SEPARATION AND CHARACTERIZATION OF RETINOIDS IN CHICK BILE

By

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RETINOIDS IN CHICK BILE

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CHAPTER I

INTRODUCTION

If vitamin A could tell its secrets, what an interesting story we would probably hear. The existence of a "fat soluble A" factor has been known for 67 years (1, 2) but little of the biological role of vitamin A is understood. Vitamin A has been shown to be involved in several biological functions, including vision (3), growth and differentiation (4), reproduction (5), and maintenance of epithelial tissues (6). More recently, vitamin A compounds have been employed in the treatment and chemoprevention of cancer (7-10), acne (11), and linked to glycoprotein synthesis (12-14). A series of publications by Olson (15-17), Dowling and Wald (4, 18), and Thomas Moore's book, Vitamin A (3), provide an excellent background on vitamin A. The term "vitamins A" as used here will include retinol and compounds similar to retinol in structure and function. Natural and synthetic analogs of vitamin A will be collectively referred to as "retinoids" (7).

The role of retinal in the visual process is well known (3), and is the only well characterized function of vitamin A. The search for an "active form" of vitamin A has been unsuccessful. This is probably due to the fact that vitamin A, with its many functions in the body, has several active forms. Retinoic acid, touted by some as an "active form" of vitamin A, replaced retinol in the support of growth in rat (4), chick (19), and pig (20), but blindness ensued if it was the sole

retinoid supplied (4). Retinoic acid had a sparing effect on liver vitamin A stores in chicks (19) and pigs (20). It did not, however, support pregnancy in female rats or spermatogenesis in male rats (21, 22). Chicks fed methyl retinoate produced normal spermatozoa and eggs, but did not produce normal embryos (23). Isolation of separate specific cellular binding proteins for retinol and retinoic acid in human uterus suggested that both forms might be necessary for normal development of those cells (24).

Vitamin A Proteins

The major stores of vitamin A in the body are as retinyl esters in the liver. Retinol is transported in the blood plasma by a specific 21,000 dalton retinol-binding protein (RBP) (25-27) associated with thyroxine-binding prealbumin. The complex apparently protects the conjugated unsaturated molecule from oxidation en route to target tissues (26). Retinoic acid is carried in the blood serum by serum albumin (28). The discovery of a cellular retinoic acid-binding protein (CRABP) in rat testes by Ong and Chytil (29) and in chick embryo metatarsel skin by Sani et al. (30) was further evidence of a cellular role for retinoic acid. Retinol did not compete with retinoic acid for binding, but instead bound tightly to a specific cellular retinol-binding protein (CRBP) (31) different from that found in serum (32-36). Both CRBP and CRABP were isolated from tissues of fetal rats (37). The CRBP was present in most tissues of the adult, but CRABP was absent in lung, liver, intestine, and kidney. The amounts of CRABP and CRBP in testes from retinol deficient rats were unchanged relative to those from pair fed control

rats (38). Ong et al. demonstrated that extracts of human carcinomas from lung and breast contained CRABP and that it was absent in normal lung or breast tissue from the same patients (38). Changes in the levels of the binding proteins at different stages of development suggest separate changing requirements for retinol and retinoic acid in organ maturation and development (37).

Vitamin A appears to be an agent in the control of mitogenic activity and cell differentiation (17, 39-42). Christophers et al. working with guinea pig epidermal cells grown in vitro found that treatment of cells with retinoic acid significantly increased cellular attachment in the first hours after seeding followed by a ten-fold increase in DNA synthesis (43, 44). Boren et al. showed that in hamster trachea epithelium, the ratios of basal cells to mucus cells to ciliated cells were altered by vitamin A deficiency and excess (45) vitamin A deficiency caused an increase in basal cells and a decrease in ciliated cells. Vitamin A excess effected an increase in ciliated cells and a decrease in mucus cells (45).

Vitamin A and Carcinogenesis

Recent studies have indicated that vitamin A and synthetic vitamin A analogs (retinoids) might be potent intermediates in the prevention of chemically induced carcinogenesis (7, 46). This is consistent with the role of vitamin A in enhancing cell differentiation since carcinogenesis involves cell de-differentiation. The rationale for employing synthetic analogs in the treatment of carcinogenesis is underlined by the toxicity of known vitamin A compounds in the body (6). Bollag reported that retinoic acid had a prophylactic or therapeutic effect on

the development of chemically induced papillomas and carcinomas of mice skin while a synthetic aromatic derivative had a therapeutic ratio (anti-carcinogenic activity/toxicity) ten times greater (47-51). The synthetic retinoids were bound by both CRABP, which is the cellular binding site for retinoic acid and serum albumin which is the transport vehicle in the blood (52, 53). Binding of the retinoids required the carboxyl on the side chain, but was not prevented by modifications on the ring (54). No correlation was found linking binding of derivatives with serum albumin to ability to inhibit tumors. The ability of analogs to bind CRABP did correlate with tumor inhibiting activity in mouse skin, hamster trachea, human breast tumor, and rat testes (55) as defined by Sporn et al. (56). This suggested that retinoid activity at the cellular level was mediated by CRABP and supported the hypothesis that retinoic acid or a derivative thereof was active in stimulating cell differentiation in those tissues.

Biological Role of Vitamin A

At least one biological role of vitamin A on a cellular level has been studied. Based on extensive studies on phosphorylated derivatives of retinol and retinoic acid, it was proposed that phosphorylation of vitamin A and linkage of the phosphorylated derivative to mannose was necessary for incorporation of mannose into manno-glycoproteins (14, 57-60). It has been reported vitamin A acts similarly as a carrier for galactose (61). Both retinol and retinoic acid were metabolized to forms that bound mannose but appeared to function in different ways. Synthetic retinyl phosphate accepted mannose <u>in vivo</u>, but synthetic retinoyl phosphate did not (58). Synthetic retinylphosphate co-chro-

matographed with the compound formed *in vivo* from retinol in rat liver membranes. It was postulated that retinoic acid formed a similar hydroxylated derivative either by decarboxylation or reduction of the carboxyl group (60). The reduction of retinoic acid has never been demonstrated *in vivo*.

A problem in vitamin A studies has been to define what constitutes a vitamin A deficient state in an intact laboratory animal. Nelson et al. developed a method for quantitation of retinoic acid and retinol in blood plasma (62) and showed that in pigs, cerebrospinal fluid pressure was an adequate and sensitive criterion for assessing vitamin A status in an animal (20). In pigs with a vitamin A deficiency, an increase in cerebrospinal fluid pressure was the first change to occur with decreasing blood plasma vitamin A levels. Retinol and retinoic acid were equally effective in reducing cerebrospinal fluid pressure (20).

Metabolism of Retinoic Acid

Retinoic acid is a normal intermediate of the metabolism of retinol, retinal, and retinyl esters. Dunagin et al. injected rats intraportally with all-*trans*-[6,7-$^{14}C_2$]retinal and in 6 hours detected small amounts of retinoic acid in liver, intestine, and bile (63). The bile fraction contained up to 6% of the injected dose of retinal as some form of retinoic acid. Other studies have demonstrated a rapid conversion of retinal to retinoic acid (64). *In vitro* incubations of β-carotene with intestinal mucosa liberated small amounts of retinoic acid (65). Addition of NAD or NADH to the system stimulated retinoic acid production ten-fold. Ito et al. (66) dosed rats intrajugularly

with all-trans-[11-^{14}C]retinyl acetate and recovered retinoic acid and a polar metabolite from all tissues examined. Retinoic acid was isolated from liver, intestine, testes, and bile 12 hours after intrajugular administration of all-trans-[15-^{14}C]retinol (67).

Retinoic acid itself is not a dead end of vitamin A metabolism. A significant finding in the study of the metabolism of retinoic acid was the discovery that 40% of injected retinoic acid was excreted in the bile of cannulated rats within 4 hours as water soluble material (68). Previously, investigators had been unable to explain the rapid disappearance of administered retinoic acid in laboratory animals. Characterization of 3 major fractions of metabolites in the bile demonstrated the presence of free retinoic acid, alkali labile esters of retinoic acid, retinoyl β-glucuronide, and an unidentified polar metabolite. An enzyme was found which catalyzed formation of retinyl and retinoyl β-glucuronide from retinol, retinoic acid, and UDP glucuronic acid (69, 70). The retinoyl β-glucuronide was found to have a biopotency 30-100% as great as all-trans-retinoic acid and to participate in enterohepatic circulation (71, 72). Ito et al. (73) quantitated the per cent dose of radioactivity in body tissues 1-48 hours after intravenous dosage of 13 micrograms of all-trans-[6,7-$^{14}C_2$] retinoic acid. Bone, muscle, liver, and small intestine accounted for 79.0% of the recovered dose in the first hour. The amounts of radioactivity in these tissues decreased over the 48 hour period. After 48 hours, 94.3% of the recovered dose was in the large intestine, feces, and urine. Sundaresan et al. reported the presence of at least 6 urinary metabolites from rats, the major one lacking Carbon #14 and Carbon #15 of retinoic acid (74).

Lehman (28) separated the metabolites of retinoic acid in chick bile into two fractions which he characterized as retinoyl β-glucuronide and a polypeptide conjugate of retinoic acid of molecular weight > 1800 daltons. Dunagin et al. (63), using extractions and gas chromatography, divided the metabolites of retinoic acid in rat bile into 3 fractions one of which contained retinoyl β-glucuronide. Roberts et al. (75-77) have reported evidence from a hamster liver microsome system and from hamster intestine that the major pathway of retinoic acid involves hydroxylation at position 4 in the ring, conversion to a 4-keto group, and subsequent conversion to more polar metabolites.

Decarboxylation of Retinoic Acid

The decarboxylation of retinoic acid *in vitro* is a well studied phenomenon. Roberts et al. (78) and Lin (79) isolated a microsomal fraction capable of decarboxylating retinoic acid. Similarly, decarboxylation has been demonstrated with tissue slices from kidney (78) and liver (80). Tissue slices from animals dosed with N,N'-diphenyl-p-phenylenediamine (DPPD), an antioxidant, showed decreased decarboxylation of retinoic acid *in vitro* (81). Rockley et al. (82, 83) identified a C-19 aldehyde as the major decarboxylation product in a model system composed of all-*trans*-retinoic acid, H_2O_2, and horseradish peroxidase. The isolation and characterization of decarboxylated retinoids produced *in vivo* has been poorly accomplished. Sundaresan et al. (84) reported up to 14% decarboxylation of all-*trans*-retinoic acid in 4 hours. The biological significance of decarboxylated retinoids was underscored in a 1964 study by Yagashita et al.

(85) who found a decarboxylated metabolite of vitamin A from the small intestine that was biologically active. Numerous studies involving radioactive all-trans-retinoic acid have suggested decarboxylation, loss of hydrogen from position 11 or 12, and cleavage of the side chain into TCA cycle intermediates. Roberts et al. (86) proposed that retinoic acid underwent 3 main reactions in the body. The first led to metabolites with intact side chains and included formation of retinoyl β-glucuronide. The second was decarboxylation of the terminal carboxyl leaving a 19-carbon retinoid. The third involved cleavage of the side chain of the decarboxylated compound into TCA cycle intermediates. The proposed alterations of retinoic acid were based on the relative amounts of ^{14}C or ^{3}H in the urine or feces of animals dosed with combinations of all-trans-[6,7-$^{14}C_2$], [15-^{14}C], [14-^{14}C], or [11,12-$^{3}H_2$]retinoic acid. No metabolites corresponding to any of these 3 proposed types have been found. Hanni et al. (87) have isolated and identified 3 decarboxylated retinoids from rat urine. They reported that each of the 3 had the side chain shortened to position 12 and contained a 4-keto group. Two had the side chain in a furanone ring and the methyl at position 1 was hydroxylated in one. The other metabolite had a hydroxylated methyl group at carbon 1 and a mono-ene side chain ending in a carboxyl group. The three decarboxylated compounds were the only urinary retinoids isolated and accounted for 26% of urinary metabolites. Hanni et al. (88) also identified 3 fecal metabolites and accounted for 61% of the fecal retinoids. All had completely intact side chains. Hanni et al. combined the results of Olson's earlier studies (63, 68-72) and their own in a proposed scheme on the pathway taken by

retinoic acid through the body. The scheme, consistent with that of Roberts et al. (86), contains two hypothesis.

The first states that retinoids without an intact side chain are removed by the kidney and excreted in the urine.

The second states that metabolites with intact side chains pass through the bile and are excreted in the feces.

Two of the cornerstone studies on which these hypotheses rest (63, 86), however, used chromatographic methods such as gas chromatography (GC), thin layer chromatography (TLC) and acid-base extractions that are not compatible with the labile nature of retinoids.

Isolation of metabolites of retinoic acid has been hampered by the labile nature of the compound, poor separatory capabilities, and impurities in radiochemical precursors. 5,8-Epoxyretinoic acid has been reported as a metabolite of retinoic acid and identified as a major impurity in stocks of ^{3}H and ^{14}C labelled all-trans-retinoic acid (89, 90). The identification of 13-cis retinoic acid as a metabolite of retinoic acid in the liver (91) remains questionable since the isolation procedure was shown to isomerize 60% of all-trans-retinoic acid to the 13-cis isomer (91). The development of high performance liquid chromatography (HPLC) technology with bonded octadecylsilane columns has improved laboratory separations capabilities. Halley and Nelson (92) isolated and identified, using Fourier transform nuclear magnetic resonance (FT-NMR), eleven photochemically produced cis-trans isomers of methyl retinoate by reverse-phase HPLC. It has recently been reported that using HPLC, Zile et al. (93) found retinoyl-β-glucuronide to be a minor bile metabolite, accounting

for only 12% of bile retinoids. Earlier studies using gas chromatography, thin layer chromatography and extractions placed the amount of retinoyl-β-glucuronide at 90% of bile retinoids (70).

The higher figure found in the earlier studies was probably due to the incomplete separation of the glucuronide derivative from other metabolites. Routine HPLC analysis of bile retinoids revealed that at least one retinoid present was decarboxylated. This generated a conflict with earlier studies that concluded that only retinoids with intact side chains were in the bile. None of the earlier studies relied on HPLC as the chromatographic method.

The objective of the present study, therefore, was to investigate the bile retinoids using HPLC as the chromatographic method.

The retinoic acid numbering system to be used in describing the retinoids is shown in Figure 1.

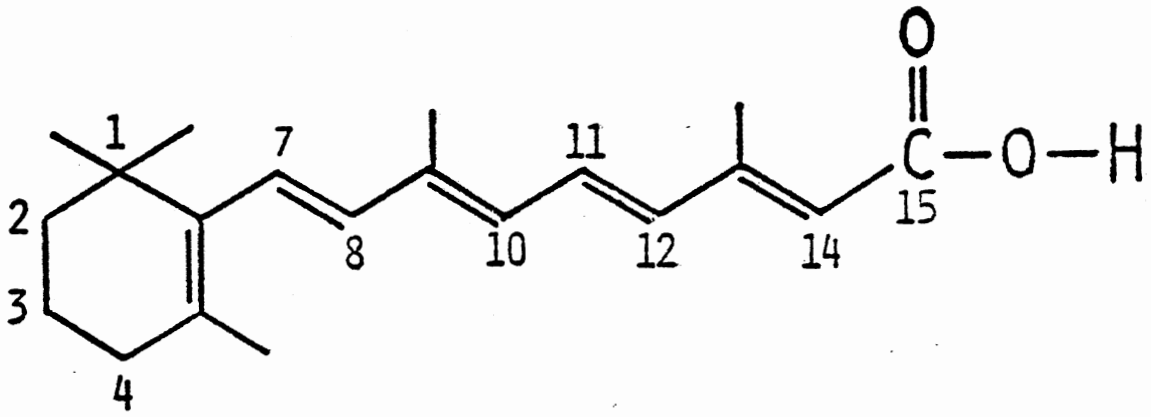

Figure 1. Numbering System for All-<u>trans</u>-Retinoic Acid

CHAPTER II

SEPARATION AND CHARACTERIZATION OF METABOLITES OF ALL-*TRANS*- AND 13-*CIS*-RETINOIC ACID IN BILE

Materials

Chemicals

All organic solvents used were low residue-distilled in glass HPLC grade solvents (Burdick & Jackson Laboratories, Inc., Muskegon, Mich.). All water was deionized and doubly distilled in glass. All-*trans*-[15-^{14}C]- (27.3 mCi/mmol), all-*trans*-[11,12-$^{3}H_2$]- (3.1 Ci/mmol), all-*trans*-[6,7-$^{14}C_2$]- (51 mCi/mmol), and nonradioactive all-*trans*-retinoic acid were gifts from Dr. W. E. Scott, Hoffmann La Roche, Inc. (Nutley, N.J.). All-*trans*-[10-^{3}H]retinoic acid (2.8 Ci/mmol) was supplied by New England Nuclear (Boston, Mass.) under contract to National Cancer Institute (NCI). 13-*Cis*-[11-^{3}H] (1.75 Ci/mmol), and all-*trans*-[13,14-$^{14}C_2$]retinoic acid (51 mCi/mmol) were supplied by Midwest Research Institute also under contract to NCI. Nonradioactive 13-*cis*-retinoic acid was produced by reactions of all-*trans*-retinoic acid with I_2 in the dark (75). Methyl retinoate was produced by reaction of all-*trans*-retinoic acid with diazomethane in the dark (78). Isomers of retinoic acid and methyl retinoate were produced by dissolving the sample in heptane, dimethylsulfoxide or methanol and placing it 6 inches from a Westinghouse FIST8/CW fluo-

rescent lamp for 8 to 72 hours. All radioactive retinoids were purified at least twice by HPLC utilizing a 0.46 x 25 cm Partisil PXS 10/25 ODS column (Whatman, Clifton, N.J.). The solvent used was 65 methanol:35 0.01 M aqueous acetic acid. All labelled retinoids were diluted with unlabelled isomerically identical retinoic acid to lower the specific activity to 90 μCi/mg and used within 1 hour of their final purification. All collections were in the dark under N_2 and were immediately extracted into carbon tetrachloride.

Nonradioactive 13-cis-retinoic acid was purified by HPLC utilizing a 0.94 x 50 cm Whatman (Clifton, N.J.) M9-ODS column. The material was eluted at 1.5 ml/min with 95 methanol:5 0.01 M aqueous acetic acid. Nonradioactive all-trans-retinoic acid was used without additional purification since it eluted as a single peak from the HPLC.

Animals

Day old chicks, obtained from Dr. R. H. Thayer, Animal Science Department, Oklahoma State University (Stillwater, Okla.), were placed on a vitamin A deficient diet until they stopped gaining weight. At that time they were divided into 2 groups. One group was dosed orally with 20 μg of all-trans-retinoic acid on Tuesdays and Fridays each week. The other group was dosed orally with 100 μg 13-cis-retinoic acid on Tuesdays and Fridays each week. The higher dosage required to keep chicks alive with 13-cis-retinoic acid is probably due to its lower biopotency relative to all-trans-retinoic acid.

HPLC Systems

Four HPLC systems, each equipped with a Valco (Houston, Tex.)

model CV-6-UHPa-N60 sample injector, were used. System #1 was used to purify nonradioactive 13-cis-retinoic acid. It consisted of a Dupont Model 830 HPLC system connected to an M9-ODS2 (C-18) column (Whatman, Clifton, N.J.). Detection was at 254 nm. Material was eluted at 1.5 ml/minute with 95 methanol:5 0.01 M acetic acid. System #2 was used to separate bile retinoids. It consisted of an Isco model 314 pump connected to a Dupont Model 830 gradient maker. The column employed for bile separations was an MCB (Cincinnati, Ohio) 0.46 x 25 cm C-8 column. Detection was at 254 nm. Additionally, a Beckman (Fullerton, Cal.) Model 25 scanning spectrometer was connected in series with the Dupont spectrophotometer. Material was eluted at 0.5 ml/minute with 30 methanol:70 water. System #3 was also used for bile separations. It consisted of 2 Waters (Millford, Mass.) Model 6000A pumps controlled by a waters Model 660 gradient maker. The same column was used as in System #2. The material was eluted at 1 ml/minute with 35 methanol:65 0.01 M acetic acid. At 40 minutes a one hour linear gradient to 100% methanol was initiated. Detection was at 254 nm with a Waters Model 440 spectrophotometer. System #4 was used for purification of radioactive retinoic acid samples. It consisted of a single Waters Model 6000A pump connected to a Whatman (Clifton, N.J.) 0.46 x 25 cm Partisil 10/25 C-18 column. Detection was at 340 nm with a Waters Model 440 spectrophotometer. Material was eluted at 1 ml/minute with 65 methanol:35 0.01 M acetic acid. All columns were protected by Whatman (Clifton, N.J.) precolumns packed with CoPell ODS.

Miscellaneous

Glucuronide assays were performed with Sigma (St. Louis, Mo.) types G4882 and G3510 β-glucuronidase from beef liver (pH 4.85) and E. coli (pH 7.0) respectively. Ultraviolet spectral analyses were performed on a Hitachi (Mount View, Cal.) Model 100-80 scanning spectrophotometer. Radioactivity was determined on a Packard (Downers Grove, Ill.) Prias PL liquid scintillation counter. Packard Insta-Gel was the scintillation fluid. Disintegrations per minute were calculated from counts per minute using computer programs developed by Bruce Halley and Kevin Ahern. Fractions off the HPLC column were collected with an Isco (Lincoln, Neb.) Model 328 fraction collector.

Methods

Chicks were injected intraperitoneally with labelled retinoids on a day when they would have been dosed orally with nonradioactive retinoids. Chicks were injected with 10 μCi (150 μg) of the same retinoid they had been fed previously. Animals dosed with less than 10 μCi of a labelled compound are noted separately. After injection of material the animals were provided with water. Six hours after injection, they were sacrificed by cervical dislocation and bile was removed with a syringe. It was immediately diluted with methanol (5 ml), filtered through a 0.5 μm filter (Millipore type FH, Bedford, Mass.) eluted through a Waters (Millford, Mass.) C-18 Sep-Pak, evaporated to dryness under N_2 and dissolved in 2 ml of methanol.

Glucuronidase assays were performed with 5000-8000 Sigma units of glucuronidase dissolved in 1 ml of the appropriate buffer.

Glucuronidase isolated from beef liver was diluted in a 0.075 M acetate buffer, pH 4.85. Glucuronidase from E. coli was dissolved in a 0.075 M phosphate buffer, pH 7. The appropriate enzyme solution was added to a dried bile sample and incubated at 37°C for 2 hours. A control was run with identical treatment and the appropriate buffer but no enzyme. After 2 hours, the reaction mixture was injected directly into HPLC system #3, described in Materials.

The bile metabolites were eluted at 1 ml/min with 35 methanol:65 0.01 M acetic acid. At 40 minutes, a 1 hour gradient to 100% methanol was begun. The UV spectrum of metabolite #2 was obtained by evaporating the solvent under N_2 and dissolving the residue in 2 ml of methanol. This was placed in a cuvette and scanned. Radioactive samples were diluted with 3-5 ml of Insta-Gel and allowed to stand for 2 hours before counting to eliminate chemiluminescence.

Results

Purity of Injected Retinoids

Two prerequisites for the use of radioactive precursors in tracing metabolic pathways are the requirement that the precursor be radiochemically and isomerically pure and that the investigator check the purity of the starting materials. If either requirement is not met, the researcher is never sure if the radioactive metabolites isolated were formed from the precursor or are contaminants. The 5,8 epoxy form of retinoic acid has been identified both as a metabolite of all-trans-retinoic acid (90) and as a major contaminant in labelled retinoic acid stocks (89).

Figure 2 illustrates the radiochemical purity of all-trans-[10-^{3}H]retinoic acid after purification by HPLC. The 13-cis isomer, eluting at 50 minutes is a typical contaminant of labelled all-trans-retinoic acid before purification. After two purifications by HPLC, the 13-cis isomer accounted for less than 0.3% of the total radioactivity.

Handling Controls

The chromatographic technique used for any metabolite purification should have as its number one priority - inertness toward sample. This has been a major problem with retinoids due to their labile nature. Previous experiments have clearly demonstrated the mild chromatographic properties of HPLC.

In any metabolic study involving labelled compounds it is important that any artifacts of handling, extracting, or storing of the compounds not be identified as metabolites of the starting material. The extent of artifact production can be very difficult to determine. Before any bile sample was injected into the HPLC system it was diluted with methanol, filtered, and eluted from a C-18 Sep-Pak. If these measures were not taken, it was not possible to perform HPLC analyses due to column damage. Because it was not possible to leave out any of the handling procedures, the following experiment was designed to determine the effect of the handling procedures on all-trans-retinoic acid. The chick was raised in the usual manner, but was injected with 0.25 ml of ethanol containing no retinoic acid six hours before sacrifice instead of with the usual radioactively labelled retinoic acid. At six hours bile was removed and diluted

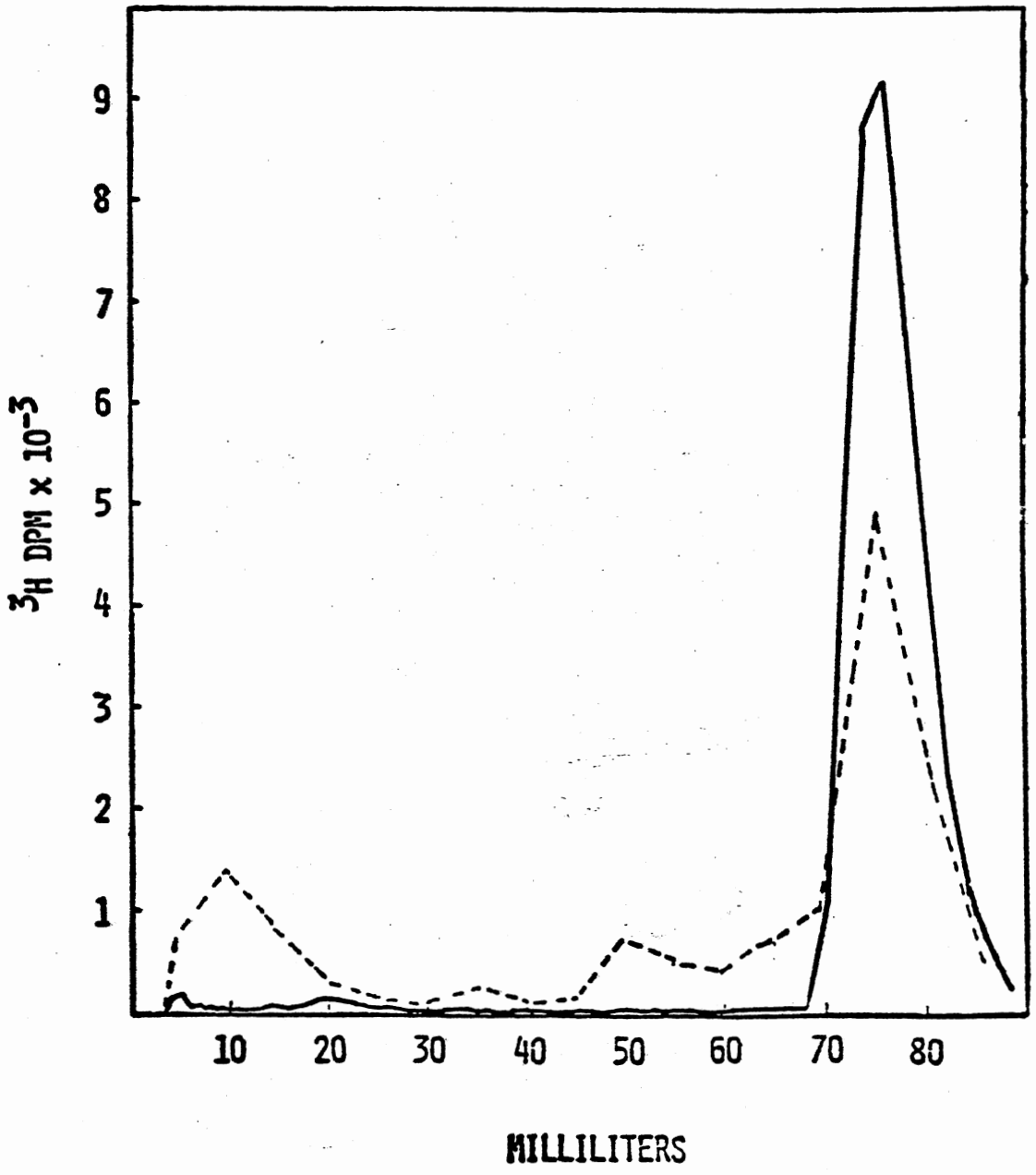

Figure 2. HPLC Profile of Retinoic Acid Before (Dashed) and After (Solid) Purification

with 5 ml of methanol containing 1 x 10^6 DPM of all-trans-[10-^{3}H]-retinoic acid. The mixture was filtered, eluted from a C-18 Sep-Pak and injected into HPLC system 3. The elution profile is shown in Figure 3. Only two regions received any significant radioactivity; the first, eluting between 5-10 mL, corresponded to the void volume of the system and might be an artifact of the injection process, the second, eluting between 97-100 mL, corresponded to free all-trans-retinoic acid. That the material eluting between 97-100 minutes was free retinoic acid was shown when unlabelled all-trans-retinoic acid was injected into HPLC system 3 under the same conditions and eluted between 97-100 mL (Figure 4). The results presented here clearly indicate that no major alterations of retinoic acid occurred when bile was routinely handled for HPLC injection. Additionally, when bile was filtered and passed through a Sep-Pak three times, it did not alter the HPLC elution pattern when compared to once treated bile.

All-trans Retinoic Acid Radiochemical Experiment

A separation of bile retinoids by HPLC from chicks injected with either all-trans-[10-^{3}H], [6,7-$^{14}C_2$], or [15-^{14}C]retinoic acid is shown in Figures 5 and 6. The chicks dosed with all-trans-[6,7-^{14}C]retinoic acid received only 2 μCi of material. Both the volumes of bile and the percentage of injected label found in the bile varied considerably. Bile volumes ranged from 0.3 to 2.0 ml. A maximum of 10% (15 μg) of the injected label was recovered in the bile although typical recoveries ranged from 1-5% (1.5-7.5 μg).

The radioactive profile (Figure 5) revealed a wide range of

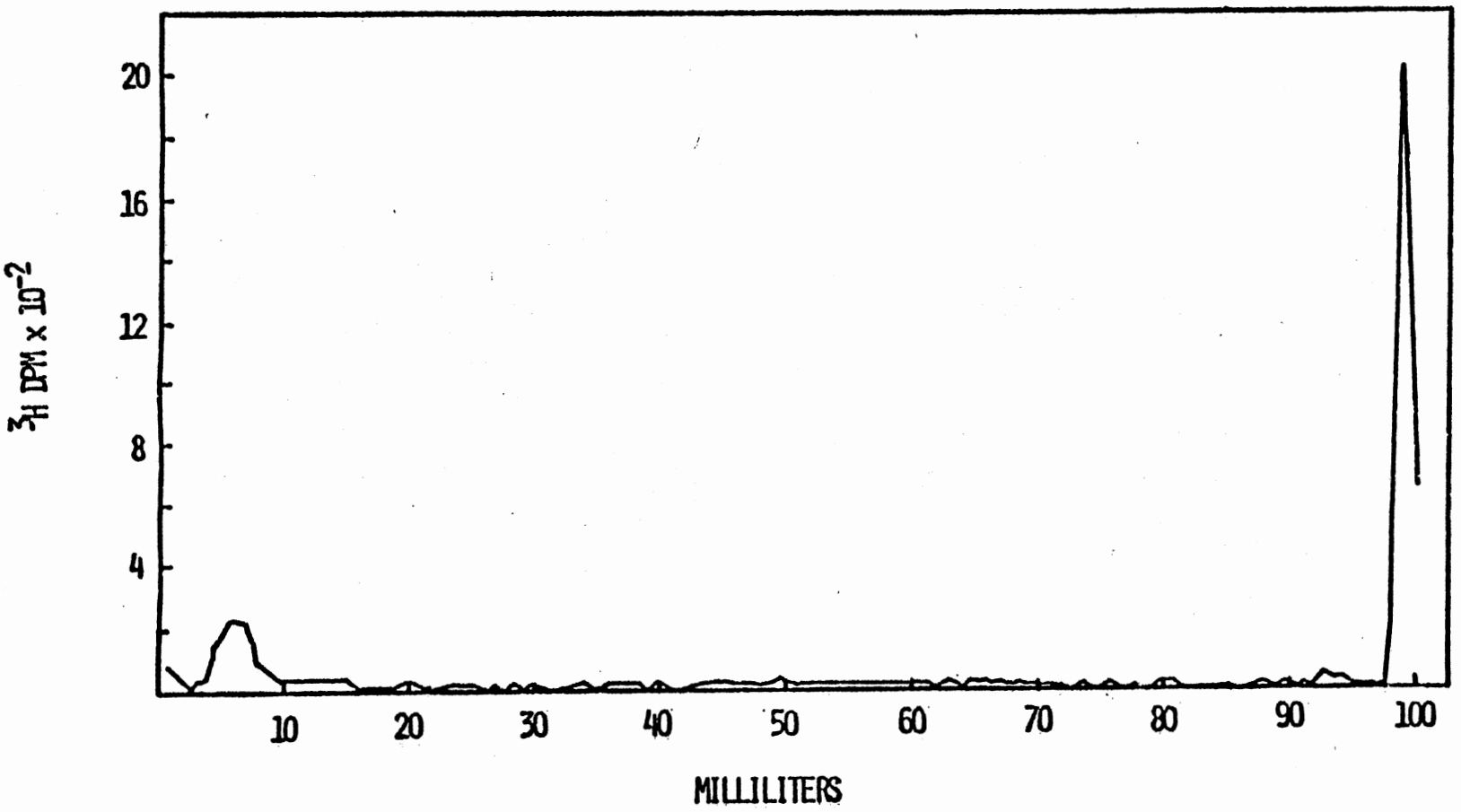

Figure 3. HPLC Profiles from System 3 of Retinoic Acid After Handling Procedures

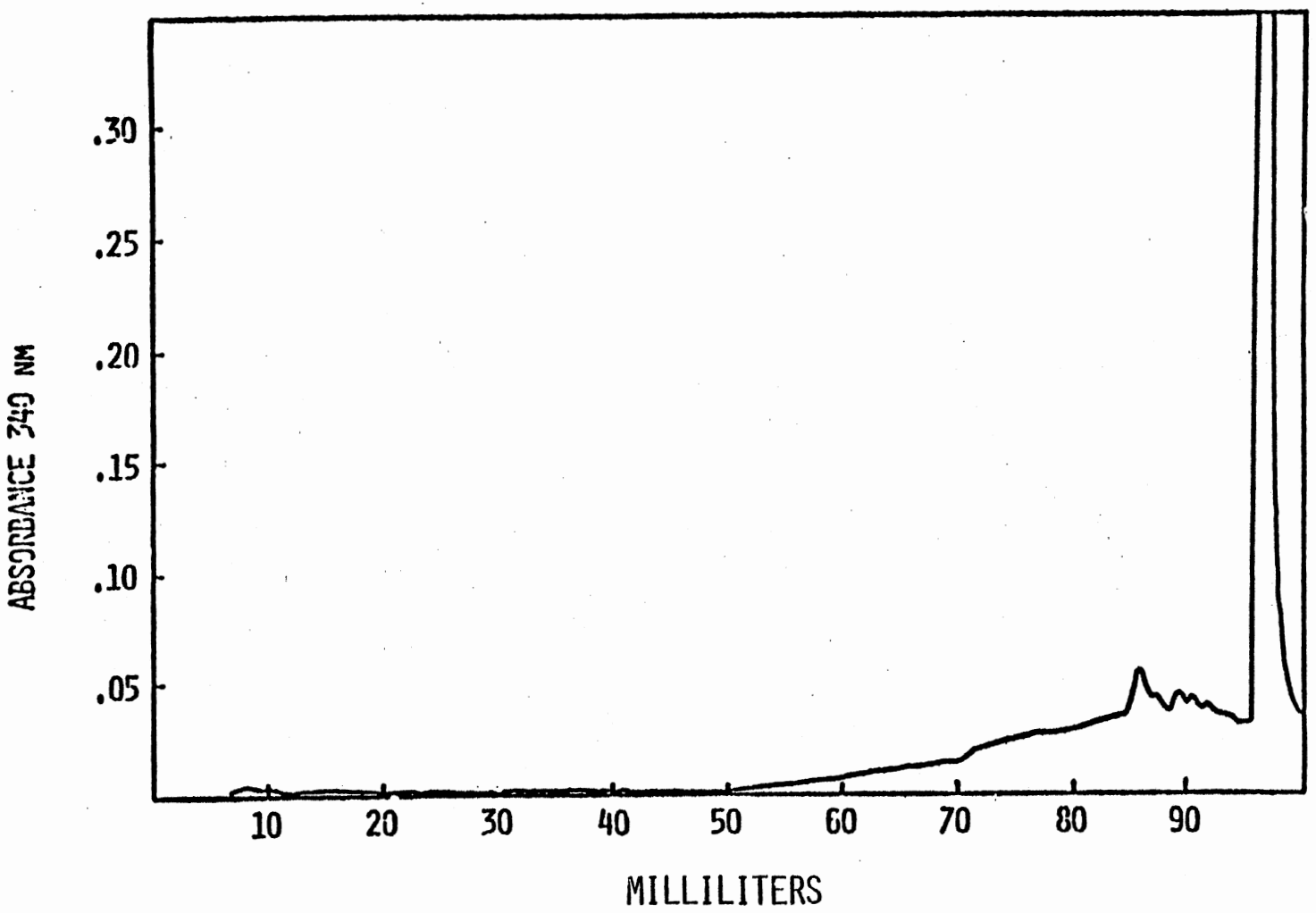

Figure 4. Elution Position of All-trans-Retinoic Acid from System 3

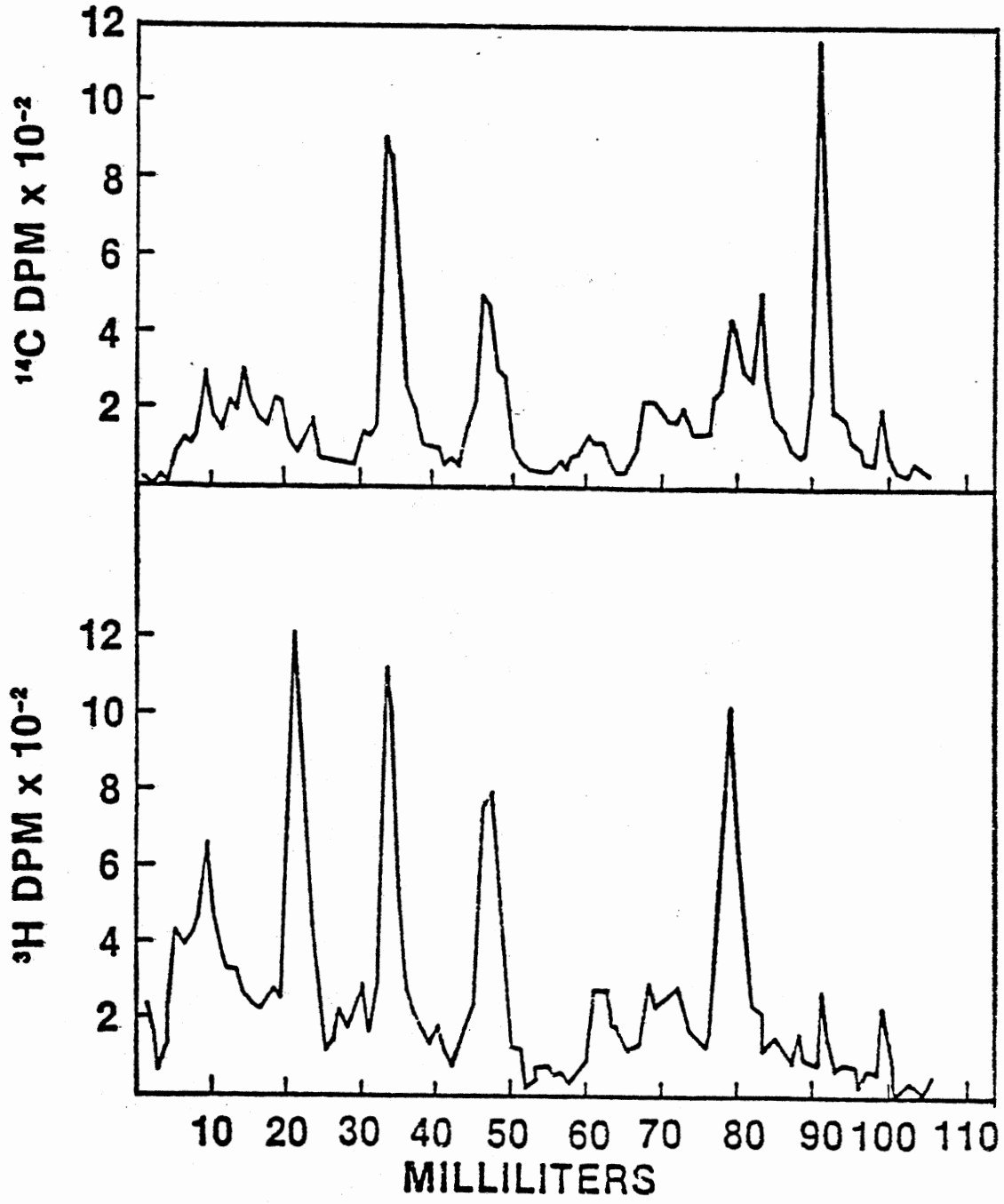

Figure 5. Comparison of HPLC Profiles from System 3 of Bile from Chicks Injected with All-<u>trans</u>-[15-^{14}C]Retinoic Acid (Top) or All-<u>trans</u>-[10-^{3}H]Retinoic Acid (Bottom)

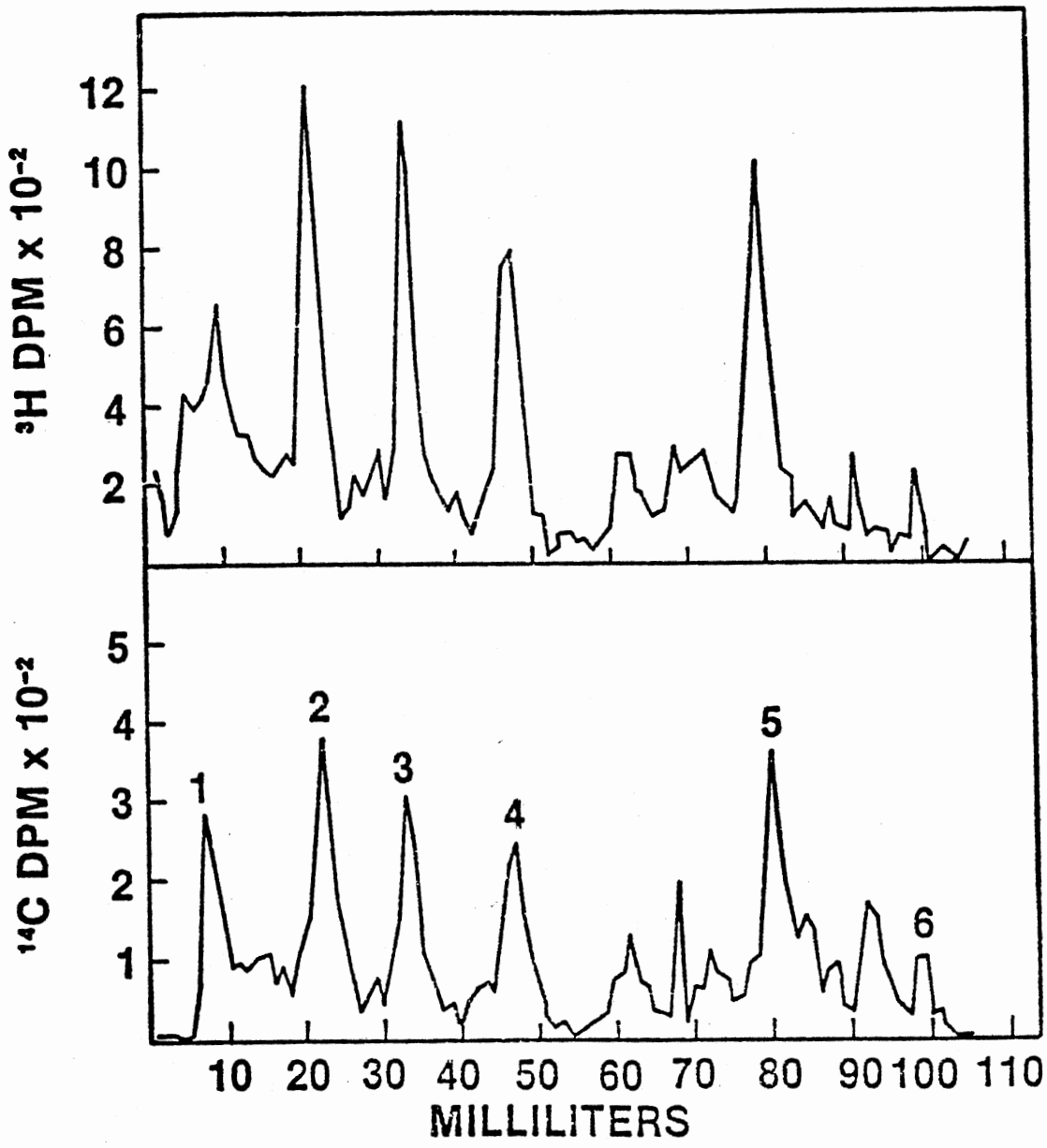

Figure 6. Comparison of HPLC Profiles from System 3 of Bile from Chicks Injected with All-trans-[6,7-$^{14}C_2$]Retinoic Acid (Top) or All-trans-[10-^{3}H]Retinoic Acid (Bottom)

polarities of retinoic acid metabolites, ranging from unchanged retinoic acid at 98-100 minutes to the very polar water soluble metabolites eluting in the first 40 minutes. One of the most abundant metabolites in the pattern (henceforth referred to as metabolite 2) eluted between 18-22 ml and lacked radioactivity from carbon #15. All other metabolites had both 10-^{3}H and 15-^{14}C. Chromatography of bile from chicks injected with all-trans-[6,7-^{14}C]-retinoic acid confirmed that all peaks that contained 10-^{3}H also contained 6,7-$^{14}C_2$ (Figure 6). The relative abundances of six bile retinoids are given in Table I. The HPLC elution pattern of bile from chicks injected with all-trans-[6,7-$^{14}C_2$]retinoic acid resembled the elution pattern of bile from chicks injected with all-trans-[15-^{14}C]retinoic acid for all peaks except metabolite 2 (Figure 7). Peak 2 was the only peak consistently lacking in [15-^{14}C]. These data indicated the ring and side chain were intact for every major retinoid except metabolite 2.

Each of the experiments described above utilized separate chicks injected with different radioactive labels, respectively, and were replicated over 30 times. A flaw inherent in such experiments is that the levels of metabolites produced in different animals could vary considerably depending on the amount of stress, physiological state or nutritional status of the organism. It could be possible that no 15-^{14}C was observed corresponding to metabolite 2 because those individual chicks injected with all-trans-[15-^{14}C]retinoic acid produced either no or small amounts of metabolite 2. To provide conclusive evidence that metabolite 2 was decarboxylated, it was necessary to examine the metabolism of all-trans-[15-^{14}C]retinoic acid and all-trans-[10-^{3}H]retinoic acid in a single animal.

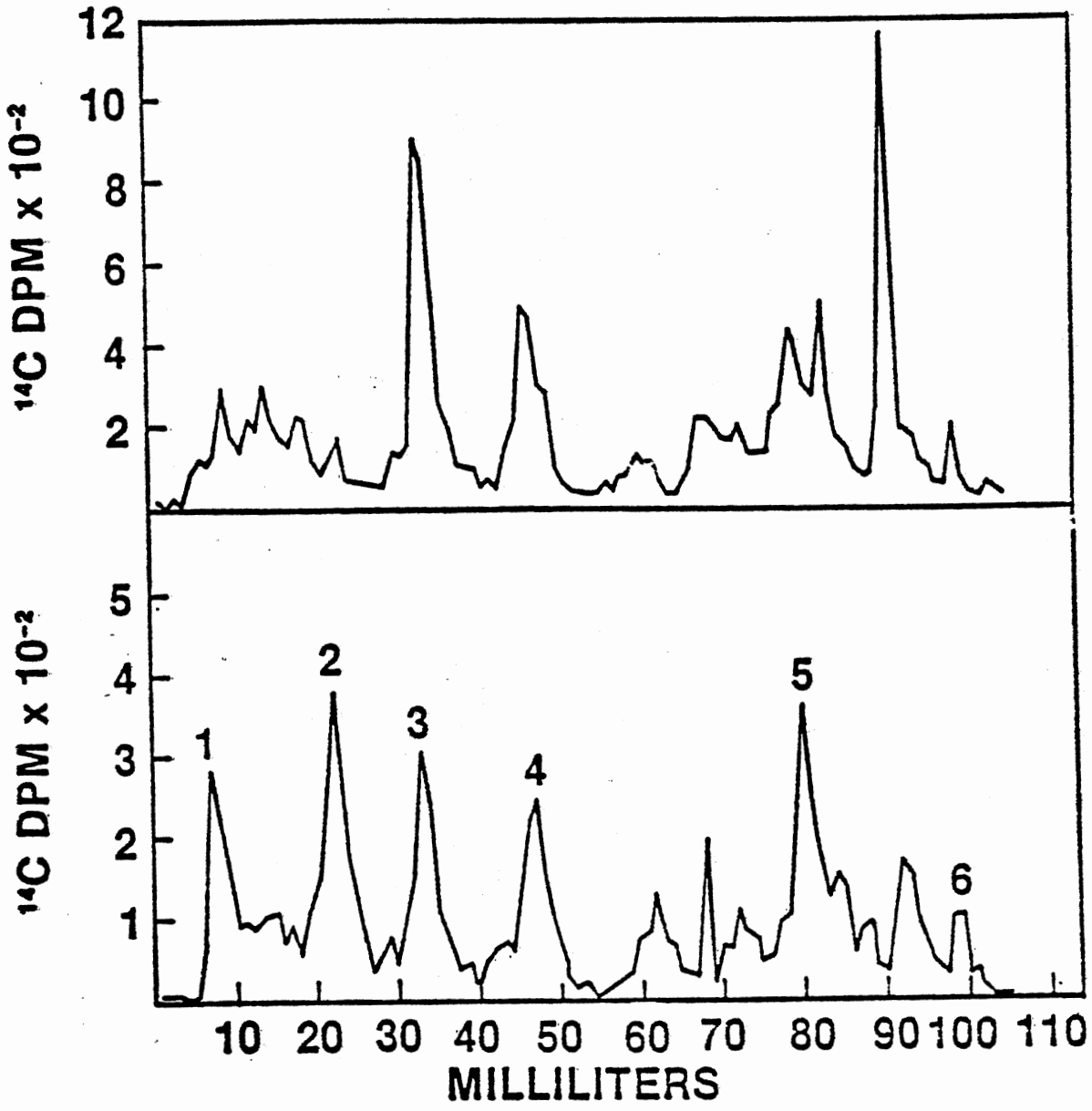

Figure 7. Comparison of HPLC Profiles from System 3 of Bile from Chicks Injected with All-trans-[15-^{14}C]Retinoic Acid (Top) or All-trans-[6,7-$^{14}C_2$]Retinoic Acid (Bottom)

TABLE I

RELATIVE ABUNDANCES OF SIX BILE RETINOIDS DERIVED FROM ALL-TRANS-[10-^{3}H]RETINOIC ACID

Metabolite No.	Per Cent of Total Bile Radioactivity
1	9.1
2	15.9
3	11.0
4	9.1
5	6.8
6	2.5

Dual Label Experiment

One chick was injected with 6 μCi of all-trans-[10-^{3}H]retinoic acid and 4 μCi of all-trans-[15-^{14}C]retinoic acid. The HPLC profile of bile metabolites from system #2 is shown in Figure 8. The HPLC column was eluted with 30 methanol:70 water. The metabolite from peak 2 which eluted under these conditions between 19-20.5 ml, clearly contained ^{3}H but no ^{14}C, confirming that the metabolite from peak 2 was decarboxylated.

Characterization of Side Chain of Metabolite #2

13,14-$^{14}C_2$

The experiments with all-trans-[6,7-$^{14}C_2$]retinoic acid, all-trans-[10-^{3}H]retinoic acid, and all-trans-[15-^{14}C]retinoic acid revealed that the side chain was intact for all the bile metabolites of retinoic acid except the metabolite eluting in peak 2. Since the 10-^{3}H position in the decarboxylated metabolite was present, it was obvious that the side chain was intact to at least carbon #10. An experiment was designed using all-trans-[13,14-$^{14}C_2$]retinoic acid to determine if radioactivity corresponding to at least one of these positions was present. A chick was injected with 10 μCi of all-trans-[13,14-$^{14}C_2$]retinoic acid and bile was prepared as described in methods. The HPLC profile (25% methanol, 75% water) from bile metabolites of retinoic acid is shown in Figure 9. The radioactivity peak observed between 42.5-45 ml corresponded to the decarboxylated metabolites. The radioactivity corresponding to the metabolite represented 26.3% of the total radioactivity in the bile. The average

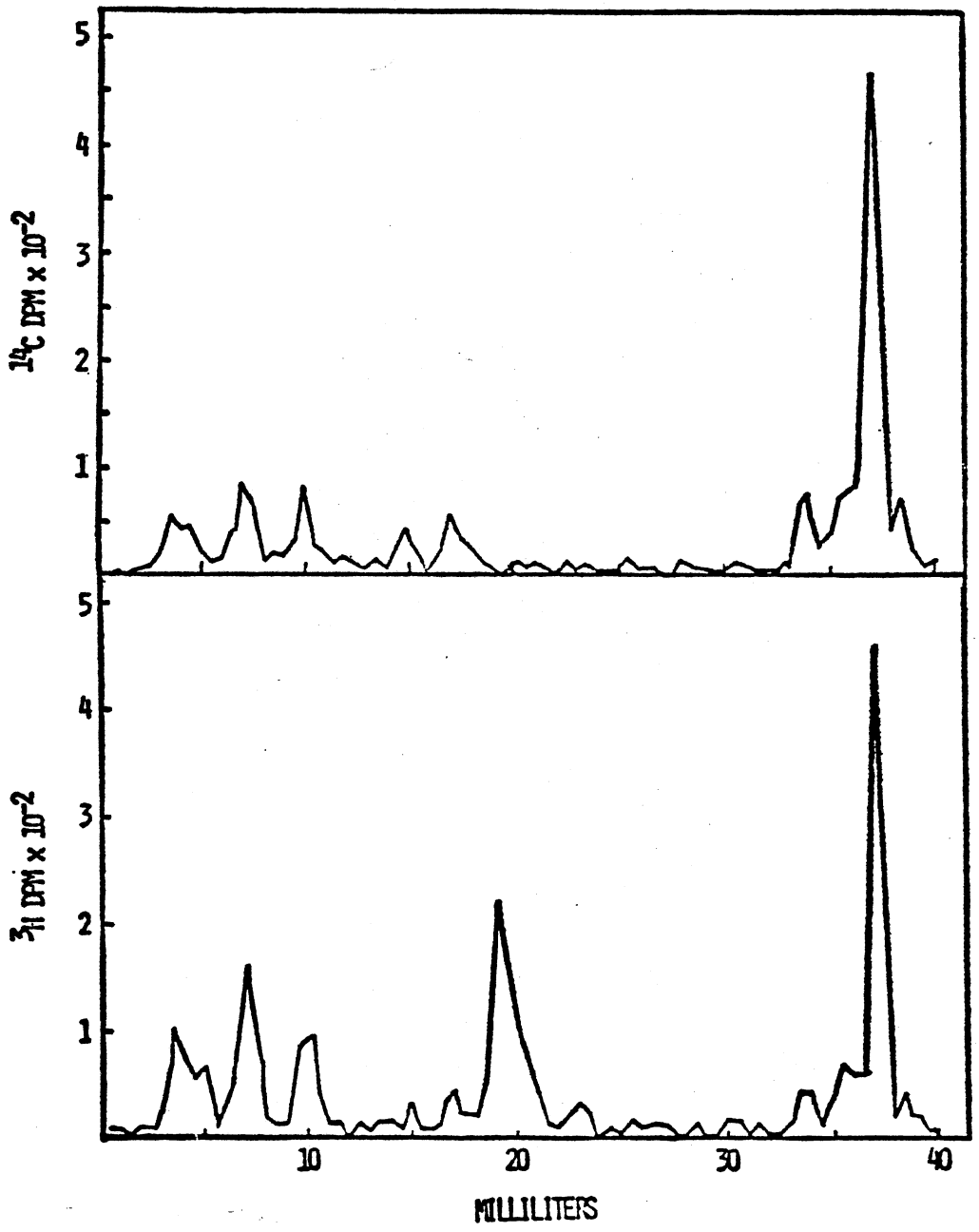

Figure 8. HPLC Profile from System 2 of Bile from a Chick Injected with All-trans-[15-^{14}C]Retinoic Acid (Top) and All-trans-[10-^{3}H]Retinoic Acid (Bottom)

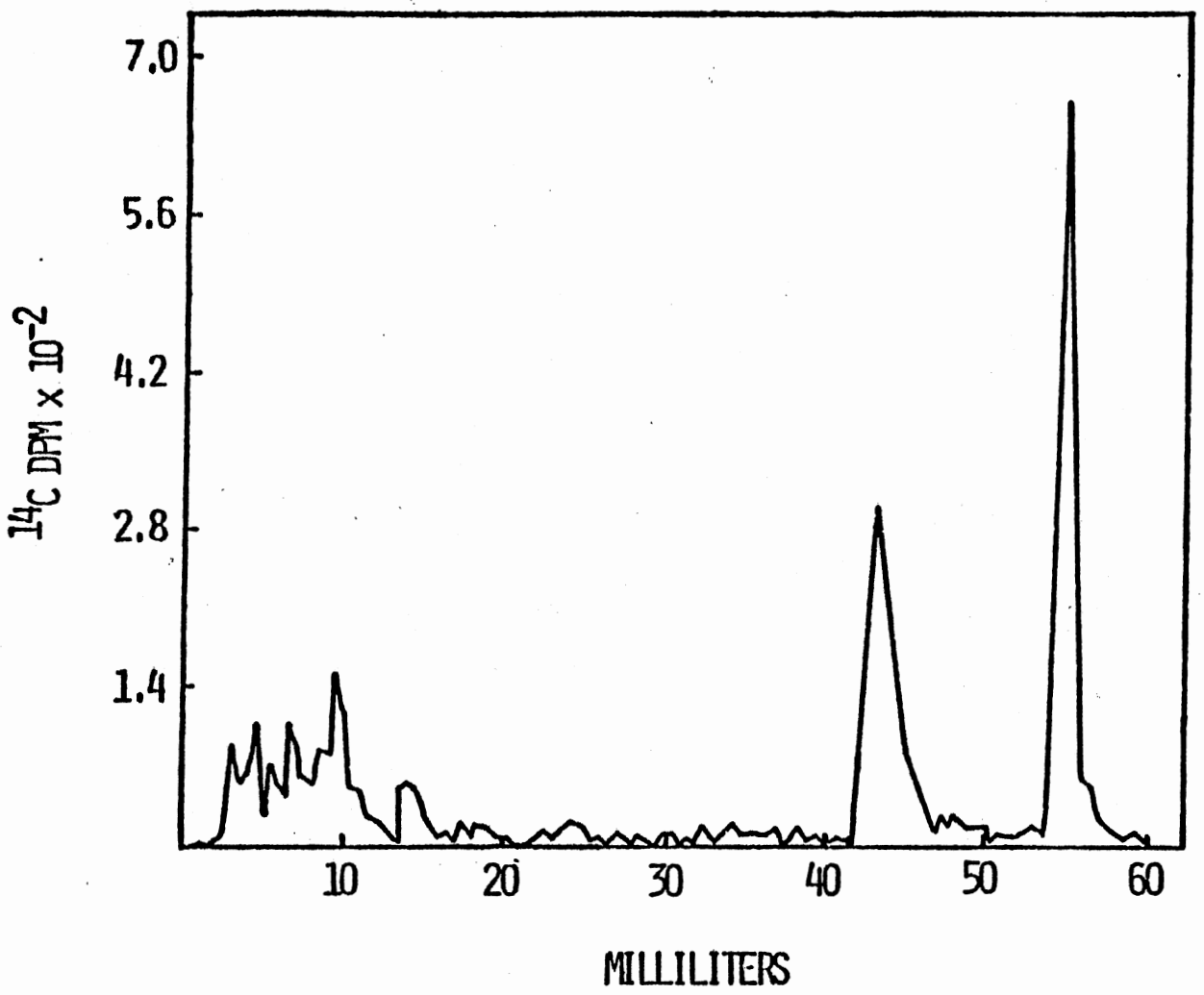

Figure 9. HPLC Profile from System 2 with 25 Methanol:75 Water of Bile from Chicks Injected with All-<u>trans</u>-[13,14-$^{14}C_2$]Retinoic Acid

percentage of the total bile radioactivity corresponding to the metabolite eluting in peak 2 for chicks injected with different radioactive labels is given in Table II. The values shown in the table are average values from six chicks for all labels but the [13,14-$^{14}C_2$] which was obtained from one chick. It was not possible to determine from these data whether or not carbon #14 was present, due to the fact that different animals could produce different amounts of metabolites. It was obvious, however, that at least carbon #13 was present.

Glucuronidase Experiments

A significant finding in understanding the pathway that retinoic acid takes in its rapid transit through the body was the discovery by Zachman et al. (68) that most retinoic acid appeared in the bile within 24 hours of injection. Subsequent studies on bile revealed that retinoyl-β-glucuronide was a metabolite that accounted for up to 90% of the bile retinoids. A more recent study has placed the figure closer to 12% (93) but, nevertheless, it was obvious from glucuronidase treatments that some metabolites of retinoic acid in the bile were linked to glucuronic acid residues. The only metabolite of retinoic acid previously identified using spectroscopic analysis in the bile was the identification by mass spectral analysis of retinoyl-β-glucuronide (93). All-trans-retinoic acid and all-trans-methyl retinoate have previously been reported present. All of these studies, however, utilized massive doses (3 mg) of retinoic acid. The formation of glucuronides under these conditions might represent a detoxification mechanism for handling the large amount of retinoids in the body.

TABLE II

AVERAGE PERCENTAGES OF TOTAL BILE RADIOACTIVITY CORRESPONDING TO METABOLITE 2 FOR DIFFERENT RADIOACTIVE LABELS

Label	Per Cent of Total Bile Radioactivity Corresponding to Metabolite 2
6,7-$^{14}C_2$	15.9
10-^{3}H	14.9
13,14-$^{14}C_2$	26.3
15-^{14}C	0.0

The following experiments were designed to locate and quantitate the glucuronic acid derivatives of all-trans-retinoic acid in the bile of chicks dosed with physiological doses of all-trans-retinoic acid.

Beef Liver β-Glucuronidase. Treatment of bile samples for 2 hours at 37°C with glucuronidase isolated from beef liver (pH 4.85) produced only minimal changes in the chromatographic profile (Figure 10) relative to a control (Figure 11). The peak between 98-100 minutes was slightly increased by the treatment. This peak, which corresponded to free all-trans-retinoic acid, would be released from retinoyl-β-glucuronide by the enzyme treatment. All-trans-retinoic acid eluted in this region as shown in Figure 4. It was not apparent from the chromatographic profile which peak corresponded to the retinoyl-β-glucuronide derivative.

Because these experiments produced inconclusive results about the existence of glucuronides among bile retinoids, experiments were conducted with glucuronidase isolated from E. coli (pH 7). The nonacidic conditions used in association with E. coli glucuronidase would be less conducive to artifact production.

E. coli Glucuronidase Treatments. Figure 12 shows the HPLC profile of radioactivity of bile retinoids isolated from chicks injected with all-trans-[10-^{3}H]retinoic acid and then treated with E. coli β-glucuronidase as shown in Figure 13. The control sample of bile retinoids was incubated with the same 0.075 M phosphate buffer pH 7.00 but without enzyme. Metabolites 1-4 showed no change in either peak positions or relative sizes on treatment with the enzyme,

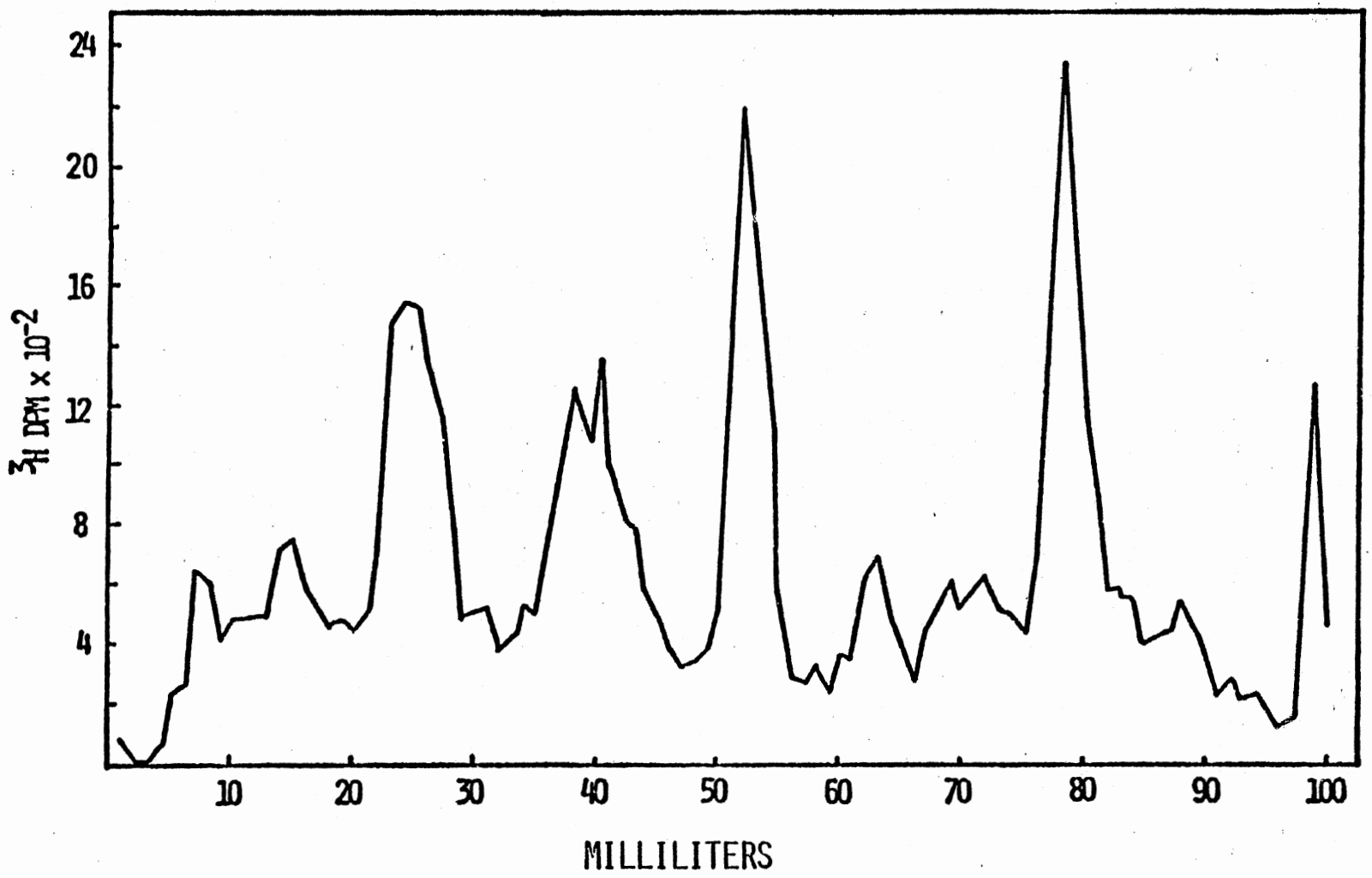

Figure 10. HPLC Profile from System 3 of Beef Liver Glucuronidase Treated Bile from Chicks Injected with All-trans-[10-^{3}H]Retinoic Acid

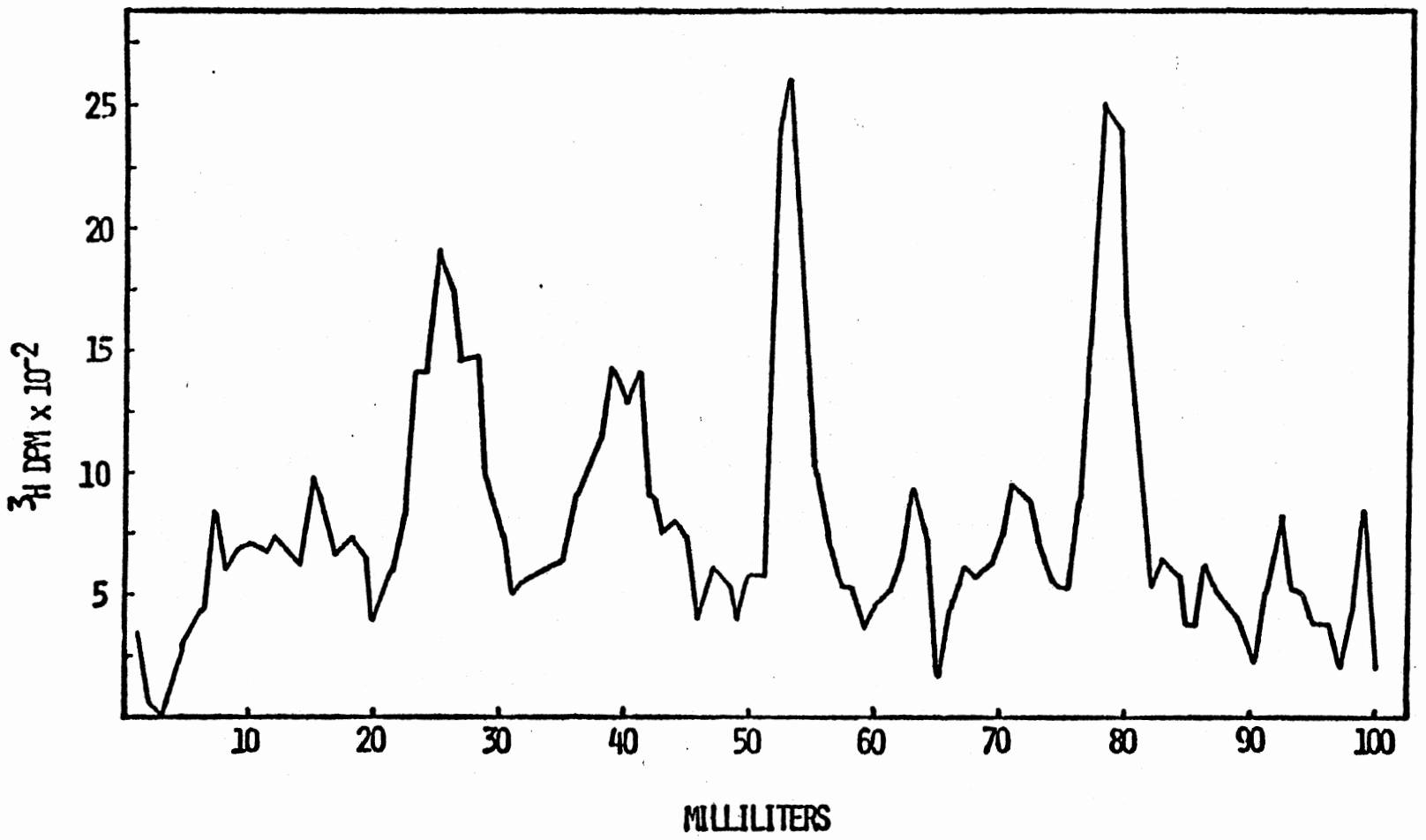

Figure 11. HPLC Profile from System 3 of Beef Liver Glucuronidase Control Bile from Chicks Injected with All-trans-[10-^{3}H]Retinoic Acid

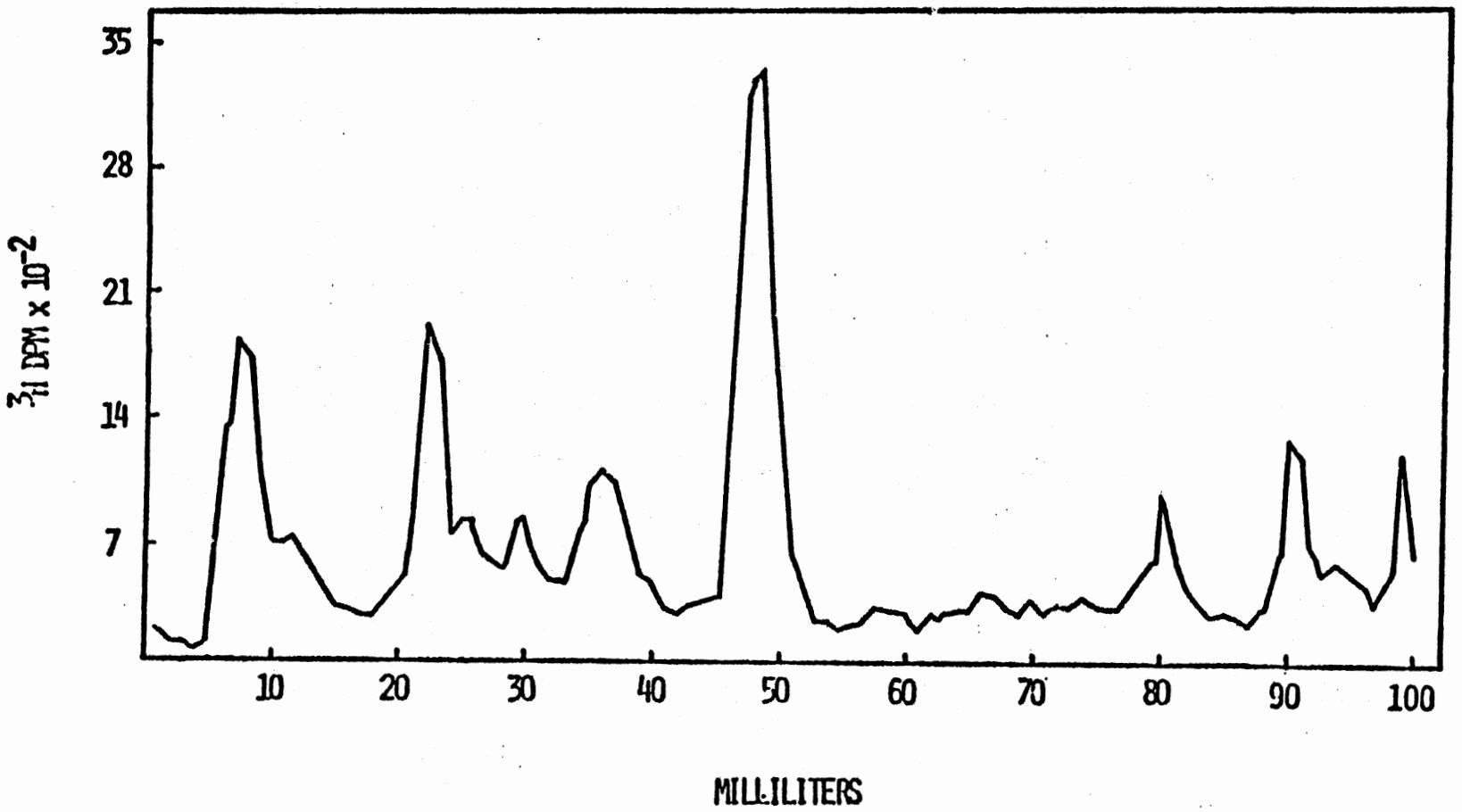

Figure 12. HPLC Profile from System 3 of E. coli Glucuronidase Treated Bile from Chicks Injected with All-trans-[10-^{3}H]Retinoic Acid

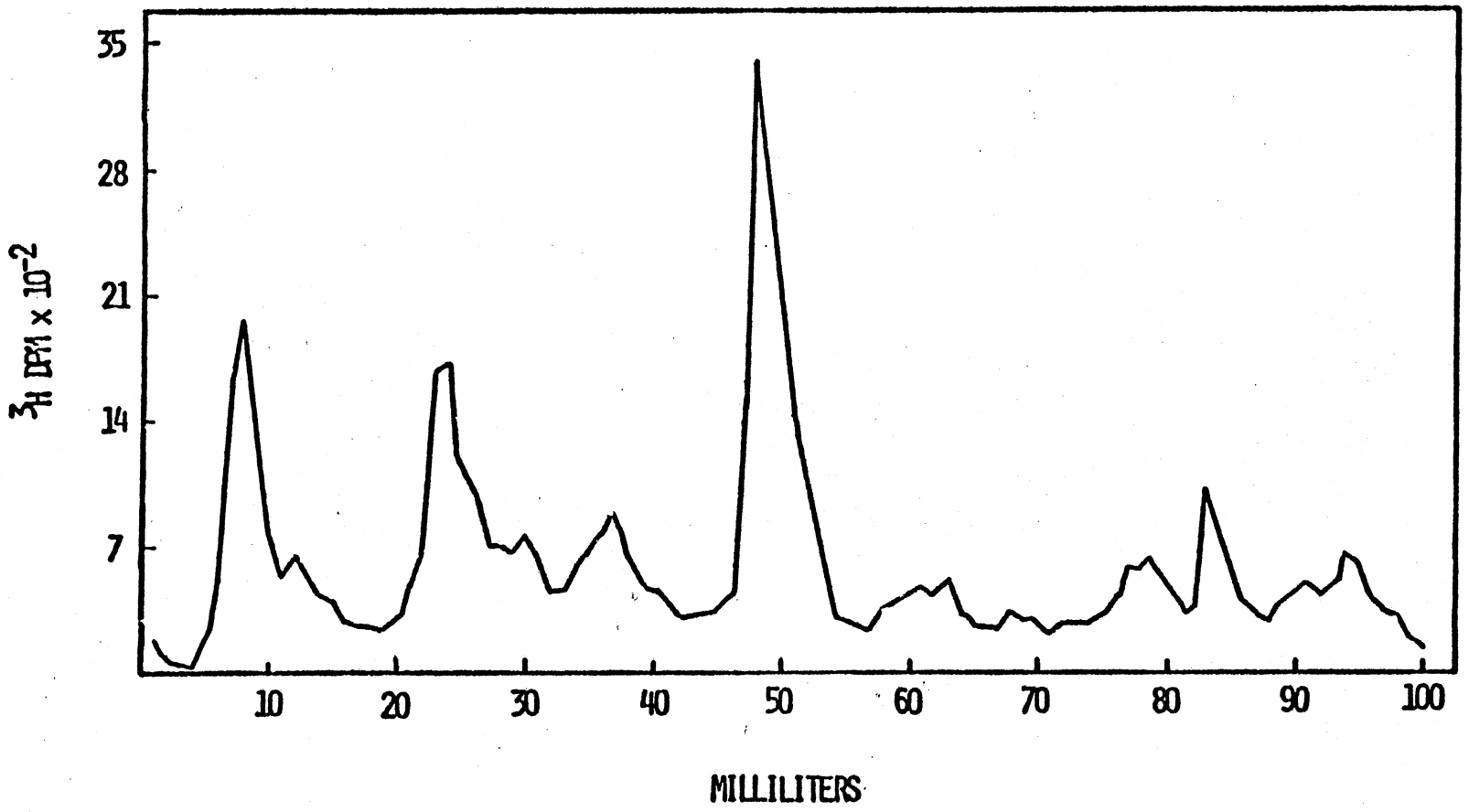

Figure 13. HPLC Profile from System 3 of E. coli Glucuronidase Control Bile from Chicks Injected with All-trans-[10-^{3}H]Retinoic Acid.

consistent with the beef liver glucuronidase treatment. The E. coli glucuronidase treatments indicated that 2 main regions of radioactivity were glucuronidase sensitive, having a reduced amount of radioactivity present relative to controls. Corresponding to the reduced amounts of metabolites between 77-80, 83-85 and 92-95 ml was the increase in the amounts of metabolites between 88-90 and 98-100 ml relative to controls. Since the radioactive peak that eluted between 98-100 ml corresponded to free retinoic acid, it was apparent that a retinoyl ester of glucuronic acid was present in the bile.

It was surprising that the glucuronic acid derivatives of retinoic acid and its metabolites would elute so late in the HPLC pattern. It was indicative that the glucuronides had polarities similar to all-trans-retinoic acid. The four bile retinoids eluting in the first 50 ml of the HPLC pattern by contrast, were very polar, essentially water soluble. No one has ever reported any polar molecule attached to a retinoid in the bile except glucuronic acid. Clearly, the presence of a glucuronic acid moiety on a retinoid did not change the polarity of the retinoid very much, yet the first four retinoids were very polar. Obviously glucuronic acid was not polar enough to account for the very polar retinoids eluting in the first 50 ml. With the exception of the metabolite eluting in peak 2, all the polar compounds had intact side chains. It was not certain whether or not the ring was totally intact. It would appear from the evidence from the glucuronidase treatments that something previously totally undescribed had happened to alter these retinoids. With respect to the decarboxylated retinoids, it is interesting to note that Luigi DeLuca (12), reviewing the work of Chen and Heller (60), postulated that a

decarboxylated derivative of retinoic acid might be phosphorylated and function to transport mannose for incorporation into manno-glycoproteins. This suggestion was based on the fact that when synthetic retinoyl phosphate was incubated with the cells, it did not function in uptake of mannose. When synthetic retinyl phosphate was used, it did function in transport of mannose. It was proposed that a terminal hydroxyl group might be present, since the compound chromatographed like retinol. For retinoic acid, this would require either reduction of the carboxyl group, which has never been demonstrated *in vivo*, or decarboxylation to an alcohol.

The polarity of the decarboxylated compound is not unlike that of a phosphate, but there is not yet any evidence to indicate that a phosphate or any moiety is or is not present.

UV Absorbance

Due to the different HPLC systems and conditions used in the separation of the decarboxylated retinoid, it was desirable to have a means to detect the decarboxylated retinoid in addition to radioactivity. Numerous collections of the decarboxylated retinoid were pooled and the UV absorption spectrum was determined as shown in Figure 14. The apparent UV absorption maximum was 318 nm. It was possible that the apparent UV absorption maximum might not correspond to the metabolite. To help resolve this question, the following experiment was designed. The HPLC elution pattern at 318 nm for bile from a chick injected with all-*trans*-retinoic acid is shown in Figure 15. Under these conditions (30 methanol:70 water) radioactivity profiles showed that the decarboxylated metabolite eluted between

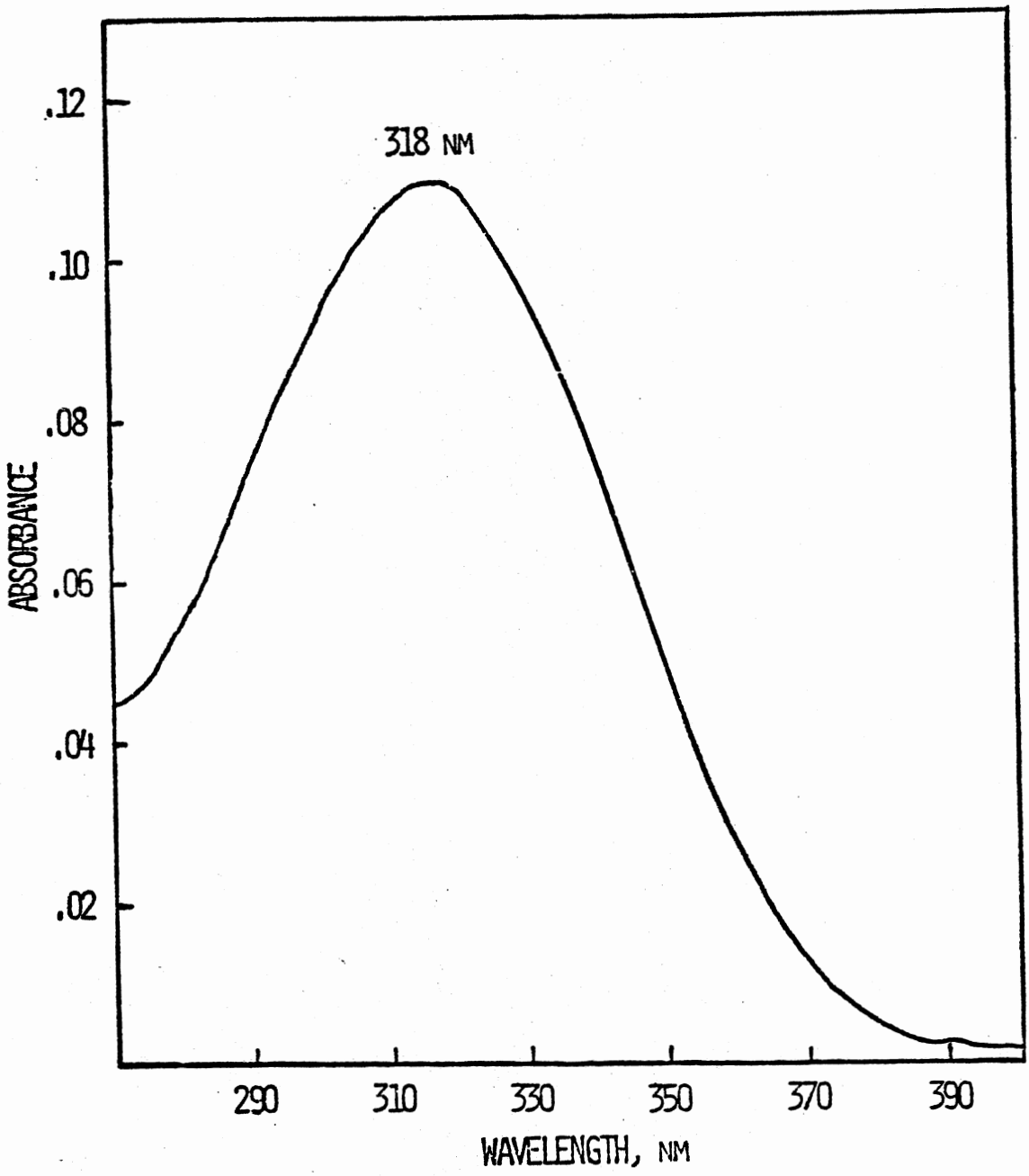

Figure 14. Ultraviolet Absorption Spectrum of Metabolite 2

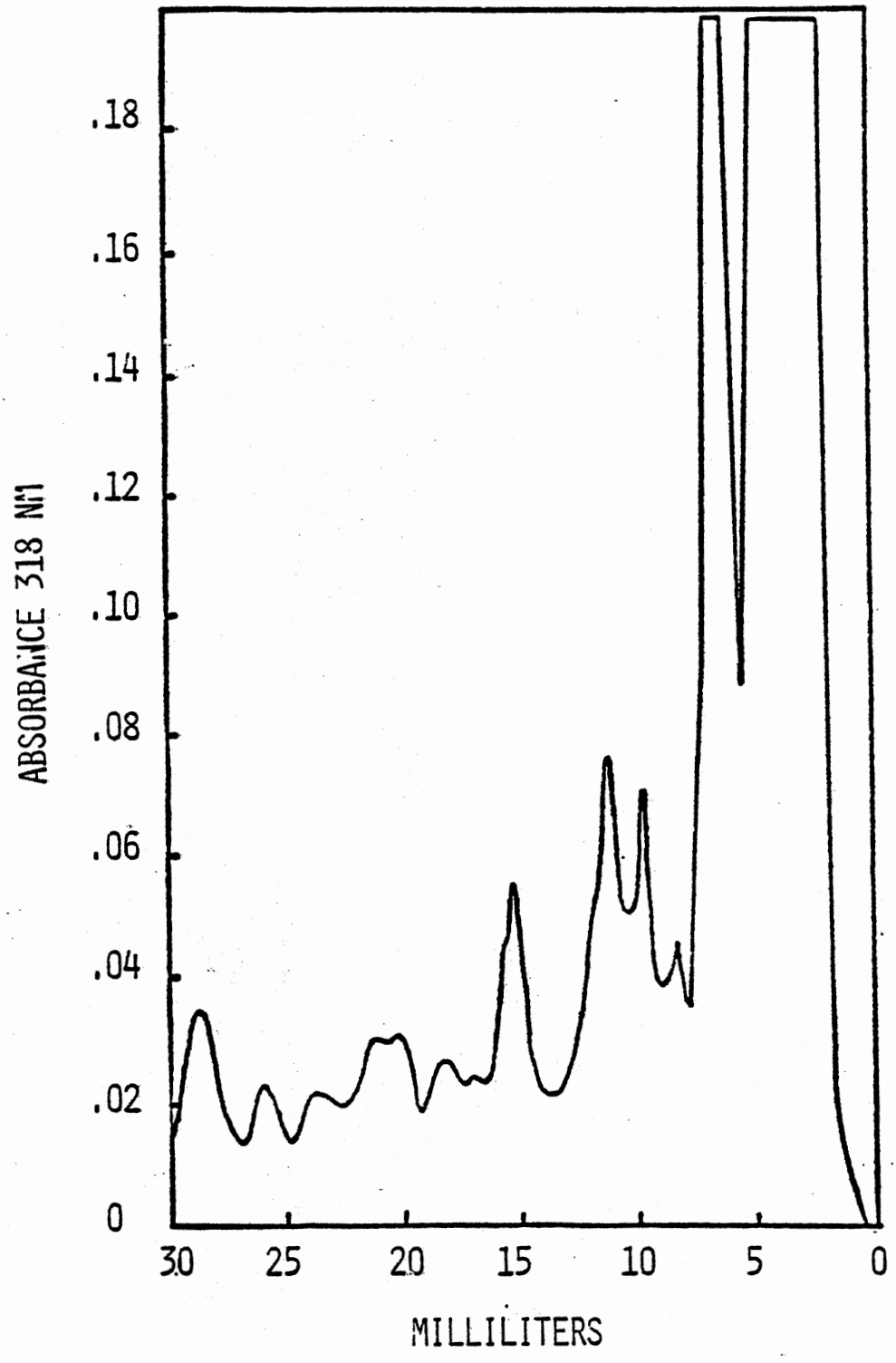

Figure 15. HPLC Profile from System 2 of Bile from Chicks Supplemented with All-trans-Retinoic Acid

27.5-30 ml and it can apparently be seen by the UV absorbance at 318 nm between 27.5 and 30 ml also. Next, bile from chicks totally deficient in vitamin A and not supplemented with retinoic acid was injected into the HPLC. The UV elution pattern at 318 nm is shown in Figure 16. The decarboxylated retinoid which would elute between 27.5-30 ml was not detected at 318 nm. The evidence that the metabolite absorbed at 318 nm is not conclusive, but bile from chicks whose diet was supplemented with all-trans-retinoic acid contained a substance which absorbed at 318 nm and eluted in the same volume as the decarboxylated metabolite of retinoic acid while bile from chicks deficient in vitamin A and unsupplemented with retinoic acid did not.

Metabolism of 13-cis-Retinoic Acid

The metabolism of 13-cis-retinoic acid is important because of its use as a chemopreventive agent in some types of epithelial cancer.

One study reported 13-cis-retinoic acid to be a metabolite of all-trans-retinoic acid (91). The chromatographic system used, however, isomerized 60% of applied all-trans-retinoic acid to 13-cis-retinoic acid (91). A recent article by Frolik et al. (94) reported that a metabolite in the liver was common to hamsters dosed with either 13-cis- or all-trans-retinoic acid. This hinted that the metabolism of 13-cis-retinoic acid had features in common with metabolism of the all-trans-isomer. The question of whether or not 13-cis-retinoic acid is a metabolite of all-trans-retinoic acid has not been determined. If 13-cis-retinoic acid were an obligatory metabolite or "active form" of all-trans-retinoic acid through which all retinoic acid metabolites were formed, then one would expect that the metabolic profiles of bile

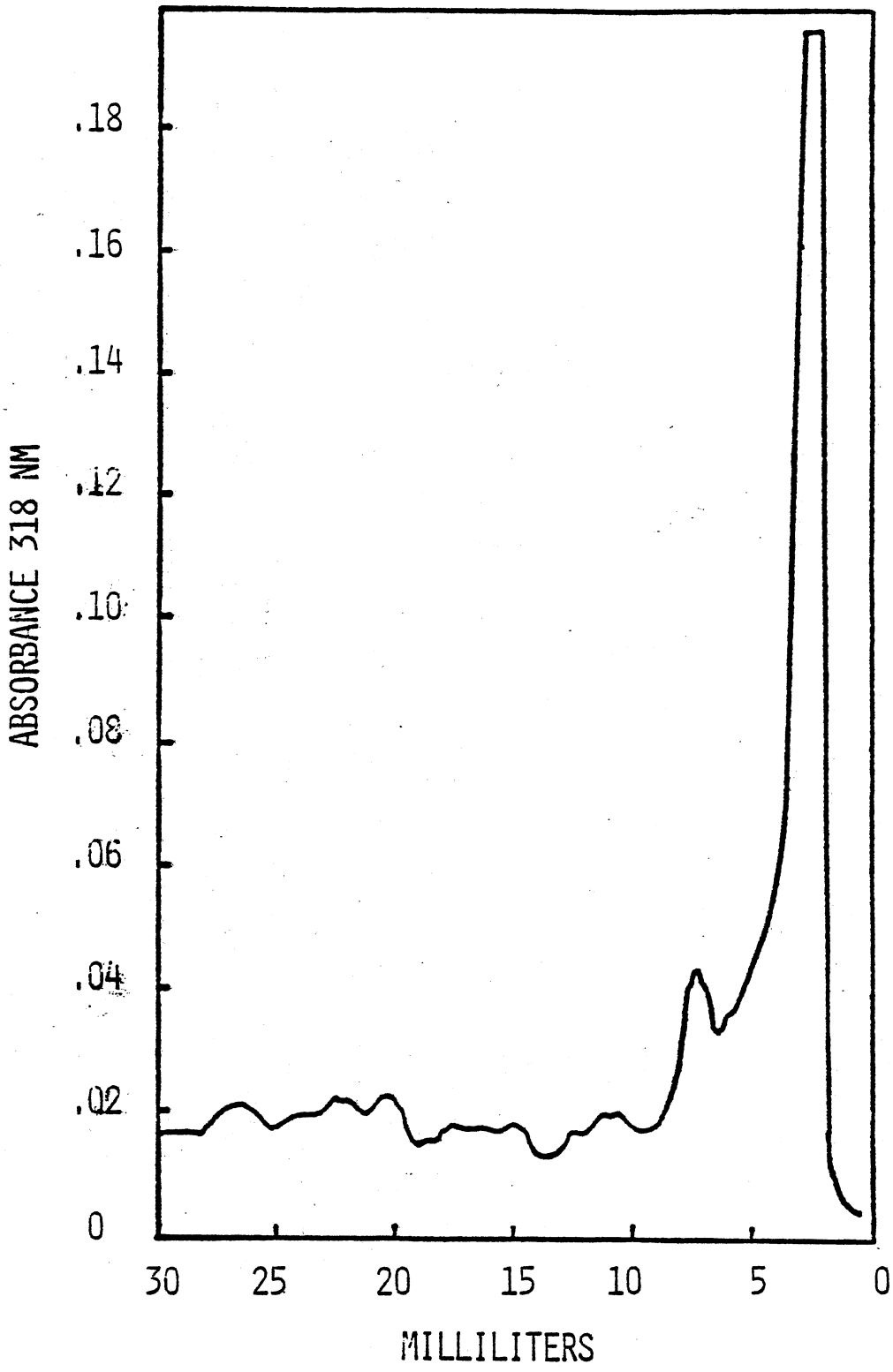

Figure 16. HPLC Profile from System 2 of Bile from Chicks Not Supplemented with All-trans-Retinoic Acid

from chicks dosed with 13-cis-retinoic acid would be identical to that from all-trans-retinoic acid treated chicks. The following experiments were designed with 3 objectives; first to determine whether or not the decarboxylated retinoid present in all-trans treated chicks was present in 13-cis-retinoic acid treated chicks; second, to compare the metabolic profile in bile from 13-cis-retinoic acid treated chicks with the profile from all-trans-retinoic acid treated chicks; and third, to determine the status of free 13-cis-retinoic acid in bile from chicks treated with all-trans-retinoic acid.

Chicks maintained on 13-cis-retinoic acid did not produce a radioactive compound corresponding to the metabolite eluting in peak 2 when injected with 13-cis-[11-^{3}H]retinoic acid. The top pattern in Figure 17 shows the metabolite eluting in peak 2 between 20.5-23 ml from a chick injected with all-trans-[13,14-$^{14}C_2$]retinoic acid, while the bottom is from a chick injected with 13-cis-[11-^{3}H]retinoic acid. The absence of radioactivity corresponding to the metabolite in peak 2 for 13-cis-retinoic acid bile did not necessarily mean nonproduction of the compound. Some experiments have suggested removal of a proton from the 11,12 position, although no such compound has been reported.

It was apparent when bile profiles from 13-cis-[11-^{3}H]retinoic acid injected chicks were compared to all-trans-[13,14-$^{14}C_2$]retinoic acid injected chicks that there were very big differences in the way the 13-cis-isomer was metabolized, compared to the all-trans. The percentages of total biliary retinoids of each of the 13-cis- and all-trans- metabolites is given in Table III. The major discrepancies between the 2 profiles were in the areas of polar retinoids at the first part of the chromatogram. Only 14% of 13-cis- metabolites eluted

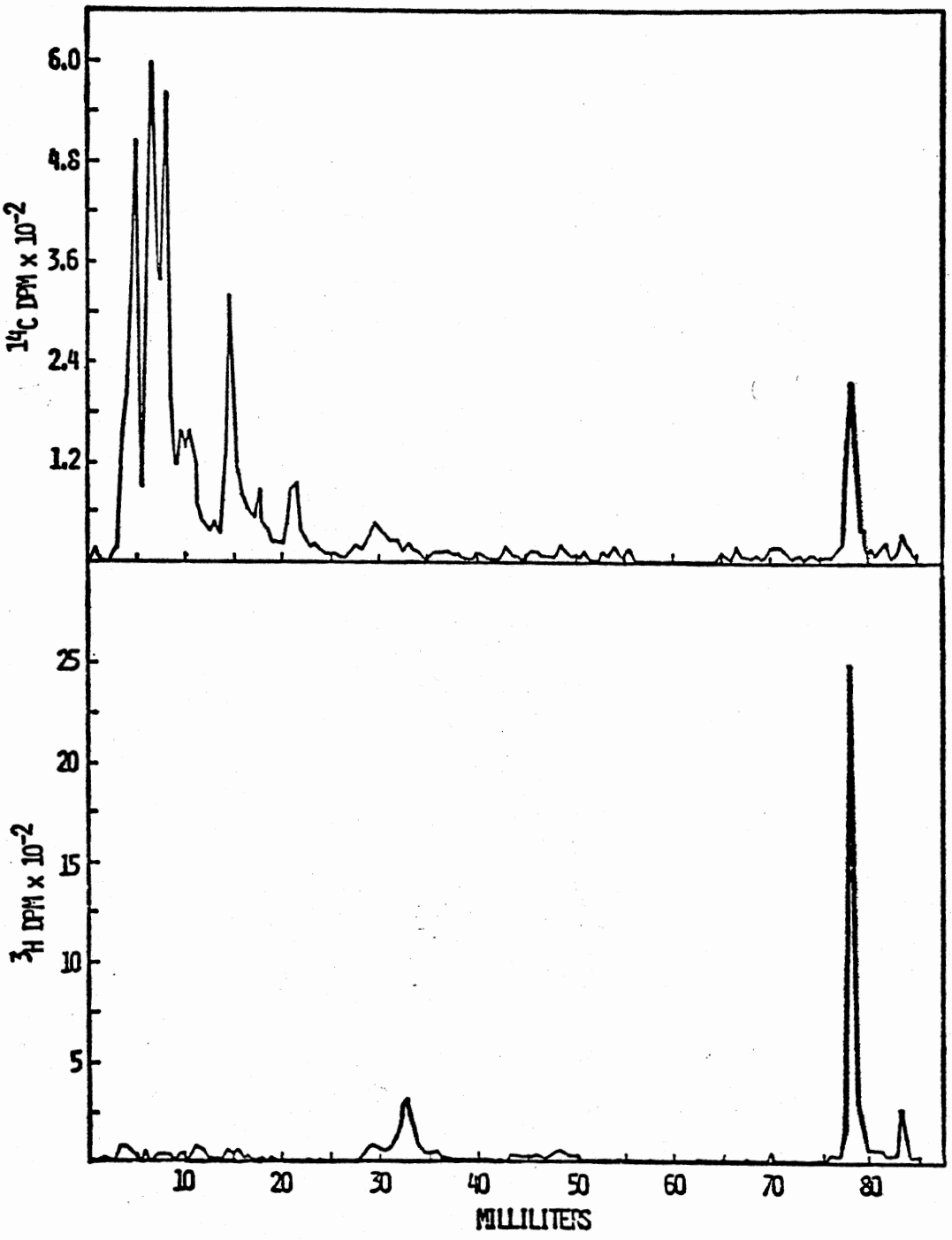

Figure 17. HPLC Profiles from System 2 of Bile from Chicks Injected with All-trans-[13,14-$^{14}C_2$]Retinoic Acid (Top) or 13-cis-[11-^{3}H]Retinoic Acid

TABLE III

COMPARISON OF ELUTION POSITIONS OF METABOLITES OF ALL-*TRANS*- AND 13-*CIS*-RETINOIC ACID

ML Elution Range	% of All-*Trans*-Metabolites	% of 13-*Cis*-Metabolites
2-5	15.4	3.5
5.5-10	37.2	3.7
10.5-15.5	16.4	4.8
20-25	4.7	1.1
31-35	1.7	16.0
77-80	7.8	49.2

during the first 50 minutes of the HPLC profile versus 81% of the all-trans- metabolites. It should be remembered that the experiments with glucuronidase revealed that the glucuronides present in the bile eluted late in the HPLC pattern. Most of the 13-cis metabolites probably represent glucuronides of 13-cis-retinoic acid. It is not known why the 13-cis- isomer was not converted to the more polar derivatives or the significance of these derivatives in the metabolism of retinoic acid. The alternative explanation that the polar metabolites were formed, but lost all tritium from C-11, however, seems unlikely.

A recent report by Zile et al. (93) stated that 13-cis-retinoic acid was indeed a metabolite of all-trans-retinoic acid in rats despite conflicting earlier reports. HPLC systems 2 and 3 used in separating metabolites in the bile were not capable of separating a mixture of all-trans- and 13-cis-retinoic acid due to the type of column used. To resolve 13-cis-retinoic acid from all-trans-retinoic acid, a modification of system 3 was made. The C-8 column was replaced by the M9-ODS-2 column from system 1. The elution conditions were changed as follows. The run solvent employed was 50 MeOH:50 0.01 M acetic acid for 10 minutes, a 1 hour 20 minute gradient to 100% methanol, followed by 30 minute elution with 100% methanol. Under all of these conditions, 13-cis-retinoic acid eluted between 159-162 ml followed by the all-trans-retinoic acid between 169.5-172.5 ml. The HPLC elution pattern of radioactivity in bile from a chick injected with all-trans-[10-^{3}H]retinoic acid is shown in Figure 18.

Very little free 13-cis between 159-162 ml or all-trans-retinoic acid between 169.5-172.5 ml was observed in the HPLC patterns, con-

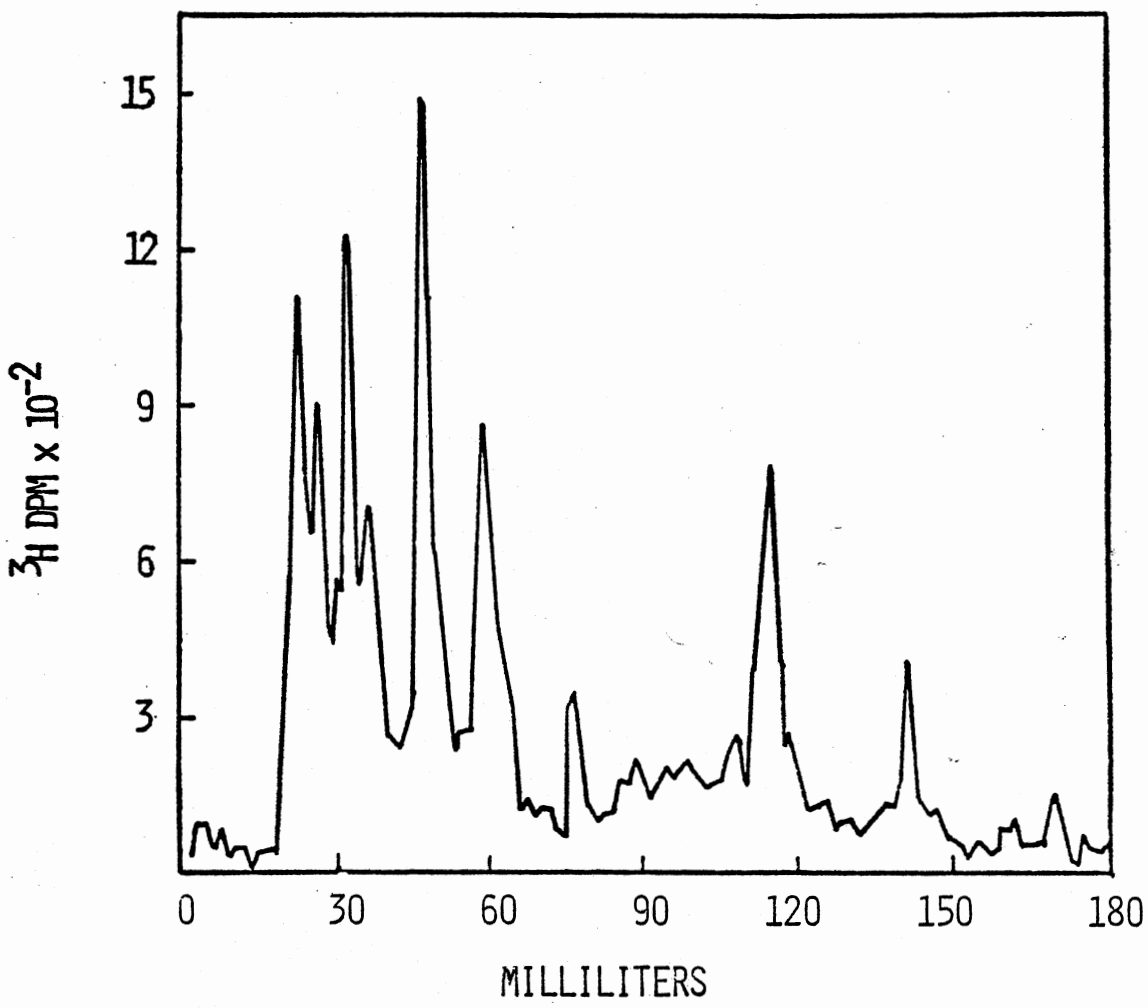

Figure 18. HPLC Profile from Modified System 3 of Bile from Chicks Injected with All-<u>trans</u>-Retinoic Acid

sistent with previous results. It was apparent, however, that a very small amount of radioactivity co-chromatographed with the 13-cis isomer between 159-162 ml, but because of the low amount of radioactivity involved, it was not possible to determine if it was significant.

Discussion

The results presented here represent the first characterization of a decarboxylated retinoid in bile. The metabolite found in bile had the side chain intact to at least carbon #13, making it the decarboxylated retinoid with the longest side chain reported to date in vivo. The decarboxylated metabolites found by Hanni et al. (87) in urine had the side chain cleaved back to position 12.

All but one of the bile retinoids had the entire side chain intact. No fragments were detected. This partly confirmed previous studies that concluded that only retinoids with intact side chains were present in the bile (63). It can only be speculated as to why previous investigations failed to discover the decarboxylated retinoid reported here. Other studies on the bile utilized massive quantities of retinoic acid (up to 3 mg vs. 150 µg used here). Under conditions like that, the body might recognize retinoic acid as toxic and rapidly eliminate it via the bile in the rapidest way possible, and the normal metabolism might be obscured. Indeed, Lippel et al. (70) characterized 90% of the bile metabolites as retinoyl-β-glucuronide. By contrast, this study characterized only 7% of the metabolites as retinoyl-β-glucuronide. Earlier studies performed on the bile utilized chromatographic techniques such as thin layer chromatography, gas

chromatography and extraction that were not as compatible with the labile nature of retinoids nor capable of achieving the necessary separation of retinoids as HPLC. To trace metabolites by radioactive precursors it is necessary that the chromatography technique employed completely and reproducibly separate the metabolites formed, lest the radioactivity of one metabolite contaminate the sample of another. Another requirement in the use of radioactive precursors is that they be radiochemically and isomerically pure. If this condition is not met then some of the so-called "metabolites" formed represent contaminants or metabolites of contaminants. Using HPLC it was possible to purify all-trans-retinoic acid so that at most 0.3% of the material injected into the animal represented 13-cis-retinoic acid, a common contaminant of radiochemical stocks of retinoids. The importance of this is emphasized by the fact that many investigations have utilized unpurified radioactively labelled retinoids in metabolic studies, yet some of the retinoids we have used were less than 10% pure prior to purification.

Glucuronidase treatments of the bile revealed that 11% of the bile retinoids are conjugated with glucuronic acid. Retinoyl-β-glucuronide represents 6.8% of the bile retinoids. This is lower than what had previously reported, but consistent with a recent report by Zile et al. (93). It was surprising to find very polar retinoids in bile that were not attached to glucuronic acid. Indeed, the glucuronides in bile eluted late in the HPLC pattern, corresponding to a polarity like that of retinoic acid. The explanation for the water solubility of the first three retinoids in the HPLC pattern of the bile is unknown. It is possible that a polar substituent such as

phosphate or sulfate is attached to the molecule. The first and the third metabolites in the pattern have intact side chains ending in a carboxyl group, presumably, since no evidence has ever been found that retinoic acid is reduced in vivo. If a phosphate or sulfate is attached at the carboxyl end, forming a mixed anhydride, the resulting high energy bond might be unstable, yet HPLC patterns of these compounds after incubation for 2 hours at 37°C indicated no decomposition.

The material eluting in peak 2, however, lacked the terminal carboxyl group. Whether or not a new carboxyl group replaced the lost carboxyl group has not been determined. Luigi DeLuca (12), reviewing the work of Chen and Heller (60), proposed that a decarboxylated retinoid with a hydroxyl group could be phosphorylated and bind mannose for transfer to mannoglycoprotein synthesis. If the decarboxylated retinoid ended in an alcohol instead of an acid, attachment of a phosphate or sulfate through an ester linkage would be more stable.

The UV maximum of 318 nm corresponds to a loss of conjugation relative to the parent all-trans-retinoic acid with a UV maximum of 350 nm. The decarboxylated C-19 aldehyde isolated by Rockley et al. (82) by contrast had a UV maximum of 280 nm. The C-19 aldehyde had conjugation interrupted at carbon #9 by hydroxylation resulting in two conjugated systems of 3 bonds each. The 3 decarboxylated retinoids found by Hanni et al. (87) in the urine had UV absorbance maxima at 262 nm, 278 nm and 264 nm, with conjugation extending over 3, 3, and 2 bonds, respectively.

This indicates the retinoid reported here either has a more

extended system of conjugation than previously reported decarboxylation products of retinoic acid or that it is attached to a chromophore.

CHAPTER III

SUMMARY

In this study the metabolites of retinoic acid in chick bile were separated by reverse phase HPLC. Comparison of the radioactive profiles of bile from chicks injected with either all-trans-[6,7-$^{14}C_2$]-, [10-^{3}H] or [15-^{14}C]retinoic acid revealed that all metabolites but one had completely intact side chains. The decarboxylated metabolite was one of the most abundant retinoids and represented 15.9% of bile retinoids. It has a UV absorbance maximum of 318 nm, a blue shift relative to all-trans-retinoic acid. No radioactivity corresponding to it was produced in animals injected with 13-cis-[11-^{3}H]retinoic acid.

Chicks maintained on 13-cis-retinoic acid have greatly reduced amounts of the very polar metabolites seen in the bile of chicks maintained on all-trans-retinoic acid. It is not clear whether or not 13-cis-retinoic acid is a metabolite of all-trans-retinoic acid in the bile.

Treatment of bile with β-glucuronidase from E. coli and beef liver revealed 2 main regions of radioactivity contained glucuronic acid residues. Both have polarities similar to all-trans-retinoic acid. None of the water soluble metabolites eluting early in the pattern were altered by incubation with glucuronidase.

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