THE EVALUATION OF THE HUMAN HEALTH ASPECTS OF USING 25-HYDROXYVITAMIN D3 AS A BROILER POULTRY FEED INGREDIENT

November 1994

Prepared for

Amoco BioProducts Corporation
Amoco Research Center
Post Office Box 3011
Naperville, Illinois 60566-7011

LIFE SCIENCES RESEARCH OFFICE
FEDERATION OF AMERICAN SOCIETIES FOR EXPERIMENTAL BIOLOGY
9650 Rockville Pike
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Prepared by
An ad hoc Expert Panel
of the
Life Sciences Research Office

and edited by
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Notice: After this report was published, a miscalculation was found in the computation of the concentration of 25(OH)D₃ used for the estimation of consumer exposure. This copy of the report contains the updated information on consumer exposure. We have also included some additional data concerning occurrence of hypercalcemia in clinical trials of the therapeutic use of 25(OH)D₃.
FOREWORD

The Life Sciences Research Office (LSRO), Federation of American Societies for Experimental Biology (FASEB), provides scientific assessments of topics in the biomedical sciences. Reports are based upon literature reviews and the scientific opinions of knowledgeable investigators engaged in work in specific areas of biology and medicine.

This report is one of a continuing series concerning the health aspects of food ingredients that may be Generally Recognized as Safe (GRAS) food substances. The Federation recognizes that the safety of GRAS substances is of national significance, and that its resources are particularly suited to marshalling the opinions of knowledgeable scientists to assist in these evaluations.

The LSRO convened an ad hoc Expert Panel to conduct an evaluation of the health aspects of using 25-hydroxyvitamin D₃ as a poultry (broiler) feed ingredient. This report was prepared for Amoco BioProducts Corporation, Amoco Research Center, Naperville, Illinois by the ad hoc Expert Panel and edited by Kenneth D. Fisher, Ph.D., formerly Director, LSRO, and Janet H. Waters, M.S., R.D., Staff Scientist, LSRO, in accordance with a contract between Amoco BioProducts Corporation and the LSRO, FASEB. Scientists selected as members of the Panel were chosen for their scientific qualifications, experience, and judgment, with due consideration for balance and breadth in appropriate professional disciplines. Members of the Panel and others who assisted in the preparation of this report are listed in Chapter VII.

In particular, the Panel and the LSRO acknowledge the cooperation of scientific staff of Amoco BioProducts Corporation who provided information, data, and studies on 25-hydroxyvitamin D₃. Specifically, the Panel and the LSRO thank James G. Yarger, Ph.D., Manager, Regulatory Affairs; Rick Gray, Ph.D., Consultant, Animal Nutrition Venture; Leonard Stark, Ph.D., General Manager, Animal Nutrition Venture; and Don Schmitt, Coordinator, Regulatory Affairs, for their efforts in collating available materials and in providing background information necessary for the Expert Panel’s deliberations and evaluation.

The Expert Panel met in April, June, and October, 1994 to obtain background information, identify and analyze pertinent literature and experimental studies, develop drafts of the report, and reach an opinion as to whether the available information and data on the health effects of 25-hydroxyvitamin D₃ are sufficient to meet the regulatory requirements of safety as a GRAS feed ingredient. The Expert Panel’s evaluation was made independently of Amoco BioProducts Corporation or of any other governmental or nongovernmental groups. The Expert Panel and the LSRO accept responsibility for the study conclusions and the accuracy of the report.

This final report was reviewed and approved by the LSRO Advisory Committee (which consists of representatives of each constituent Society of FASEB) under authority delegated by the Federation Board. Upon completion of these review procedures, the report was approved and transmitted to the Amoco BioProducts Corporation by the Executive Director, FASEB.

While this is a report of the Federation of American Societies for Experimental Biology, it does not necessarily reflect the opinion of each individual member of the constituent Societies.

November 30, 1994
Date

Sue Ann Anderson, Ph.D.
Acting Director
Life Sciences Research Office
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I. INTRODUCTION

This report concerns the potential human health aspects of using 25-hydroxyvitamin D₃ (25-hydroxycholecalciferol) as an ingredient in poultry feed, specifically for broiler chickens. The report is based on information contained in a compilation of pertinent scientific literature, a product briefing book prepared by Amoco BioProducts Corporation, and a series of special studies provided to the LSRO by Amoco BioProducts Corporation (Amoco BioProducts Corporation, 1994).

To ensure completeness and accuracy of the evaluation of safety of 25(OH)D₂, this information has been supplemented by the use of generally available scientific and statistical reference sources and compendia; new, relevant books and reviews, and the literature citations contained in them; current literature citations obtained through computer retrieval systems of the National Library of Medicine and the National Agricultural Library; relevant data in the files of the LSRO; relevant regulatory documents of the Food and Drug Administration (FDA); and, the combined knowledge and experience of members of the Expert Panel and the LSRO staff.

As indicated in the Food, Drug, and Cosmetic Act (21 USC 321 [s]), (U.S. Congress, 1992), generally recognized as safe (GRAS) substances are exempt from the premarketing clearance that is required for food and feed additives. This Act and the Code of Federal Regulations (21 CFR 170.3 and 170.30) (Office of the Federal Register, 1994a,b) state that GRAS can mean general recognition of safety by experts qualified by scientific training and experience to evaluate the safety of substances on the basis of scientific data derived from published literature. These sections of the Code also indicate that expert judgment is to be based on evaluation of results of publicly available, credible toxicological testing, which may be corroborated by unpublished studies and data. Further, the Code specifies that expert judgment is to take into account reasonably anticipated patterns of consumption, cumulative effects in the diet, and safety factors appropriate for the utilization of animal experimentation data.

In regard to substances used in animal feed, the current Code of Federal Regulations (21 CFR 582.1) (Office of the Federal Register, 1994c) states that GRAS feed ingredients must be used for the purposes intended in accordance with good manufacturing or feeding practices. Good manufacturing or feeding practices are defined with the following restrictions:

1. The quantity of a substance added to animal food does not exceed the amount reasonably required to accomplish its intended physical, nutritional, or other technical effect in food.

2. The quantity of a substance that becomes a component of animal food as a result of its use in the manufacturing, processing, or packaging of food, and which is not intended to accomplish any physical or other technical effect in the food itself, shall be reduced to the extent reasonably possible.

3. The substance is of appropriate grade and is prepared and handled as a food ingredient. Upon request the Commissioner will offer an opinion, based on specifications and intended use, as to whether or not a particular grade or lot of the substance is of suitable purity for use in food and would generally be regarded as safe for the purpose intended, by experts qualified to evaluate its safety.

---

1 In this report, 25(OH)D₂ is the abbreviation used for 25-hydroxyvitamin D₂ (25-hydroxycholecalciferol). The term vitamin D₃ without a subscript, is used generically in this report to represent either or both vitamin D₃ or vitamin D₃ metabolites.
Currently, both vitamin D₃ and vitamin D₂ are listed as GRAS feed ingredients (21 CFR 582.5950 and 21 CFR 582.5953, respectively) (Office of the Federal Register, 1994d,e). Both are also listed as GRAS food ingredients (21 CFR 182.5950 and 21 CFR 182.5953, respectively) (Office of the Federal Register, 1994f,g). In all cases, the two forms of the vitamin are considered GRAS when used in accordance with good manufacturing practices. 25(OH)D₃ is not currently listed as a GRAS substance; however, it is marketed as a drug for human use (see page 45-46).

The FDA also specifies that persons seeking affirmation of GRAS status of substances that directly or indirectly become components of human food must submit a petition for GRAS affirmation that contains all relevant chemical, physical, and biological data related to the intended uses (21 CFR 170.35) (Office of the Federal Register, 1994h). These data must include a description of the physical and chemical properties of the substance; past, current, or intended use in foods; methods for detecting the substance in foods; information that supports safety and functionality; a statement attesting to the balance and representative nature of the submitted information; and, a statement affirming that non-clinical laboratory studies were conducted in compliance with Good Manufacturing Practice (GMP) regulations (Office of the Federal Register, 1994i). The petition must also include an environmental assessment of justification for exclusion. Finally, the FDA recognizes (21 CFR 170.30) (Office of the Federal Register, 1994b) that it is impossible to provide assurance that any substance is absolutely safe for human consumption.

The LSRO ad hoc Expert Panel reviewed and evaluated the available information on the safety of 25(OH)D₃ in full recognition of the foregoing provisions. In reaching its conclusion on safety, in accordance with FDA's guidelines, the Expert Panel relied primarily on directly related published data as well as on unpublished studies and other information and data. This report was prepared for use by Amoco BioProducts Corporation in submitting a GRAS affirmation petition to the FDA Commissioner on 25(OH)D₃ under the Federal Food, Drug, and Cosmetic Act. The Expert Panel anticipates that its conclusions would be reviewed if new information on the safety of the substance becomes available.
II. BACKGROUND INFORMATION

A. CHEMICAL IDENTITY

25-Hydroxyvitamin D₃ [25(OH)D₃], also called 25-hydroxycholecalciferol or calcifediol, is a normal metabolite found in vertebrate animal species that convert vitamin D₃ (cholecalciferol) to the metabolically active form 1α,25-dihydroxyvitamin D₃ (Bouillon et al., 1995; Combs, 1992; Norman, et al., 1979; Norman, 1990; Reichel et al., 1989). Chemically, the substance is 9,10-secocholesta-5,7,10(19)-trien-3β, 25-diol (CAS Registry No. 25631-40-7). The monohydrate form (C₂₇H₄₄O₂•H₂O) (CAS Registry No. 63283-36-3) has a molecular weight of 418.66 (Figure 1).

![Structure of 25(OH)D₃ monohydrate.](image)

Figure 1. Structure of 25(OH)D₃ monohydrate.
B. SYNTHESIS AND PRODUCTION

In vertebrate and other animals, the cholesterol precursor, 7-dehydrocholesterol (cholesta-5,7-dien-3β-ol), is photolytically converted to vitamin D₃. The yeast sterol, ergosterol (ergosta-5,7,22-trien-3β-ol), has the same 5,7-diene as cholesterol and is photolytically converted to vitamin D₃. Ergosterol is a cheap and convenient source of the delta 5,7-sterols, which may be used to generate vitamin D₃ for use in animal feed. However, ergosterol contains a C-22 unsaturation and C-24 methylation substituents on the side chain which are different from the cholesterol side chain present in vitamin D₃. As an alternative, and using conventional genetic and molecular biological manipulations, Amoco BioProducts Corporation has constructed a strain of Saccharomyces cerevisiae that produces cholesta-5,7,24-trien-3β-ol in high yield and without measurable ergosterol (European Patent 0486290A2; ATCC Designations #74027, #74090). The cholestatrienol is photolytically and chemically modified to form 25(OH)D₃.

1. Yeast strain development

Ergosterol is the normal endproduct for the sterol branch of the isoprenoid pathway in yeast, whereas cholesterol derivatives are normally very minor components in that organism. The two enzymes of ergosterol biosynthesis that introduce the side chain modifications of the yeast sterol are zymosterol-24-methyltransferase (CAS Registry No. 37287-07-1) and ergosta-5,7,24(28)-trien-22-dehydrogenase (CAS Registry No. 110183-45-4); the structural genes are ERG6 and ERG5, respectively. A major regulatory point in the control of precursors through the isoprenoid pathway is an early step in the pathway, the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) to mevalonate. This conversion step is feedback regulated and is mediated by two isozymes, HMG-CoA reductases (EC 1.1.1.34 and 1.1.1.68), whose structural genes are HMG1 and HMG2. Using a newly developed and patented process, Amoco BioProducts Corporation has substantially augmented the expression of HMG1 to effect a large increase in the amount of the reductase isozyme designated by the HMG1 gene, Hmg1p. The result is a major increase in the production of mevalonic acid and subsequent formation of steroids.

The Amoco Technology Company (ATC) yeast strain carrying the erg5,erg6 double mutations (genes inactivated) was constructed by crossing S. cerevisiae strain pol5 (mat α, erg5) to strain M610-12 Beta (mat α, erg5-5, ilv3, trp1, lys7) using classical yeast genetics. The diploids were sporulated and a haploid strain ATC04030MU (mat α, erg5, erg6-5, trp1) was obtained. This was crossed to strain DBY745 (mat α, ade1, ura3-52, leu2-100, leu2-112). The resulting diploid was sporulated to yield the haploid strain ATC0402MU (mat α, erg5, erg6-5, trp1). Strain ATC0402MU failed to produce ergosterol. A similar erg6 mutant produced no measurable ergosterol, the limits of detection being less than 10⁻¹¹ moles of ergosterol per 10⁸ cells (McCammon et al., 1984). Thus, deviating from the normal yeast sterol biosynthetic pathway at zymosterol, strain ATC0402MU produces cholesta-5,7,24-trien-3β-ol. The terminal steps in sterol synthesis in strain ATC0402MU are shown in Figure 2.

The concentration of the cholestatrienol was increased by amplifying the HMG1-reductase. Two plasmids containing the Hmg1p gene regulated by high expression promoters were integrated into the genome of strain ATC0402MU. The first plasmid, pARC3000D, contained a cloned copy of the yeast HMG1-reductase coding region fused to the yeast phosphoglycerate kinase (PGK1) promoter. The plasmid also carried the Escherichia coli origin of replication, the ampicillin resistance gene, and the yeast TRP1 gene. Plasmid pARC3000D was integrated into the trp1 locus on yeast chromosome IV. The second plasmid, pARC304S, contained the same HMG1-reductase coding region fused to the yeast alcohol dehydrogenase (ADH1) promoter. In addition, plasmid pARC304S contained the E. coli origin of replication, the ampicillin resistance gene, and the yeast URA3 gene. Plasmid pARC304S was
Figure 2. Modified Sterol Synthesis in the *Saccharomyces cerevisiae* Production Strain ATC1562.

*Structures provided by W. H. Okamura, Department of Biochemistry, University of California, Riverside.*
integrated into the ura3 locus of yeast chromosome V. These two integrations generated the yeast strain ATC1561, which had an approximately 200-fold overproduction of HMG1-reductase activity and an increased accumulation of cholesta-5,7,24-trienol. By using classical yeast genetic procedures, strain ATC2551 was diploidized to create the production strain ATC1562 (mat α/mat α, erg5/erg5, erg6-5, erg8-5, TRP1/trp1, URA3/ura3).

2. Production of 25(OH)D₃.

The S. cerevisiae yeast strain ATC1562 is grown fermentatively on a medium consisting of yeast extract, peptone, mineral salts, vitamins, and glucose. Following fermentation, the yeast cells are heat killed and harvested as a slurry. This slurry is saponified with caustic soda and extracted with heptane to provide a crude sterol extract. This extract contains squalene and multiple yeast sterols including cholesta-5,7,24-trien-3β-ol, cholestadienol, lanosterol, zymosterol, methylzymosterol, and dimethylzymosterol.

The desired cholestatrienol is separated from the other yeast sterols in the extract by chromatography after a Diehls-Alder adduct is prepared. By using lead tetraacetate and phthalhydrazide, the adduct is formed specifically for cholestadienol that facilitates product separation. Following chromatography of the adduct, a 24,25-epoxide of the adduct is formed by using m-chloroperoxybenzoic acid. This is reduced to 25-hydroxyprovitamin D₃ (3,25-dihydroxy-cholesta-5,7-dienol) with lithium aluminum hydride. The 25-hydroxyprovitamin D₃ is purified from the reaction mixture by crystallization, yielding solids containing greater than 90 percent pure 25-hydroxyprovitamin D₃. This product may also contain other isomerization-linked sterol compounds such as cholesta-5,7,24-trienol, epoxyprovitamin D₃, and/or 24,25-dihydroxyprovitamin D₃.

The 25(OH)D₃ is formed from 25-hydroxyprovitamin D₃ by erythrosin-mediated photoconversion and thermal isomerization and is purified from the other photoreactants by crystallization to obtain a product that is greater than 94 percent pure. The remainder of the solids, present at not more than 1 percent each, may include 25-hydroxyvitamin D₃, 25(OH)D₃, 25-hydroxyprevitamin D₃, epoxyprovitamin D₃, cholesta-5,7,24-trienol, and photoproducts of 25-hydroxyprovitamin D₃ and 25-hydroxylumisterol. The outline for the process of synthesizing 25(OH)D₃ is shown in Figure 3.

C. PRODUCT CHARACTERISTICS AND SPECIFICATIONS

The photoconversion/thermal isomerization process results in the formation of 25(OH)D₃ which is separated from the other photoproducts by crystallization. The 25(OH)D₃ product has a minimum purity of 94 percent as calculated from HPLC analyses and contains a maximum of 5 percent H₂O as measured by the Karl Fisher titration method.

The product is a white to slightly pink, odorless, crystalline substance containing one molecule of water of hydration. Data provided by Amoco BioProducts Corporation on analyses of three lots of the feed grade 25(OH)D₃ are given in Table 1. Samples of two of these lots of feed grade 25(OH)D₃ were submitted to Hazleton Wisconsin laboratories for independent analyses (Morrissey, 1994). (See Table 2.) Data from both laboratories are similar, indicating that the 25(OH)D₃ is relatively pure (> 96 percent).

The identity of impurities in the feed grade 25(OH)D₃ was examined via HPLC assays by both the manufacturer (Amoco BioProducts Corporation, 1994) and by independent laboratory analysis (Morrissey, 1994). Analyses of two separate lots indicated the presence of several substances closely
Figure 3. Outline of the Process of 25(OH)D₃ Synthesis.
Table 1. Chemical Analysis of 25(OH)D₃ Samples.¹

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>LOT Number</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PS-P02</td>
<td>PS-P05</td>
<td>PS-P07</td>
</tr>
<tr>
<td>25(OH)D₃ Content by HPLC</td>
<td>96.1%</td>
<td>97.8%</td>
<td>96.6%</td>
</tr>
<tr>
<td>Water by Karl Fisher Titration</td>
<td>4.4%</td>
<td>4.5%</td>
<td>4.3%</td>
</tr>
<tr>
<td>Solvent Content by Gas Chromatography</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Acetone</td>
<td>0.35%</td>
<td>0.18%</td>
<td>0.39%</td>
</tr>
<tr>
<td>- 2-Propanol</td>
<td>0.44%</td>
<td>0.10%</td>
<td>0.37%</td>
</tr>
<tr>
<td>- Triethylamine</td>
<td>&lt; 0.10%</td>
<td>&lt; 0.10%</td>
<td>&lt; 0.10%</td>
</tr>
<tr>
<td>Heavy Metals</td>
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<tr>
<td>- Al (ppm)</td>
<td>2.1</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>- Pb (ppm)</td>
<td>2.8</td>
<td>4.2</td>
<td>2.6</td>
</tr>
</tbody>
</table>


Table 2. Chemical Analysis of 25(OH)D₃ Samples.¹

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>LOT Number</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PS-P02</td>
<td>PS-P05</td>
</tr>
<tr>
<td>25(OH)D₃ Content by HPLC</td>
<td>97.3%</td>
<td>98.7%</td>
</tr>
<tr>
<td>Water by Karl Fisher Titration</td>
<td>4.6%</td>
<td>4.5%</td>
</tr>
<tr>
<td>Solvent Content by Gas Chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Acetone</td>
<td>0.351%</td>
<td>0.168%</td>
</tr>
<tr>
<td>- 2-Propanol</td>
<td>0.442%</td>
<td>0.122%</td>
</tr>
<tr>
<td>- Triethylamine</td>
<td>0.066%</td>
<td>0.023%</td>
</tr>
<tr>
<td>Heavy Metals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Al (ppm)</td>
<td>0.724</td>
<td>&lt; 0.500</td>
</tr>
<tr>
<td>- Pb (ppm)</td>
<td>4.41</td>
<td>5.18</td>
</tr>
<tr>
<td>Erythrosin (ppm)</td>
<td>3.9</td>
<td>0.4</td>
</tr>
</tbody>
</table>

¹ Analysis by Hazleton Wisconsin (Morrissey, 1994).
related to 25(OH)D₃. The HPLC data are noted in Table 3. These data suggest that the process for production of 25(OH)D₃ results in synthesis of closely related substances that would be expected to be present in any mammalian biosynthetic system.

Based upon these analyses of several lots, Amoco BioProducts Corporation is proposing the following specifications in their petition for the 25(OH)D₃ feed grade product:

<table>
<thead>
<tr>
<th>Purity (by HPLC):</th>
<th>25(OH)D₃</th>
<th>minimum</th>
<th>94 percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>other sterols</td>
<td>maximum, each</td>
<td>1 percent</td>
<td></td>
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<table>
<thead>
<tr>
<th>Water (by Karl Fisher):</th>
<th>maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 percent</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Heavy metals:</th>
<th>Lead</th>
<th>maximum</th>
<th>20 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminum</td>
<td>maximum</td>
<td>20 ppm</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Erythrosin:</th>
<th>maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 ppm</td>
<td></td>
</tr>
</tbody>
</table>

For use as a poultry feed supplement, the 25(OH)D₃ product is stabilized through formulation into feed grade, hydrated vegetable oil-based beadlets containing butylated hydroxytoluene (BHT) and citric acid as antioxidants. The 25(OH)D₃ is formulated at a concentration of 12.5 to 50 mg per g of beadlets (20 to 80 times dilution). The formulated product is stable for periods of 5 months when stored in a cool, dark location or at temperatures up to 40°C.

The beadlets are removed from storage and mixed with rice hulls at a concentration of 2.5 to 10 g/lb of rice hulls. Thus, one pound of formulated rice hulls contains 125 mg of 25(OH)D₃. This commercial product will be sold to feed manufacturers directly or to formulators who will incorporate the beadlet/rice hull carrier complex into vitamin/mineral premixes and subsequently into broiler feed. By either means, feed formulators will determine the quantity of 25(OH)D₃ to be included in feed or premixes on the basis of the quantity in the formulated beadlet/rice hull carrier complex (125 mg/lb). Amoco BioProducts Corporation proposes to add 25(OH)D₃ to broiler feed at a rate of 62.5 mg/ton of feed. At this proposed rate of addition, formulators would add 0.5 lb of rice hulls containing the 25(OH)D₃ mixture to each ton of feed.

D. USE IN FEED

The production of poultry for human consumption is a highly complex agribusiness. Feed preparation and composition depend upon the type of bird (e.g., turkeys versus chickens) and the purposes for which the birds are produced. For example, diets for broiler chickens, pullets, laying hens, and breeder stock in commercial production vary not only with market goal but also with age of the bird. Without exception, commercially prepared feed is carefully formulated to contain appropriate levels of nutrients and energy. In addition, feed contains premix vitamin and mineral supplements as well as other substances such as antibiotics, if considered necessary.
Table 3. HPLC Analyses of Two Feed Grade Lots of 25(OH)D₃.¹

<table>
<thead>
<tr>
<th>PRODUCT</th>
<th>wt Percent of 25(OH)D₃/lot</th>
<th>PS-P02</th>
<th>PS-P05</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D₃</td>
<td></td>
<td>100.00</td>
<td>100.00</td>
</tr>
<tr>
<td>25(OH)-Isotachysterol₃, 25(OH)-isovitamin D₃, and 25(OH)-5,6 transvitamin D₃ ²</td>
<td>0.11</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.21</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>25(OH)-Lumisterol₃</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25(OH)-Previtamin D₃ ³ ⁴</td>
<td>0.40</td>
<td>0.49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.90</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td>25(OH)-Provitamin D₃ ⁴</td>
<td>0.13</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.09</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>25(OH)-Tachysterol₃</td>
<td>0.21</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>25(OH)-5,6-transvitamin D₃ ³ ⁵</td>
<td>0.12</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>0.27</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.28</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>Total Impurities (%)</td>
<td>1.24</td>
<td>1.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.63</td>
<td>1.85</td>
<td></td>
</tr>
</tbody>
</table>

¹ The upper figures were provided by Amoco BioProducts Corporation (1994); the lower figures are the Hazleton Wisconsin (Morrissey, 1994) data from the same lots.

² Analyzed separately by Amoco BioProducts Corporation; peaks for 25(OH)-isotachysterol₃, 25(OH)-isovitamin D₃, and 25(OH)-5,6 transvitamin D₃ were considered together as a single HPLC peak (Fig.1) rather than as separate HPLC peaks (Morrissey, 1994).

³ All vitamin compounds (vitamin D₃, 25(OH)D₃, and 1α,25(OH)₂D₃) containing the 5(6), 7(8), 10(19) triene system are in thermal equilibrium with their cognate previtamin structures which possess a 5(10), 6(7), 8(9) triene system. The equililibrium between vitamin D₃ and previtamin D₃ is temperature and time dependent; thus, the proportion of previtamin D₃ present in any preparation can vary depending on the temperature and time of storage (Curtin and Okamura, 1991; Tian et al., 1993).

⁴ The epidermal cutaneous reservoir of 7-dehydrocholesterol (previtamin D₃) is converted to previtamin D₃ photochemically during sunlight exposure. Previtamin D₃ is thermally labile and must undergo an isomerization to form vitamin D₃ (Norman et al., 1982).
Amoco BioProducts Corporation anticipates the marketing of 25(OH)D₃ as a substitute for vitamin D₃ in broiler feed providing vitamin D activity. The Corporation requested that the LSRO evaluate the safety of 25(OH)D₃ in broiler feed; thus, the LSRO ad hoc Expert Panel has focused its review of the literature and discussions on safety solely on potential use in broiler feed.

In commercial production, broiler chickens are grown within the confines of buildings with large numbers of birds per building and are supplied with bulk feed and water for ad libitum consumption. Various strains of chickens have been developed for broiler production. They have been bred primarily for rapid weight gain and efficient feed utilization. Broilers grow rapidly and are harvested on or before 9 weeks of age. Typically, broilers are harvested at 6 to 7 weeks if size and weight requirements are attained. Crumbled starter feed is supplied during weeks 1 to 3, pelleted grower feed during weeks 4 to 6, and finisher feed until harvest. In some operations, grower feed is replaced earlier by finisher feed and finisher feed is replaced by withdrawal feed a short time before slaughter. The major differences among the four types of feed is the levels and sources of protein and supplements of essential amino acids, minerals, and vitamins provided in the feed, and in the instance of withdrawal feed, the exclusion of medications and drugs. Vitamin D₃ is added as a supplement to all four types of feed. Amoco BioProducts Corporation plans to market 25(OH)D₃ as a vitamin D supplement for all types of broiler feed.

The Subcommittee on Poultry Nutrition, Committee on Animal Nutrition, Board of Agriculture, National Research Council (1994) has recommended that the vitamin D₃ requirement of broilers can be met by diets that contain 200 IU of vitamin D₃/kg of feed (90 percent dry matter) when birds are fed diets containing 3200 kcal metabolizable energy per kg of feed over the typical 8-week growing period. A supplement of 200 IU/kg of feed is equivalent to about 4.5 mg of vitamin D₃/ton of feed. However, this vitamin D₃ requirement was established on the basis of research done before 1970 and under conditions in which chickens may have been exposed to ultraviolet (UV) light. Commercial broiler strains currently grow much faster and utilize feed more efficiently; in addition, they are reared in confinement with little exposure to UV light. Thus, the National Research Council (1994) recommendation of 200 IU of vitamin D₃/kg of feed is virtually never used in the broiler industry and supplementation of broiler feed with vitamin D₃ is typically at a considerably higher level.

As pointed out by McNaughton (1990a), the level of vitamin supplementation provided in the industry is based on type of diets fed, species, age of the animal, antagonists, form of vitamin product, requirement status (optimum or minimum requirements), marketing program requirements, disease status, complexity of the ration, and environmental factors, primarily ambient temperature. Only after all these factors are considered can the optimal vitamin requirements for poultry be estimated. Thus, in practice, the quantities of vitamin supplements fed are based on field experience and not on extensive research at levels in which vitamins are utilized. As McNaughton (1990a) has observed, the poultry nutritionist practices the art of balancing maximum performance, minimum risk of deficiency, and benefits with a host of dietary economical considerations.

Ward (1993) recently surveyed commercial practices in regard to vitamin supplementation of poultry feed. He collected data from 62 companies that supply feed or actually feed broilers. Levels of vitamin D₃ fortification of feed are presented in Table 4. As evidenced by this survey, the range of vitamin D₃ supplementation per ton of broiler feed is 32.5 to 84.5 mg/ton (short ton = 2000 lbs), excluding withdrawal feed data which represent only a small fraction of the total poultry feed manufactured. As expected, vitamin D₃ fortification is highest in young broilers and is progressively reduced as birds mature. This is, in part, a reflection of the quantity of feed consumed. As birds mature, feed consumption increases significantly; however, the amount of feed consumed by male broilers is greater than that consumed by female broilers. By using figures developed by the Poultry
Table 4. Levels of Vitamin D₃ Fortification in mg/ton of Feed for Commercial Broiler Production.¹

<table>
<thead>
<tr>
<th>Type of Feed</th>
<th>Lowest² 25%</th>
<th>Mean³</th>
<th>Highest² 25%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starter</td>
<td>49.0</td>
<td>63.8</td>
<td>84.5</td>
</tr>
<tr>
<td>Grower</td>
<td>46.2</td>
<td>58.2</td>
<td>70.0</td>
</tr>
<tr>
<td>Finisher</td>
<td>32.5</td>
<td>50.0</td>
<td>68.2</td>
</tr>
<tr>
<td>Withdrawal⁴</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>all</td>
<td></td>
<td>18.0</td>
<td></td>
</tr>
<tr>
<td>fortified only</td>
<td></td>
<td>36.2</td>
<td></td>
</tr>
</tbody>
</table>

¹ Modified from Ward (1983).

² Mean of lowest or highest 15 reported values.

³ Mean of 62 reported values.

⁴ Total number of reported values not identified.
Nutrition Committee (National Research Council, 1994) for average feed consumed per broiler per week and data from Ward (1993), daily vitamin D₃ intake can be estimated. (See Table 5.) After a 7-week growth period, average body weights of male and female birds are 2.6 and 2.1 kg, respectively. Vitamin D₃ consumption totals on average about 308 and 266 μg/bird for male and female birds, respectively, over a 7-week period. Where high vitamin D₃ supplement levels are used, the male and female vitamin D₃ intakes over a 7-week period are 371 and 322 μg/bird, respectively.

Amoco BioProducts Corporation proposes to market 25(OH)D₃ for use in broiler feed at about 62.5 mg/ton (58.0 to 67.0 mg/ton) of feed. This level of fortification, equivalent to 68.8 μg (63.8 to 73.7 μg) of 25(OH)D₃/kg of feed,² is somewhat higher than the estimated mean levels for finisher feed, but similar to the estimated mean levels for starter and grower feed, as reported by Ward (1993). This level of broiler feed supplementation for 25(OH)D₃ was selected somewhat empirically, based on knowledge of current practice for vitamin D₃ feed supplementation. Starting with this concentration (equivalent to current vitamin D₃ usage), Amoco BioProducts Corporation had several field trials performed at fortification levels of 1.7 to 13,751.4 μg/kg of feed. (These field trials are discussed in Chapter IV.)

Table 6 provides estimated intakes of 25(OH)D₃ over a 7-week period assuming an average supplementation level of 68.8 μg/kg of feed and a higher supplementation level of 73.7 μg/kg of feed. Table 6 provides predicted intake levels of 25(OH)D₃ that are comparable to the vitamin D₃ intake levels estimated from data on current practice in Table 5. Inasmuch as the 25(OH)D₃ feed supplementation rate was selected empirically from current practice of vitamin D₃ supplementation, it is logical that estimated total 25(OH)D₃ consumption over a 7-week period would be essentially equivalent to that of vitamin D₃, as presented below (figures are μg/bird):

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th></th>
<th>Females</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>average</td>
<td>high</td>
<td></td>
<td>average</td>
</tr>
<tr>
<td>Vitamin D₃ (Table 5)</td>
<td>308</td>
<td>371</td>
<td></td>
<td>266</td>
</tr>
<tr>
<td>25(OH)D₃ (Table 6)</td>
<td>345</td>
<td>368</td>
<td></td>
<td>297</td>
</tr>
</tbody>
</table>

It should be noted that the estimates derived in Tables 5 and 6 do not take into account the differences in molecular weight and water of hydration as well as the relative biological potency of 25(OH)D₃. The relative biological potency of 25(OH)D₃ was not a component of the initial decision to supplement broiler feed at 68.8 μg/kg. The issue of relative biological potency of 25(OH)D₃ versus that of vitamin D₃ in growth and development of poultry is discussed in Chapter IV (page 25).

² Throughout this report, all references to 25(OH)D₃ and vitamin D₃ levels provided in feed are presented in μg of 25(OH)D₃ or vitamin D₃/kg of feed. Providing this information as a consistent unit measure will allow comparisons to be made across studies administering different levels of 25(OH)D₃ or vitamin D₃ levels in feed.
Table 5.  Estimated Daily Feed and Vitamin D₃ Consumption of Broilers Fed Average and High Levels of Starter, Grower, and Finisher Feed Over a 7-Week Period.¹ ²

<table>
<thead>
<tr>
<th>Age In Weeks</th>
<th>Daily Feed Consumption (g)³</th>
<th>Daily Vitamin D₃ Consumption (µg)¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>High</td>
</tr>
<tr>
<td>1</td>
<td>19.3</td>
<td>18.7</td>
</tr>
<tr>
<td>2</td>
<td>41.4</td>
<td>39.0</td>
</tr>
<tr>
<td>3</td>
<td>69.6</td>
<td>63.4</td>
</tr>
<tr>
<td>4</td>
<td>100.6</td>
<td>91.7</td>
</tr>
<tr>
<td>5</td>
<td>137.1</td>
<td>105.4</td>
</tr>
<tr>
<td>6</td>
<td>163.0</td>
<td>143.0</td>
</tr>
<tr>
<td>7</td>
<td>183.0</td>
<td>154.4</td>
</tr>
</tbody>
</table>

¹ Data on vitamin D₃ supplementation levels from Ward (1993). Respective amounts of vitamin D₃ in average and high diets were 70.1 and 53.0 µg/kg in starter feed, 64.1 and 77.0 µg/kg in grower feed, and 50.0 and 55.0 µg/kg in finisher feed.

² Starter feed weeks 1-3; grower feed weeks 4-6; finisher feed week 7.

³ National Research Council (1994).
Table 6.  Estimated Daily Feed and 25(OH)D₃ Consumption of Broilers Fed Average and High Levels of 25(OH)D₃ over a 7-Week Period.¹

<table>
<thead>
<tr>
<th>Age In Weeks</th>
<th>Daily Feed Consumption (g)²</th>
<th>Daily 25(OH)D₃ Consumption (µg)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
<td>Males</td>
</tr>
<tr>
<td>1</td>
<td>19.3</td>
<td>18.7</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>41.4</td>
<td>39.0</td>
<td>2.9</td>
</tr>
<tr>
<td>3</td>
<td>69.6</td>
<td>63.4</td>
<td>4.8</td>
</tr>
<tr>
<td>4</td>
<td>100.6</td>
<td>91.7</td>
<td>6.9</td>
</tr>
<tr>
<td>5</td>
<td>137.1</td>
<td>105.4</td>
<td>9.5</td>
</tr>
<tr>
<td>6</td>
<td>163.0</td>
<td>143.0</td>
<td>11.2</td>
</tr>
<tr>
<td>7</td>
<td>183.0</td>
<td>154.4</td>
<td>12.6</td>
</tr>
</tbody>
</table>

¹ The level of 25(OH)D₃ was assumed to be constant during the 7-week period when starter, grower, and finisher feed was fed. Feed was assumed to contain 68.8 µg and 79.7 µg of 25(OH)D₃/kg of feed for average and high diets, respectively.

² National Research Council (1994).
III. CONSUMER EXPOSURE

Amoco BioProducts Corporation requested that the LSRO examine the health effects of using 25(OH)D\textsubscript{3} as an alternative source of vitamin D\textsubscript{3} activity in broiler feed. In response, an evaluation of consumer exposure to 25(OH)D\textsubscript{3} from consumption of broiler chickens raised on 25(OH)D\textsubscript{3}-supplemented feed was conducted by the LSRO and the ad hoc Expert Panel. This evaluation, consisting of an assessment of usual exposure, was considered in the context of the total intake of 25(OH)D\textsubscript{3} in the diet. In addition to this evaluation, a discussion of usual vitamin D\textsubscript{3} intakes of adults and children and of vitamin D contributions from dietary supplements and sunlight is presented in this chapter.

A. USUAL EXPOSURE

1. Approach used to estimate usual exposure

The concept of "usual exposure," as described by Anderson (1988), was utilized to estimate consumer exposure to 25(OH)D\textsubscript{3} from consumption of broiler chickens raised on 25(OH)D\textsubscript{3}-supplemented feed. The term "usual exposure" is used in this chapter to refer to mean and selected percentiles of intake for persons over extended, but indefinite, periods of time.

Estimates of usual exposure were calculated based on national food disappearance data and weighted averages of 25(OH)D\textsubscript{3} tissue concentrations found in breast meat and leg meat with skin from broilers raised on feed supplemented with 25(OH)D\textsubscript{3}. Food disappearance data represent the annual food supply and utilization of most major agricultural commodities. Total food supply is based on records of commodity flows from production to end uses and is calculated as the sum of production, beginning inventories, and imports (Putnam, 1993). For most food commodities, exports, industrial uses, farm inputs (seed and feed), and ending inventories represent measurable nonfood uses. The amount of food that remains after subtracting these uses from the total available food supply represents food that "disappears" into the marketing system. Hence, this residual component is often referred to as food disappearance. The U.S. Department of Agriculture, Economic Research Service, annually calculates the amounts of several hundred foods that "disappear" in the marketing system.

Food disappearance data represent all food used by households, restaurants, fast-food chains, and other eating institutions. Per capita food "consumption" is calculated by dividing total food disappearance by the U.S. population using U.S. Census data. The most recent update of data on per capita consumption of food commodities is available from 1970 through 1992 (Putnam and Alishouse, 1993).

In general, disappearance data are useful as indicators of trends in consumption over time rather than as measurements of absolute levels of foods eaten (Putnam, 1993). In other words, these data provide an indication as to whether Americans on the average are using more or less of various foods over time. This situation holds true as long as changes in food production and marketing practices or consumer behavior over time do not alter the relative disparity between food disappearance and the amount of food actually eaten. A limitation of disappearance data is that they tend to overestimate the amount of food available for consumption because spoilage and waste accumulated through the marketing system and food that is thrown away at home or fed to pets are included. These overestimates, however, are assumed to be consistent over time because methodology has remained relatively constant (Welsh and Marston, 1982). Since it can be expected that persons of different age
and sex groups will consume different amounts of food and because per capita estimates are calculated without adjusting for age or sex, comparisons of data over time or between populations reflect differences in demographic structure. Another limitation of disappearance data is that they do not provide consumption data for population subgroups of concern, such as children, or for persons who consume large amounts of food. When estimating consumption for children, it is generally assumed that children consume about half the portion size of adults (U.S. Department of Agriculture, 1985, 1986). Consumption for heavy consumers, i.e., consumers at the 90th percentile, has been reported to be 2 to 3 times the mean consumption of average consumers (Food and Drug Administration, 1993).

A main reason for using disappearance data to estimate consumer exposure for this evaluation was that the ad hoc Expert Panel considered that these data provided the closest representation of current chicken consumption in the United States. Another reason was that these data provided a reasonable estimate of current chicken consumption because a large proportion of the population eats chicken. Furthermore, these data most closely matched the form of chicken (raw chicken) for which 25(OH)D₃ tissue concentration levels were available.

According to food disappearance data, chicken consumption has steadily increased in this country over the past two decades. Between 1977 and 1992, consumption increased nearly 60 percent, from 29.0 to 45.9 pounds per capita (Putnam and Allahouse, 1993). These per capita figures are based on the consumption of chicken parts commonly eaten by consumers, i.e., boneless, trimmed chicken including the skin, as well as chicken parts not commonly eaten by consumers, i.e., neck meat and giblets. Adjustments, however, were not made to correct for the inclusion of neck meat and giblets in the consumer exposure estimates because these chicken parts represent only a small portion of the total weight of the chicken. It should be noted that these per capita figures exclude the amount of ready-to-cook chicken going to pet food and some fluid leakage that occurs when chicken is cut up before packaging.

The use of chicken consumption data collected in the Nationwide Food Consumption Surveys (NFCS) 1977-78 and 1987-88 was considered as a possible alternative to using food disappearance data for estimating consumer exposure; however, because of limitations of data collected in these two surveys, these data were not used. A major limitation of the NFCS 1977-78 data was that these data were collected more than 15 years ago. As stated earlier in this chapter, per capita consumption of chicken more than doubled since the NFCS 1977-78 was conducted. Thus, if the NFCS 1977-78 data were used to estimate consumer exposure, the amount of chicken consumed at the present time would be underestimated.

Another reason for not using the NFCS 1977-78 data was that these data, as reported by Pao et al. (1982), did not account for all sources of chicken that might be eaten. While information was provided in the Pao et al. (1982) tables for the amount of chicken parts, breaded and floured chicken, chicken sticks, and baby food chicken consumed, information was not provided for the amount of Cornish game hen, chicken loaf, chicken liver, or chicken spread consumed. Furthermore, for mixtures that included chicken as an ingredient, such as pot pies, frozen plate meals, and soups, the quantity of chicken contained in each of these mixtures could not be reasonably estimated based on the information compiled by Pao et al. (1982). For example, data indicated that 69 percent of the pot pies consumed contained chicken; 45 percent of the frozen plate meals consumed were poultry entrees; and 27 percent of the soups consumed were grain mixtures, such as chicken noodle soup. To estimate the amount of chicken consumed based on this information would require making assumptions about the amount of chicken contained in each of these products. Because the amount of chicken contained in each of these products can vary greatly from product to product, reasonable estimates about the amount of chicken contained in each product would be difficult to make without having information about the chicken content for each product by brand name and market share.
Although more recent chicken consumption data were collected in the NFCS 1987-88, these data also were not considered for estimating consumer exposure. Because this survey had a response rate less than 38 percent, the possibility of nonresponse bias would be present through the use of these data (Life Sciences Research Office, 1991; U.S. Department of Agriculture, 1992). Food consumption data from the more recent Continuing Survey of Food Intakes by Individuals (CSFII) 1989-1991 and from the first phase of the Third National Health and Nutrition Examination Survey (NHANES III) 1988-1991 were considered for calculating consumer exposure; however, these data were not publicly available when this report was prepared.

2. Estimates of usual exposure

The ad hoc Expert Panel considered that an estimate of 25(OH)D₃ consumption by heavy users of broiler chickens raised on 25(OH)D₃-supplemented feed and other foods that contain 25(OH)D₃ was a key element in evaluating the safety of 25(OH)D₃ consumption among humans. A consumption model was created for estimating daily intakes of 25(OH)D₃ from foods that contain this vitamin D metabolite for average and heavy consumers. The model was developed to include foods that serve as major contributors of 25(OH)D₃ to the diet, i.e., milk, eggs, and beef, as well as broiler chickens raised on 25(OH)D₃-supplemented feed. Market penetration of 25(OH)D₃-supplemented broiler chicken was assumed to be 100 percent, i.e., all broiler chickens in this country would be raised on feed supplemented with 25(OH)D₃ at levels proposed by Amoco BioProducts Corporation (62.5 mg of 25(OH)D₃/ton of feed). By assuming 100 percent market penetration, a conservative estimate of consumer exposure would be achieved.

Some of the foods that were potential sources of 25(OH)D₃ or 25(OH)D₃ in the diet were excluded from the model included liver and fish liver oils, and fortified grains, pasta, and cereals, respectively. Liver and fish liver oils were not included in the model because these two foods are consumed in relatively small quantities in this country. Fortified grains, pasta, and cereals were not included because these foods were assumed to contain small amounts of 25(OH)D₃ compared to foods selected for inclusion in the model. Another reason for excluding these foods from the model was that food composition data were not available on the 25(OH)D₃ content of these foods.

a. Diets containing 25(OH)D₃-fed broiler chicken

The most current food disappearance data available for 1992 from Putnam and Allshouse (1993) were used to identify per capita consumption of milk, eggs, and beef in pounds per year. These per capita consumption values subsequently were used to estimate per capita consumption for each of these food items in pounds per day and in grams per day. Mean per capita intake of 25(OH)D₃ in nanograms per day was then calculated for adults and children for each food item based on the mean daily gram intake of each food item and the 25(OH)D₃ content of the food item in nanograms per 100 grams. Table 7 presents these calculations for milk, eggs, and beef.

The amount of 25(OH)D₃ supplied in the diet from 25(OH)D₃-fed broiler chicken was calculated based on results of field trial Colorado AM-1-93 (Quarles, 1993a) conducted by Colorado Quality Research of Fort Collins, Colorado for Amoco BioProducts Corporation. For this study, tissue samples from the breast, leg, and skin of broilers raised on feed supplemented with 68.8 µg of 25(OH)D₃/kg of feed were analyzed for tissue concentrations of 25(OH)D₃ [this level of 25(OH)D₃ supplementation has been proposed by Amoco BioProducts Corporation for broiler feed]. When calculating 25(OH)D₃ tissue concentrations in a piece of boneless chicken breast with skin, it was assumed that 59 percent of the chicken breast is meat and 11 percent is skin. When calculating 25(OH)D₃ concentrations in a
Table 7. Estimated Daily Per Capita Intake of 25(OH)D₃ for Adults and Children Based on Diets Containing Broiler Chickens Raised on 25(OH)D₃-Supplemented Feed.

<table>
<thead>
<tr>
<th>Food</th>
<th>Per capita consumption</th>
<th>25(OH)D₃ level (ng/100g)</th>
<th>Adults Per capita intake of 25(OH)D₃ (ng/d)</th>
<th>Children Per capita intake of 25(OH)D₃ (ng/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lb/yr¹</td>
<td>lb(g)/d</td>
<td>mean²</td>
<td>90th percentile¹⁰</td>
</tr>
<tr>
<td>Milk</td>
<td>218.5²</td>
<td>0.599 (272.0)</td>
<td>38⁶</td>
<td>104</td>
</tr>
<tr>
<td>Whole eggs</td>
<td>30.2</td>
<td>0.083 (37.7)</td>
<td>300⁶</td>
<td>113</td>
</tr>
<tr>
<td>Beef tissue</td>
<td>62.8⁸</td>
<td>0.172 (78.1)</td>
<td>253⁷</td>
<td>196</td>
</tr>
<tr>
<td>Chicken tissue, 25(OH)D₃ fed</td>
<td>45.9⁴</td>
<td>0.126 (57.2)</td>
<td>607⁸</td>
<td>347</td>
</tr>
<tr>
<td><strong>Total per capita intake of 25(OH)D₃</strong></td>
<td></td>
<td></td>
<td>762</td>
<td>1524</td>
</tr>
</tbody>
</table>

¹ Per capita consumption (lbs/yr) was based on 1992 food disappearance data from Putnam and Allshouse (1993).
² Value includes plain whole milk, low fat milk (1 and 2 percent), skim milk, flavored whole and low fat milk, and buttermilk.
³ Value was based on boneless, trimmed beef.
⁴ Value includes all types of chicken, including broiler chicken. Value is based on boneless, trimmed chicken and includes the skin, neck meat, and giblets, and excludes the amounts of ready-to-cook chicken going to pet food and some water leakage that occurs when chicken is cut up before packaging.
⁵ 25(OH)D₃ level was calculated from the 379 pg/mL value reported in Hollis et al. (1981) based on cows maintained on a daily supplement of 4000 IU of vitamin D₃. This value was converted to a ng/100mL basis and then multiplied by 1.038 (density of milk).
⁶ 25(OH)D₃ level was taken from Kassinen and Vahtonen (1985).
⁷ 25(OH)D₃ level was calculated from the three 25(OH)D₃ levels (ng/g) for beef muscle reported in Kooby and VanDerSlik (1977) and then converted to a ng/100g basis.
⁸ A weighted 25(OH)D₃ level was calculated from 25(OH)D₃ concentrations in tissue samples of breast, leg, and skin from broiler chickens raised on feed supplemented with 88.8 μg of 25(OH)D₃/kg of feed. Analysis of 25(OH)D₃ concentrations was conducted by Colorado Quality Research of Fort Collins, Colorado under field trial Colorado AM-1-93 for Amoco BioProducts Corporation (Quarles, 1993a).
⁹ Mean daily per capita intake of 25(OH)D₃ for adults was estimated based on the 25(OH)D₃ level in the food item and the per capita consumption (g/d) of the food item.
¹⁰ Mean daily per capita intake of 25(OH)D₃ for 90th percentile consumers was assumed to be twice the mean daily per capita intake (Food and Drug Administration, 1993).
¹¹ Mean daily per capita intake of 25(OH)D₃ for children was assumed to be one-half of the mean daily per capita intake of adults (U.S. Department of Agriculture, 1985, 1986).
chicken leg, it was assumed that 85 percent of the chicken leg is meat and 15 percent is skin. These percentages were based on yields of breast meat, thigh meat, and skin from male and female broilers 6 to 7 weeks of age (Moran and Orr, 1969). Based on these assumptions, a weighted level of 25(OH)D₃ contained in the 25(OH)D₃-supplemented breast and leg meat with skin was estimated to be 607 ng/100 g. (See Table 7.)

Estimated daily mean per capita intakes of 25(OH)D₃ for adults and children are shown in Table 7. Based on daily mean per capita intakes of milk (104 ng/d), whole eggs (113 ng/d), beef (198 ng/d), and meat and skin from broiler chickens raised on 25(OH)D₃-supplemented feed (347 ng/d), mean per capita intakes of 25(OH)D₃ for average adult consumers were estimated at 762 ng/d. Intakes for adults at the 90th percentile were estimated at twice the mean per capita intake or 1524 ng/d. Mean per capita intakes of 25(OH)D₃ for children were estimated at one-half the mean per capita intake of average adult consumers or 382 ng/d, and children at the 90th percentile were estimated to consume twice this amount or 762 ng/d. Intakes for children were calculated based on the assumption that children consume about half the portion size of adults (U.S. Department of Agriculture, 1985, 1986).

b. Diets containing vitamin D₃-fed broiler chicken

An additional evaluation was conducted to compare daily mean per capita intakes of 25(OH)D₃ in the diets of adults and children that included meat and skin from 25(OH)D₃-fed broiler chicken with daily mean per capita intakes of 25(OH)D₃ in the diets of adults and children that included meat and skin from vitamin D₃-fed broiler chicken. For this evaluation, data from the Colorado AM-1-93 field trial (Quarles, 1993a) were used to determine 25(OH)D₃ concentrations in breast and leg meat with skin from broiler chickens raised on feed supplemented with 62.5 mg of vitamin D₃/tan (the normal commercial level of vitamin D₃). Based on a diet that consisted of milk (104 ng/d), eggs (113 ng/d), beef (198 ng/d), and meat and skin from vitamin D₃-fed broiler chicken (120 ng/d), mean per capita intakes of 25(OH)D₃ were estimated at 535 ng/d for average adult consumers and per capita. (See Table 8.) Intakes for heavy consumers at the 90th percentile were estimated at twice this amount or 1069 ng/d. For children, mean per capita intakes were estimated at 266 ng/d, and 90th percentile heavy consumers were estimated to have intakes of 535 ng/d.

As shown in Tables 7 and 8, a comparison of the two diets indicated that the percentage increase in 25(OH)D₃ intakes from diets containing 25(OH)D₃-fed broiler chicken instead of vitamin D₃-fed broiler chicken was about 42 percent for both adults and children. The increase in estimated per capita intakes of 25(OH)D₃ by adults from diets containing 25(OH)D₃-fed broiler chicken compared to diets containing vitamin D₃-fed broiler chicken was 227 ng/d (mean) and 455 ng/d (90th percentile). Comparable values for children were 114 ng/d (mean) and 227 ng/d (90th percentile).

Although vitamin D₃ is generally stable to heat, acids, and oxygen, significant destruction of the vitamin can occur during cooking (Harris, 1988). Maximum cooking losses have been estimated at 40 percent. Vitamin D₃ also can be slowly destroyed in foods and feed that are slightly alkaline, especially in the presence of air and light. It is likely that 25(OH)D₃ would experience the same cooking losses and destruction in alkaline foods and feed as vitamin D₃. Thus, actual daily mean per capita intakes of 25(OH)D₃ can be expected to be somewhat lower than the daily mean per capita intakes stated above. Reasons for this are that cooked chicken rather than raw chicken is normally consumed by humans [25(OH)D₃ tissue concentrations presented above were based on analysis of raw chicken], chicken as consumed may be processed or prepared into mixtures with foods that may destroy vitamin D activity, and/or less than ideal handling or storage of chicken may destroy the vitamin.
Table 8. Estimated Daily Per Capita Intake of 25(OH)D₃ for Adults and Children Based on Diets Containing Broiler Chickens Raised on Vitamin D₃-Supplemented Feed.

<table>
<thead>
<tr>
<th>Food</th>
<th>Per capita consumption</th>
<th>25(OH)D₃ level (ng/100g)</th>
<th>Adults Per capita intake of 25(OH)D₃ (ng/d)</th>
<th>Children Per capita intake of 25(OH)D₃ (ng/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>218.5³</td>
<td>36⁶</td>
<td>mean⁹ 104</td>
<td>mean¹¹ 52</td>
</tr>
<tr>
<td>Whole eggs</td>
<td>30.2</td>
<td>300⁹</td>
<td>113</td>
<td>57</td>
</tr>
<tr>
<td>Beef tissue</td>
<td>62.8³</td>
<td>253⁷</td>
<td>198</td>
<td>99</td>
</tr>
<tr>
<td>Chicken tissue, vitamin D₃ fed</td>
<td>45.9⁴</td>
<td>209⁸</td>
<td>120</td>
<td>60</td>
</tr>
<tr>
<td>Total per capita intake of 25(OH)D₃</td>
<td></td>
<td></td>
<td>535</td>
<td>268</td>
</tr>
</tbody>
</table>

¹ Per capita consumption (lbs/yr) was based on 1992 food disappearance data from Putnam and Allshouse (1993).
² Value includes plain whole milk, low fat milk (1 and 2 percent), skim milk, flavored whole and low fat milk, and buttermilk.
³ Value was based on boneless, trimmed beef.
⁴ Value includes all types of chicken, including broiler chicken. Value is based on boneless, trimmed chicken and includes the skin, neck meat, and giblets, and excludes the amounts of ready-to-cook chicken going to pet food and some water leakage that occurs when chicken is cut up before packaging.
⁵ 25(OH)D₃ level was calculated from the 372 pg/mL value reported in Hollis et al. (1981) based on cows maintained on a daily supplement of 4000 IU of vitamin D₃. This value was converted to a ng/100mL basis and then multiplied by 1.052 (density of milk).
⁶ 25(OH)D₃ level was taken from Koskinen and Valtanen (1985).
⁷ 25(OH)D₃ level was calculated from the three 25(OH)D₃ levels (ng/g) for beef muscle reported in Koshy and VanDerSlik (1977) and then converted to a ng/100g basis.
⁸ A weighted 25(OH)D₃ level was calculated from 25(OH)D₃ concentrations in tissue samples of breast, leg, and skin from broiler chickens raised on feed supplemented with 68.8 µg of vitamin D₃/kg of feed. Analysis of 25(OH)D₃ concentrations was conducted by Colorado Quality Research of Fort Collins, Colorado under field trial Colorado AM-1-93 for Amoco BioProducts Corporation (Quarles, 1993a).
⁹ Mean daily per capita intake of 25(OH)D₃ for adults was estimated based on the 25(OH)D₃ level in the food item and the per capita consumption (g/d) of the food item.
¹⁰ Mean daily per capita intake of 25(OH)D₃ for 90th percentile consumers was assumed to be twice the mean daily per capita intake (Food and Drug Administration, 1993).
¹¹ Mean daily per capita intake of 25(OH)D₃ for children was assumed to be one-half of the mean daily per capita intake of adults (U.S. Department of Agriculture, 1985, 1986).
B. SOURCES OF VITAMIN D

1. Diet

Because most foods contain very small amounts of vitamin D, foods fortified with vitamin D serve as major sources of the vitamin in the diet. Vitamin D in the form of vitamin D₃ or vitamin D₃₃ is used for fortification purposes (Combs, 1992; National Research Council, 1989). Other foods contain vitamin D indirectly through supplementation of animal feed (Combs, 1992). Tissue concentrations in animals also are determined by the amount of sunlight exposure received by the animal.

A major source of vitamin D₃ in the diet of children is processed cow's milk (National Research Council, 1989). In the United States, almost all milk is fortified with 10 µg (400 IU) of vitamin D₃ per quart² (1.0 IU = 0.025 µg vitamin D₃); thus, an 8-ounce glass of milk contains 2.5 µg (100 IU) of vitamin D₃ (American Academy of Pediatrics, 1993a). Most infant formulas are fortified at the same level as cow's milk. Human milk contains only about 0.52 µg (21 IU) of vitamin D₃ per quart (American Academy of Pediatrics, 1993b).

In the United States, usual dietary intakes of vitamin D₃ have been estimated primarily for infants and children (National Research Council, 1989). Infant formulas have been estimated to provide 6.75 µg (270 IU) of vitamin D₃/d for infants from birth to 3 months of age and 8.5 µg (340 IU) of vitamin D₃/d for infants 4 to 6 months of age (Fomon and Ziegler, 1993). Breast-fed infants receive only about 0.4 µg (16.5 IU) of vitamin D₃/d from 750 mL of human milk (American Academy of Pediatrics, 1993b). Children who drink three 8-ounce glasses of milk per day ingest about 7.5 µg (300 IU) of vitamin D₃ and a small amount of additional vitamin D from other foods.

According to the CSFII 1985, the average adult male obtains about 2.1 µg (84 IU) of vitamin D₃ from milk per day (U.S. Department of Agriculture, 1986), whereas the average adult female obtains about 1.5 µg (60 IU) of vitamin D₃ from milk per day (U.S. Department of Agriculture, 1987). The NFCS 1977-78 found that usual dietary intakes of vitamin D₃ in this country range from 1.25 to 1.75 µg (50 to 70 IU) per day (U.S. Department of Agriculture, 1983). Omdahl et al. (1982) found that males and females 60 to 93 years of age had median daily vitamin D₃ intakes of 1.95 and 1.35 µg (78 and 54 IU), respectively.

Establishing a Recommended Dietary Allowance (RDA) for vitamin D₃ is difficult because sunlight serves as a valuable source of the vitamin. Holick (1994) reported that the current 1989 RDA of 5 µg (200 IU) for adults appears to be reasonable for those exposed to sunlight. Because aging does not appear to alter vitamin D absorption, the RDA for elderly adults and younger adults is the same. According to the American Academy of Pediatrics (1993a), the RDA of 10 µg (400 IU) for infants provides an ample margin of safety even in the absence of sunlight exposure.

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² One International Unit (IU) was defined in 1935 to be the activity of 1 mg of the international standard solution of irradiated ergosterol, which has been found to be equivalent to 0.025 µg of crystalline vitamin D (League of Nations, 1936). This definition was subsequently modified in 1949 by the World Health Organization to be as follows: the International Unit of vitamin D recommended for adoption is the vitamin D activity of 0.025 µg of the international standard preparation of crystalline vitamin D₃ (World Health Organization, 1950).
2. **Dietary supplements**

Data from the 1986 National Health Interview Survey (Moss et al., 1989) showed that 43.3 percent of children 2 to 6 years of age and 36.3 percent of adults 18 years of age and older take some type of nonprescription multivitamin and mineral supplement. Females 18 years of age and older were more likely than their male counterparts to report such practices, 41.3 and 31.2 percent, respectively. Among children who were reported vitamin and mineral supplement users, 88.1 percent had vitamin D (form unspecified) included in their supplements. About two-thirds of adults who reported using vitamin and mineral supplements said that vitamin D (form unspecified) was included in their supplements.

Currently, product labels indicate that many over-the-counter multivitamin preparations contain 10 μg (400 IU) of vitamin D per capsule or dose. According to Holick (1994), most pharmaceutical companies add 1.5 to 2 times the amount of vitamin D stated on the label; thus, users of multivitamin supplements could receive 15-20 μg (600-800 IU) of vitamin D₃ per day from supplements, in addition to vitamin D intakes from the diet.

Toxic levels of vitamin D₃ have not been well established in humans (National Research Council, 1989). In young children, hyperparathyroidism D has been associated with daily dietary intake only about 5 times the RDA – 45 μg (1800 IU) (American Academy of Pediatrics, 1963). Overuse of vitamin D in Britain and Europe during and after World War II led to frequent episodes of idiopathic hypercalcemia in infants at intakes between 70 and 100 μg/d (American Academy of Pediatrics, 1988a). Thus, use of supplements containing vitamin D may be detrimental for the normal child who drinks two or more glasses of vitamin D₃-fortified milk per day and is exposed to regular sunlight (American Academy of Pediatrics, 1963). In adults, adverse effects have been reported from consumption of 1250 μg/d (50,000 IU/d) of vitamin D₃ (Davies and Adams, 1978; Davies et al., 1986; Harrison, 1978).

3. **Sunlight**

Sunlight exposure results in the synthesis of vitamin D₃ through the action of 7-dehydrocholesterol on the skin (MacLaughlin et al., 1982). The efficiency of the action of ultraviolet light in producing vitamin D is affected by the physical properties of the skin and the environment. Physical factors that reduce skin exposure to vitamin D-producing ultraviolet radiation are skin pigmentation, clothing, indoor living, and use of sunscreens. Environmental factors, such as time of day, season, and latitude, affect exposure to vitamin D-producing ultraviolet radiation. Exposure is greatest at midday, reaches an annual peak in midsummer, and decreases with the distance from the earth’s equator. Together these factors are responsible for the wide variation of vitamin D biosynthesis occurring in individuals.

Under appropriate conditions, individuals who are regularly exposed to sunlight do not have a dietary requirement for vitamin D (National Research Council, 1989). However, individuals who are exposed to very little sunlight, particularly those living in extreme latitudes during the winter months, require a dietary supply of the vitamin.

Hypervitaminosis D has never been reported from excessive exposure to sunlight (Combs, 1992). Although high oral doses of vitamin D₃ can lead to very high levels of plasma 25(OH)D₃ (more than 400 ng/mL) associated with toxicity, intensive UV-radiation can rarely produce plasma concentrations more than one-fifth this level. Most persons show seasonal fluctuations in circulating plasma levels of 25(OH)D₃. Normal plasma 25(OH)D₃ concentrations in humans are 15 to 38 ng/mL (Combs, 1992).

According to Holick (1994), a combination of sunlight exposure and 5 μg (200 IU) of vitamin D₃ appears to be adequate in meeting an adult’s daily requirement for vitamin D₃. Inadequate data are
available to determine the exact amount of vitamin D₃ required by adults who are not regularly exposed to sunlight. Because of the low vitamin D₃ content of human milk, a supplement of 10 μg (400 IU) is recommended to prevent deficiency in breast-fed infants who are deeply pigmented and do not get adequate sunlight exposure (American Academy of Pediatrics, 1993b). Although the vitamin D₃ content of infant formula is higher than the vitamin D₃ content of human milk, deficiency also may occur in formula-fed infants who are not exposed to sunlight and do not obtain sufficient amounts of vitamin D₃ in the diet.
IV. BIOLOGICAL STUDIES

A. OVERVIEW OF VITAMIN D METABOLISM

The formation, metabolism, and metabolic roles of vitamin D have been studied extensively (DeLuca, 1993; Holick, 1994; Norman et al., 1982a). In vertebrate animals, vitamin D activity is essential for skeletal formation and maintenance of calcium and phosphorus homeostasis. By controlling intestinal absorption of calcium and mobilization of bone stores through the hormonal action of 1α,25(OH)₂D₃, vitamin D regulates the calcium and phosphorus concentrations necessary for cellular metabolism, neuromuscular function, and bone ossification (Norman et al., 1982b). (See Figure 4). In addition, the active form of vitamin D₃, 1α,25-dihydroxyvitamin D₃ [1α,25(OH)₂D₃], is now considered a hormone involved in many physiological functions of the immune system, reproductive system, insulin secretion, and differentiation of keratinocytes and promyelocytes (DeLuca, 1993; Holick, 1994; Norman et al., 1982a; Norman, 1990).

The active hormone, 1α,25(OH)₂D₃, is formed from vitamin D₃ (cholecalciferol). Vitamin D₂ (ergocalciferol) is found only in plants and is formed from ergosterol as a result of ultraviolet light photoconversion. The quantity of vitamin D₃ ingested from foods of plant origin and converted to the active hormone is limited. The major source of vitamin D₃ activity in humans, as in other vertebrates, is formation in the skin from 7-dehydrocholesterol photochemically upon exposure to ultraviolet radiation (280-310 nm) (MacLaughlin et al., 1982) or ingestion of foods such as liver, eggs, fish liver oils, and vitamin D-fortified foods such as milk, grains, pasta, and cereals.

Once formed in the skin or ingested, vitamin D₃ is transported to the liver where it is converted by hydroxylation to the major circulating form, 25(OH)D₃, which is considered a prohormone. This form is transported to the proximal convoluted tubule cells of the kidney where it is further hydroxylated to 1α,25(OH)₂D₃. This form of the vitamin is considered to be the hormone that controls various functions in the several target organ systems. The 25(OH)D₃ intermediate is also converted into other dihydroxycholecalciferols and derivatives that have some hormonal activity.

1α,25(OH)₂D₃ has the same functions in poultry as in other vertebrate animals (Bouillon et al., 1995; Norman, 1990). (See Figure 4.) It regulates calcium and phosphorus homeostasis, osteocalcin formation, bone growth, egg shell formation, as well as other endocrine system functions (Ameenuddin et al., 1985; Arnold, 1977; National Research Council, 1994).

In the chicken, 25(OH)D₃ is the major circulating form of vitamin D₃ followed by vitamin D₂ and three dihydroxy derivatives (1α,25; 24,25; and 25,26 dihydroxyvitamin D₃) (Haussler and Rasmussen, 1972). The initial hydroxylation of vitamin D₃ occurs primarily in the liver and is catalyzed by a 25-hydroxylase enzyme which is a mixed-function oxidase of the cytochrome P-450 class of hydroxylases. In the liver, two hydroxylases have been identified, one microsomal and the other mitochondrial (Bergman and Postlind, 1990; Su et al., 1990).

Hughes et al. (1977) observed that 1-day-old chicks fed vitamin D₃-depleted diets for 3 to 4 weeks had plasma concentrations of 25(OH)D₃ and 1α,25(OH)₂D₃ that were essentially undetectable (radioassay limit of detection 20 ng/mL and 20 pg/mL, respectively). However, birds fed diets with 35 µg/kg of feed had plasma concentrations of 29 ng/mL for 25(OH)D₃ and 0.06 ng/mL for 1α,25(OH)₂D₃. Further, chicks fed diets with 1750 µg/kg of feed had plasma concentrations of 104 ng/mL for 25(OH)D₃ and 0.42 ng/mL for 1α,25(OH)₂D₃. Hughes et al. (1977) suggested that the concentration of circulating 25(OH)D₃ was correlated with the availability of dietary vitamin D and amount of sunlight exposure,
Figure 4. Schematic Diagram of Metabolism and Endocrine Functions of Vitamin D (Bouillon et al., 1995).
while the circulating levels of 1α,25(OH)₂D₃ were under more stringent control. Hughes et al. (1977) observed that lack of stringent regulation of the 25-hydroxylase enzyme and more stringent regulation of the 1α-hydroxylase were the probable cause. Because dietary and environmental conditions are known to influence fluctuations in plasma concentrations of 25(OH)D₃, it is utilized as an index or measure of vitamin D nutritional status.

The conversion of 25(OH)D₃ to 1α,25(OH)₂D₃ and 24,25(OH)D₃ is dependent on the physiological state of the bird (Horst and Littlefield, 1982). Hypocalcemia stimulates the release of parathyroid hormone (PTH) from the parathyroid glands, and PTH receptors in the kidney cause a signal transduction which results in activation of the 1α-hydroxylase enzyme. The mechanism is thought to be mediated via activation of protein kinase-C (Janulis et al., 1992). The 24-hydroxylase is elevated by 1α,25(OH)₂D₃ through a receptor-mediated process (Mandla et al., 1990). PTH is also thought to suppress 24-hydroxylase (Shinkai et al., 1992). Hypophosphatemia also stimulates renal 1α,25(OH)₂D₃ synthesis (Gray and Napoli, 1983). This stimulation of the 1α-hydroxylase by hypophosphatemia is not PTH mediated but was shown to be completely blocked by hypophysectomy (Gray, 1981).

In summary, the metabolic role and intermediary metabolism of vitamin D₃ in poultry are well established and are consistent with knowledge of vitamin D metabolism in other species.

B. VITAMIN D ACTIVITY IN POULTRY FEED SUPPLEMENTS

In the commercial broiler chicken industry, vitamin D₃ is added to feed as the primary source of vitamin D activity (National Research Council, 1994). Vitamin D is added to poultry feed because most birds are raised in houses with minimal exposure to sunlight and artificial light containing ultraviolet radiation. Thus, a dietary source of vitamin D activity assures an adequate supply for growth and development. Husbandry conditions in the commercial broiler industry are such that feed is essentially the sole source of vitamin D activity. The quantity provided in feed typically exceeds that known to be required nutritionally because the broiler industry desires to obtain uniform growth of birds in 45 to 55 days to a weight of 4 to 5 pounds.

Vitamin D₃ is not used as a source in poultry feed because its biological activity is only 10 percent that of vitamin D₉ (Chen and Bosmann, 1964; Drescher et al., 1969). Hoy et al. (1968) demonstrated that the vitamin D binding protein affinity for vitamin D₃ is about 5 times that of vitamin D₉.

Amoco BioProducts Corporation has developed a 25(OH)D₃ product as a potential source of vitamin D activity for broiler poultry feed. This effort is based on the availability of 25(OH)D₃ from a potentially cost effective manufacturing process.

Shortly after 25(OH)D₃ became available for investigational use, Myrtle and Norman (1971) conducted studies with White Leghorn chicks in which the relative activities of vitamin D₃ and 25(OH)D₃ were determined. Chicks were fed a rachitogenic diet for 3 weeks. During the 4th week, the influence of vitamin D₃ and 25(OH)D₃ on in vivo ⁴⁶Ca²⁺ transport across the duodenum was evaluated 24 hours after the test compounds were given to the chicks. Although the number of comparisons was few, the data indicated that 25(OH)D₃ was 1.4 to 1.8 times as active as vitamin D₃ in promoting ⁴⁶Ca²⁺ transport when doses were low (0.0325 to 0.0425 μg/chick). However, when vitamin D₃ and 25(OH)D₃ doses were 0.125 to 0.130 μg/chick, no difference in activity was evident. Haussler and Rasmussen (1972) used a similar approach except that they fed rachitogenic chicks a low-calcium diet (< 0.1 percent) for 3 days before giving a single oral dose of the test compounds. On the basis of incremental changes in plasma calcium determined 24 hours after the sterols were administered, it was concluded that 25(OH)D₃ was 4 times as active as vitamin D₃.
McNutt and Haussler (1973) fed White Leghorn chicks a rachitogenic diet from 1 to 28 days of age. Starting at day 8, chicks were given an oral dose of about 0.02 or 0.15 μg of vitamin D₃, 25(OH)D₃, or 1α,25(OH)₂D₃ per chick every other day for 21 days. Differences in relative activities of vitamin D₃ and its two metabolites were most evident when low doses were given. Both 25(OH)D₃ and 1α,25(OH)₂D₃ were between 1.5 and 2.2 times as active as vitamin D₃ with regard to stimulation of weight gain and maintenance of plasma calcium concentrations. 1α,25(OH)₂D₃ was found to be 1.3 times more potent than vitamin D₃ in terms of supporting normal calcium and bone metabolism.

Norman and Wong (1972) reported that when 0.75 μg of vitamin D₃ or 25(OH)D₃ were given per os daily for 21 to 23 days to chicks fed a rachitogenic diet, 25(OH)D₃ was twice as active as vitamin D₃ in support of weight gain. However, no differences were observed in relative activities in the instances of in vivo ⁴⁶Ca²⁺ transport in the intestine nor in enhancement of bone ash. In contrast to the latter observation, Boris et al. (1977) found that when relatively low daily doses (0.03 μg) of the sterols were given to rachitic chicks, 25(OH)D₃ was 4 times as active as vitamin D₃ in promotion of bone ash. But, in agreement with the observations of Norman and Wong (1972), relative activities were not different when greater amounts of sterols (0.1 μg/chick) were given daily for 21 to 23 days. The data presented by Boris et al. (1982) supported the previous observations (Boris et al., 1977) that the relative activity of 25(OH)D₃ for bone ash development was greater than that of vitamin D₃ when these sterols were given in such low doses to rachitic chicks.

In one of the few studies that did not involve the use of rachitic chicks, McNaughton et al. (1977) found that approximately 50 percent less dietary 25(OH)D₃ than vitamin D₃ was required to maximize bone ash in broiler chicks. The concentrations required for maximum bone ash were 6.6 and 8.9 μg/kg of diet for 25(OH)D₃ and vitamin D₃, respectively.

Sunde (1970) evaluated 25(OH)D₃ in practical diets of turkey poults, using the incidence of leg disorders as the primary criterion. Generally, lower dietary concentrations of 25(OH)D₃ than of vitamin D₃ were needed to minimize incidence of leg disorders in 3- to 4-week-old poults. This difference was most evident when vitamin D₃ or 25(OH)D₃ dietary concentrations ranged from 10 to 25 μg/kg of feed. In these instances, incidence of leg disorders was reduced by 50 percent or more when 25(OH)D₃ was fed compared with vitamin D₃.

In summary, although several studies have been done to compare the biological activities of 25(OH)D₃ with vitamin D₃ for poultry, the findings have not resulted in definitive information that could be used to establish relative activity of 25(OH)D₃ versus vitamin D₃. Relative activities derived by using rachitic chicks varied considerably depending on the doses of the sterols administered and response criteria used. Besides using rachitic animals, most studies used single or multiple oral dosings of the sterols instead of testing 25(OH)D₃ and vitamin D₃ as dietary constituents. Nevertheless, data obtained on bone development with broiler chicks and on incidence of leg disorders with poults showed that the activity of 25(OH)D₃ was greater than that of vitamin D₃, especially when the sterols were present in low concentrations. This could be expected on the basis that the absorption of 25(OH)D₃ is more efficient than that of vitamin D₃ in chicks (Bar et al., 1980) and in rats (Sitrin et al., 1982) and the need for hydroxylation of vitamin D₃ in the liver is unnecessary when 25(OH)D₃ is given.
C. SAFETY OF 25(OH)D₃

An evaluation of the safety of 25(OH)D₃ as a broiler feed ingredient and subsequently as a component of human food is necessary in order to assess its potential toxicity in the target species and in humans who consume the target species raised on 25(OH)D₃-supplemented feed.

1. Toxicity in poultry

Pertinent data on the toxicity of 25(OH)D₃ in broiler chickens are limited. Most published studies on this vitamin D₃ metabolite have reported investigations on efficacy rather than possible adverse effects on growth and development.

a. Chickens

A key paper by Morrissey et al. (1977) presented data on the possible toxicity of 25(OH)D₃ in chickens. In this study, chicks of an unnamed type from the age of 14 days were fed diets supplemented with 0, 10, 100, 1000, 10,000, or 100,000 μg of vitamin D₃ or 25(OH)D₃/kg of feed. The group of chicks that received the diet containing 10 μg vitamin D₃/kg of feed (equivalent to 400 IU/kg of feed) was designated as the control. Chicks were killed after 3, 6, or 14 days. The parameters that were evaluated included growth, feed consumption, tibia weight and ash content, and kidney weight, as well as the histology of liver, terminal duodenum, aorta, tibia, and kidney. The results indicated that weight gain was reduced with 100,000 μg of 25(OH)D₃/kg of feed after 3 days (P < 0.05) and 10,000 μg vitamin D₃ or 25(OH)D₃/kg of feed after 6 or 14 days (P < 0.05). Feed consumption followed a similar pattern. One chick that was fed the diet containing 100,000 μg of 25(OH)D₃/kg of feed died on day 1 of the study; by day 14, all chicks that were fed 25(OH)D₃ at this level had died. In the group fed the diet containing 100,000 μg of vitamin D₃/kg of feed, one chick died by day 14 of the study. Tibial, hepatic, and aortic tissues from all chicks were examined and found to be histologically normal. However, epithelial necrosis and mineralization in the distal convoluted tubules of the kidney were noted in chicks fed 10,000 μg of vitamin D₃/kg of feed for 3 or more days. Similar lesions were found after 3 days in chicks fed 100 μg of 25(OH)D₃/kg of feed. Renal calcium concentration increased significantly (P < 0.05) when 25(OH)D₃ was administered at 1000 and 10,000 μg/kg of feed and when vitamin D₃ was administered at 10,000 μg/kg of feed. Morrissey et al. (1977) concluded that 25(OH)D₃ was approximately 100 times as toxic as vitamin D₃ and that the maximum "safe" dietary levels of vitamin D₃ and 25(OH)D₃ were 1000 and 10 μg/kg of feed, respectively. A safe upper dietary level of 1000 μg vitamin D₃/kg of feed for chickens is consistent with other findings (National Research Council, 1987). This level represents 200 times the dietary requirement of vitamin D₃ for chickens.

A contributing factor to the higher toxicity of 25(OH)D₃ relative to vitamin D₃ may be an increased absorption. According to Bar et al. (1980), vitamin D-deficient chicks absorbed a higher percentage of 25(OH)D₃ than vitamin D₃, 83.6 and 66.5 percent, respectively, from a diet containing 15 μg of 25(OH)D₃ or vitamin D₃/kg of feed.

Other published findings on the effects of administering 25(OH)D₃ to growing chickens either in the diet or as an oral dose are summarized in Table 9. In general, no signs of toxicity were found when 25(OH)D₃ was provided at dietary levels up to 10 μg/kg of feed for periods up to 56 days or at dietary levels of 5 μg/48 hours over a 35-day period.
Table 9. Research Findings on the Effects of 25(OH)D₃ in Growing Chickens.

<table>
<thead>
<tr>
<th>Species/type</th>
<th>Number</th>
<th>Age</th>
<th>Amount (µg/kg)</th>
<th>Duration</th>
<th>Route</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chickens/broiler</td>
<td>420</td>
<td>1 d</td>
<td>1-6.6¹</td>
<td>21 d</td>
<td>Diet</td>
<td>Growth rate not affected significantly. Linear effect of dietary level on growth and tibia ash content</td>
<td>McNaughton et al. (1977)</td>
</tr>
<tr>
<td>Chickens/not stated</td>
<td>180</td>
<td>14 d</td>
<td>0, 10, 100, 1000, 10,000, 100,000</td>
<td>14 d</td>
<td>Diet</td>
<td>Renal calcification with 100 µg/kg after 3 d. Weight gain reduced with 100,000 µg/kg after 3 d and with 10,000 µg/kg for 6 d. Mortality at 5 d with 100,000 µg/kg and at 14 d with 10,000 µg/kg</td>
<td>Morrissey et al. (1977)</td>
</tr>
<tr>
<td>Chickens/broiler</td>
<td>432</td>
<td>1 d</td>
<td>1.25 - 5</td>
<td>56 d</td>
<td>Diet</td>
<td>Linear improvement in production with increasing 25(OH)D₂. Production better with 25(OH)D₃ than with D₂. No effects on bone strength</td>
<td>Cantor &amp; Bacon (1978)</td>
</tr>
<tr>
<td>males</td>
<td></td>
<td>(?)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chickens/Arbor Acre</td>
<td>360</td>
<td>28 d</td>
<td>1.5¹</td>
<td>21 d</td>
<td>Diet</td>
<td>Production lower with 25(OH)D₃ than with D₃</td>
<td>Soares et al. (1978)</td>
</tr>
<tr>
<td>broiler males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chickens/Arbor Acre</td>
<td>357</td>
<td>1 d</td>
<td>1.5¹</td>
<td>21 d</td>
<td>Diet</td>
<td>Production lower with 25(OH)D₃ than with D₃</td>
<td>Soares et al. (1978)</td>
</tr>
<tr>
<td>broiler males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chickens/broiler</td>
<td>250</td>
<td>1 d</td>
<td>0.005-5 µg/bird/48 h</td>
<td>35 d</td>
<td>Oral</td>
<td>Better growth with D₃ than with 25(OH)D₂, at similar dosage; poorer growth with 1α,25(OH)₂D₃</td>
<td>Masoni et al. (1984)</td>
</tr>
<tr>
<td>males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chickens/broiler</td>
<td>Not stated</td>
<td>1 d</td>
<td>10</td>
<td>16 d</td>
<td>Diet</td>
<td>Reduced weight gain in 1 of 3 experiments</td>
<td>Edwards (1989)</td>
</tr>
<tr>
<td>males</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Calculated (see text).
McNaughton et al. (1977) compared the toxicity of 25(OH)D₃ and vitamin D₃ in starting broiler diets. At levels providing up to 6.6 μg of 25(OH)D₃ or vitamin D₃/kg of feed, no toxic effects were found. Growth rate was not significantly different between the two sources of vitamin D, and a linear effect of the dietary level was evident on body weight and tibia ash content. The authors concluded that the potency of 25(OH)D₃ was 1.5 times greater than vitamin D₃.

Soares et al. (1978) fed diets containing 25(OH)D₃ and vitamin D₃ to male broiler chicks from 1-day-old or from 28 days of age for 21 days. Levels up to 3.8 μg of vitamin D₃ and 1.5 μg of 25(OH)D₃/kg of feed were provided. The authors estimated that 25(OH)D₃ was 2.5 times as potent as vitamin D₃. Production and a biological index of phosphorus availability were found to be lower with 25(OH)D₃ than with vitamin D₃. These results were attributed to a feedback mechanism on 25(OH)D₃ or to a loss in activity during the assay period. It is also possible that the relative activity of 25(OH)D₃ had been overestimated because the authors concluded that 25(OH)D₃ was 2 times as effective as vitamin D₃ in growing broiler chicks. In view of the findings of other researchers, it is unlikely that the results can be attributed to toxicity.

Cantor and Bacon (1978) fed diets supplemented with vitamin D₃ or 25(OH)D₃ at levels up to 5 μg/kg of feed to male broiler chickens from 1 day to 8 weeks of age. Production was improved with increasing levels of supplementation and was better in chicks fed 25(OH)D₃ than in those fed vitamin D₃. Incidence of broken bones associated with processing cage-reared broilers was unaffected by the vitamin D source provided.

Masoni et al. (1984) fed a rachitogenic diet containing 1.85 percent calcium, 0.4 percent phosphorus, and 0.25 percent magnesium to 250 1-day-old broiler cockerels for 3 to 5 weeks. Oral doses of vitamin D₃, 25(OH)D₃, or 1α,25(OH)₂D₃ were administered on alternate days. No growth depression was observed during the 3- to 5-week period with the highest dose of 25(OH)D₃ administered (5 μg/48 hours).

Edwards (1989) fed diets containing 27.5 μg of vitamin D₃ or 10 μg of 25(OH)D₃/kg of feed to 1-day-old broiler cockerels and reported a significantly lower (P < 0.05) 16-day weight with the 25(OH)D₃-fed group in one experiment. The effect, however, was not observed in other experiments, and weight gain was not depressed when this vitamin D metabolite was added to a diet containing vitamin D₃.

Estimates of tolerance of 25(OH)D₃ by broiler chickens can also be assessed from a number of field trials conducted for Amoco BioProducts Corporation. Results of these studies are summarized in Table 10 and are discussed below.

Field trial PARC 89-51 (McNaughton, 1989) set out to determine how well 25(OH)D₃ could functionally replace vitamin D₃ in broiler feed containing marginal levels of vitamin D activity and available phosphorus. In this trial, 3040 broilers from 1 day to 46 days of age were fed either vitamin D₃ or 25(OH)D₃ to provide 6.2 μg of vitamin D₃ or 25(OH)D₃/kg of feed. Results showed that better growth, feed efficiency, and lower mortality were evident in chickens fed 25(OH)D₃ than in those fed the same unit-dose level of vitamin D₃ at all levels of available phosphorus, 0.140, 0.215, 0.290, and 0.365 percent in starter feed (day 1-21) and 0.132, 0.207, 0.282, and 0.357 percent in grower feed (day 22-46).

The aim of field trial PARC 90-11 (McNaughton, 1990b) was to determine whether 25(OH)D₃ exhibited toxicity effects when added to broiler diets at levels up to 75 times the NRC dietary requirement for vitamin D₃, and to compare the effects of 25(OH)D₃ with vitamin D₃ when provided at the same concentration. In this study, 4800 broilers from 1 day to 46 days of age were fed diets to which either vitamin D₃ or 25(OH)D₃ were added at six different levels ranging from 6.2 to 450 μg/kg of feed.
Table 10. Amoco BioProducts Corporation Research Studies on the Effects of 25(OH)D₃ in Growing Chickens.

<table>
<thead>
<tr>
<th>Species/type</th>
<th>Number</th>
<th>Age</th>
<th>Amount (µg/kg)</th>
<th>Duration</th>
<th>Route</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler/Petersen x Arbor Acre</td>
<td>3,040</td>
<td>1 d</td>
<td>6.2 as D₃ or 25(OH)D₃</td>
<td>46 d</td>
<td>Diet</td>
<td>Production better and mortality lower with 25(OH)D₃</td>
<td>McNaughton (1989)</td>
</tr>
<tr>
<td></td>
<td>4,800</td>
<td>1 d</td>
<td>6.2 - 450 as D₃ or 25(OH)D₃</td>
<td>46 d</td>
<td>Diet</td>
<td>No effects on production or mortality</td>
<td>McNaughton (1990b)</td>
</tr>
<tr>
<td></td>
<td>3,200</td>
<td>1 d</td>
<td>6.2 or 56.2 as D₃ or 25(OH)D₃</td>
<td>46 d</td>
<td>Diet</td>
<td>No effects on production or mortality</td>
<td>McNaughton (1990c)</td>
</tr>
<tr>
<td></td>
<td>2,400</td>
<td>1 d</td>
<td>6.2 or 25(OH)D₃</td>
<td>46 d</td>
<td>Diet</td>
<td>No effects on production or mortality</td>
<td>McNaughton (1990d)</td>
</tr>
<tr>
<td></td>
<td>3,200</td>
<td>1 d</td>
<td>6.2 or 56.2 as D₃ or 25(OH)D₃</td>
<td>46 d</td>
<td>Diet</td>
<td>No effects on production or mortality</td>
<td>McNaughton (1990e)</td>
</tr>
<tr>
<td></td>
<td>2,400</td>
<td>1 d</td>
<td>56.2</td>
<td>46 d</td>
<td>Diet</td>
<td>No effects on production or mortality</td>
<td>McNaughton (1990f)</td>
</tr>
<tr>
<td>Broiler/Petersen x Arbor Acre</td>
<td>7,200</td>
<td>1 d</td>
<td>2.5 - 20 as D₃ or 25(OH)D₃</td>
<td>46 d</td>
<td>Diet</td>
<td>Improved production and higher bone ash content with 25(OH)D₃</td>
<td>McNaughton (1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reduced mortality with 25(OH)D₃ at the highest level</td>
<td>PARC 91-40</td>
</tr>
<tr>
<td>Broiler males/Arbor Acre</td>
<td>2,000</td>
<td>1 d</td>
<td>66.8 in Starter and Grower then 41.3 in Finisher, as D₃ or 25(OH)D₃</td>
<td>49 d</td>
<td>Diet</td>
<td>No effects on production or mortality</td>
<td>Quarles (1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Colorado AM-1-91</td>
</tr>
<tr>
<td>Broiler/Arbor Acre</td>
<td>3,600</td>
<td>1 d</td>
<td>34.4 - 137.5 D₃ or 25(OH)D₃, separately or in combination</td>
<td>48 d</td>
<td>Diet</td>
<td>No effects on production or mortality</td>
<td>Quarles (1992a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Colorado AM-1-92</td>
</tr>
<tr>
<td>Species/type</td>
<td>Number</td>
<td>Age</td>
<td>Amount (µg/kg)</td>
<td>Duration</td>
<td>Route</td>
<td>Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------</td>
<td>--------</td>
<td>-----</td>
<td>-------------------------------------------------------------------------------</td>
<td>----------</td>
<td>-------</td>
<td>------------------------------------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Broiler/Arbor Acre</td>
<td>3,921</td>
<td>1 d</td>
<td>68.8 or 137.5 D₃ or 25(OH)D₃, separately or in combination</td>
<td>50 d</td>
<td>Diet</td>
<td>No effects on production or mortality although mortality higher than in previous trials</td>
<td>Quarles (1992b) Colorado AM-2-92</td>
</tr>
<tr>
<td>Broiler/Petersen x Arbor Acre</td>
<td>5,920</td>
<td>1 d</td>
<td>1.7 - 68.8 as D₃ or 25(OH)D₃</td>
<td>47 d</td>
<td>Diet</td>
<td>Improved production, higher bone ash content, and reduced mortality with 25(OH)D₃</td>
<td>McNaughton (1993a) PARC 93-AMO-07-B</td>
</tr>
<tr>
<td>Broiler/Petersen x Arbor Acre</td>
<td>7,200</td>
<td>1 d</td>
<td>1.7 - 68.8 as D₃ or 25(OH)D₃</td>
<td>46 d</td>
<td>Diet</td>
<td>Improved production and higher bone ash content with 25(OH)D₃</td>
<td>McNaughton (1993b) PARC 93-AMO-08-B</td>
</tr>
<tr>
<td>Broilers/Arbor Acre</td>
<td>1,120</td>
<td>1 d</td>
<td>68.8 as D₃, 68.8 - 687.6 as 25(OH)D₃</td>
<td>49 d</td>
<td>Diet</td>
<td>No effects on production or mortality</td>
<td>Quarles (1993a) Colorado AM-1-93</td>
</tr>
<tr>
<td>Broilers/Arbor Acre</td>
<td>3,600</td>
<td>1 d</td>
<td>34.4 - 86.0 as 25(OH)D₃, 68.8 as D₃</td>
<td>53 d</td>
<td>Diet</td>
<td>No effects on production or mortality</td>
<td>Quarles (1993b) Colorado AM-3-93</td>
</tr>
<tr>
<td>Broiler/Petersen x Arbor Acre</td>
<td>3,500</td>
<td>1 d</td>
<td>68.8, 687.6, 3437.8, 6875.7, and 13,751.4 as D₃ or 25(OH)D₃</td>
<td>48 d</td>
<td>Diet</td>
<td>Renal tubular degeneration and mineralization and bone lesions with 687.6 µg 25(OH)D₃ at 22 d. Renal tubular degeneration and mineralization with 3437.8 µg D₃/kg and bone lesions with 13,751.4 µg D₃/kg at 22 d. Mortality higher at 22 d with 25(OH)D₃ than D₃.</td>
<td>Quarles (1994a,b) Colorado AM-2-94</td>
</tr>
</tbody>
</table>
No treatment effects were noted for weight gain, feed efficiency, or mortality. However, a trend toward increased mortality was evident in broilers fed diets containing 225 to 450 μg of 25(OH)D₃/kg of feed. No other parameters were studied.

The PARC 90-12 (McNaughton, 1990c) field trial compared the effects of using vitamin D₃ and 25(OH)D₃ on mycotoxin toxicity in broilers. This study involved 3200 broilers from 1 day to 46 days of age. Diets supplemented with either vitamin D₃ or 25(OH)D₃ were fed to provide vitamin D levels at the NRC minimum dietary requirement of 6.2 μg/kg of feed or at a typical commercial level of 56.2 μg/kg of feed with and without the addition of 100 ppb aflatoxin. Source or level of vitamin D supplementation of the diet had no effect on body weight or feed efficiency; however, the presence of aflatoxin did adversely affect weight gain after 46 days among birds fed 6.2 μg of vitamin D₃ or 25(OH)D₃/kg of feed. Mortality was highest (10.3 percent) during the first 21 days when the diet contained 6.2 μg of vitamin D₃/kg of feed and aflatoxin. Lower mortality rates of about 2 percent were found during the first 21 days among birds fed diets containing 6.2 μg of 25(OH)D₃/kg of feed and 56.2 μg of vitamin D₃ and 25(OH)D₃/kg of feed with and without the addition of aflatoxin. Few additional toxic effects were found during the subsequent 22 to 46 day period with either source of vitamin D. During this time, mortality increased to 11.5 percent among birds fed 6.2 μg of vitamin D₃/kg of feed and aflatoxin and increased to a range of 2.1 to 5.6 percent among birds fed diets containing 6.2 μg of 25(OH)D₃ and 56.2 μg of vitamin D₃ and 25(OH)D₃/kg of feed with and without the addition of aflatoxin.

The purpose of the PARC 90-13 (McNaughton, 1990d) field trial was to determine how well 25(OH)D₃ could functionally replace vitamin D₃ in broiler feed at marginal levels of vitamin D providing 6.2 μg of vitamin D₃ or 25(OH)D₃/kg of feed and 0.282, 0.357, and 0.432 percent of available phosphorus. A total of 2400 birds were involved in this study from 1 day to 46 days of age. The vitamin D source had no effect on body weight, feed efficiency, or mortality.

Field trial PARC 90-14 (McNaughton, 1990e) set out to determine if feed phosphorus could be utilized more efficiently when 25(OH)D₃ replaced vitamin D₃ in broiler feed at 6.2 μg/kg of feed and at commercially used levels of 56.2 μg/kg of feed. This study involved 3200 birds from 1 day to 46 days of age. Marginal levels of available phosphorus were used at both levels of vitamin D activity. Starter feed (day 1-21) contained 0.356 or 0.44 percent available phosphorus, whereas grower feed (day 22-46) contained 0.357 or 0.44 percent available phosphorus. No apparent differences in body weight, feed efficiency, or mortality were found when 25(OH)D₃ or vitamin D₃ were fed at both levels of supplementation.

A similar 46-day study was conducted with 2400 broilers in the PARC 90-15 (McNaughton, 1990f) field trial. The purpose of this study was to determine how well 25(OH)D₃ could functionally replace vitamin D₃ at commercially used levels of vitamin D activity that provided 56.2 μg of vitamin D₃ or 25(OH)D₃/kg of feed, and at marginal levels of available phosphorus (0.29, 0.365, and 0.44 percent in the starter feed and 0.282, 0.357, and 0.432 percent in the grower feed). No effects of vitamin D source on body weight, feed efficiency, or mortality were noted.

The PARC 91-40 (McNaughton, 1991) field trial was designed to compare the biological availability of 25(OH)D₃ with a commercially used vitamin D₃ preparation as a dietary supplement for broiler production. In this study, 7200 broilers were fed diets containing vitamin D₃ or 25(OH)D₃ at 0, 2.5, 5, 10, and 20 μg/kg of feed for 46 days. Results indicated that 25(OH)D₃ improved body weight, feed efficiency, and bone ash content compared to vitamin D₃. Mortality was highest (23.1 percent) when no source of vitamin D activity was fed and when vitamin D₃ or 25(OH)D₃ were fed at 2.5 μg/kg of feed (25.9 percent and 24.7 percent, respectively). Mortality was lowest (9.2 percent) when 25(OH)D₃ was fed at 20 μg/kg of feed.
The objective of the PARC 93-AMO-07-B (McNaughton, 1993a) field trial was to determine the levels of 25(OH)D₃ required to equal the performance of different levels of vitamin D₃ when diets were used that met or exceeded NRC recommendations. In this study, 5920 broilers from 1 day to 46 days of age were fed diets supplemented with 25(OH)D₃ or vitamin D₃ at 1.7 to 68.8 μg/kg of feed. Production was better and mortality was lower with 25(OH)D₃ than with vitamin D₃. No effects of vitamin D source on blood calcium content were noted at the higher levels of supplementation. Bone ash content was higher with increasing levels of supplementation of 25(OH)D₃ than with vitamin D₃. The PARC 93-AMO-08-B (McNaughton, 1993b) field trial followed the same protocol as the PARC 93-AMO-07-B (McNaughton, 1993a) study but involved 7200 broilers. Similar results were found.

Field trial Colorado AM-1-91 (Quarles, 1991) was designed to determine if 25(OH)D₃ could functionally replace vitamin D₃ in broiler feed. This study involved 2000 birds from 1 day to 49 days of age. The treatments provided 25(OH)D₃ at the same level as vitamin D₃ in all feed types. Starter and grower feed contained 68.8 μg of vitamin D₃ or 25(OH)D₃/kg of feed. Finisher feed provided 41.3 μg of vitamin D₃ or 25(OH)D₃/kg of feed. No treatment effects were found on production or mortality.

The purpose of field trial Colorado AM-1-92 (Quarles, 1992a) was to evaluate the vitamin D activity of 25(OH)D₃ at various levels. It involved 3600 broilers starting at one day of age. Vitamin D₃ and 25(OH)D₃ were added to 6 diets separately and in combination to provide 34.4 to 137.5 μg of vitamin D₃ or 25(OH)D₃/kg of feed. After 48 days, no effects on production or mortality were noted.

Colorado AM-2-92 (Quarles, 1992b) investigated the effects of substituting vitamin D₃ with 25(OH)D₃ on broiler performance and carcass quality. The trial studied 3921 broilers from 1 day to 50 days of age. The two vitamin D sources were added to 7 diets, separately and in combination, to provide 68.8 μg/kg of feed, except for treatment 2 which provided twice this amount. No apparent differences were noted in production or mortality.

Colorado AM-3-93 (Quarles, 1993b) investigated the effects of administering varying levels of 25(OH)D₃ and a commercially used level of vitamin D₃ on broiler production. A total of 3600 broilers from 1 day to 53 days of age were fed diets providing 68.8 μg of vitamin D₃/kg of feed or 34.4 to 86.0 μg of 25(OH)D₃/kg of feed. No adverse effects were observed on production and mortality.

The Colorado AM-1-93 (Quarles, 1993a) field trial was a safety study that investigated the effects of providing 25(OH)D₃ in the diet at levels up to 10 times the maximum NRC recommended level on broiler production and mortality. In this study, 1120 broilers from 1 day to 49 days of age were fed diets supplemented with 68.8 μg of vitamin D₃/kg of feed or with 68.8, 206.3, or 687.6 μg of 25(OH)D₃/kg of feed. Body weight and feed efficiency were similar across all treatments. Mortality was lower with 25(OH)D₃ (12.5 to 14.3 percent) than with vitamin D₃ (16.8 percent). The high mortality recorded in this trial might have been related to the low starting weight. No gross pathological effects were noted, and no histopathological effects were found in birds fed the highest concentrations of 25(OH)D₃.

Because most of the aforementioned studies conducted for Amoco Bioproducts Corporation have dealt primarily with efficacy rather than the possible adverse effects of 25(OH)D₃, the Expert Panel requested that an additional toxicity study be conducted. This study, Colorado AM-2-94 (Quarles, 1994a,b), focused on determination of occurrence of toxic effects when 25(OH)D₃ is administered in poultry broiler feed at 1, 10, 50, 100, and 200 times the commercial level proposed by Amoco BioProducts Corporation (68.8 μg/kg).

This study, involving 3500 broiler chickens (1750 male, 1750 female) from 1 day to 49 days of age, included 10 treatments. Each treatment consisted of 350 birds randomly assigned to 70 pens (7 pens per treatment, 50 birds per pen). Feed was supplemented with either 25(OH)D₃ or vitamin D₃ at
levels providing 68.8, 687.6, 3437.8, 6875.7, and 13,751.4 µg/kg of feed. Specific treatments are presented in Table 11. Feed and water were provided ad libitum throughout the study, and all feed added and removed from pens was weighed.

Table 11. Treatments for Safety Study AM-2-94.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>25(OH)D₃ (µg/kg of feed)</th>
<th>Vitamin D₃ (µg/kg of feed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>68.8</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>687.6</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>3437.8</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>6875.7</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>13,751.4</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>68.8</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>687.6</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>3437.8</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>6875.7</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>13,751.4</td>
</tr>
</tbody>
</table>

All birds were observed twice daily for signs of toxicity and mortality. All abnormal conditions were recorded. Periodic observations of feathering, litter conditions, and leg disorders were made throughout the study.

Weight gain was determined for each bird by pen and sex at the end of the study (day 48). Feed efficiency was calculated for days 1 to 48 and adjusted for mortalities and removed birds. All birds that died during the study were recorded and necropsied to determine probable cause of death. Those unable to get to feed and water were culled at the discretion of the investigator, and records were made of weights, day of removal, and necropsy findings of all dead and culled birds.

A complete gross necropsy was conducted on two randomly presented birds (1 male, 1 female) from each pen on approximately day 21 and day 48 or 49 of the study. Kidney, heart, aorta, and tibia tissues, as well as any other tissues with gross lesions were deposited in neutral buffered formalin and then examined histologically. Ten birds (5 male, 5 female) per pen were sacrificed after final weights were recorded and examined for gross pathology. An examination of heart, kidney, bursa of Fabricius, pancreas, eye, intestine, liver, spleen, lungs, and skin tissue was conducted for gross pathology.

After final weights were recorded and tissues were collected, 8 male birds were selected from treatments 1, 2, 6, and 7 on approximately day 49 or 50 of the study. The selected birds weighed ±5 percent of the mean body weight of each pen's male birds. A total of 56 birds were processed from each of above treatments for a total of 224 birds. From each bird, live weights (individual), hot and chilled weights (by pen), drained weight (individual), and breast weight (individual) were determined.

Results of Colorado AM-2-94 indicated that a 5.1 percent reduction in mean body weight was found among broiler chickens at 22 days of age when 25(OH)D₃ was fed at 687.6 µg/kg of feed. At higher levels of supplementation, reduction in mean body weight was more pronounced. When 25(OH)D₃ was fed at 3437.8 µg/kg of feed, a 60.7 percent reduction in mean body weight was found, and when 25(OH)D₃ was fed at 6875.7 and 13,751.4 µg/kg of feed, a 65.1 and 72.3 percent reduction in mean body weight was found, respectively. In the groups fed vitamin D₃, a 13.2 percent reduction in mean
body weight was found at 22 days when 6875.7 µg of vitamin D₃/kg of feed was fed. A more pronounced reduction (24.9 percent) in mean body weight was found when vitamin D₃ was fed at 13,751.4 µg/kg of feed.

By 48 days of age, mean body weight decreased 11.6 percent among birds fed 687.6 µg of 25(OH)D₃/kg of feed. At higher levels of 25(OH)D₃ supplementation, all birds died or were removed by day 48. In the vitamin D₃-fed groups, a 12.1 percent reduction in mean body weight was found when feed contained 3437.8 µg/kg. A more pronounced reduction in mean body weight was found at higher levels of vitamin D₃ supplementation. At levels of 6875.7 and 13,751.4 µg of vitamin D₃/kg of feed, mean body weight decreased 50.6 and 65.0 percent, respectively. Reduction in mean body weight was comparable when 25(OH)D₃ was fed at 687.6 µg/kg of feed and when vitamin D₃ was fed at 3437.8 µg/kg of feed.

Toxicity accounted for some of the mortality observed in the treatment groups fed 687.6 µg of 25(OH)D₃/kg of feed and higher. At 22 days, mortality increased from 1.7 percent in the groups fed 68.8 and 687.6 µg of 25(OH)D₃/kg of feed to 9.7 percent in the group fed 3437.8 µg of 25(OH)D₃/kg of feed, and to 17.4 percent in the group fed 13,751.4 µg of 25(OH)D₃/kg of feed. In contrast, mortality among vitamin D₃-fed birds at 22 days ranged from 0.9 to 1.4 percent across all levels of supplementation. By 48 days, mortality increased to 4.0 percent in the group fed 68.8 µg of 25(OH)D₃/kg of feed and to 6.0 percent in the group fed 687.6 µg of 25(OH)D₃/kg of feed. No birds fed more than 687.57 µg of 25(OH)D₃/kg of feed survived to day 48. In the groups fed vitamin D₃, mortality at 48 days was 5.1 percent at the 68.8 µg/kg of feed level, 9.1 percent at the 687.6 µg/kg of feed level, and 12.6 percent at the 13,751.4 µg/kg of feed level. Toxicity accounted for part of the mortality, but only at levels of 3437.8 µg of vitamin D₃/kg of feed and higher. The number of removed birds was similar in each of the vitamin D₃-fed groups.

Histopathological data showed that renal tubular degeneration and mineralization and bone lesions were found at 22 days when 687.6 µg or more of 25(OH)D₃/kg of feed was given. Male birds fed at 13,751.4 µg of 25(OH)D₃/kg of feed had a lower incidence of mineralization of the aorta than female birds fed at this level of 25(OH)D₃. The incidence of renal tubular degeneration and mineralization only became marked at levels of 3437.8 µg of vitamin D₃/kg of feed and higher. Mineralization of the aorta was not observed across all levels of vitamin D₃ supplementation. At 22 days, bone lesions were observed only in birds receiving 13,751.4 µg of vitamin D₃/kg of feed. At 48 days, bone lesions were also found in birds that received half this amount of vitamin D₃.

Based on these findings, it can be concluded that 25(OH)D₃ produces signs of toxicity in broiler chickens when fed at 10 times the proposed commercial usage rate (687.6 µg of 25(OH)D₃/kg of feed). Data were not collected to determine the level between 68.8 and 687.6 µg of 25(OH)D₃/kg of feed at which toxic effects became apparent. Vitamin D₃ showed no signs of toxicity when fed at levels 10 times the commercial usage rate (687.6 µg of vitamin D₃/kg of feed); however, signs of toxicity began to surface when birds were fed at 50 times the commercial usage rate (3437.8 µg of vitamin D₃/kg of feed). Thus, the margin of safety is greater with vitamin D₃ than with 25(OH)D₃. Based on these results, 25(OH)D₃ appears to be about 5 to 10 times as toxic as vitamin D₃ to the broiler chicken.

b. Other poultry

Published findings on the effects of administering 25(OH)D₃ to other poultry are summarized in Table 12. Some of these studies involved a comparison of several metabolites. These reports indicated
Table 12. Research Findings on the Effects of 25(OH)D$_3$ in Other Poultry.

<table>
<thead>
<tr>
<th>Species/type</th>
<th>Number</th>
<th>Age</th>
<th>Amount $\mu$g/kg</th>
<th>Duration</th>
<th>Route</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chickens/layers</td>
<td>Not stated</td>
<td>224 d</td>
<td>22</td>
<td>Not stated</td>
<td>Diet</td>
<td>Improved Ca$^{2+}$ deposition compared with D$_3$</td>
<td>Marrett et al. (1976)</td>
</tr>
<tr>
<td>Chickens/layers</td>
<td>120</td>
<td>434 d</td>
<td>6 or 30$^1$</td>
<td>28 d</td>
<td>Diet</td>
<td>Linear reduction in egg production when diets based on limestone. Opposite result when diets based on oyster shell. Feed consumption lower with 6 $\mu$g/kg and oyster shell</td>
<td>McLoughlin and Soares (1976)</td>
</tr>
<tr>
<td>Chickens/layers</td>
<td>30</td>
<td>518 d</td>
<td>6</td>
<td>56 d</td>
<td>Diet</td>
<td>Improved shell quality and shell thickness</td>
<td>McLoughlin and Soares (1976)</td>
</tr>
<tr>
<td>Chickens/layers</td>
<td>400</td>
<td>147 d</td>
<td>3 or 9</td>
<td>91 d</td>
<td>Diet</td>
<td>No effects on production or egg shell quality. Hatchability better than with other metabolites</td>
<td>Abdulrahim et al. (1979)</td>
</tr>
<tr>
<td>Japanese quail/layers</td>
<td>50</td>
<td>182 d</td>
<td>3.1 or 6.2</td>
<td>42 d</td>
<td>Diet</td>
<td>Tibia breaking strength higher with 3.1 $\mu$g/kg than with same level of D$_3$. No deleterious effects with 6.2 $\mu$g/kg. No effects on tibia ash content or serum Ca$^{2+}$</td>
<td>Kaelzel and Soares (1979)</td>
</tr>
<tr>
<td>Chickens/layers</td>
<td>576</td>
<td>646 d</td>
<td>8, 16, or 24</td>
<td>84 d</td>
<td>Diet</td>
<td>No effects on production or on internal or external egg quality</td>
<td>Hamilton (1980)</td>
</tr>
<tr>
<td>Chickens/layers</td>
<td>2208</td>
<td>126 d</td>
<td>25 or 50</td>
<td>448 d</td>
<td>Diet</td>
<td>Production unaffected but egg weight higher than with D$_3$</td>
<td>Janssen et al. (1981)</td>
</tr>
<tr>
<td>Chickens/layers</td>
<td>5</td>
<td>476 d</td>
<td>12</td>
<td>70 d</td>
<td>Diet</td>
<td>No deleterious effects on egg production, blood chemistry, or toe ash content</td>
<td>Soares et al. (1982)</td>
</tr>
<tr>
<td>Chickens/layers</td>
<td>10</td>
<td>392 d</td>
<td>6</td>
<td>84 d</td>
<td>Diet</td>
<td>No data on production. No effects on egg shell quality</td>
<td>Soares et al. (1982)</td>
</tr>
</tbody>
</table>
Table 12. (Continued).

<table>
<thead>
<tr>
<th>Species/type</th>
<th>Number</th>
<th>Age</th>
<th>Amount μg/kg</th>
<th>Duration</th>
<th>Route</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkeys/starting</td>
<td>280</td>
<td>1 d</td>
<td>16.6 with or without 4.1 μg/kg 1α,25(OH)<em>{2}D</em>{3}</td>
<td>21 d</td>
<td>Diet</td>
<td>No effect on body weight at 14 d. Plasma Ca\textsuperscript{2+} at 14 d lowest with 25(OH)D_{3} than with D_{3} or with 25(OH)D_{3} in combination with 1α,25(OH)<em>{2}D</em>{3}. Highest tibin breaking strength obtained at 21 d with 25(OH)D_{3} in combination with 1α,25(OH)<em>{2}D</em>{3}</td>
<td>Stevens and Blair (1987)</td>
</tr>
</tbody>
</table>

\^ Calculated (see text)
no signs of toxicity in laying hens when 25(OH)D$_3$ was provided at dietary levels up to 50 µg/kg of feed for periods up to 448 days. Laying Japanese quail and growing turkeys were fed diets containing up to 16.6 µg of 25(OH)D$_3$/kg for periods up to 42 days with no adverse effects recorded.

McLoughlin and Soares (1976) studied the effects of 25(OH)D$_3$ and vitamin D$_3$ on egg shell quality in White Leghorn laying hens. Dietary levels of the vitamin D source provided were calculated based on the authors' statement that 25(OH)D$_3$ was approximately 2.5 times more active than vitamin D$_3$. In one experiment, 82-week-old hens were fed diets containing vitamin D$_3$ at 15 µg/kg of feed or 25(OH)D$_3$ at 6 and 30 µg/kg of feed for 28 days. The calcium content of diets was 3.5 percent using pulverized limestone or oyster shell as the calcium source. Compared with hens consuming diets containing 15 µg of vitamin D$_3$/kg of feed, egg production was significantly higher ($P < 0.05$) among hens consuming diets containing both levels of 25(OH)D$_3$ when oyster shell was used as the calcium source. A significant increase ($P < 0.05$) in egg shell thickness was found at the fourth week of the experiment when diets contained 6 µg of 25(OH)D$_3$/kg of feed in combination with oyster shell; however, egg shell thickness decreased with 30 µg of 25(OH)D$_3$/kg of feed and oyster shell and with 15 µg of vitamin D$_3$/kg of feed and limestone. Feed consumption was significantly lower ($P < 0.05$) when 6 µg of 25(OH)D$_3$/kg of feed was fed in combination with oyster shell than when the other three treatments were fed. In a subsequent experiment, 74-week-old hens were fed diets containing 6 µg of 25(OH)D$_3$/kg of feed and 3.5 percent calcium as either limestone or oyster shell for 56 days. Shell quality and shell thickness were improved compared with eggs from hens fed diets containing 15 µg of vitamin D$_3$/kg of feed and limestone.

Hamilton (1980) fed diets containing two levels of available phosphorus (0.34 and 0.6 percent) and either vitamin D$_3$ or 25(OH)D$_3$ at 8, 16, and 24 µg/kg of feed to 576 force-molted laying hens for 84 days. No significant effects of source or level of vitamin D were found on body weight, feed intake, egg production, shell weight, percentage shell in the egg, or in internal egg quality.

In a 22-week trial designed to evaluate the toxic effects of vitamin D metabolites in laying hens, no effects on egg production, feed consumption, or egg shell quality were recorded when 1-year-old Single Comb White Leghorn laying hens were fed diets containing 15 and 30 µg of vitamin D$_3$ or 6 and 12 µg of 25(OH)D$_3$/kg of feed (Soares et al., 1982). During the initial 12-week period, groups of 10 hens were fed diets containing vitamin D$_3$, 25(OH)D$_3$, or 1α,25(OH)$_2$D$_3$ at 15, 6, or 3.4 µg/kg of feed, respectively (described as being equivalent to 15 µg of vitamin D$_3$/kg of feed). Half of the birds in each group were then fed their respective vitamin D source at twice the original level for an additional 10 weeks. No treatment effects on egg shell quality were found in the first 12-week period (data on the other parameters were not presented). During the subsequent 10-week period, egg production, feed consumption, and egg shell quality declined when diets were supplemented with 1α,25(OH)$_2$D$_3$ at the higher level. No significant effects on serum calcium or phosphorus or on toe ash content were noted. It was concluded that a dietary level of 6.8 µg of 1α,25(OH)$_2$D$_3$/kg of feed was toxic. A second experiment confirmed that toxicity occurred with 10 or 15 µg of this metabolite with continued feeding. However, no toxic effects were reported when 25(OH)D$_3$ was supplemented to diets at 6 µg/kg of feed for 22 weeks or at 12 µg/kg of feed for 10 weeks.

Stevens and Blair (1987) compared the antirachitic effects of vitamin D$_3$, 25(OH)D$_3$, and 1α,25(OH)$_2$D$_3$ in starting turkeys. Over a 21-day period, four concentrations of available phosphorus (0.2, 0.3, 0.45, and 0.6 percent) and five vitamin D treatments were fed. Per kg of feed, the five vitamin D treatments supplied: (1) 22.5 µg of vitamin D$_3$; (2) 67.5 µg of vitamin D$_3$; (3) 16.6 µg of 25(OH)D$_3$; (4) 4.1 µg of 1α,25(OH)$_2$D$_3$; and (5) 16.6 µg of 25(OH)D$_3$ plus 4.1 µg of 1α,25(OH)$_2$D$_3$. Body weight was not significantly different among the five treatment groups at day 7 and 14; however, at day 21, pouls receiving 25(OH)D$_3$ in combination with 1α,25(OH)$_2$D$_3$ were significantly heavier than those receiving 22.5 µg of vitamin D$_3$. Plasma calcium concentrations were lowest among pouls receiving 25(OH)D$_3$ at day 14, but no significant differences were evident among the five treatment groups at day 21. The
highest tibia breaking strength was found at day 21 when 25(OH)D₃ was fed in combination with 1α,25(OH)₂D₃. At day 14, body weight, feed consumption, and tibia breaking strength increased as the level of available phosphorus increased. Plasma calcium levels decreased as available phosphorus increased. Low available phosphorus diets containing 25(OH)D₃ and 1α,25(OH)₂D₃ provided no apparent benefits compared to low available phosphorus diets providing sufficient vitamin D₃.

c. Summary

The published data suggest that dietary levels up to 10 μg of 25(OH)D₃/kg of feed are safe for broiler chickens for prolonged feeding. A dietary level of 10 times that amount (100 μg/kg of feed) has been reported to be acutely toxic; however, an oral dose of 5 μg of 25(OH)D₃/growing broiler/48 hours for 35 days appears to be safe. There are insufficient data to allow an assessment of the safety of dietary levels between 10 μg and 100 μg of 25(OH)D₃/kg of feed. Preliminary field trials conducted for Amoco BioProducts Corporation where dietary levels of 25(OH)D₃ up to 687.6 μg/kg of feed were fed resulted in no overt toxicity. However, a subsequent field trial found evidence of toxicity of 25(OH)D₃ at 687.6 μg/kg of feed over a 48-day growing period. The suggested usage level of 25(OH)D₃ at 68.8 μg/kg of feed for commercial broiler production as proposed by Amoco BioProducts Corporation, appears to be within the tolerable range for growing broilers.

For other poultry, the published data suggest that dietary levels up to 50 μg of 25(OH)D₃/kg of feed are safe for prolonged feeding, at least in adult birds. No toxic level appears to have been established for these classes of poultry, and the published data are in agreement with the broiler data cited above.

2. Toxicity in other animal species

a. Rats

(1) Absorption of 25(OH)D₃ in rats

As cited above, Bar et al. (1980) showed that in avian species the absorption of 25(OH)D₃ was significantly (P < 0.01) higher than that of vitamin D₃. In chickens, the respective values were 83.6 and 66.5 percent. Sitrin et al. (1982) conducted similar studies in rats. In the presence of bile salts, the values for the absorption (amount of administered dose absorbed in 6 hours) of 25(OH)D₃ and vitamin D₃ were 74.1 and 33.9 percent, respectively. In the absence of bile salts, the respective values were 78.5 and 33.4 percent. This study also indicated that 25(OH)D₃ was more rapidly absorbed than vitamin D₃. These results suggest that the absorption of 25(OH)D₃ is higher than that of vitamin D₃ in both the mammalian and avian species.

(2) Metabolism of 25(OH)D₃ in rats

Rojanasathit and Haddad (1976) studied the metabolism of 25(OH)D₃ in rats and concluded that circulating 25(OH)D₃ had a minimal potential for hepatic accumulation in comparison with vitamin D₃. Rachitic rats were given [³⁵Cl] vitamin D₃ or [³⁵Cl] vitamin D₃ and 25(OH)[³⁵H] D₃. In rats administered [³⁵Cl] vitamin D₃ alone, the percentage of the administered dose in the liver after 15 and 45 minutes, was 27 and 43 percent, respectively. When the rats received doses of [³⁵Cl] vitamin D₃ and 25(OH)[³⁵H] D₃, 25 and 35 percent of the vitamin D₃ was found in the liver after 15 and 45 minutes, respectively. However, only 7 or 5 percent of the 25(OH)[³⁵H] D₃ was found in the liver after 15 and
45 minutes, respectively. Thus, while hepatic vitamin D₃ increased over time, hepatic 25(OH)D₃ fell. A similar decline took place in the serum, where an administered dose of 25(OH)D₃ fell from 37 percent after 15 minutes to 29 percent after 45 minutes. Co-administration of 25(OH)D₃ at a 30-fold higher amount (by weight) than vitamin D₃ did not increase the serum content of 25(OH)D₃ over that obtained with vitamin D₃ alone.

These data suggest that 25(OH)D₃ has a low potential for accumulation in the liver or the serum of rats. The kinetics of 25(OH)D₃ accumulation and elimination in the rats have not been reported; however, these results suggest that exogenous 25(OH)D₃ is cleared rapidly from the plasma and liver of the rat. The fate of the administered dose is not clear, and no renal data similar to those obtained with chickens by Morrissey et al. (1977) appear to be available.

(3) Plasma responses and morbidity studies in rats

Shepard and DeLuca (1980) compared the short-term responses of rats to oral doses of vitamin D₃ and 25(OH)D₃. The experimental period was 14 days, and intoxication was measured by hypercalcemia and morbidity. It was found that intoxication was produced with 650 nmol of vitamin D₃/d and with 4600 nmol of 25(OH)D₃/d. While no histopathological studies were done, gross morphology at autopsy suggested that renal calcification occurred at these dose levels. Shepard and DeLuca (1980) concluded that vitamin D₃ was more toxic to rats than 25(OH)D₃ because about sevenfold more 25(OH)D₃ than vitamin D₃ had to be ingested to cause hypercalcemia in the rat in 14 days.

(4) Toxicity studies

In tests reported to the Food and Drug Administration in support of a new drug application for 25(OH)D₃ by The Upjohn Company, an oral LD₅₀ value of 320 mg/kg bw was estimated for the rat (strain not specified) (Dutta, 1979; Food and Drug Administration, 1979). In further tests, 5 male and 5 female rats (strain not specified) received 0.12 mg/kg bw/d in the diet for 5 days. No macroscopic evidence of effects was observed.

Based on the above results, a 6-month feeding trial was conducted. Groups of 15 male and 15 female rats (strain not specified) received 0.012, 0.040, or 0.120 mg of 25(OH)D₃/kg bw/d in the diet. At 180 days, five rats of each sex were autopsied. There was evidence of increased incidence of nephrocalcinosis in the females receiving 0.120 mg/kg bw/d and uroliths in males at this highest dosage regimen. No other effects were noted.

b. Dogs

The Upjohn Company also conducted a 6-month feeding trial with dogs (breed not identified) (Dutta, 1979; Food and Drug Administration, 1979). 25(OH)D₃ was administered to 2 male and 2 female dogs in gelatin capsules daily. Dosages were 0, 3, 6, and 12 μg/kg bw/d. A second equivalent group of dogs received vitamin D₃ similarly at 4.5, 9, and 18 μg/kg bw/d. Feeding 25(OH)D₃ or vitamin D₃ at these levels had no effect on food consumption, body weight, hematology, blood chemistry, or urinalysis. Postmortem studies revealed no 25(OH)D₃ or vitamin D₃ effects on organ weights, gross pathology, or histopathology of major organs.
c. Summary

Data from these drug studies indicate that rats and dogs were fed significantly higher doses of 25(OH)D₃ (The Upjohn Company, 1980) than broiler chickens and other poultry. The only adverse effects observed in female and male rats were nephrocalcinosis and urolithiasis, respectively, when 25(OH)D₃ was administered at doses of 0.120 mg/kg bw/d for six months. This dosage regimen is approximately 25 μg/d, a level at which broiler chickens exhibit no adverse effects.

3. Potential toxicity to humans

Pertinent data on the toxicity of 25(OH)D₃ in humans or other mammals are limited. Most of the published reports on 25(OH)D₃ have dealt with potency or efficacy, not toxicity. In the absence of these data, one approach to answer this question is to suggest that the dietary exposure to 25(OH)D₃ would be so low that it would present no danger to the human consumer.

An analysis of acute toxicity to 25(OH)D₃ was conducted by the LSRO and the ad hoc Expert Panel to estimate the amount of 25(OH)D₃-fed broiler chicken that would need to be consumed every day to reach toxic levels of 25(OH)D₃. A similar evaluation was conducted to determine the amount of vitamin D₂-fed broiler chicken that would need to be consumed every day to reach toxic levels of vitamin D₂. Although it can be expected that a very large amount of either 25(OH)D₃ or vitamin D₂-fed broiler chicken would need to be consumed over a relatively short period of time in order to produce adverse effects, reliable data upon which to base consumption estimations are not available. Furthermore, questions remain, particularly for 25(OH)D₃, as to dietary levels that need to be consumed to produce adverse effects. Regardless, assumptions were made in order to arrive at conservative estimates of acute toxicity.

a. Estimates of acute toxicity

Based on usual exposure estimates calculated in Chapter III, mean per capita intakes of 25(OH)D₃ from a diet containing milk, whole eggs, beef, and meat and skin from 25(OH)D₃-fed broiler chicken were estimated at 762 ng/d for average adult consumers and twice this amount or 1524 ng/d (1.52 μg/d) for heavy consumers at the 90th percentile. (See Table 7.) If the assumption is made that 25(OH)D₃ is as toxic for the human as it appears to be for the one species that has been examined in detail (the chicken, in which it appears to be 5 to 10 times as toxic as vitamin D₃), the following calculations can be made:

1. Toxic dose of vitamin D₃ for the human: 1.25 mg/d (chronic) (Davies and Adams, 1978; Davies et al., 1986; Harrison, 1978). The range of toxic dose in these reports is 50,000 to 200,000 IU/d, and the lowest figure has been taken.

2. Estimated intake of 25(OH)D₃ (90th percentile): 1.52 μg/d

3. Relative toxicity of 25(OH)D₃: less than 10 times as toxic according to the Quarles (1994a,b) toxicity trial. However, a 10 times factor has been used to provide a safety margin.

4. Adjusted intake of 25(OH)D₃ relative to 3 above: 15.2 μg/d

45
5. Ratio of 25(OH)D₃/vitamin D₃ intakes on a comparable toxicity basis:

\[
15.2 \, \mu g/1.25 \, mg \, \text{vitamin D₃} = 1.216\% 
\]

Based on an assumed toxic level for 25(OH)D₃ of 15.2 \( \mu g/d \), an adult would have to eat about 5.5 pounds of raw, boneless chicken meat (including skin) per day in order to experience adverse effects from 25(OH)D₃. However, persons consume cooked rather than raw chicken meat and skin. According to Harris (1988), up to 40 percent of vitamin D₃ can be destroyed during cooking. It is likely that 25(OH)D₃ would experience similar cooking losses. Thus, it would appear that more than 5.5 pounds of cooked, boneless chicken meat (including skin) could be consumed per day before reaching toxic levels of 25(OH)D₃; however, the actual amount of cooked, boneless chicken that could be consumed before adverse effects become apparent is not known. In the absence of this information, a conservative estimate of chicken consumption would be achieved by assuming that none of the 25(OH)D₃ is destroyed during cooking. Thus, up to 5.5 pounds of cooked, boneless chicken meat (including skin) could be consumed by adults per day without experiencing adverse effects.

Field trial Colorado AM-2-94 (Quarles, 1994a,b) found that 25(OH)D₃ toxicity in broiler chickens became evident when fed at more than 68.8 but less than 687.6 \( \mu g/kg \) of feed. Because the exact dose level at which the onset of toxicity surfaces is not known, caution should be exercised when identifying a maximum safe consumption level of 25(OH)D₃-fed broiler chicken for adults.

In regard to acute toxicity of vitamin D₃, data from various sources (Davies and Adams, 1978; Davies et al., 1986; Harrison, 1978) indicated adverse effects from vitamin D₃ toxicity for adult humans at 1.25 mg/d or 1250 \( \mu g/d \) (50,000 IU/d). Tissue concentrations of vitamin D₃ in vitamin D₃-fed broiler chicken (or in other types of poultry) are not known. In the absence of these data, the assumption was made that 25(OH)D₃ and vitamin D₃ tissue concentrations in vitamin D₃-fed broiler chicken were the same. Thus, tissue concentrations of vitamin D₃ were assumed to be 209 \( \mu g/100g \) (0.209 \( \mu g/100g \)). (See Table 8.) Based on this assumption and the documented vitamin D₃ toxicity level of 1.25 \( \mu g/d \) or 1250 \( \mu g/d \) (60,000 IU/d), an adult would have to consume over 1300 pounds of vitamin D₃-fed broiler chicken in a day to reach toxic doses of vitamin D₃. Daily human consumption of this amount of chicken is inconceivable.

b. Summary

These calculations suggest that the maximum intake of 25(OH)D₃ at the 90th percentile of chicken consumption with a 10 times safety factor would be equivalent to about 1.2 percent of the chronic toxic dose of vitamin D₃ and about half that for the typical human consumer of chicken. Thus, risk to the typical consumer of broiler meat (including skin) appears to be very small. The Expert Panel made the above calculations based on data provided by Quarles (1994a,b), even though the earlier study of Morrissey et al. (1977) concluded that 25(OH)D₃ was 100 times as toxic as vitamin D₃ in the chicken. In due course, a further investigation of toxicity of 25(OH)D₃ in various animal models is warranted.

The above estimation pertains to adults. Some evidence, as noted on page 23, suggests that children have a lower tolerance level for vitamin D₃ and, by inference, for 25(OH)D₃. Even so, intakes of 25(OH)D₃ from diets containing 25(OH)D₃-fed chickens by children is several fold lower than the assumed toxic level of 25(OH)D₃. The latter is based on the 1963 report of the American Academy of Pediatrics which indicates that young children with dietary intakes of 45 \( \mu g \) vitamin D₃/d experience hypervitaminosis D. With the assumed 10-fold greater toxicity of 25(OH)D₃ versus vitamin D₃, i.e., 4.5 \( \mu g/d \), the safety factor calculates to about 6 for children consuming 0.76 \( \mu g \) of 25(OH)D₃/d (90th percentile).
D. SPECIAL STUDIES

Extensive human studies on 25(OH)D₃ were conducted in the late 1970s by The Upjohn Company as required for a New Drug Application (#18,312). The 25(OH)D₃ trade name, Calderol®, was marketed for use in treatment of metabolic bone disease secondary to chronic renal failure. The information reported in the following paragraphs is derived from The Summary Basis of Approval (Food and Drug Administration, 1979) and Clinical Review and Evaluation of NDA (Dutta, 1979) for Calderol®. (Both memoranda were obtained from files of the Food and Drug Administration.) The Expert Panel has not examined the original data; however, the absence of adverse effects in clinical trials, as reported by Coburn et al. (1976) and Teitelbaum (1976), at dosages exceeding by many fold those anticipated in the use of 25(OH)D₃ in broiler feed is pertinent to safety considerations.

1. Acute toxicity

The LD₉₀ values reported for the mouse (strain not specified) and rat (strain not specified) were 210 and 320 mg/kg bw. Rats (sex and strain not specified) fed diets containing 0.12 mg of 25(OH)D₃/kg of feed for 5 days exhibited no evidence of toxicity.

2. Short-term toxicity tests

Rats (strain not specified) fed 120 µg of 25(OH)D₃/kg bw/d for 6 months exhibited uroliths in males and nephrocalcinosis in females at autopsy. No toxicity was noted in rats fed 40 µg of 25(OH)D₃/kg bw/d. Two male and two female dogs (strain not specified) fed 12 µg of 25(OH)D₃/kg bw for 6 months exhibited no gross or histopathological evidence of toxicity.

3. Developmental toxicology

25(OH)D₃ was not teratogenic in studies with rats (strain and sex unknown) fed 12, 40, or 60 mg/kg by gavage in corn oil from day 6 to day 15 of gestation. In Dutch Belted rabbits, skeletal abnormalities were observed in newborns when dams received 6.2 and 12.5 mg/kg but not 1.2 mg/kg. The Expert Panel considers these dosage levels, 6 to 13 times the human dose (4 mg/kg), not pertinent. In further studies, The Upjohn Company (1980) reported that rats (strain and sex unknown) fed 12, 40, or 60 mg/kg/d from day 15 of gestation to weaning at day 21 showed no evidence of teratogenic or other toxic effects.

4. Mutagenicity and carcinogenicity

The Upjohn Company (1980) conducted bacterial mutagenicity tests using the Salmonella reversion test with and without metabolic activation. Results with three Salmonella strains (not identified) were uniformly negative. Based on these results, no rodent carcinogenicity studies were conducted.

5. Allergenicity

Reports of clinical trials with 57 patients at 6 centers in controlled studies and records of 91 patients in 14 uncontrolled research protocols contained no reference to occurrence of allergic hypersensitivity to 25(OH)D₃ when administered orally for periods of up to 89 weeks at doses of 21 to 86 µg/d.
6. **Neurobehavioral toxicology**

No reports describing neurobehavioral changes resulting from consumption of 25(OH)D₃ by any animal model appear to be available in the scientific literature. In addition, no such adverse effects were noted in the data reported by The Upjohn Company (1980) in its new drug application for Calderol® (#18,312).

7. **Other**

In the Upjohn Company’s new drug application for Calderol® (#18,312), occurrence of hypercalcemia was reported in patients given 21 to 86 μg/d of 25(OH)D₃ for up to 89 weeks during the controlled clinical trials described above. Serum calcium concentrations greater than 13 mg/dl occurred on one or more occasions in three patients, two of whom had to discontinue the study. Serum calcium concentrations greater than 11 mg/dl occurred on one or more occasions in about 76 percent of patients. Fifty-one percent of these occurred at dosages of 86 μg/d and 49 percent occurred at lower dosages.
V. OPINION OF THE EXPERT PANEL

A. CONCLUSIONS

25(OH)D₃, a normal component of vitamin D metabolism, is formed in the liver from vitamin D₃ and transported via the blood to the kidney where it is further hydroxylated to the hormonally active form, 1α,25(OH)₂D₃. Available scientific data indicate that the pathways of vitamin D metabolism in chickens and other vertebrate animals, including humans, are similar.

Amoco BioProducts Corporation prepares 25(OH)D₃ biosynthetically using a modified strain of \textit{S. cerevisiae} that produces cholesta-5,7,24-trien-3β-ol via the isopenoid pathway. This product is hydroxylated and photoconverted to 25(OH)D₃ in a manner analogous to that which occurs in nature. Amoco BioProducts Corporation plans to market 25(OH)D₃ as an alternative source of vitamin D activity in broiler feed. The proposed use level for 25(OH)D₃ in broiler feed would be 68.8 μg/kg of feed, the same approximate level as currently practiced with vitamin D₃.

Based on extrapolation from feed consumption by broiler chickens and from human consumption of chicken, the Expert Panel estimated that mean usual per capita intakes of 25(OH)D₃ from chickens raised on 25(OH)D₃-supplemented feed would be about 347 ng/d for adults and 174 ng/d for children. At the 90th percentile for chicken consumption, usual intakes would be about 694 and 347 ng/d for adults and children, respectively. These intake estimates are about 175 percent of those derived from data on usual per capita intakes of beef and about 160 percent of those derived from data on usual per capita intakes of both vitamin D₃-fortified milk and whole eggs. Intakes of 25(OH)D₃ at the 90th percentile from meat and skin from broiler chickens fed 25(OH)D₃ (694 ng/d) would be about 65 percent of the estimated 90th percentile intakes of 25(OH)D₃ from diets consisting of vitamin D₃-fortified milk, whole eggs, beef, and meat and skin from broiler chickens fed vitamin D₃ (1069 ng/d).

25(OH)D₃ has been used as a human drug in treatment of metabolic bone disease secondary to chronic renal failure. The drug has been administered at dosages of 21 to 86 μg/d for periods of up to 89 weeks. At higher dosages over prolonged periods, hypercalcemia was observed in some patients. Dosages of 21 to 86 μg/d are 30 to 124 times and 60 to 248 times the 90th percentile estimated usual intakes for adults and children consuming chickens fed 25(OH)D₃ at 68.8 μg/kg of feed, respectively.

A number of studies conducted for Amoco BioProducts Corporation establish that when 25(OH)D₃ is fed at average levels of 68.8 μg/kg of feed, no observable adverse effects on growth and finished weight of broilers were observed during the typical 7-week production period. However, these studies also established that the margin of safety for 25(OH)D₃ in broiler production is more limited than that for vitamin D₃ when administered at similar levels in feed. That is, adverse effects are evident in birds fed 637.6 μg of 25(OH)D₃/kg of feed for 7 weeks.

Estimated tissue levels of 25(OH)D₃ in broiler meat and skin are such that at 90th percentile intakes, diets that include vitamin D₃-fortified milk, whole eggs, beef, and meat and skin from 25(OH)D₃-fed broiler chicken would be about 42 percent higher in 25(OH)D₃ content than analogous diets containing meat and skin from vitamin D₃-fed broiler chicken (1524 ng/d versus 1069 ng/d). Based on current available information, even though 25(OH)D₃ is more toxic than vitamin D₃, subsequent human exposure to 25(OH)D₃ from consumption of meat and skin from 25(OH)D₃-fed broiler chicken is sufficiently low that it is considered to be of little practical significance. Because exposure of broilers to 25(OH)D₃ would be limited to 68.8 μg/kg of feed, humans who consume broiler chickens raised on feed containing 25(OH)D₃ would be ingesting an incremental increase in total 25(OH)D₃ from dietary sources compared to humans who consume broiler chickens raised on feed containing vitamin D₃.
Toxicity studies with broilers suggest that 25(OH)D₃ in broiler feed has a lower margin of safety than vitamin D₃ and the exact concentrations of 25(OH)D₃ in broiler feed that may elicit toxic effects is yet to be determined.

Based on the available information and evaluation of the biological effects of 25(OH)D₃, the Expert Panel concludes that:

*The available information supports a Generally Recognized as Safe (GRAS) classification of 25-hydroxyvitamin D when supplied as a source of vitamin D activity in broiler feed at the intended level of use of about 68.8 µg (63.8 to 73.7 µg) per kg of feed.*

**B. RECOMMENDATIONS**

The Expert Panel recognizes that the level of addition of 25(OH)D₃ to broiler feed is based on the current industry practice of adding vitamin D₃ to poultry feed. In due course, as time permits, future studies on 25(OH)D₃ might be conducted to determine whether less than 68.8 µg/kg of feed might be as effective in broiler production.

Finally, the Expert Panel recommends that if 25(OH)D₃ is marketed for its proposed uses, a post-market surveillance mechanism should be provided for user comment via labeling information. Comments to the manufacturer, which are also made available to the Food and Drug Administration, would provide an additional follow-up measure to the assurance of safety evidenced by the information and data evaluated by the Expert Panel.
VI. LITERATURE CITED


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