-trans-retinoic acid was not toxic and resulted in a significant physiological response in the vitamin A-deficient embryo. The data are selected results from Tables 2, 4, and 8, and are presented in graphic form for ease of comparison. Retinol had the greatest biological activity among the retinoids tested,

Table 8: Effects of 0.1 μg of all-trans-, di-dehydro-, and 13-cis-retinoic acid on cardiovascular development of vitamin A-deficient quail embryos

Embryonic development at 65-75 hours of incubation

Hours of incubation at time of treatment	Retinoid given[2]	n[3]	% of embryos classified as:[1] Normal	Vitamin A-deficient	Abnormal[4]
0	at-RA	5	20.4 (+7.5,-4.5)[5,6]	72.6 (+4.1,-8.2)	7.0 (+2.9,-4.1)
	dd-RA	3	8.8 (+2.2,-3.8)	89.7 (±5.7)	1.5 (+2.8,-0.1)
	13c-RA	3	11.9 (+1.5,-3.8)	88.1 (+3.5.-4.9)	0
24	at-RA	8	33.9 (+2.6,-7.25)	63.2 (+6.8,-3.8)	2.9 (+0.8,-2.4)
	dd-RA	3	17.1 (+4.4,-4.0)	80.0 (± 0.1)	2.9 (+4.5,-2.6)
	13c-RA	3	4.9 (+2.7,-2.6)	90.5 (+4.5,-2.7)	4.9 (+3.1,-4.3)
36	at-RA	3	6.1 (+4.0,-3.9)	90.2 (+3.5,-4.3)	3.7 (+3.9,-2.9)
	dd-RA	3	1.4 (+2.4,-0.010	95.7 (+2.7,-2.4)	2.9 (+2.7,-1.9)
	13c-RA	3	0	89.8 (+4.3,-3.1)	10.2 (+3.1,-4.3)

[1] Experimental details and classification of embryonic development are described in **Materials and Methods**

[2] at = all trans-; dd = di-dehydro-; 13c = 13-cis-retinoic acid

[3] Number of replications at each treatment time; mean number of embryos/replication = 23

[4] Unrelated to vitmain A-deficiency

[5] Mean ± SE; statistical analysis was done on arc sine square root transformed data and, therefore, the SE is asymmetrical around the untransformed mean

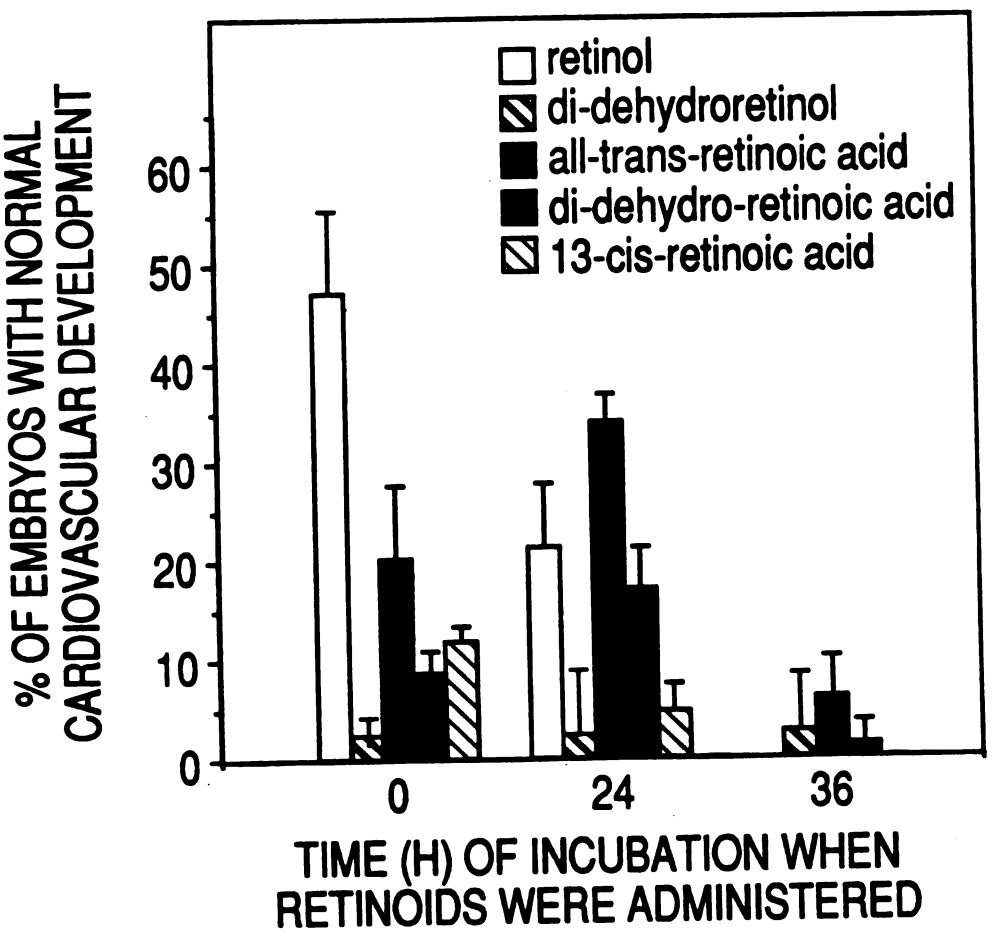
[6] Statistically significant differences ($P \leq 0.05$) are:

Single compound at different treatment times:
- at-RA: significant differences between 0 and 36 h, 24 and 36 h
- dd-RA: significant differences between 0 and 24 h, 24 and 36 h
- 13c-RA: no significant differences between any treatment times

Three compounds at the same treatment time:
- 0 h: at-RA is different from both dd-RA and 13c-RA
- 24 h: at-RA is different from both dd-RA and 13c-RA
- 36 h: there are no significant differences between any compounds

Figure 6: Comparison of the biological activities of five retinoids for induction of normal cardiovascular development

A single dose of 0.1 μg of retinoid was injected into the air sac of the egg. Embryos were examined after 65-75 hours of incubation. Data are from Tables 2, 4, and 8.


% OF EMBRYOS WITH NORMAL CARDIOVASCULAR DEVELOPMENT
retinol
di-dehydroretinol
all-trans-retinoic acid
di-dehydro-retinoic acid
13-cis-retinoic acid
60
50
40
30
20
10
0
0
24
36
TIME (H) OF INCUBATION WHEN RETINOIDS WERE ADMINISTERED

inducing normal development in 47% of treated embryos when the retinoids were administered at 0 hours of incubation. All-trans-retinoic acid was the most active form of these physiologically occurring retinoids tested at the time of development (24 hours of incubation) when vitamin A activity is required to insure the subsequent normal cardiovascular development. The biological activity of di-dehydroretinol was negligible at all time points studied.

Metabolism of [^{3}H]-retinol in the quail embryo

Vitamin A metabolism in the vitamin A-deficient quail embryo was studied using a single dose of [^{3}H]-retinol. After 3 hours of incubation, the retinoids were extracted from the embryo tissues and separated by HPLC. Although it was possible to quantitate radioactivity from both retinol and di-dehydroretinol, the sample size was insufficient to quantitate radioactivity from polar metabolites, including retinoic acid, even in samples of pooled embryos (56 embryos in 10 pooled samples of 3, 6, 8, or 10 embryos/sample). While some radioactivity was present in fractions corresponding to known retinoic acids, the results were inconsistent, and radioactivity was also present in fractions from the area of polar retinoids but did not coelute with known standards. Retinol was found in all 3 sections of tissue, i.e. the embryo, the area vasculosa, and the area opaca. Analysis of these tissues revealed the presence of a metabolite, identified as di-dehydroretinol by

coelution with authentic standard. The results of these experiments are presented in Tables 9, 10, and 11. The tissue sections were different in size, and this is reflected in the amount of protein measured for each section. The percentage of total radioactivity from retinol and di-dehydroretinol in each tissue section correlates closely to the percentage of protein present in that section (Table 9).

Comparing the amount of total radioactivity in each of the three tissue sections, shown in Table 10, it is evident that the majority of the retinol was found in the area opaca (88%), with a considerably smaller amount (10%) in the area vasculosa, and very little (1.5%) in the embryo, as shown in Table 10. The largest amount of di-dehydroretinol was also found in the area opaca (56%), but the proportions of retinol and di-dehydroretinol shift; while there is more retinol than di-dehydroretinol in the area opaca, both the area vasculosa and the embryo contain more of the di-dehydro- form than retinol. Retinol accounted for almost all of the radioactivity in the area opaca (94%), but only 54% of the total radioactivity for both forms of retinol in the embryo. The proportion of the two retinol forms in the area vasculosa is intermediate compared to the area opaca and to the embryo, with 80% of the radioactivity found in the area vasculosa attributed to retinol. Table 11 shows the quantities of retinol and di-dehydroretinol relative to protein. The amount of di-dehydroretinol was 4-times

Table 9: Distribution of radioactivity and protein in the embryo and extraembryonic membranes[1]

	Embryo	Area vasculosa	Area opaca
% of total DPM[2]	2.6	12.1	85.3
% of total protein	4.7	10.3	85.0

[1] Experimental details and description of tissue sections are described in **Materials and Methods**
[2] Combined radioactivity of retinol and di-dehydroretinol in all tissue sections; quantitation based on radioactivity coeluting with authentic standards; mean = 8056 dpm/sample
[3] Mean protein for combined embryo and membranes = 0.62 mg

Table 10: Distribution of retinol and di-dehydroretinol in the embryo and extraembryonic membranes[1]

	% of total radioactivity for each retinoid in all sections combined		
	Embryo	Area vasculosa	Area opaca
Retinol	1.5	10.5	88.0
Di-dehydroretinol	14.0	29.6	56.4
	% of total radioactivity for each retinoid within each section		
Retinol	54	79.6*	94.5*
Di-dehydroretinol	46	20.4	5.5

[1] Experimental details and description of tissues sections are described in **Materials and Methods**; quantitation is based on radioactivity coeluting with authentic standards;
* Indicates significant difference between the amount of retinol and di-dehydroretinol within section

Table 11: Distribution of retinol and di-dehydroretinol relative to protein in embryo and extraembryonic membranes[1]

	DPM x 10^{-3}/mg of protein		
	Embryo	Area vasculosa	Area opaca
Retinol	4.4 ± 3.5[2]	11.9 ± 3.1*	14.8 ± 3.3*
Di-dehydroretinol	3.3 ± 3.1	3.8 ± 3.1	0.8 ± 3.3

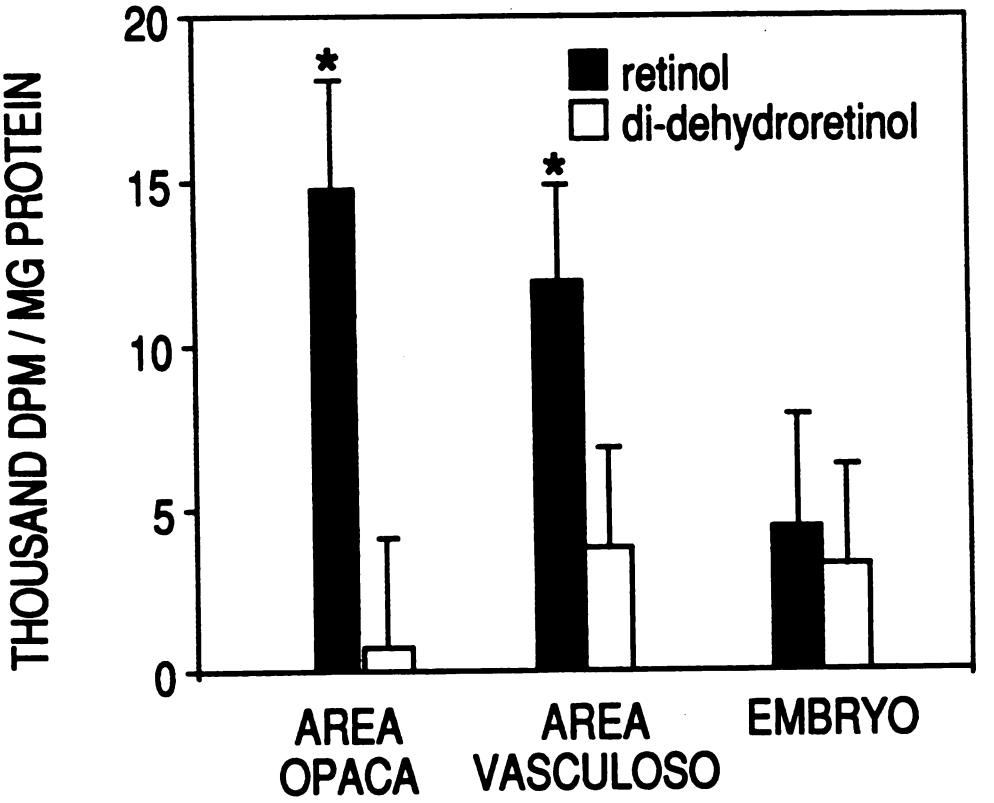
[1] Experimental details and description of tissue sections are described in **Materials and Methods**. Quantitation is based on radioactivity coeluting with authentic standards;
[2] Mean ± SE
* Indicates significant difference ($P \leq 0.05$) between retinol and di-dehydroretinol within the same tissue section

greater in the embryo compared to that of the area opaca; in contrast, these quantities were almost reversed when comparing the amount of retinol, which was 3½-times higher in the area opaca than in the embryo. The comparison of retinol to di-dehydroretinol within each tissue section is shown graphically in Figure 7. In both the area opaca and area vasculosa, the amount of retinol is significantly greater than that of di-dehydroretinol.

Figure 7: Distribution of retinol and di-dehydroretinol after treatment with [^{3}H]-retinol

Distribution of retinol and di-dehydroretinol relative to protein within each of the three tissue sections after a 3-hour pulse dose of [^{3}H]-retinol. Quantitation is based on radioactivity coeluting with authentic standards. Data are from Table 11.

* indicates a significant difference ($P \leq 0.05$) between retinol and di-dehydroretinol within the same section


THOUSAND DPM / MG PROTEIN
20
15
10
5
0
*
*
retinol
di-dehydroretinol
AREA OPACA
AREA VASCULOSO
EMBRYO

DISCUSSION

It is well known from the pioneering work of Thompson JN *et al.* (1969) and the subsequent studies by Sporn *et al.* (1985) and Heine *et al.* (1985) that the vitamin A-deficient avian embryo cannot form a normal heart and cardiovascular system. Most importantly, these researchers demonstrated that the administration of vitamin A-active compounds to the vitamin A-deficient embryo enables the subsequent normal cardiovascular development. Thompson JN *et al.* (1969) and Sporn *et al.* (1985) demonstrated the reversibility of vitamin A-deficiency by injecting vitamin A-active compounds into vitamin A-deficient eggs at the beginning of incubation. Heine *et al.* (1985) demonstrated the prevention of vitamin A-deficiency using culture conditions in which early quail embryos were dissected from vitamin A-deficient or control eggs and cultured for 24 hours on medium containing whole egg extract from either control or vitamin A-deficient eggs. Normal heart development resulted when either control or vitamin A-deficient embryos were cultured on normal control medium, and some normal control embryos developed a normal heart when cultured on the deficient medium. Deficient embryos did not develop normally when cultured on deficient medium.

The studies of these researchers indicated that a vitamin A-requiring process, which enables cardiogenesis to proceed normally, takes place between 24-48 hours of development. However, the exact time when the active form of vitamin A must be present in the embryo has not been determined in the *in vivo* model.

Retinol was effective in reversing vitamin A-deficiency while retinoic acid was toxic at the dose levels tested (Thompson JN *et al.*, 1969). No further studies have been conducted to determine the effectiveness of retinoic acid to support normal cardiovascular development in the vitamin A-deficient avian embryo. However, approximately 25% of vitamin A-deficient embryos had normal cardiovascular development when the methyl ester of retinoic acid (methyl retinoate) in doses of 4-50 μg/egg were injected into vitamin A-deficient chicken embryos (Thompson JN *et al.* 1969). Sporn *et al.* (1985) observed 70% and 41% normal cardiovascular development in vitamin A-deficient quail embryos using 3.0 μg and 1.0 μg of methyl retinoate, respectively. These studies suggested that the active form of vitamin A most likely is retinoic acid since methyl retinoate is known to be hydrolyzed to retinoic acid and the metabolic conversion of retinoic acid to retinol or retinal is not possible (Sporn *et al.* 1984).

The present studies were designed to ascertain the time during early embryonic development when the active form of vitamin A must be present in the embryo for

subsequent normal development of the heart and the cardiovascular system. Although retinoic acid has been implicated as the active form in avian cardiovascular development (see above), no experimental evidence exists to confirm this hypothesis.

Biological activity of retinol

The results of this study demonstrate that the biological activity of retinol in supporting normal cardiovascular development in the vitamin A-deficient quail embryo is both time and dose dependent. The finding that retinol is most active when administered at the beginning of incubation, inducing 97% normal development (Table 2), is consistent with the studies of Thompson JN *et al.* (1969) who found that 2.0 μg of retinol injected into the vitamin A-deficient chicken egg at the beginning of incubation resulted in 74% of embryos developing normally. The percentage of normally developing chick embryos decreased to 56% - 35% when the retinol was administered at later times (7 - 36 hours of incubation), and decreased further (35% - 0%) when the injection was at 41 - 89 hours of incubation (Thompson JN *et al.*, 1969). These results are in general agreement with those of the present study, although, in the quail embryo, the optimal dose was 1.0 μg rather than 2.0 μg, and only 7% or fewer of the embryos had normal development when retinol was administered at 36 hours and later. The smaller optimal dose for quail may

be related to the smaller size of the embryo compared to chicken embryo. Sporn *et al.* (1985) found that 2.0 μg and 1.0 μg of retinol were approximately equal in activity in the vitamin A-deficient quail embryo, enabling normal development in 73% and 69% of embryos, respectively. The more rapid decrease in activity of retinol in quail compared to chick embryos as the time of incubation before injection is lengthened may relate to the slightly faster development of quail compared to chickens (17-18 day vs 21 days of incubation required for hatching). The decrease in activity with low doses and with later administration of retinol, until it is no longer active in preventing the abnormal cardiovascular development of vitamin A-deficient embryos, is consistent with a requirement for some metabolic activation process such as conversion of retinol to retinoic acid. Conversion of retinol to retinoic acid is thought to take place in a 2-step process: 1) dehydrogenation by alcohol dehydrogenases to retinal, and 2) further oxidation to retinoic acid by retinal oxidase (reviewed by Blomhoff *et al.*, 1992). The first step appears to be the rate-limiting step and only a small portion of the total retinol (< 5%) may be converted to retinoic acid (Bhat *et al.*, 1988). Retinal is a transient intermediate metabolite in this conversion and is not known to have physiological functions other than in vision (Blomhoff *et al.*, 1992). Retinol may also be converted to other compounds, such as 3,4-didehydroretinol (Thaller and

Eichele, 1990; Torma and Valquist, 1990). Furthermore, most cellular retinol is bound to cellular retinol binding protein which may play a role in delivering retinol to the proper enzyme for metabolism (Blomhoff *et al.*, 1992).

The above considerations support the conclusion that when retinol is administered early during incubation of the chick or quail embryo, this allows the maximal amount of time for the metabolic reactions to take place leading to conversion to retinoic acid. There is evidence that the normal chick embryo limb bud can convert exogenous retinol to retinoic acid (Thaller and Eichele, 1988). It was also demonstrated that the endogenous level of retinol in the limb bud is 20-25 fold that of retinoic acid, which suggests that retinol could be a source of retinoic acid in this tissue (Thaller and Eichele, 1988). Since the affinity of retinol for CRABP is very low (Maden and Summerbell, 1986), retinol itself would have low activity for any vitamin A function involving CRABP. It is currently thought that vitamin A functions through retinoic acid receptors. This process involves retinoic acid transport to the nucleus via CRABP where the retinoic acid binds to its specific nuclear receptor, RAR; the ligand-receptor interaction results in gene activation (Blomhoff *et al.*, 1992).

Comparison of the biological activities of 0.1 and 1.0 μg of all-trans-, and di-dehydroretinol

The finding that retinol was much more active than di-dehydro-retinol, also called vitamin A_2, supports the view that the active form of vitamin A in this embryonic system is the more common form of retinol, also known as vitamin A_1. If di-dehydro-retinoic acid were the active form, as has been suggested (Thaller and Eichele, 1990), it seems logical that di-dehyroretinol would be at least as active as all-trans-retinol. The results presented here suggest that metabolism of di-dehyroretinol to some other active form is necessary since the activity of di-dehydroretinol was lower than that of retinol at the dose levels and time points studied.

Effect of citral on retinol activity

Citral did not significantly decrease the activity of retinol when injected into vitamin A-deficient eggs at either the beginning of incubation, when retinol is usually most active, or at 24 hours of incubation (Table 3). This is in contrast to several other studies of the effect of citral on retinol conversion to retinoic acid. However, differences in the methodology of those studies and the present experiments could account for the differences in results. In *in vivo* studies using mouse epidermis, citral was an effective inhibitor of retinol conversion to retinoic acid, but the citral was administered 15 - 30

minutes prior to the application of retinol (Connor, 1988; Connor and Smit, 1987). In the current study, a single solution containing both retinol and citral was used due to concern that injection of two separate solutions into the air sac of the egg could result in differential diffusion of the two compounds to the embryo. The application of citral prior to that of retinol could allow the citral to bind with enzymes, decreasing the conversion of retinol to retinoic acid to a greater extent than the concurrent application of retinol and citral as was used in the present study. Connor (1988) and Connor and Smit (1987) analyzed the results of enzymatic action on retinol after a short (½ - 1 hour) incubation period; the results are difficult to compare to the morphological changes in embryonic development which take place over a period of 1 - 3 days. Citral was found to inhibit the conversion of retinol to retinal by 43 - 67% compared to control values in *in vitro* experiments with rat liver microsomes (Shih and Hill, 1991). The completion of the conversion from retinal to retinoic acid was not reported in that study. Microsomal enzyme activity toward retinol was found only in liver and not in several other tissues examined. Furthermore, the possible role and physiological significance in normal cellular retinoid metabolism of the microsomal enzymatic conversion of retinol to retinal are unknown (Shih and Hill, 1991). *In vitro* studies of epithelial tissue cultures from vitamin A-sufficient chicks

demonstrated that citral alone produced effects similar to vitamin A-deficiency which could be ameliorated by the simultaneous use of citral and vitamin A (Aydelotte, 1963a, 1963b, 1963c). The intensity of the effects varied according to the relative concentrations of the two compounds as well as the sensitivity of the particular tissue to retinol (Aydelotte, 1963b, 1963c). None of the studies with citral included data regarding the metabolism of citral itself. It may be that, administered in a single dose, citral is subject to metabolic degradation processes, and that within the time of retinoid sensitivity for normal cardiovascular development in the embryo, conversion of retinol to retinoic acid could take place after citral was no longer active as an inhibitor. In the present study, response of the vitamin A-deficient quail embryo to retinol in experiments involving citral was lower than that to retinol observed in the first retinol experiments of this study (Table 2 and Table 3). This may have been due to a difference in the vehicles which were used. The vehicle for all of the egg injection experiments, except those using citral, was 95:5 saline:whole, homogenized vitamin A-deficient egg. However, because citral was not soluble in this vehicle, dimethyl sulfoxide (DMSO) was used as a carrier for both retinol and citral in the experiments involving citral; DMSO was used in previous studies of the effect of citral on retinol metabolism (Connor and Smit, 1987). This

change of vehicle may have been a factor in the lower activity of retinol (60% normal development of treated embryos, Table 3) compared to those experiments using retinol in the saline:vitamin A-deficient egg vehicle (97% normal development, Table 2). In earlier studies using the injection technique for administering retinoids to vitamin A-deficient chick embryos, Thompson JN *et al.*, (1969) used saline with Tween or an oil based vehicle; the highest level of normal cardiovascular development in response to retinol reported in those studies was 74%. It seems reasonable that some of the variation in results may be due to the influence of the vehicle. The saline:vitamin A-deficient egg vehicle used for the majority of the experiments in the present study was chosen to provide albumen and phospholipid as physiological carriers for the retinoids, which are lipid soluble and which can non-specifically bind serum albumin (Goodman, 1973; Smith *et al.*, 1973). More extensive dose-response studies should be conducted to establish whether citral inhibits retinol activity in the vitamin A-deficient quail model to determine if conversion of retinol to retinoic acid is obligatory for normal cardiovascular development.

Embryotoxicity of all-trans-retinoic acid

The high biological activity of retinoic acid is associated with high embryotoxicity (Creech-Kraft *et al.*, 1991). In their pioneering studies, Thompson JN *et al.*

(1969) observed that retinoic acid was toxic to 20% of chick embryos when injected into chicken eggs at a level of 0.1 μg/egg; toxicity increased to 40% at a dose of 0.5 μg, to 66% with 1.0 μg and to 100% with 5 μg. The results of the present study are similar to that obtained by Thompson JN *et al.* (1969). Toxicity ranged from 8.3% to 100% in embryos treated with all-trans-retinoic acid in doses of 0.2 μg and higher. The wide range of toxicity at different dose levels and at the different times of administration which were used in this study (0 and 24 hours of incubation) may be related to the variability in placement of the injected solution in the air sac of the egg relative to the actual position of the embryo.

Time response of all-trans-retinoic acid

The major development of the avian cardiovascular system takes place during 20-30 hours of incubation (Patten, 1951), the time "window" when injection of retinoic acid is active in preventing the vitamin A-deficiency associated abnormality of cardiovascular development. The primordial cells of the cardiovascular system are determined by 24 hours of incubation and the paired heart tubes are present soon afterward. By 30 hours of incubation there is a single heart tube and by 33 - 36 hours of incubation the omphalomesenteric veins are extending toward the developing extra-embryonic vascular system (Patten, 1951). In order for a retinoid to directly

affect the morphological development of the heart and vasculature, its action must come within this time period. Heine *et al.* (1984) identified failure of development of the omphalomesenteric veins as the first abnormality of cardiogenesis in the vitamin A-deficient quail embryo. It is therefore logical to assume that the responsiveness of vitamin A-deficient embryos to retinoic acid during this period is associated with a specific role of retinoic acid in cardiovascular development of the quail embryo. The percent of embryos with normal development was similar (and not statistically different) when retinoic acid was given at 0 hours of incubation or within the time period of 24 - 28 hours of incubation. However, retinoic acid given at 14 - 18 hours of incubation resulted in a significantly lower percentage of embryos with normal development. A possible explanation for the decrease in apparent biological activity of retinoic acid when given at 14 - 18 hours of incubation may be the presence at this time of enzymes capable of degrading retinoic acid to less active or inactive forms. In addition, it may be that some components necessary for biological activity of retinoic acid, such as the cellular binding protein or nuclear receptors, are not yet present. Thus the retinoic acid could be degraded without initiating the sequence of events which results in the morphological changes leading to normal cardiovascular development, and which only take place if retinoic acid is administered at 24 - 28 hours of

incubation. The higher biological activity of retinoic acid when given at 0 hours of incubation may be due to a related but slightly different sequence of events. If the enzymes which degrade retinoic acid are not present until 14-18 hours of incubation, there would be time for retinoic acid administered at 0 hours of incubation to bind to other proteins or compounds which could serve to protect it from degradation when those enzymes appear, but still allow the usual metabolism of retinoic acid once the specific binding proteins and nuclear receptors are present, after 24 hours of incubation.

In the present study, a time "window" of 24-28 hours of incubation was observed during which injected retinoids elicited the highest level of morphological response for a normal cardiovascular development of the vitamin A-deficient embryo. There are several inherent features of the injection technique used in this study which may influence the width of this window of retinoid responsiveness. There is variation in exact stage of development of the embryos from eggs which have been incubated the same length of time and are, therefore, within the same treatment time group. This may be due to several factors. There is natural variation in the state of development between embryos incubated for the same length of time. In addition, the eggs were collected only once daily; some development could take place before the eggs were collected and placed in cool storage, causing

differences in the embryonic stage of development between eggs laid earlier or later in the day. Because the embryos were treated in ovo, it was not possible to know the exact stage of development of any given embryo. If the retinoid could be applied to embryos of an exactly known stage of development, it might be possible to more narrowly define the time of retinoid requirement.

Another source of variability in apparent biological activity of the retinoids may be the influence of the mode of administration. The retinoid solution was injected into the air sac of the egg; the exact location of the embryo relative to the site of injection could not be precisely determined. The retinoid solution must diffuse from the injection site through both a thin layer of albumen and the vitellin membrane which cover the embryo. The vitellin membrane is permeable to water and to a variety of molecules including amino acids and large proteins; however, the precise role of the membrane in the transport of molecules has not been determined (Burley and Vadehra, 1989). The time required for the retinoid solution to reach the embryo and the exact amount of the retinoid available to the embryo were not determined.

Finally, it is important to note that the embryo changes very rapidly in this period of early embryogenesis. During the time of retinoid effect on embryonic development examined in the present study (0-48 hours), the embryo obtains nutrients by diffusion (Patten, 1951). Both

retinol and its esters are present in the yolk of normal eggs (Moore, 1957). Retinol is bound to its transport protein, retinol-binding-protein (RBP); thus retinol from the egg yolk would be available to cells of the embryo in a manner similar to that of the adult animal in which retinol is transported in plasma bound to RBP. During early development injected retinoids would most likely also be available to the embryo through diffusion, but specific transport mechanisms may also develop which could alter the availability of a retinoid to the embryo.

The problems discussed above are inherent in the model and may account for the variability in responses obtained in the present study, as well as for the results of Thompson JN *et al.* (1969) who found that, in some embryos, retinol was active when administered even later than 40 hours of incubation. Similarly, in the present study some retinoids demonstrated biological activity in a small percentage of embryos when administered at 36 hours of incubation.

Mechanism of vitamin A activity

The proposed mechanism of vitamin A action involves the recognition of the retinol-binding protein complex by receptors on the cell surface membrane (Wolf, 1984). Retinol, but not the binding protein, is transferred into the cytoplasm where it is bound to cellular binding protein (Sporn *et al.*, 1984). Within the cell, retinol may be

converted to various metabolic forms of vitamin A including retinoic acid, thought to be the active form of vitamin A for many functions (Wolf, 1984). Similar metabolic conversion of retinol to retinoic acid takes place in the embryonic limb bud (Thaller and Eichele, 1987, 1988).

In the cytosol, retinoic acid is bound to a specific cellular retinoic acid binding protein (CRABP) which is thought to transport retinoic acid to the cell nucleus for interaction with nuclear receptors (Ross, 1991). Several researchers have found CRABP to be present in embryonic chick tissues (Maden and Summerbell, 1986, Maden *et al.*, 1988; Singh, 1990). CRABP was present in chick limb buds of embryos of stage 20-35 (3-9 days of incubation), with the highest amount of CRABP present during stage 24 (4 days of incubation) of development (Maden and Summerbell, 1986, Maden *et al.*,1988). The level of CRABP in the limb bud cells during development appears to be strictly regulated and could play a regulatory role in establishing the effective concentration of retinoic acid reaching the nucleus (Maden *et al.*, 1988). Only 4% of the available CRABP needs to be bound to retinoic acid to have a morphological effect in the limb bud (Maden and Summerbell, 1986), i.e. a very low level of retinoic acid is required for action. CRABP has also been found in other chick embryonic tissues, including the heart (Singh, 1990). Since the present study was conducted in embryos of earlier stages (approximately stages 3-18 or 12-68 hours of

incubation), some of the differences in the results may be explained on the basis of different levels of CRABP.

The beta-retinoic acid receptor (RAR-β) is present in chick embryos at about 24 hours of incubation (Smith and Eichele, 1991). Exogenous retinoic acid induces this receptor in the chick limb bud (Noji *et al.*, 1991), the gene for which contains a retinoic acid response element in its promoter region (deThe *et al.*, 1990). Studies with transgenic mouse embryos demonstrated retinoic acid-inducible reporter gene expression in several tissues including the heart tube (Balkan *et al.*, 1992). These studies indicate that retinoic acid affects the embryo via gene action during the time of cardiogenesis and supports the conclusions reached by Thompson JN *et al.* (1969) and Heine *et al.* (1984). The responsiveness of vitamin A-deficient embryos to exogenous retinoic acid given between 24 and 28 hours of incubation parallels the time of early development of the cardiovascular system in the normal embryo and the time of demonstrated gene response to retinoic acid (Noji, 1991).

Retinoic acid administration after 30 hours of incubation was ineffective in preventing the vitamin A-deficiency-related abnormality (Table 7). In the normal embryo this time is critical for the development of the omphalomesenteric veins, which are established by 33 hours of incubation (Patten, 1951). Failure of these veins to develop is the first morphological abnormality of the

vitamin A-deficient embryo, indicating that the time of vitamin A action in normal cardiogenesis is prior to 33 hours of incubation. In the present study, retinoids were tested at 36 hours of incubation and later to determine whether there would be any subsequent development of heart structures or whether any additional development would occur such as branching of vessels, and to ascertain a control point beyond which the retinoids were no longer active. While the work of Thompson JN *et al.* (1969) demonstrated activity of retinol in the chick embryo, even when administered later than 40 hours of incubation, its activity decreased with the length of prior incubation. In the present study, retinoids applied after 33 hours of incubation were inactive in regard to cardiovascular development in the quail embryo; the gross anatomical appearance of vitamin A-deficient embryos treated with retinoids after 33 hours of incubation was not different from that of the vitamin A-deficient embryos that did not receive any retinoids.

Comparison of the biological activities of 0.1 μg of all-trans-, 13-cis-, and di-dehydro-retinoic acids

While the three forms of retinoic acid tested in this study, all-trans-, 13-cis-, and 3,4-di-dehydro-retinoic acids, all prevented the vitamin A-deficiency-associated abnormality of the cardiovascular system of the quail embryo, these different forms were not equally active.

The biological activity of 13-cis-retinoic acid in cardiovascular development of embryos in the present study was only 58% of that of all-trans-retinoic acid when administered at the beginning of incubation, and 14.5% of that when administered at 24 hours of incubation. These results parallel those of Sporn *et al.* (1985) who similarly found a greater decrease in activity of 13-cis isomers of two other retinoids compared to their all-trans forms in similar studies with vitamin A-deficient quail embryos. While 3.0 μg of all-trans-methyl retinoate was active in 70% of treated embryos, the same amount of the 13-cis isomer was active in only 4% of embryos. Their study demonstrated a dose response: increasing 13-cis-methyl retinoate by 3-fold to 9 μg resulted in an increase of normal embryos to 18%; however, this was still only 26% of the percentage of embryos with normal development when treated with 3.0 μg of all-trans-methyl retinoate. Similarly, at a 6 μg dose, 13-cis-ethylretinamide was 25% as active as its all-trans form, while doses of 18 μg of the two isomers of ethylretinamide were approximately equal in enabling normal cardiovascular development (in 27% and 24% of embryos, respectively). The researchers did not report toxicity of these doses of the retinoids, although the amounts necessary to induce normal development were higher than the level at which retinoic acid produced toxic effects in embryos in the present study and that of Thompson JN *et al.* (1969), i.e. <1.0 μg of retinoic acid.

The biological activity of 13-cis-retinoic acid has been attributed to its isomerization to all-trans-retinoic acid (Creech-Kraft *et al.*, 1989a, 1991; Lee *et al.*, 1991). In the present study 13-cis-retinoic acid was most active when injected at the beginning of incubation, and less active when injected at 24 hours of incubation. This observation suggests that isomerization of 13-cis-retinoic acid to all-trans-retinoic acid may be necessary for activity and that induction, or presence of, an isomerase may be required. Isomerization of 13-cis-retinoic acid to all-trans-retinoic acid has been demonstrated in other systems. Treatment of a pregnant women with 13-cis-retinoic acid during early gestation resulted in a level of all-trans-retinoic acid 2-fold higher than that of 13-cis-retinoic acid in the human embryo at the limb bud stage of development (Creech-Kraft *et al.*, 1989b). In studies of retinoic acid toxicity in rat embryos, all-trans-retinoic acid was 3-10 times more toxic than 13-cis-retinoic acid, depending on the dose level; the researchers concluded that the toxic effect of 13-cis-retinoic acid was due to isomerization to all-trans-retinoic acid (Creech-Kraft *et al.*, 1991; Klug *et al.*, 1989; Lee *et al.*, 1991). These toxicity studies involved mammalian species, therefore the site of isomerization could be either maternal or embryonic. However, these data are consistent with our conclusions that 13-cis-retinoic acid itself is less active than all-trans-retinoic acid in the quail embryo. Maden

and Summerbell (1986) reported the binding affinity of 13-cis-retinoic acid for CRABP to be 33% that of all-trans-retinoic acid. Trown *et al.* (1980) reported the binding affinity of 13-cis-retinoic acid to CRABP to be 60% that of the all-trans form but the biological activity to be only 25% that of all-trans-retinoic acid in other assays. Those authors suggest that the apparent discrepancy between binding affinity to CRABP and biological activity might be due to insufficient concentrations of 13-cis-retinoic acid in the target cells as a consequence of such factors as cell uptake and metabolism. A lower binding affinity for CRABP provides one possible explanation for the lower activity of the 13-cis isomer of retinoic acid if the activity of retinoic acid requires binding to CRABP as is currently believed (Ross, 1991).

In the present study, di-dehydro-retinoic acid had approximately ½ the biological activity of all-trans-retinoic acid for preventing the vitamin A-deficiency-associated cardiovascular abnormality (Table 8). This contrasts to the results reported by Thaller and Eichele (1990) who found di-dehydro-retinoic acid to be equal in activity to all-trans-retinoic acid in the chick limb bud, and suggested that di-dehydro-retinoic acid may be the active form of retinoid in that system. However, an important difference between that study and the present study is the fact that the quail embryo in the present

study was vitamin A-deficient. The exogenous retinoic acid, all-trans-, or di-dehydro-, was the only retinoid available to initiate all vitamin A-dependent functions involved in normal cardiovascular development. In the chick limb bud, vitamin A is already present and the endogenous active form can initiate all vitamin A-dependent functions. The exogenous all-trans- or di-dehydro-retinoic acid is in addition to the normal requirement. It may be that only a small amount of additional retinoic acid is needed to produce the duplication of digits in the chick limb bud model which already has sufficient endogenous vitamin A for normal development. Exogenous all-trans- and di-dehydro- retinoic acids were equally active in causing digit duplication, suggesting equal biological activity of these two forms of retinoic acid. However, the vitamin A-deficient quail embryo model used in the present study would give a more accurate assessment of biological activity because a single form of retinoid is present, compared to the chick limb bud model in which an unknown but sufficient amount of the endogenously active vitamin A is present in addition to the added retinoic acid.

There may also be differences between the cells of the cardiovascular system that are retinoid-responsive during stages 3-18 of embryonic development, and the cells of the limb bud which respond to retinoids at slightly later stages.

In the present study, all-trans-retinoic acid was the most active form of the retinoic acids, a finding that is consistent with the hypothesis that the all-trans form of retinoic acid is the most active form. All-trans-retinoic acid has the highest affinity for CRABP; Trown *et al.* (1980) found that only compounds with a high binding affinity to CRABP also had a high biological activity.

Comparison of the biological activities of 0.1 μg of various retinoids

It is important to note in Figure 5 that retinol is less active than all-trans-retinoic acid at this 0.1 μg level when administered at 24 hours of incubation. This is indirect evidence for the hypothesis that retinoic acid is the active form and that retinol needs some processing to be active. The finding that retinol is more active when given in the larger doses, and at earlier times indicates that time is required for some processes related to vitamin A activity to take place. These could include conversion of retinol to retinoic acid, and induction of enzymes, binding proteins and receptors; when enough retinoic acid can be synthesized from retinol, a physiological response occurs and cardiovascular development is normal. However, when the amount of retinol is limiting, or the retinol is administered later, these processes are not accomplished and the overall result is that retinol is not as active as retinoic acid.

Single, larger doses of retinol are also more active in growth than single doses of retinoic acid (Zile and DeLuca, 1968)). When the amount of retinoic acid is comparable to that of retinol, but the retinoic acid is given in small, multiple doses to compensate for its faster metabolism and lack of storage, retinoic acid is as active as retinol (Zile and DeLuca, 1968). When given as a single, pulse dose, the full amount of the retinoic acid is only available for a short time since retinoic acid is rapidly metabolized to inactive forms. This interpretation is compatible with the observation that the ½-life of exogenous retinoic acid in the chick limb bud is 20 minutes (Eichele, 1989).

It is apparent from the above discussion that various factors account for the relatively wide time window of response to retinoic acid, as well as for the fact that maximal biological activity was not obtained in the vitamin A-deficient quail embryo system. However, the fact that normal development was achieved in some embryos with only 0.025 μg of retinoic acid indicates that the embryo is very sensitive to retinoic acid.

Metabolism of [^{3}H]-retinol by the quail embryo

If retinoic acid is synthesized by the embryo from retinol, the administration of a pulse dose of radiolabeled retinol would result in the appearance of radiolabeled retinoic acid in embryonic tissues. However, in the

present study, all-trans-retinoic acid was not consistently found as a metabolite of retinol in the HPLC fractions corresponding to the retinoic acids. The primary factor for this result was probably the very small size of the embryo at this stage of development. Other researchers, using chick limb buds from stage 20 embryos, pooled as many as 2000 for one sample to detect retinoic acids (Thaller and Eichele, 1990). The fact that a few samples in the present study demonstrated radioactivity coeluting with all-trans-, 13-cis-, and di-dehydro-retinoic acids, suggests that combining a larger number of embryos for one sample would enable quantitation of the metabolic conversion of retinol to retinoic acid. The 3-hour uptake of 0.67% of the pulse dose of retinol observed in this study is similar to the 0.24 - 0.56% of available vitamin A in the 3-day chick embryo calculated from the embryo vitamin A content and the decrease in egg yolk vitamin A content (Romanoff, 1967). The concentration of vitamin A in the quail embryo following the 3-hour incubation with [^{3}H]-retinol was 12.6 nM. This is 2-3 times higher than the value calculated for the chick embryo (4-6.5 nM during days 3-8) (Romanoff, 1967), but the level in the quail embryo may not represent an equilibrium level. The concentration of endogenous retinol measured in the limb bud of 3-day chick embryos was 25 nM (Thaller and Eichele, 1987). The levels of retinoids found in tissues following the pulse dose of [^{3}H]-retinol used in this study are

within the physiological range reported by others, and suggest that the mechanisms for uptake of the labeled retinol could be similar to those for uptake of endogenous retinol from the yolk.

Di-dehydroretinol was identified as a metabolite of retinol in the embryo; this finding is consistent with the data obtained by others who demonstrated the conversion of retinol to di-dehydroretinol in the limb bud and neural tube of chick embryos (Wagner *et al,* 1990). The authors proposed that this conversion was the first step in the formation of di-dehydro-retinoic acid as the ultimate active form of vitamin A in the chick embryo limb bud and neural tube (Thaller and Eichele, 1990; Wagner *et al.*, 1990). This hypothesis was based on the finding that di-dehydro-retinoic acid was as active as all-trans-retinoic acid in directing pattern formation of the limb bud and was present at a 6-fold higher level than all-trans-retinoic acid (Thaller and Eichele, 1990). However, in the present study with the quail embryo during the early stages of cardiovascular development, neither di-dehydroretinol nor di-dehydro-retinoic acid, injected into the quail egg, was as active as their respective all-trans forms in inducing normal cardiovascular development. It is possible that different forms of retinoids serve different functions, determined by developmental stage, cell type and tissue function.

The high concentration of di-dehydroretinol relative to retinol within the embryo (Table 10, 11) suggests that either the embryo converts retinol to di-dehyroretinol, or accumulates the di-dehydro form which probably comes from the extraembryonic area vasculosa and/or area opaca. The accumulation in the embryo could be the result of slow degradation and/or removal of the di-dehydroretinol. The enzymes necessary for the conversion of retinol to di-dehyroretinol have not been identified (Torma *et al.*, 1991); virtually nothing is known about the function or metabolism of di-dehydroretinol (Torma and Vahlquist, 1991).

Citral has been shown to inhibit the conversion of retinol to di-dehydroretinol in epidermal cells; the reaction occurred predominately in undifferentiated skin cell types while the conversion was slow in differentiated cells (Torma and Vahlquist, 1991). Furthermore, the authors showed that di-dehyroretinol was present in these cells at levels of 8-17% that of retinol (Torma and Vahlquist, 1991).

The significance of di-dehydroretinol in the avian embryo is not clear. While it may be a precursor to di-dehydro-retinoic acid in the limb bud and the neural tube of the chick embryo (Thaller and Eichele, 1990; Wagner *et al.*, 1990), it has not been conclusively demonstrated that di-dehydro-retinoic acid is the active form in these embryonic tissues. The finding of the present study of

significant amounts of di-dehydroretinol in the embryo and extraembryonic tissues, can not be explained at this time. In the present study, the low biological activity of di-dehydro-retinoic acid in cardiovascular development suggests that the metabolism of retinol and retinoic acid to their di-dehydro forms may be a mechanism to inactivate or remove the all-trans-retinoic acid that is present in the embryo and its membranes in amounts above those required for physiological functions.

Conclusions

The hypothesis of this study was that retinoic acid is active in preventing the vitamin A-deficiency-associated abnormalities of the cardiovascular system in the vitamin A-deficient quail embryo. It is clear that retinoic acid can be reintroduced into the embryonic environment at non-toxic levels which induce normal development. The data also suggest that the all-trans- form of retinoic acid is the most active natural form of vitamin A in this model system and that retinol is converted to retinoic acid.

A time window of retinoid responsiveness was demonstrated, although it is likely that conditions of the experimental model and the techniques used influenced the width of that time period of response. Likewise, the small sample size for analysis of metabolism of retinol to retinoic acid was a major factor in the inconclusive evidence that this conversion takes place in the embryo.

Future Research

Future research could expand several areas of the findings of the present study. In order to confirm the physiological requirement of endogenous retinoic acid for embryonic cardiovascular development, it will be important to demonstrate that retinol can be converted to all-trans-retinoic acid by the embryo. Identification of retinoic acid in the vitamin A-deficient embryo after administration of other retinoids will help elucidate the pattern of metabolic conversion of these retinoids. The results from the present work are consistent with the hypothesis that retinoic acid is the active form of vitamin A in the avian embryo and that other retinoids exert their activity by being converted to all-trans-retinoic acid. Although it could not be demonstrated that the conversion of retinol to retinoic acid takes place in the embryo, this was most likely due to the small numbers of embryos available to pool for analysis. In future experiments a larger number of embryos should be pooled in order to detect and quantitate the physiological amounts of retinoids in embryonic tissues, particularly retinoic acid and its metabolites and to assess the conversion of retinol and other retinoids to retinoic acid.

Localization of retinoic acid within embryonic tissues by use of an monoclonal antibody will also help to establish the role of retinoic acid in embryogenesis. Localization of retinoic acid in the normal embryo at

specific sites and at certain time points during development will provide information regarding both the spatial distribution of retinoic acid and the time frame when retinoic acid is first needed for such subsequent events as receptor induction. Addition of retinoids to vitamin A-deficient embryos and determination of the pattern of distribution of retinoic acid over time will help to identify the specific cell types and tissues which require retinoic acid during various stages of development.

The time window of retinoid sensitivity shown in this study was influenced by use of the injection technique with intact eggs in which the exact stage of embryonic development and location of the embryo were not known. The culture technique use for the [^{3}H]-retinol metabolism in this study also has potential for future experiments to measure the time window of retinoid sensitivity more precisely because it would be possible to select embryos of similar developmental stage for treatment. This could decrease the width of the time period identified in this study as the time of vitamin A requirement for normal cardiovascular development.

The presence of retinoic acid receptors during embryogenesis suggests that the mode of vitamin A action is through the effect of retinoic acid on its nuclear receptors which induce gene expression. Another area of research is identification of the spatial and temporal pattern of retinoic acid receptor expression during the

period of embryonic development when vitamin A is active in cardiogenesis. The vitamin A-deficient quail model offers unique opportunities for this work. Retinoic acid receptor-β is known to be inducible by retinoic acid. In the normal embryo, receptor induction takes place naturally making measurement of induction difficult since it must be assessed by the increase above the normal level which can be elicited by additional, exogenous retinoids. With the vitamin A-deficient model used in the present study it is possible to define the pattern of receptor induction because the endogenous receptor level will initially be low if retinoic acid is required for its induction. Addition of low levels of exogenous retinoic acid can approximate the events normally regulated by endogenous retinoids.

APPENDICES

APPENDIX A

Composition of Vitamin A-deficient diets with methyl retinoate for Japanese quail

Vitamin A Deficient Starter/Grower Diet for Japanese Quail
(with Methyl Retinoate)

TD 83079

		g/Kg
Soybean Meal (47.5%)		505.0
DL-Methionine		3.5
L-Lysine HCl		2.5
Dextrose, monohydrate		383.4144
Soybean Oil		40.0
Fiber (cellulose)		10.0
Mineral Mix (TD 83075)		44.856
Calcium Phosphate, dibasic	$CaHPO_4 \cdot 2H_2O$	4.5
Calcium Carbonate	$CaCO_3$	1.5
Manganese Sulfate	$MnSO_4 \cdot H_2O$	0.123
Choline Dihydrogen Citrate		4.2
Thiamin HCl		0.025
Riboflavin		0.015
Calcium Pantothenate		0.03
Niacin		0.08
Pyridoxine HCl		0.015
Vitamin B_{12} (0.1% trituration in mannitol)		0.03
Folic Acid		0.005
Biotin		0.0006
Menadione Sodium Bisulfite Complex		0.016
DL-alpha-Tocopheryl Acetate (1000 U/g)		0.05
Vitamin D_3 in oil (100,000 U/g)		0.03
BHT (antioxidant)		0.1
Methyl Retinoate (supplied by customer)		0.01

This formula is designed to provide the nutrients required by Japanese Quail (except vitamin A) 0-6 weeks of age.

Vitamin A Deficient Breeder Diet for Japanese Quail
(with Methyl Retinoate)

TD 83080

		g/Kg
Soybean Meal (47.5%)		464.0
DL-Methionine		3.5
L-Lysine HCl		1.0
Dextrose, monohydrate		380.3344
Soybean Oil		40.0
Mineral Mix (TD 83075)		44.856
Calcium Phosphate, dibasic	$CaHPO_4 \cdot 2H_2O$	13.9
Calcium Carbonate	$CaCO_3$	48.7
Manganese Sulfate	$MnSO_4 \cdot H_2O$	0.123
Choline Dihydrogen Citrate		3.2
Thiamin HCl		0.025
Riboflavin		0.015
Calcium Pantothenate		0.03
Niacin		0.06
Pyridoxine HCl		0.015
Vitamin B_{12} (0.1% trituration in mannitol)		0.03
Folic Acid		0.005
Biotin		0.0006
Menadione Sodium Bisulfite Complex		0.016
DL-alpha-Tocopheryl Acetate (1000 U/g)		0.05
Vitamin D_3 in oil (100,000 U/g)		0.03
BHT (antioxidant)		0.1
Methyl Retinoate (supplied by customer)		0.01

This formula is designed to provide the nutrients required (except vitamin A) by Japanese quail breeding hens.

Mineral Mix

TD 83075

		g/Kg
Calcium Phosphate, dibasic	$CaHPO_4 \cdot 2H_2O$	557.339
Calcium Carbonate	$CaCO_3$	142.6788
Sodium Chloride	NaCl	156.0549
Potassium Citrate, monohydrate		60.1926
Magnesium Sulfate	$MgSO_4$	55.0651
Ferric Citrate		13.3761
Manganese Sulfate	$MnSO_4 \cdot H_2O$	7.5798
Zinc Carbonate		5.3505
Cupric Sulfate	$CuSO_4 \cdot 5H_2O$	1.4045
Chromium Potassium Sulfate	$CrK(SO_4)_2 \cdot 12H_2O$	0.6465
Potassium Iodate	KIO_3	0.2007
Sodium Molybdate	$Na_2MoO_4 \cdot 2H_2O$	0.0557
Cobalt Chloride	$CoCl_2 \cdot 6H_2O$	0.0446
Sodium Selenite	$Na_2SeO_3 \cdot 5H_2O$	0.0112

This formula was designed for use in TD 83077. It also can be used with other diets in this series when calcium and phosphorus are increased to appropriate levels.

APPENDIX B

Retinoid equivalents in μg and moles

	all-trans-retinol (mw=286)[1]	di-dehydro-retinol (mw=284)	all-trans- and 13-cis-retinoid acid (mw=300)	di-dehydro retinoic acid (mw=298)
μg	moles			
0.025	0.09	0.09	0.08	0.08
0.050	0.18	0.18	0.17	0.17
0.100	0.35	0.35	0.33	0.34
0.500	1.75	1.76	1.67	1.68
0.750	2.62	2.64	2.50	2.52
1.000	3.50	3.52	3.33	3.36
1.125	3.93	3.96	3.75	3.78
1.500	5.25	5.28	5.00	5.03

[1] mw = molecular weight

APPENDIX C

Effect of retinol administered at 40 hours of incubation on the cardiovascular development of the vitamin A-deficient quail embryo

		Embryonic development at 65-75 hours of incubation		
		% of embryos classified as[1]		
μg of retinol	n[2]	Normal	Vitamin A deficient	Abnormal[3]
0.1	20	0	100	0
0.5	20	0	100	0
1.0	17	0	100	0
2.0	20	0	100	0

[1]Experimental details and classification of embryonic development are described in **Material and Methods**
[2]Number of embryos
[3]Unrelated to vitamin A-deficiency

APPENDIX D

Effect of 0.75-1.5 μg of all-trans-retinoic acid on quail embryos

Effect of retinoic acid on normal quail embryos

μg of retinoic acid	retinoic acid injected at 0 h			retinoic acid injected at 24 h		
	% of embryos classified as:[1]					
	n[2]	Normal	Abnormal[3] or dead	n	Normal	Abnormal or dead
0.75	14	64.3	35.7	36	91.7	8.3
1.125	-	-	-	9	66.7	33.3
1.5	-	-	-	3	0	100

Effect of retinoic acid on vitamin A-deficient quail embryos

μg of retinoic acid	Retinoic acid injected at 0 h				Retinoic acid injected at 24 h			
	% of embryos classified as:[1]							
	n[2]	Normal	Vitamin A-deficient	Abnormal[3] or dead	n	Normal	Vitamin A-deficient	Abnormal or dead
0.75	8	0	100	0	33	39.4	51.5	9.1
1.125	-	-	-	-	6	33.3	16.7	50
1.5	-	-	-	-	4	0	50	50

[1]Experimental details and classification of embryonic development are described in Materials and Methods; embryos were examined after 65-75 hours of incubation
[2]Number of embryos
[3]Abnormalities unrelated to vitamin A-deficiency

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