METABOLITES OF RETINOIC ACID IN CHICK BILE

By

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IN CHICK BILE

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NOMENCLATURE

cpm	–	counts per minute
cAMP	–	adenosine 3',5'-cyclic monophosphate
DPM	–	disintegrations per minute
Fe^{2+}	–	ferrous ion
CGC/MS	–	capillary gas chromatograph/mass spectrometer/data analysis system
HPLC	–	high performance liquid chromatograph
KOH	–	potassium hydroxide
$KMnO_4$	–	potassium permanganate
µCi	–	micro Curie
mg	–	milligram
ml	–	milliliter
M	–	molar
NADH	–	nicotinamide adenine dinucleotide (reduced form)
NADPH	–	nicotinamide adenine dinucleotide phosphate (reduced form)
ODS	–	octadecylsilane
ODC	–	ornithine decarboxylase
^{1}H-NMR	–	proton nuclear magnetic resonance
RBP	–	retinol-binding protein
TPA	–	12-O-tetradecanoyl-phorbol-13-acetate

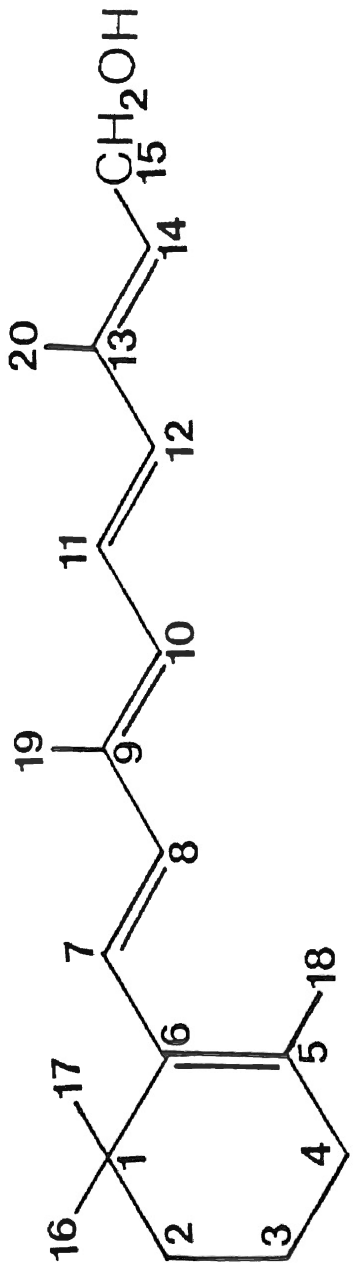
CHAPTER I

INTRODUCTION

In 1915 McCollum and Davis found that growth factors in food fell into two classes according to how soluble they were in fat and water (1). In 1916 McCollum and Kennedy (2) called these fat-soluble A and water-soluble B. Drummond in 1920 (3) renamed the fat-soluble A, "Vitamin A," and for many years it continued to be called vitamin A or vitamin A alcohol. This usage has not completely died out. In 1931, Karrer *et al.* (4) deduced the structure and in accordance with the principle that once the structure of a vitamin is known, a specific chemical name should be given to the molecule, it was designated retinol (5). The term vitamin A is now used generically to describe all derivatives having the same (β-ionone) ring and showing qualitatively the biological activity of retinol, excluding the provitamin carotenoids (6). Another term that has been in use is "retinoids" which refers to natural and synthetic analogs of retinol. The structure and numbering system of retinol to be used in describing the retinoids is shown in Figure 1 (7).

Even though the existence of the "fat-soluble A" has been known for decades (8, 9), little of its biological activity is understood. The only function of vitamin A reasonably well understood at the molecular level is as retinaldehyde in the chromophoric group of visual pigments, the photoreceptor molecules of the eyes (7). Aside from vision, vitamin A has been shown to be involved in several biological functions which include: reproduction (10), maintenance of epithelial tissues (11), and growth and differentiation (12). Cutaneous lesions due to vitamin A deficiency in humans have been reported since the first quarter of this century (13). Recently, the most active area of intensive research is

Figure 1. Structure of Retinol Showing the Numbering System


CH2OH
15
14
13
20
12
11
10
19
9
8
7
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6
5
17
1
4
16
2
3

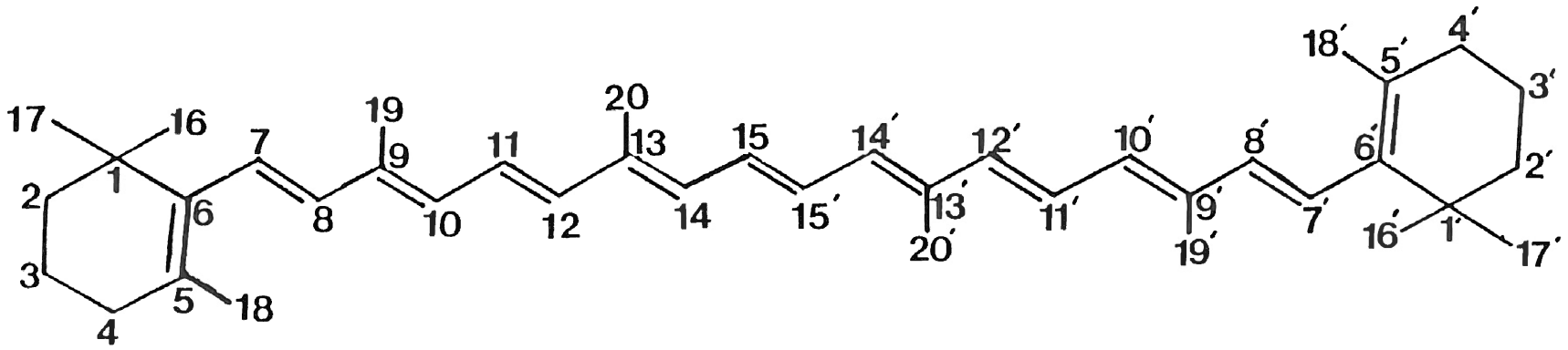
efforts towards the possible relationship between retinoids and cancer prevention. Earlier histopathological studies on retinol-deficient animals revealed in many tissues the replacement of the columnar and transitional epithelial cells that duplicate rapidly (14).

The support of many biological activities by vitamin A triggered the search for an "active form" of this fat-soluble factor. This search has proved futile and may be due to the fact that, with its many functions in the body, it may have several active forms. Retinoic acid derived from naturally occurring all-*trans*-retinol has been accepted by some as the "active form" of vitamin A. Chickens fed a retinol-deficient diet supplemented with all-*trans* -retinoic acid grew normally (15), but showed impairment of reproductive ability in the male as well as the female (16, 17) and impairment of vision (18).

Sources of Retinol and Retinoic Acid

The major natural sources of vitamin A are from animals, long-chain fatty acid retinyl esters and from plants, the carotenoid pigments that can serve as precursors of vitamin A (7). To manifest provitamin A activity, a carotenoid must have at least half of its molecule common to that of retinol. This makes β-carotene the most common and most effective provitamin A molecule (7). The structure of β-carotene, showing the numbering system, is shown in Figure 2. The carotenoids are synthesized exclusively by photosynthetic microorganisms and by members of the plant kingdom. Animals are incapable of *de novo* synthesis of vitamin A-active substances (19). Among members of the animal kingdom, therefore, herbivores derive their retinol by biosynthesis from ingested carotenoids, and omnivores and carnivores obtain their retinol by biosynthesis from ingested plant carotenoids and from ingestion of animal products that contain the preformed vitamin (19). For practical nutritional purposes, vitamin A occurs naturally only in animals. The major sources include the liver, fish liver oils, dairy products, kidneys and eggs. Carotenoid precursors of vitamin A are widely found in plants; in high amounts in carrots, green and yellow vegetables, and some fruits. β-Carotene is also

Figure 2. Structure of β-Carotene Showing the Numbering System


17
16
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14′
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20′
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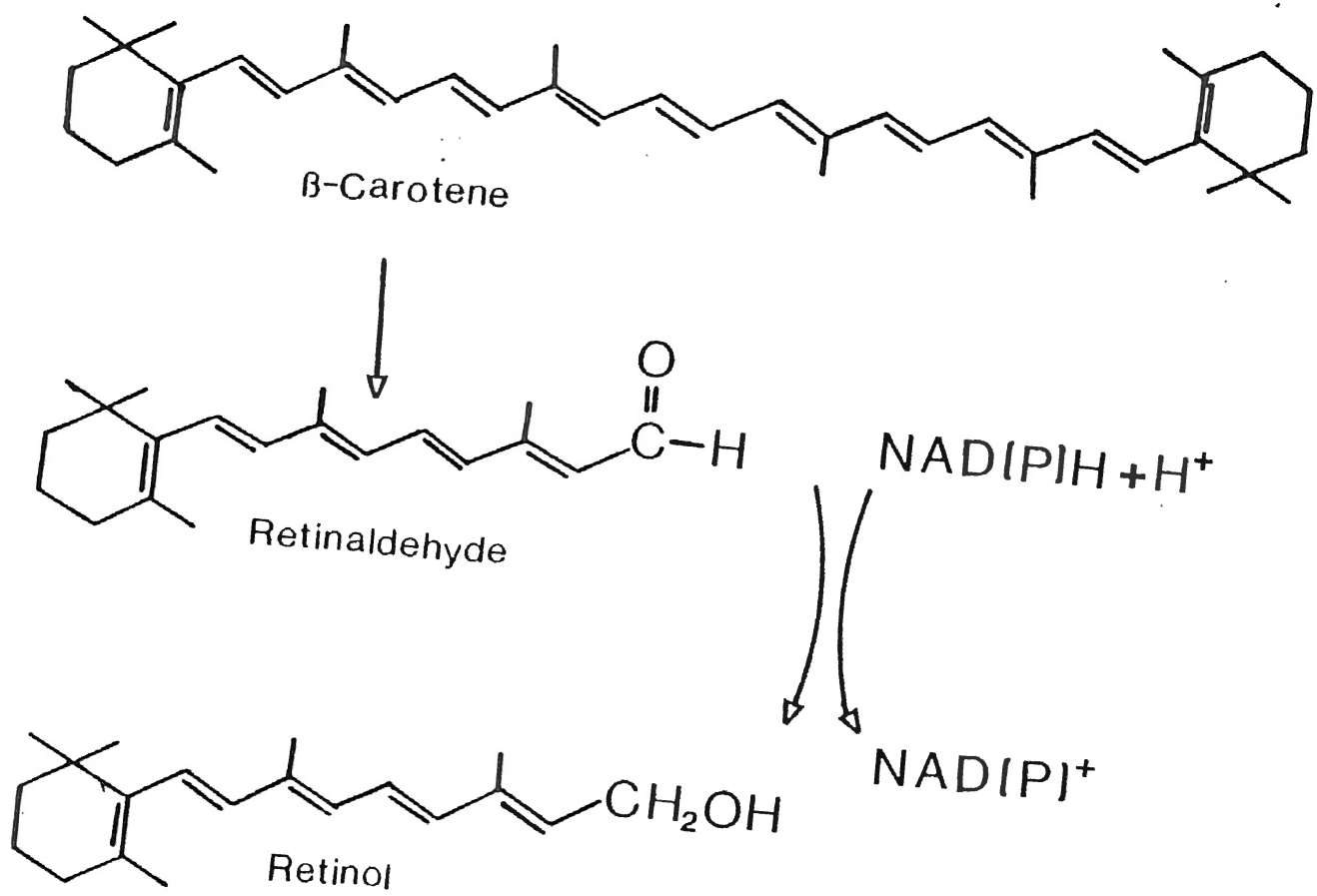
10′
9′
19′
8′
7′
6′
5′
18′
4′
3′
2′
16′
1′
17′

synthesized commercially (20) and added to foods and animal feeds (21). Many animals, including most humans in developed countries of the world, receive their vitamin A as retinol instead of the provitamin carotenoids.

β-Carotene, after absorption from the diet, can be broken down by 15,15'-dioxygenase in the intestinal wall (Figure 3). One molecule of oxygen reacts across the central double bond of the 15 and 15' carbon atoms to form an unstable 15-15'-peroxide that rapidly cleaves to give two molecules of retinaldehyde (22, 23). A little of this retinaldehyde formed in the intestinal wall from carotenoids is oxidized to retinoic acid while the majority is reduced to retinol by an enzyme which, in the rat intestine, can use either NADH or NADPH (24).

Retinol, when obtained from the diet, is usually in the esterified form. Normally, it is completely hydrolyzed in the small intestine by either a pancreatic hydrolase or by a hydrolytic enzyme, probably on the brush border of the intestinal mucosal cell (25). Whether obtained by hydrolysis of dietary retinyl esters or by reduction of retinaldehyde formed in the cleavage of carotenoids, retinol is re-esterified inside the mucosal cells with long-chain fatty acids, mainly palmitate and to a lesser extent, stearate and oleate (26). These retinyl esters are incorporated into lymph chylomicrons (26). Shortly after a meal, the newly absorbed retinyl esters can be found in the blood, mainly with the low-density lipoproteins (27, 28). When retinol is in excess of the body's immediate needs, it is mainly stored in the liver, esterified with long chain fatty acids, predominantly palmitate (29). These retinyl esters can be hydrolyzed (30), resynthesized (31), and undergo a steady turnover (32, 33). The liver reserve esters function to maintain a constant level of retinol in the blood to supply the vitamin to peripheral tissues. Goodman *et al.* (34) isolated and purified retinol-binding protein (RBP) and showed that retinol circulates in blood as a retinol-protein complex. RBP has a molecular weight of 21,000 and binds one molecule of retinol. Transthyretin (TTR), formerly known as prealbumin (PA), has a molecular weight of about 50,000, and forms 1:1 protein complex with plasma RBP.

Figure 3. Formation of All-*trans*-retinol from β-Carotene


ß-Carotene
O
C–H
Retinaldehyde
NAD(P)H + H+
NAD(P)+
CH2OH
Retinol

Retinol-binding protein serves as a vehicle to deliver retinol to the tissues where it can be oxidized reversibly to its aldehyde which in turn can be further oxidized irreversibly to retinoic acid (Figure 4) (35).

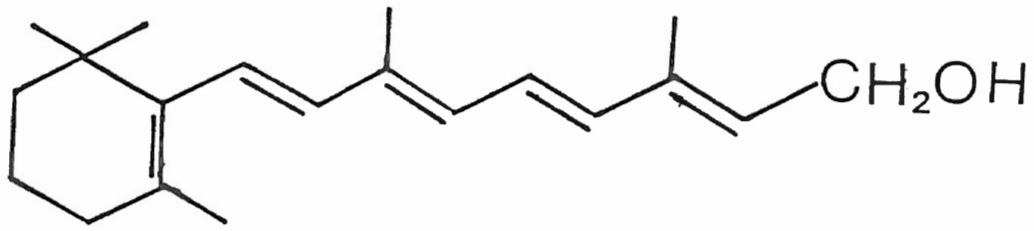
Biological Functions of Vitamin A

Early investigations concerning vitamin A functions placed emphasis upon describing physical pathological lesions due to vitamin A deficiency. Unfortunately, other than in vision, in which retinal (vitamin A aldehyde) interacts with visual pigments, there has been little success in correlating any of the physical signs of vitamin A deficiency with specific biochemical lesion. Wolf and De Luca (36) tried to explain this by proposing that fat-soluble vitamins function at a hormonal level to control protein synthesis rather than as a coenzyme.

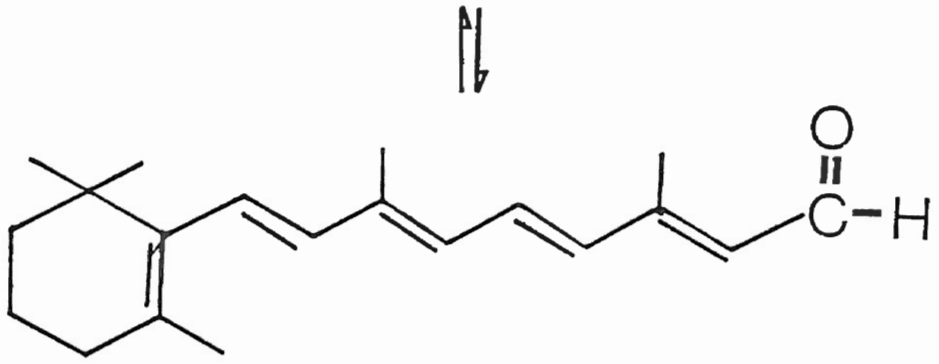
A problem in vitamin A studies has been to define what constitutes a vitamin A-deficient state in an intact laboratory animal. Nelson *et al.* (37) developed a method for quantitation of retinoic acid and retinol in blood plasma. Using pigs, they also showed that cerebrospinal fluid pressure was an adequate and sensitive criterion for assessing vitamin A status in an animal (38). 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 (38).

The part that vitamin A plays in vision, though of high importance, is not its principal activity. No animal dies of blindness. It is clear that vitamin A must play some very general role in cellular metabolism or cell structure, a role perhaps particularly associated with epithelial cells, since these undergo such marked changes early in vitamin A deficiency (18). Wasting away of glands and arrest of growth in many cases characterize retinol deficiency in animals (14). For instance, in the rat intestinal mucosa, the number of goblet cells decreases (39). In the testis, spermatogenesis is halted and

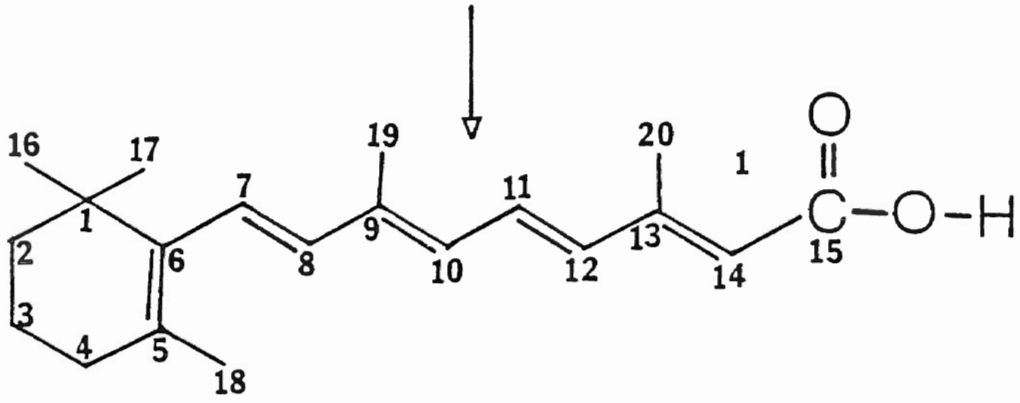
Figure 4. Oxidation of Retinol to Retinoic Acid



all-trans-retinol



all-trans-retinal



all-trans-retinoic acid

germinal epithelium disappears (14). In vitamin A-deficient female mice, morphologic studies showed large areas of adipose tissue, greatly reduced ductal and lobule-alveolar development, and decreased total secretory activity in the mammary glands (40). One biological role of vitamin A that has been studied at the cellular level involves the phosphorylated derivatives of retinol and retinoic acid. Researchers have proposed that phosphorylation of vitamin A and linkage of the phosphorylated derivative to mannose is essential for incorporation of mannose into manno-glycoproteins (41-45). Another vitamin A activity that has been studied involves retinoic acid and cAMP-dependent protein kinase activity (46). Ludwig *et al.* (46) studied the influence of all-*trans*-retinoic acid on cyclic AMP metabolism in B_{16}-F_1 mouse melanoma cells. They proposed that cAMP-protein kinase is involved in mediating at least some of the actions of retinoic acid in the B_{16}-F_1 murine melanoma cell line. Retinoic acid significantly increased the activity of cAMP-dependent protein kinase which is the major receptor responsible for the effects of this nucleotide. Retinoic acid does not inhibit growth or enhance protein kinase activity in a variant of B_{16}-F_1 cells (MR-4) which is resistant to the growth inhibiting effects of melanocyte-stimulating hormone and has deficient protein kinase activity (44).

Retinoids and Cancer

Presently, one of the most exciting area of intensive research involving retinoids is the possible relationship between retinoids and cancer. Many histopathological studies on vitamin A-deficient animals have revealed that in many tissues, columnal and transitional epithelium have been replaced by squamous, frequently keratinizing, epithelium that multiplies rapidly (14). This metaplasia is suggestive of neoplastic potentiality (14). The dramatic changes occurring in vitamin A-deficient animals could be fully reversed by a diet rich in retinol (47). The rationale for attempting to use retinoids in the prevention of cancer is due to the fact that the great majority of human cancers arise in epithelial target sites, such as lungs, bladder, breast, colon, pancreas, esophagus, stomach, uterus, and

prostate (48). Retinoids are known to be required for maintenance of normal epithelial cell differentiation at these sites. Now it is well established that retinoids have the following important properties with respect to chemoprevention of cancer (49). They suppress malignant transformation *in vitro*, whether it is caused by chemical carcinogens (50), ionization radiation (51), or transforming peptides from virally transformed cells (52). Some retinoids are potent inhibitors of the tumor-promoting effects of phorbol esters (53-59) which are among the most potent tumor-promoting substances known (58-59). Mainly, the search for a relationship between retinoids and cancer has been towards elucidating the mechanism of action of retinoids. Many have suggested that retinoids act by activating or inhibiting an intermediary enzyme. Particular advances have been made in the elucidation of the structure and activity of phorbol esters, such as 12-0-tetradecanoyl-phorbol-13-acetate (TPA). This ester causes an immense increase in the activity of ornithine decarboxylase (ODC) in mouse skin and is believed to be intimately related to tumor promoting activity of TPA (60). Recently, the important observation has been made that many retinoids are capable of inhibiting the increase in ODC activity caused by TPA in mouse skin (54). Michael *et al.* found that monolayer cultures of F9 tetracarcinoma stem cells and P19 stem cells differentiate into endoderm and fibroblast-like cells, respectively, when treated with retinoic acid (61). They demonstrated that this differentiation is associated with a large increase (greater than 40-fold) in the activity of an enzyme, prolyl-4-hydroxylase, involved in the post translational modification of collagens. This large increase in prolyl-4-hydroxylase activity occurs between 42 and 72 hours after retinoic acid addition (61).

Many side effects of natural retinoids greatly reduce their usefulness in prevention of cancer. Toxic effects may include the likelihood of liver injury due to excessive deposition of high doses of vitamin A and the inadequate tissue distribution of natural retinoids. Studies have shown that the therapeutic margin is very narrow in both animals and patients with skin diseases and tumors (49, 62, 63). Researchers have, therefore, aimed to

develop new retinoids with a pronounced dissociation between therapeutic effect and toxic side effects. Theoretically, an almost unlimited number of retinoids can be synthesized since it has already been shown that variations in the ring, side chain, or polar terminal group of retinoids can all lead to molecules with useful biological activity (64).

Synthetic retinoids were highly active in mouse prostate organ culture in inhibiting the effects of methylcholanthrene (65) and of N-methyl-N'-nitro-N-nitrosoguanidine (66). Bollag (63) demonstrated that an aromatic retinoic acid derivative with a modified polar end group exerted a prophylactic as well as a therapeutic effect on the tumors and was more effective than all-*trans*-retinoic acid. Two synthetic retinoids were successfully used to inhibit mammary cancer in the rat. Retinyl-methyl-ether inhibited the incidence and decreased the number of mammary tumors induced by 7,12-dimethylbenz(a)-anthracene (67, 68), while N-(4-hydroxyphenyl)-all-*trans*-retinamide inhibited the development of mammary cancer induced by N-nitroso-N-methylurea (69). Furthermore, many isomers of retinoic acid have been synthesized and tested. From these, 13-*cis*-retinoic acid was the most useful (70).

Retinoic Acid Metabolism

Retinoic acid is an irreversibly formed metabolite of retinol that supports growth and epithelial differentiation (71-73). It is at least equipotent with retinol in promoting growth and in maintaining differentiation of epithelial tissue in organ culture (74, 75, 65). However, retinoic acid is unlike retinol, a precursor of the retinal which is needed for the biosynthesis of visual pigments (12). Although it has been found to be a normal metabolite of both retinal and retinol (76-79), blindness results if it is the sole retinoid supplied to the animal (18).

Unprecedented search for metabolites of retinol and retinoic acid occurred in the recent past after suggestions were made that retinol and retinoic acid could be precursors of an unknown active metabolite. After oral administration to rats, retinoic acid was

rapidly excreted in the bile as retinoyl-β-glucuronide (80). Zachman *et al.* (81) injected retinoic acid-^{14}C into bile duct-cannulated rats. Later analysis showed that less than 10% of the radioactivity was recovered in the liver, intestine, and kidneys. Within 6 hours, 40% of the radioactivity had appeared in the bile. By 24 hours, practically no radioactivity remained in the liver, intestine and kidney. The rapid disappearance of retinoic acid from tissues followed by the appearance of retinoic acid metabolites in the bile after either a physiological or pharmacological dose suggests that conjugation in the liver may be a major route of metabolism.

The metabolism of retinoic acid has been studied in several tissues including the testes, liver, intestine, serum, kidney, and the trachea. The findings showed that retinoic acid metabolism *in vivo* is a complex process that occurs through multiple metabolites, which are at least partially tissue specific (82). As a result of experiments done with retinoic acid radioactively labelled in various positions, DeLuca and Roberts suggested that the metabolites in the urine of rats had a shortened side chain (83, 84). Hänni *et al.* (85), after the intraperitoneal administration of high doses of ^{14}C- and ^{3}H-labelled retinoic acid to rats isolated three major metabolites in the urine. The structures of these metabolites were elucidated by mass spectrometry and Fourier transform ^{1}H-NMR spectroscopy. In these metabolites, the tetraene side chain of retinoic acid was shortened and the cyclohexene ring oxidized (85). Chaudhary and Nelson (86) studied the metabolism of all-*trans*-[15-^{14}C] retinoic acid in vitamin A-adequate or vitamin A-deficient rats fed retinoic acid following intratesticular injection. Analysis of testicular metabolites showed that the retinoic acid was isomerized to 13-*cis*-retinoic acid and metabolized to polar metabolites in both groups. Three major metabolites as well as intact retinoic acid were isolated and identified from rat feces, following intraperitoneal administration of retinoic acid (87). Hydroxylation of the methyl group at C-5, oxidation of the ring at C-4, and *cis-trans* isomerization of the side chain produced these metabolites (87). It was proposed that metabolites with a shortened side chain are mainly eliminated by the kidney while

metabolites with an intact side chain and retinoic acid are eliminated in feces via the liver and bile (83, 84). Zachman *et al.* (81) reported extensive enterohepatic circulation of biliary metabolites of retinoic acid-^{14}C.

Recently, several biologically active metabolites of retinoic acid have been identified and characterized. McCormick *et al.* (88) isolated and identified 5,6-epoxyretinoic acid in pure form from intestinal mucosa of vitamin A-deficient rats given [^{3}H]retinoic acid. This metabolite has proven to be highly biologically active. If given intraperitoneally, synthetic 5,6-epoxide has 100-157% of the activity of retinyl acetate in supporting growth in vitamin A-deficient rats (89). The biological activity of retinoyl-β-glucuronide has been assessed both in growth assay (90) and in the rat vaginal smear assay (91, 92). The growth assay showed retinoyl-β-glucuronide to possess 30-40% of the biological activity of all-*trans*-retinoic acid. However, in the rat vaginal smear assay, its biological activity was determined to be higher than that of retinoic acid. Retinoic acid also may be metabolized to a 4-hydroxyretinoic acid and 4-oxoretinoic acid in a hamster tracheal organ culture system (93, 94). These compounds displayed one-tenth the biological activity of all-*trans*-retinoic acid when tested in a vitamin A deficient hamster tracheal organ culture assay (93, 94).

Retinoic acid metabolism will not be complete without mentioning the decarboxylated metabolites. The decarboxylation of retinoic acid has been under study since the discovery of a decarboxylated product that was shown to be biologically active (95). Several investigators have studied the breakdown of ^{14}C-retinoic acid labelled at different positions on the side chain by analysis of the radioactivity in urine, carbon dioxide and feces following intravenous injections of substrate into retinol-deficient rats (83, 96, 97). With the 15-^{14}C and 14-^{14}C labelled retinoic acid, a significant amount of radioactivity evolved as $^{14}CO_2$ (10-20%). Roberts and DeLuca (98, 99) demonstrated decarboxylation of retinoic acid with tissue slices of rat liver and kidney. In the presence of microsomes, NADPH, and Fe^{2+} and further stimulated by phosphate, they achieved

50% decarboxylation (99). Lin (97) and Nelson *et al.* (100) observed a requirement for O_2 and Fe^{2+} for a partially purified preparation of microsomal enzyme to decarboxylate retinoic acid. McKenzie and Nelson (101) investigated decarboxylation of retinoic acid by horseradish peroxidase. They demonstrated that the requirements for phosphate, oxygen and ferrous ion could be eliminated when hydrogen peroxide was present in the incubation medium. DeLuca and Roberts suggested that the *in vivo* metabolic degradation of the retinoic acid side-chain starts with oxidation at C(14) and loss of the terminal C-atom (C(15)) by decarboxylation. This decarboxylation would lead to an acid with 19 C-atoms (83, 84).

The isolation and characterization of retinoic acid metabolites produced *in vivo* has been poorly accomplished. This has been because of the labile nature of the retinoids, poor separation techniques and impurities in radiochemical precursors. The development of high-performance liquid chromatographic systems and mild extraction procedures have allowed the isolation and identification of retinoic acid metabolites without the production of artifacts (102, 103). Skare *et al.* (104), using Sephadex LH-20 chromatography and several HPLC procedures, recently isolated and purified to homogeneity a major decarboxylated metabolite of all-*trans*-retinoic acid from normal rat given either pharmacological or physiological doses. This polar metabolite was positively identified as retinotaurine.

The purpose of this study, therefore, was to investigate the biliary metabolites of all-*trans*-retinoic acid using HPLC as the chromatographic method and to isolate, purify the decarboxylated metabolite and identify it using mass spectrometry.

For structure and numbering system of retinoic acid used throughout this paper, refer to Figure 4.

CHAPTER II

MATERIALS AND METHODS

Chemicals

All organic solvents except ethanol used for HPLC and sample preparation were of HPLC grade, low residue, distilled in glass (Burdick & Jackson, Inc., Muskegon, Mich.). Aldehyde-free ethanol, used to dissolve retinoic acid for injection into chicks, was prepared by distilling approximately 1500 ml of 95% ethanol vigorously from 4-5 g $KMnO_4$ and 15-20 g KOH in the dark. The first 250-300 ml and the last 350 ml were discarded. All water was deionized, doubly distilled in glass and filtered through 0.5 μm filter (Millipore type HA, Bedford, Mass.).

Non-radioactive all-*trans*-retinoic acid and all-*trans*-[15-^{14}C]retinoic acid of specific activity 30.6 μCi/mg 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 prepared by New England Nuclear (Boston, Mass.) and all-*trans*-[10,11-$^{3}H_2$]retinoic acid, 11.25 mCi/mg, was prepared by SRI International (Menlo Park, Calif.).

High Performance Liquid Chromatograph

Purification of retinoids and biliary metabolites was performed on three separate high-performance liquid chromatography (HPLC) system. HPLC System A was used to determine the purity of retinoids. It consisted of a Waters Associates Model 680 automated gradient controller, Model 440 dual wavelength absorbance detector and two Model 6000A chromatography pumps. This system was equipped with an Omniscribe Recorder (Houston Instruments, Austin, Tx.) and connected to a HP 1040A HPLC

photodiode array detector and a data processing system (Hewlett-Packard, Palo Alto, Calif.). The detector allowed eight wavelength signals to be monitored simultaneously and absorbance spectra are obtained on the upslope, apex, downslope and baseline of each chromatographic peak without stopping the eluent flow. The column employed was a reverse-phase analytical column, Partisphere C_8 .79 x 14.6 cm (.47 ID)(Whatman Inc., Clifton, N.J.).

HPLC System B was used to purify radioactive retinoic acid and consisted of a single Waters Model 6000A pump connected in series to a Whatman (Clifton, N.J.) 0.46 x 25 cm Partisil PXS 10/25 ODS-3 C-18 column. Detection was at 254 nm with a Waters Model 440 dual wavelength absorbance detector connected to a Fisher Recordal Series 5000 recorder (Fisher Scientific Company, Pittsburgh, Penn.). Samples were eluted at 1.5 ml/min with 75% methanol, 25% 0.01 M acetic acid.

System C consisted of Waters Model 660 solvent programmer, a Model 440 dual wavelength absorbance detector, two Model M 6000A chromatography pumps, and a Fisher Recordal Series 5000 recorder. This system was used to separate biliary metabolites using reverse phase analytical Partisphere C_{18} .79 x 14.6 cm (.47 ID) column. Materials were eluted at 1 ml/min with 25% methanol, 75% 0.01 M acetic acid for 40 minutes followed by a 1 hour 20 minutes linear gradient to 100% methanol. All systems were equipped with Valco (Houston, Tx.) model CV-6-UHPa-N60 six port injectors. Each column was guarded by an Uptight precolumn (Upchurch Scientific, Inc., Oak Harbor, Wash.). All columns used were analytical reverse phase. For collection of fractions, an ISCO Model 328 fraction collector (Instrumentation Specialities Company, Lincoln, Nebr.) was used.

Samples were dried or volume reduced by using a Büchi Rotavapor-R (Brinkman Instruments, Westbury, N.Y.) or Multi-Block Heater Model H2025-1A (American Scientific Products, McGraw Park, Ill.) at 40°C.

Liquid Scintillation

Radioactivity was determined by using liquid scintillation counting with either Prias PL or DLD Tri-carb liquid scintillation counters (Packard Instrument Co., Downers Grove, Ill.). Packard Insta-Gel was the scintillation cocktail employed. DPM was calculated from CPM using a quench correction curve and a program developed on a MacIntosh Computer by Jerry Merz (105).

Animals

Four- to ten-week old chickens were obtained form the Oklahoma State University Agricultural Experiment Station, Department of Animal Science Poultry Farm. Chicks usually weighed between two and five pounds. Chicks were not placed on any special diet. Initially they were taken off feed and water the night before they were used for experiments, but, later it was found that there was no notable difference whether they ate and drank before the experiments or not.

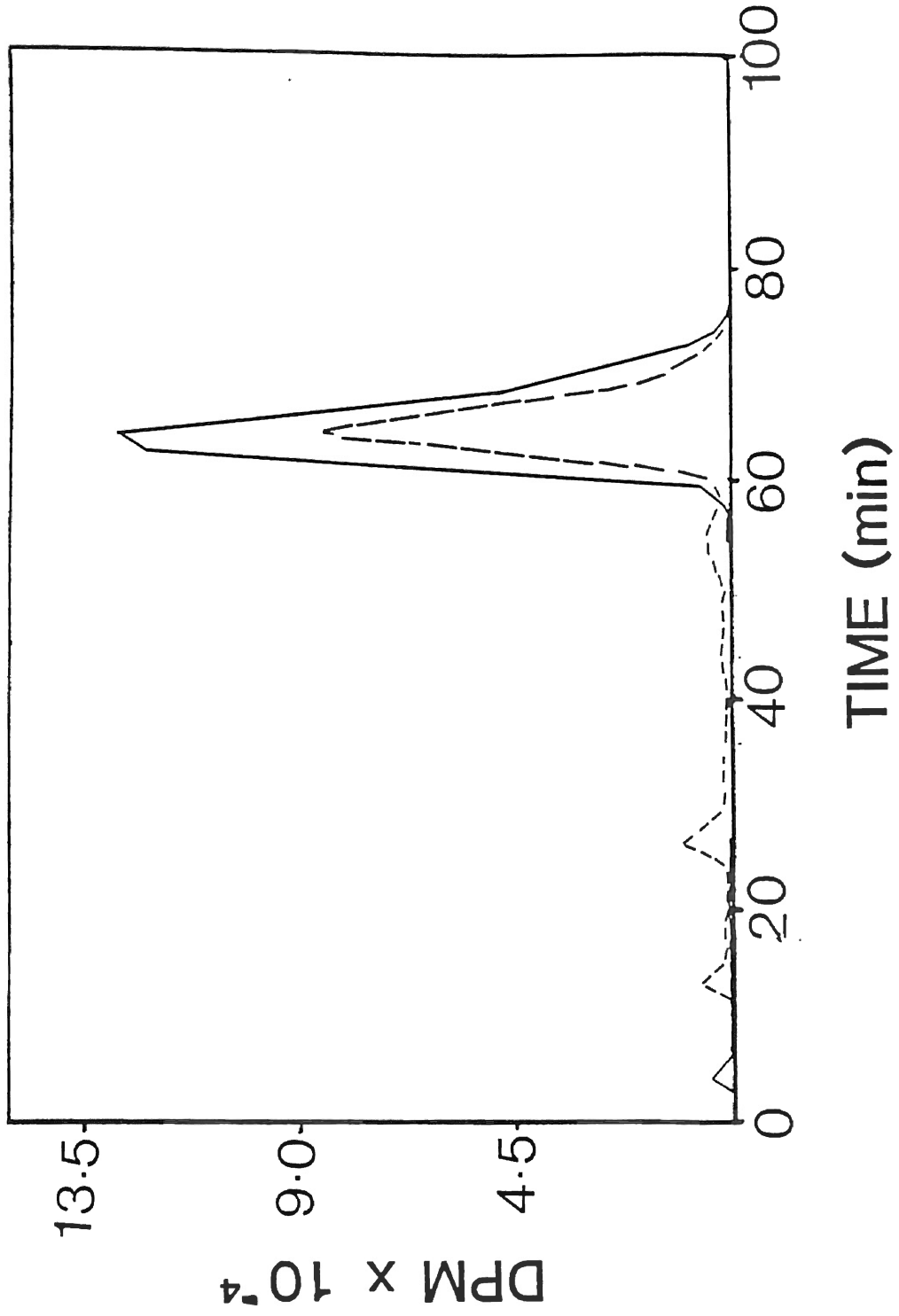
Methods

Purification of Retinoic Acid and Preparation of Dose

To make sure that labelled all-*trans* retinoic acid used in this project did not contain contaminants that could be identified as metabolites, the labelled retinoic acid was purified two times on HPLC System B. In metabolism experiments, it is imperative that the investigator be sure that the precursors used are pure. This ensures that the metabolites identified were not introduced into the system as contaminants in the first place. 5,6-Epoxyretinoic acid was the first target tissue metabolite of retinoic acid to be isolated and identified (88, 82). McKenzie *et al.* (106) identified this compound as the major contaminant of stored radiolabelled all-*trans*-retinoic acid.

Figure 5 shows the purity of the radio-labelled all-*trans* retinoic acid before and after

Figure 5. HPLC Profile of All-*trans*-retinoic Acid Before (---) and After (—) Purification. Sample was purified on HPLC System B. Materials were eluted with 75% methanol, 25% 0.01 M acetic acid at a flow rate of 1 ml/min on Partisil PXS 10/25 ODS-3 column.


DPM x 10⁻⁴
13.5
9.0
4.5
0
20
40
60
80
100
TIME (min)

two purifications. The dashed lines represent the elution profile of retinoic acid prior to purification while the solid line shows profile after two purifications. All-*trans*-[10-^{3}H]retinoic acid and all-*trans*[15-^{14}C]retinoic acid were only 80% pure prior to the purification step. However, following HPLC purification, substrates were routinely 98% pure. The 13-*cis*-retinoic acid eluting between 55 and 58 minutes and the oxidation products eluting at 15 and 27 minutes accounted for about 20% of the total radioactivity. After the second purification they were totally absent. The peak eluting between 5 and 7 minutes corresponded to the void volume of the system and might be an artifact of the injection. For an additional check of purity, after the retinoids were injected into the chicken, the syringe was rinsed and eluted on HPLC System B. Unlabelled all-*trans*-retinoic acid was not purified before use in the experiment since analysis on the photo diode array detector and data processing system showed it to be pure.

The dose was prepared by dissolving dried, twice purified labelled retinoic acid and unlabelled all-*trans*-retinoic acid in aldehyde-free ethanol. To this, an equal volume of saline solution was added. The labelled retinoic acid was not diluted with unlabelled retinoic acid prior to injection. All-*trans*[15-^{14}C] had a specific activity of 30.6 μCi/mg and all-*trans*-[10-^{3}H]- and [10,11-$^{3}H_2$]retinoic acid had specific activity of 2.8 Ci/mmol and 11.25 mCi/mg, respectively. The activity injected into a chicken depended on the size of the chicken and it ranged from 5 μCi of each radionuclide for small chickens to 10 μCi for each of the bigger ones.

Bile Collection and Sample Preparation

Chicks were injected intraperitoneally with labelled retinoids. Following the injection of retinoids, the chicks were placed in individual cages and were allowed water *ad libitum*. Six hours after injection, they were sacrificed by ether anaesthesia and then cervical dislocation. Bile was removed with a syringe. It was then immediately diluted with methanol (5 ml), eluted through a Waters (Milford, Mass.) C-18 Sep-Pak to seclude

large denatured protein aggregates. This made it possible to filter it through a 0.5 μm filter (Millipore type FH, Bedford, Mass.). The bile was then evaporated to dryness under a gentle stream of nitrogen at 30°C. The dried bile was then redissolved in 50% methanol, 50% 0.01 M acetic acid.

Biliary metabolites were eluted on HPLC System C at 1 ml/min with 25% methanol; 75% 0.01 M acetic acid. At 40 minutes, a 1 hour 20 minute linear gradient to 100% methanol was started.

Retinoids throughout the experiment were handled under yellow lights to prevent isomerization. The retinoids were exposed to air for a minimum time needed to prepare the samples. Morgan and Thompson have documented that retinoids are sensitive to light and to air oxidation (107). They reported that epoxides may be produced by atmospheric oxidation of retinol, retinyl esters and retinoic acid derivatives during chemical manipulation and storage of tissue extracts. Retinoids were kept in solution under N_2 and stored in a freezer until needed.

CHAPTER III

RESULTS

Controls

Investigations of vitamin A metabolism have been hampered by the labile nature of vitamin A compounds and poor separation techniques. Dunagin *et al.* (80), using ion-exchange and silicic acid chromatography, identified retinoyl β-glucuronide as a major metabolite in rat bile. Zile *et al.* (108) used HPLC to obtain a better resolution of retinoic acid metabolites and characterized retinoyl β-glucuronide as rather a minor metabolite of retinoic acid in rat bile. The development of reverse phase HPLC has provided the advantages of simple, rapid, mild and reliable separation procedure for natural and synthetic retinoids (103).

In preparation of the samples for HPLC analysis, the bile had to be diluted with methanol, eluted through a C-18 Sep-Pak, filtered, dried and redissolved. All these measures were imperative since injecting the bile into the HPLC unit without these mild precautions would clog the system. To make sure that the metabolites isolated were not produced as a result of the handling procedures, the following experiment was carried out. A chick from the same source as those used in other experiments was injected with 1.5 ml of aldehyde-free ethanol and saline containing no retinoic acid. Six hours later, the chick was sacrificed and bile was removed and diluted with 5 ml of methanol. This denatured the proteins in the bile. Then, all-*trans*[10,11-$^{3}H_2$]retinoic acid [33,000 cpm] was added to the bile. The bile was then eluted through a Sep-Pak, filtered and dried under N_2 at 30°C. The bile extract was redissolved and injected into HPLC System C immediately after handling procedures and eluted under similar conditions as the other biliary

metabolites. To further prove that the retinoic acid was pure before it was added to the bile, the syringe used was rinsed and eluted on HPLC System B. The sample was eluted with 75% methanol, 25% 0.01 M acetic acid at a flow rate of 1.5 ml/min. Figure 6 shows the profile of the syringe rinse. Materials eluted at 42 minutes and since it eluted as a single peak, it can be concluded that retinoic acid used in the experiment was pure. The elution profile of the bile-treated retinoic acid is shown in Figure 7. The retinoic acid added to the bile eluted as a single peak between 110 and 113 minutes. To show that the material that eluted between 110 and 115 was all-*trans*-retinoic acid, unlabelled all-*trans*-retinoic acid was injected into HPLC System 3 and eluted under similar conditions. All-*trans*-retinoic acid eluted between 108 and 110 (Figure 8). The two minutes difference was shown to be due to the lag time between the appearance of the absorbance on the detector and the fraction collector. The results shown here undoubtedly proves that the HPLC and handling procedures have no effect on the retinoic acid.

HPLC Columns

Ahern (109) in unpublished data elucidated the HPLC profile of radiolabelled all-*trans*-retinoic acid in chick bile. The profile showed a spectrum of metabolites ranging from polar, water soluble metabolites to unchanged all-*trans*-retinoic acid. To confirm his findings, the HPLC column needed was one that could at least separate the biliary metabolites that could be compared to his findings. To choose a suitable column, a chick was injected intraperitoneally with 6 μCi of all-*trans*-[10,11-$^{3}H_{2}$]retinoic acid and bile was prepared for analysis on a number of different columns. Bile was injected into the HPLC unit immediately after collection and handling procedures.

The HPLC profile of the metabolites on the columns are shown in Figures 9 and 10. Analysis was performed on HPLC System C replacing columns as needed. Figure 9 shows comparison between Partisphere C-18 .79 x 14.6 cm column (Whatman, Clifton, N.J.) and MCB, 0.46 x 25 cm C-8 column (Cincinnati, Oh.). With the Partisphere C-18

Figure 6. Elution Profile of Syringe Rinse of the All-*trans*-[10,11-$^3H_2$]Retinoic Acid Used in the Control Experiment. Sample was eluted on HPLC System B with 75% methanol, 25% 0.01 M acetic acid at 1.5 ml/min. Partisil PXS 10/25 ODS-3 column was used.

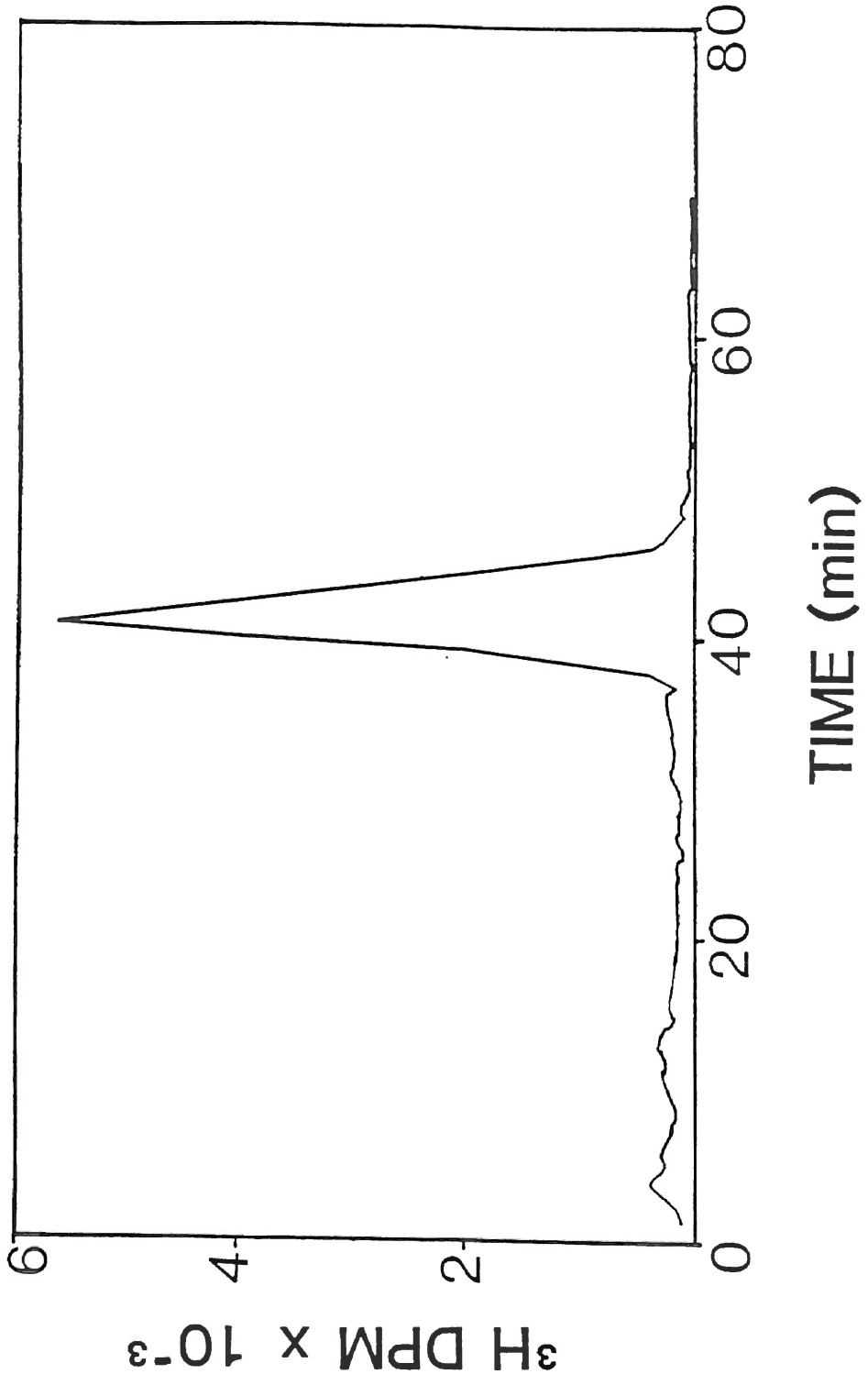

3H DPM x 10-3
0
2
4
6
0
20
40
60
80
TIME (min)

Figure 7. HPLC Profile of All-*trans*-[10,11-$^{3}H_2$]Retinoic Acid on HPLC System C with Partisphere C-18 .79 x 14.6 cm Column After Treatment with Bile. Materials were eluted with 25% methanol, 75% 0.01 M acetic acid for 40 min followed by 1 hr 20 min linear gradient to 100% methanol at 1 ml/min.

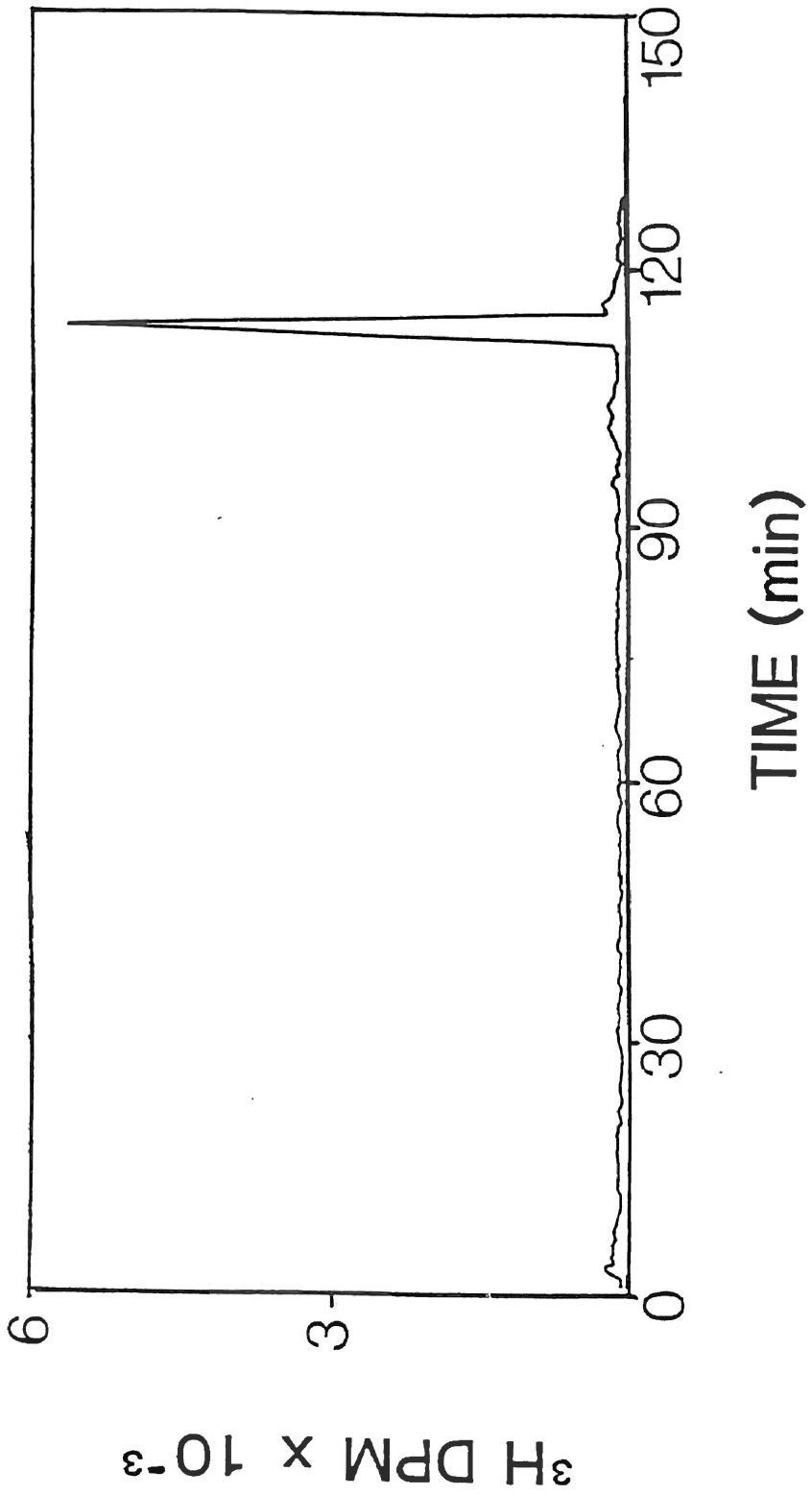

3H DPM x 10-3
6
3
0
30
60
90
120
150
TIME (min)

Figure 8. Elution Time of All-*trans*-Retinoic Acid on HPLC System C with Partisphere C-18 .79 x 14.6 cm Column. Sample was eluted with 25% methanol, 75% 0.01 M acetic acid for 40 min, then a 1 hr 20 min linear gradient to 100% methanol at 1 ml/min.

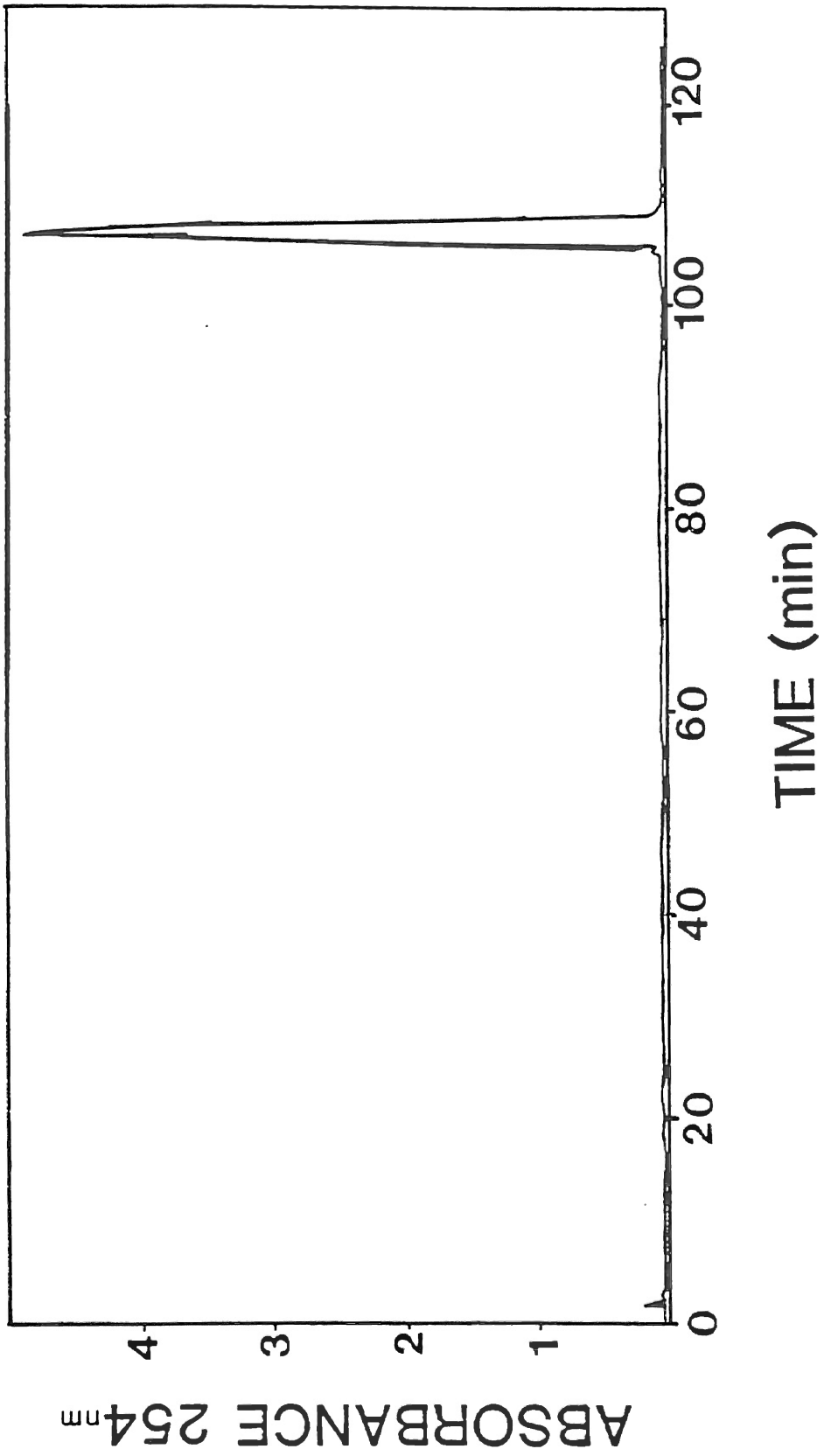

ABSORBANCE 254nm
4
3
2
1
0
20
40
60
80
100
120
TIME (min)

Figure 9. Comparison of HPLC Profile of Bile Metabolites on HPLC System C with Partisphere C-18 .79 x 14.6 cm Column (Top) and MCB C-8 .46 x 25 cm Column (Bottom). Materials were eluted with 30% methanol, 70% 0.01 M acetic acid for 40 min. followed by 1 hr 20 min linear gradient to 100% methanol at 1 ml/min using the Partisphere C-18 column. With the MCB C-8 column, solvent was 35% methanol, 65% .01 M acetic acid for 40 min followed by 1 hr 20 min linear gradient to 100% methanol at 1 ml/min.

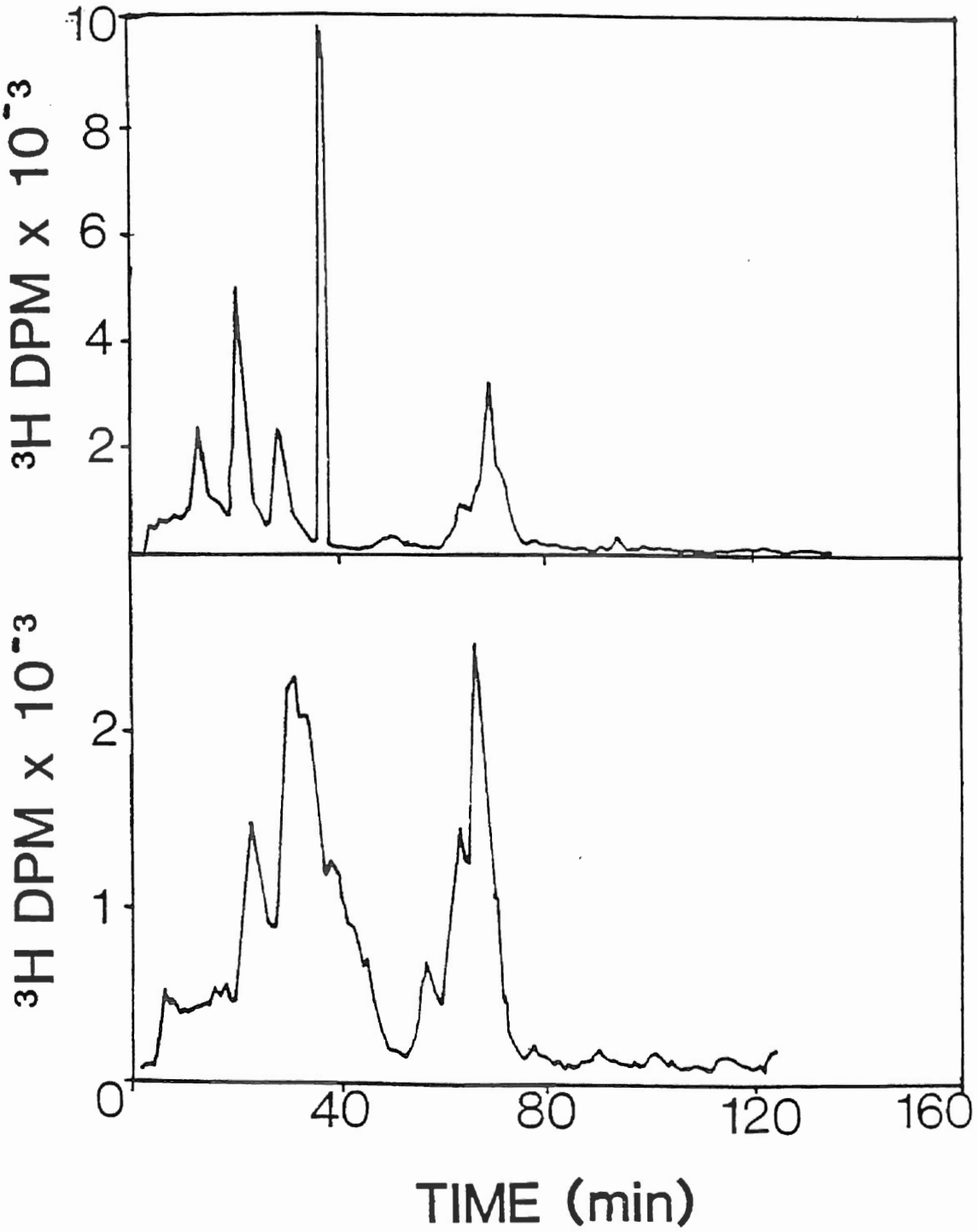
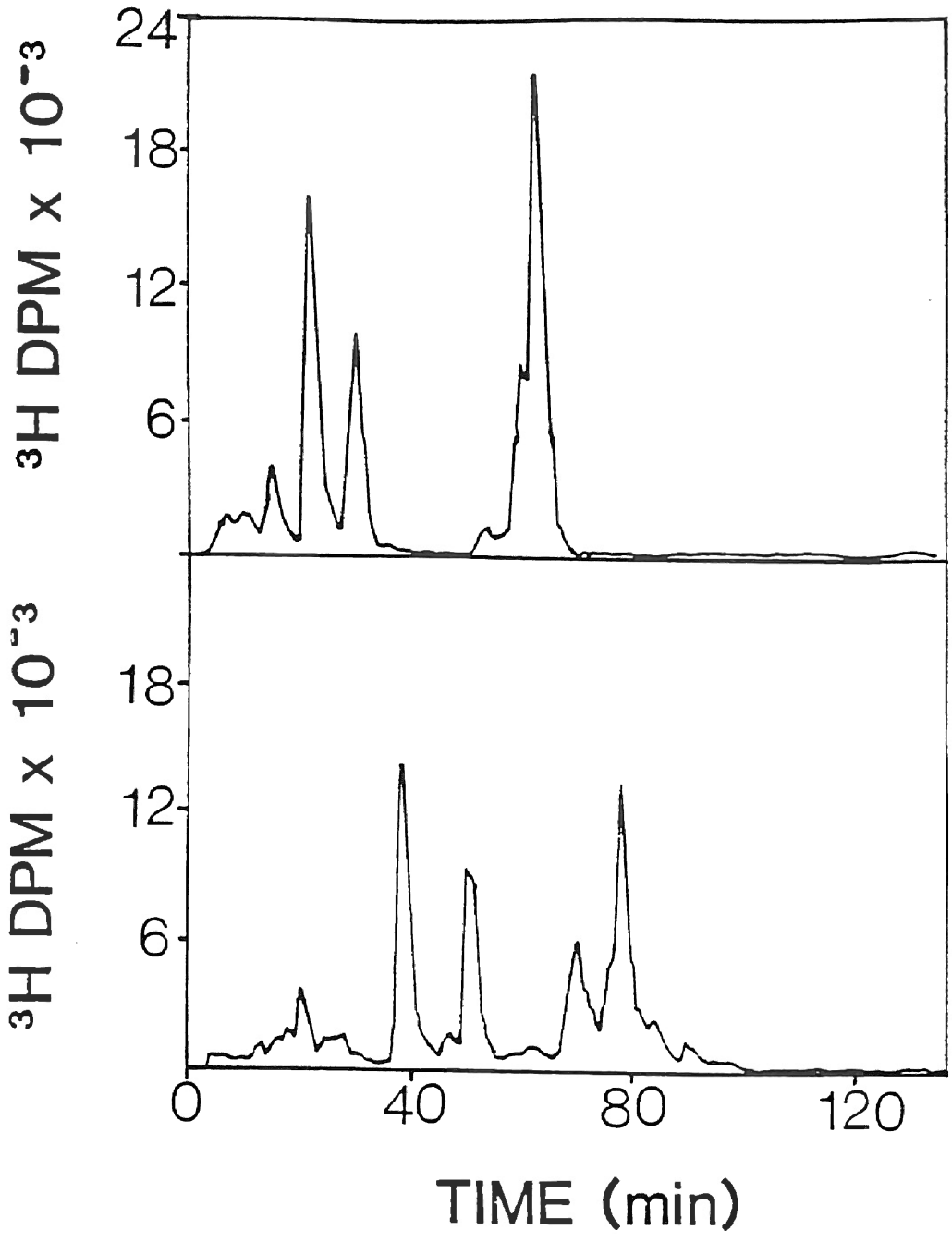

^{3}H DPM x 10^{-3}
10
8
6
4
2
^{3}H DPM x 10^{-3}
2
1
0
40
80
120
160
TIME (min)

Figure 10. Comparison of HPLC Profile of Bile Metabolites on HPLC System C with Partisphere C-8 .79 x 14.6 cm Column (Top) and C-18 .79 x 14.6 cm Column (Bottom). Materials were eluted with 30% methanol, 70% 0.01 M acetic acid for 40 min followed by 1 hr 20 min linear gradient to 100% methanol at 1 ml/min.


24
18
12
6
^{3}H DPM x 10^{-3}
18
12
6
^{3}H DPM x 10^{-3}
0
40
80
120
TIME (min)

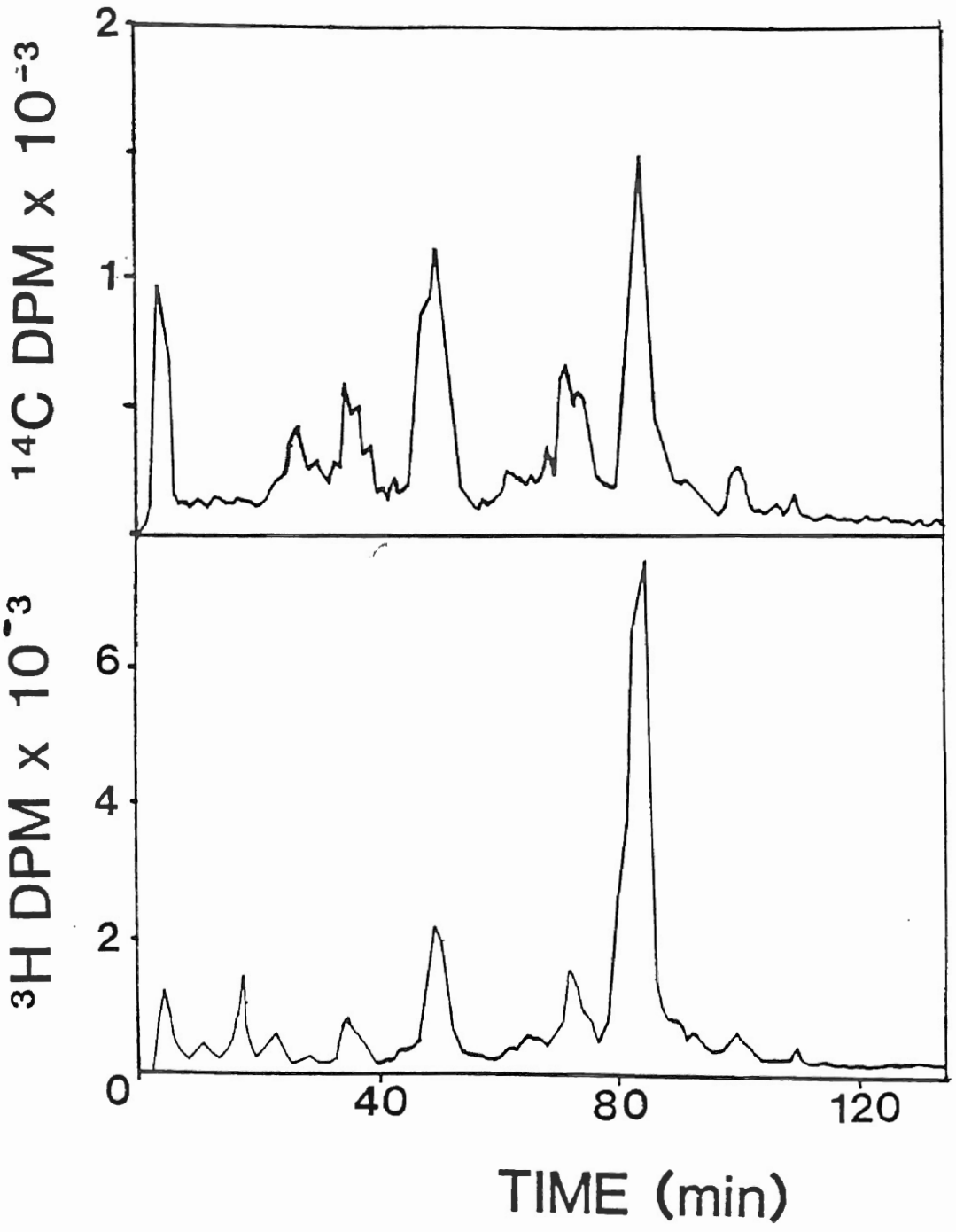
column profile (Figure 9, top), materials were eluted with 30% methanol, 70% 0.01 M acetic acid at 1 ml/min for 40 minutes. Then a 1 hour 20 minutes linear gradient to 100% methanol was started. Sample was eluted on the MCB C-8 column with 35% methanol, 65% 0.01 M acetic acid at 1 ml/min for 40 minutes followed by 1 hour 20 minutes linear gradient to 100% methanol. Ahern (109), using the same MCB column and similar conditions, was able to separate six well resolved peaks. The radioactive profile here (Figure 9, bottom) showed rather poorly resolved peaks. The peak that eluted between 20 and 50 minutes seemed to consist of several unresolved peaks. The Partisphere C-18 column resolved these peaks into four peaks as shown in the top of the figure.

A comparison was also made between Partisphere C-18 and C-8 columns. Here materials in both cases were eluted under the same conditions with 30% methanol, 70% .01 M acetic acid for 40 minutes followed by 1 hour 20 minutes linear gradient to 100% methanol. Figures 10 shows the elution profile of both columns. Even though the C-8 column (Top) gave a resolution comparable to that of the C-18 (Bottom), the C-18 was chosen over the C-8 column. The C-18 column resolved the peak that eluted between 70 minutes and 80 minutes while the C-8 column could not. The results shown here clearly show that the Partisphere C-18 column was most suitable for separation of bile retinoids.

Single Label Experiments

HPLC profiles of biliary metabolites from chicks injected with either all-*trans*-[10,11-$^{3}H_2$]retinoic acid or all-*trans*-[15-^{14}C]retinoic acid are shown in Figure 11. Chicks injected with all-*trans*-[10,11-$^{3}H_2$]retinoic acid received 9 μCi while those injected with all-*trans*-[15-^{14}C]retinoic acid received 6 μCi. Bile was injected into HPLC System C immediately after the handling procedures. The amount of bile collected and percentage of radioactivity recovered varied. For the chicken dosed with ^{3}H-labelled retinoic acid, the bile collected was 1.2 ml and activity recovered was 6.4% (9.8 μg) of the activity injected. The ^{14}C-labelled chicken gave 1 ml of bile and 3% (5.5 μg) activity recovery. Typical

Figure 11. Comparison of HPLC Elution Profiles of Bile from Chicks Injected with Either All-*trans*-[15-^{14}C]Retinoic Acid (Top) or All-*trans*-[10,11-$^{3}H_2$]Retinoic Acid (Bottom). Materials were eluted on HPLC System C with Partisphere C-18 .79 x 14.6 cm column with 25% methanol, 75% 0.01 M acetic acid for 40 min, followed by 1 hr 20 min linear gradient to 100% methanol at a flow rate of 1 ml/min.


2
1
^{14}C DPM x 10^{-3}
6
4
2
0
^{3}H DPM x 10^{-3}
40
80
120
TIME (min)

recovery for both radiolabelled retinoic acid ranged form 0.5% to 7%.

The elution profile showed a spectrum of metabolites ranging from polar water soluble metabolites eluting in the first 50 minutes to the unchanged retinoic acid eluting between 108 and 112 minutes. The most abundant metabolite in the profile corresponded to the peak eluting between 80 and 90 minutes. Ahern (109), using β-glucuronidase, identified this peak as a glucuronide conjugate of retinoic acid. The HPLC profile of both radio-labelled retinoic acids showed that all the metabolites had an intact side chain except the peak that eluted between 17 and 23 minutes. Every peak that occurred in the ^{3}H-labelled retinoic acid had a corresponding peak with the ^{14}C-labelled retinoic acid except the peak that eluted between 17 and 23 minutes. This observation tends to confirm Ahern's finding (109) that the metabolite that eluted between 17-23 minutes was decarboxylated. The peak eluting at 100 minutes and 110 minutes corresponded to the elution time of 13-*cis*- and all-*trans*-retinoic acid, respectively, under similar conditions.

The results obtained in these experiments were inconclusive since separate chicks were used in the experiments. The experiment was repeated eight times and while the HPLC profile of the metabolites followed the same pattern, the amount of each metabolite produced by each chicken varied. Since the chicken used in these experiments originally were reared for disease control experiments, the question arose as to whether the level of metabolite produced depended on stress, physiological and nutritional status of the animal. One could argue that the absence of ^{14}C peak that eluted between 17 and 23 minutes was due to the fact that even though the chick produced it, it was too small to be detected due to biological variations in the chicks. To answer this question, an experiment was devised such that a single chicken was injected with both all-*trans*-[15-^{14}C]retinoic acid and all-*trans*-[10,11-$^{3}H_2$]retinoic acid.

Double Label Experiments

One chick was injected with 6 μCi each of all-*trans*-[10,11-$^{3}H_2$]retinoic acid and all-*trans*-[15-^{14}C]retinoic acid. For additional check of purity after the retinoids were injected into the chicken, the syringe was rinsed and eluted on HPLC System B. The sample was eluted with 80% methanol and 20% 0.01 m acetic acid at a flow rate of 1 ml/min. Materials eluted as a single peak (Figure 12) between 20 and 30 minutes showing that retinoids injected into the chick were pure.

Bile extract was eluted with 25% methanol, 75% 0.01 M acetic acid for 40 minutes. Then a 60 minute linear gradient to 100% was started. Materials were eluted on HPLC System C immediately after the bile was collected and prepared for HPLC analysis. HPLC profile of the metabolites is shown in Figure 13. The profile showed a similar pattern as in the single-labelled experiments. Each all-*trans*-[10,11-$^{3}H_2$]retinoic acid peak had a corresponding all-*trans*-[15-^{14}C]retinoic acid peak except the peak eluting between 17 and 23 minutes which will be referred to as peak number 2 henceforth. The data shown here undoubtedly confirms that this peak lacked the C # 15; here the major peak eluted between 72 and 77 minutes. Under these conditions, 13-*cis*-retinoic acid eluted between 88 and 93 minutes, while all-*trans*-retinoic acid eluted between 95 and 101 minutes.

Table I shows the relative abundance of bile metabolites derived from all-*trans*-[10,11-$^{3}H_2$]retinoic acid. The numbering of the peaks is as shown in Figure 13. This represents the average of eight experiments. The bile collected ranged from 0.5 ml to 2.0 ml and the activity recovered ranged from 30.0% (1.80 μCi) to 5.6% (.335 μCi) for all-*trans*-[10,11-$^{3}H_2$]retinoic acid and 12.0% (.730 μCi) to 2.00% (.116 μCi) for all-*trans*-[15-^{14}C]retinoic acid. The radioactivity corresponding to peak number 2 represented 1.69% of total biliary all-*trans*-[10,11-$^{3}H_2$]retinoic acid activity. The major peak (#7a) represented 3.92% while 13-*cis*-retinoic acid and all-*trans*-retinoic acid represented 0.67 and 0.60%, respectively.

Figure 12. Purity Check on All-*trans*-[10,11-$^{3}H_2$]- (—) and All-*trans*-[15-^{14}C]Retinoic Acid (---) Injected into the Same Chicken. Materials were eluted on HPLC System B with 80% methanol, 20% 0.01 M acetic acid with Partisil PXS 10/25 ODS-3 column at 1 ml/min.

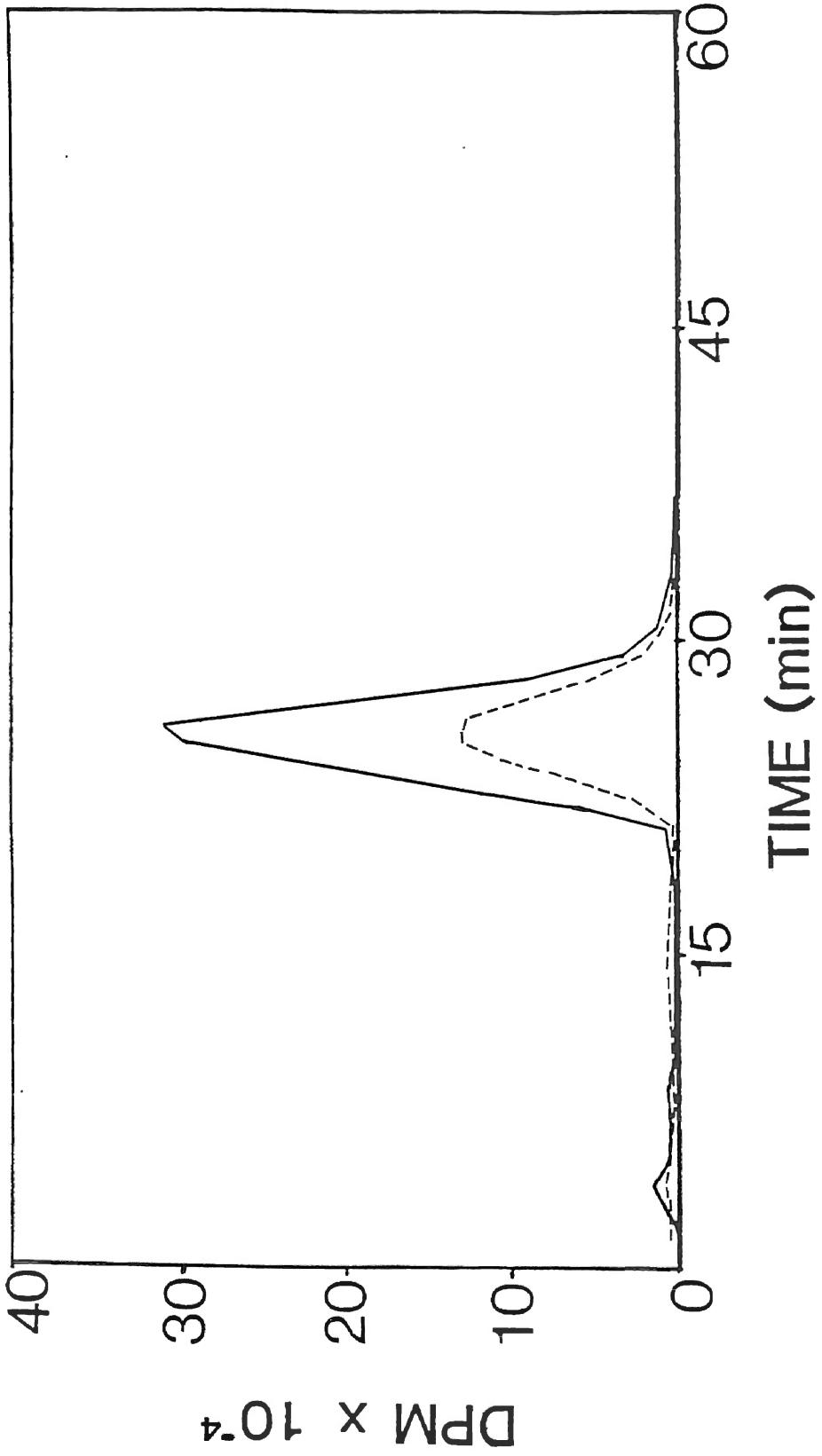

DPM x 10⁻⁴
40
30
20
10
0
15
30
45
60
TIME (min)

Figure 13. HPLC Profile of Bile Retinoids from Chicken Injected with Both All-*trans*-[10,11-$^{3}H_2$]-Retinoic Acid (—) and All-*trans*-[15-^{14}C]Retinoic Acid (---). Sample was eluted on HPLC System C with Partisphere C-18 .79 x 14.6 cm column. Solvent was 25% methanol, 75% 0.01 M acetic acid for 40 min then 60 min linear gradient to 100% methanol at 1 ml/min.

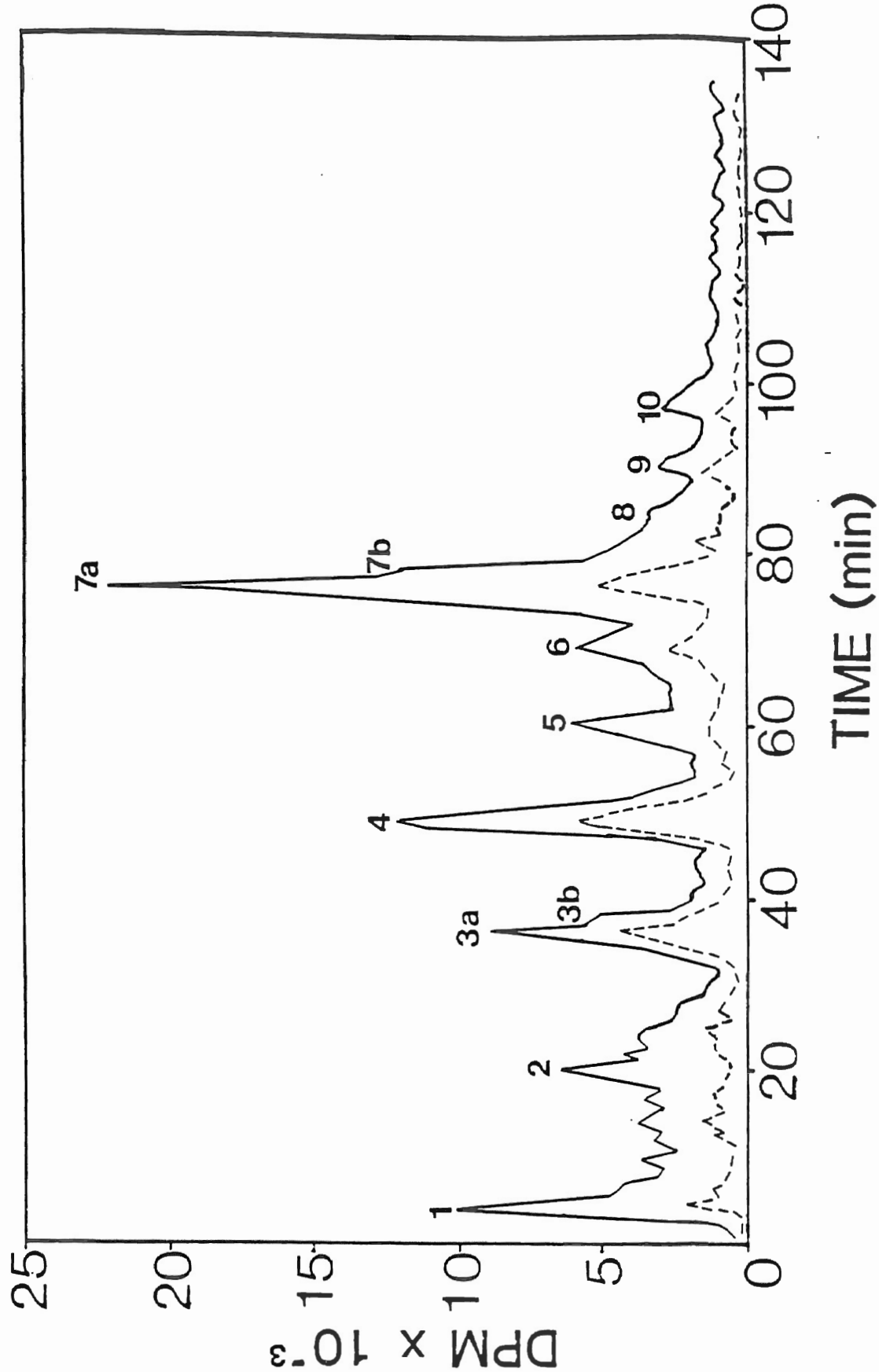

DPM x 10⁻³
25
20
15
10
5
0
20
40
60
80
100
120
140
TIME (min)
1
2
3a
3b
4
5
6
7a
7b
8
9
10

TABLE I

RELATIVE ABUNDANCE OF THE BILE METABOLITES OF ALL-*TRANS*-[10,11-$^{3}H_2$]RETINOIC ACID FROM CHICK

Metabolite Number	Elution Time (min)	Per Cent of Total Bile Activity Recovered
1	3-9	1.90
2	17-23	1.69
3a	33-37	1.49
3b	38-41	0.70
4	46-54	3.01
5	58-63	1.31
6	65-71	1.72
7a	72-77	3.92
7b	78-81	1.52
8	82-85	0.77
9	88-93	0.67
10	95-101	0.60

TABLE II

RELATIVE ABUNDANCE OF BILE METABOLITES OF ALL-*TRANS*-[15-^{14}C]RETINOIC ACID FROM CHICK

Metabolite Number	Elution Time (min)	Per Cent of Total Bile Activity Recovered
1	3-9	1.36
2	Absent	Absent
3a	33-37	2.85
3b	38-41	1.18
4	46-54	5.33
5	58-63	1.56
6	65-71	3.00
7a	72-77	3.53
7b	78-81	2.30
8	82-85	0.97
9	88-93	1.16
10	95-101	0.83

Table II shows the relative abundance of the bile metabolites from all-*trans*-[15-^{14}C]retinoic acid. Here 7a represented 3.53% of total biliary ^{14}C-labelled activity. 13-*Cis*-retinoic acid represented 1.16% and all-*trans*-retinoic acid represented 0.83%.

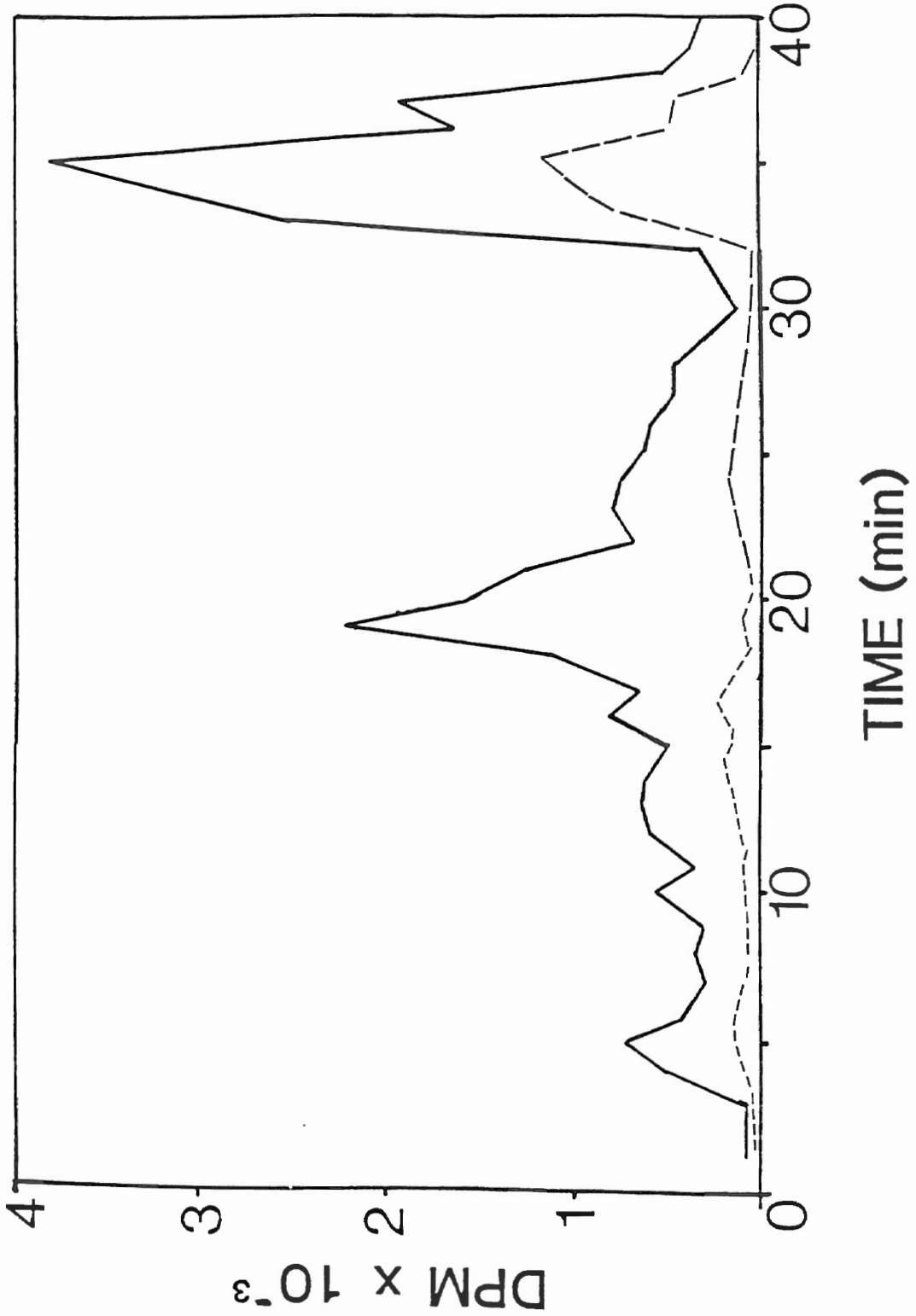
The data presented here shows clearly that all the bile retinoids have intact side chains except peak 2 which lacks the carboxylic carbon on C # 15.

Characterization of the Decarboxylated Retinoids

The on-going experiments with all-*trans*-[10,11-$^{3}H_2$]retinoic acid and all-*trans*-[15-^{14}C]retinoic acid have revealed that the metabolite in peak 2 was decarboxylated. To characterize this peak, separate chicks were injected with both labelled and unlabelled all-*trans*-retinoic acid. Chicks that received labelled retinoic acid received between 7 and 10 μCi each of ^{3}H-labelled and ^{14}C-labelled all-*trans*-retinoic acid. Those that received the unlabelled retinoic acid received 250 μg per chick. The radiolabel was used to identify the elution time for the peak (Figure 14). The peak was eluted on HPLC System C with 25% methanol, 75% 0.01 M acetic acid.

The peak was collected and stored in solution under nitrogen in a freezer until 5 μg was collected. The sample was then pooled and dried using a Rotavapor at 35°C. It was then rechromatographed on HPLC System A with Partisphere C-8 column. The solvent employed was isocratic 20% methanol, 80% 0.01 M acetic acid at a flow rate of 1 ml/min. Here the peak eluted between 15 and 20 minutes. The sample was dried and stored in a freezer under nitrogen for about 30 days since the CGC/MS was not functioning at that time. After the storage period, the sample was put on the HPLC System A again to check the purity and absorption spectrum fo the metabolite using Hewlett Packard 1040A diode array detector and information processing system. Ahern (109) had shown the UV absorption spectrum of this metabolite with maximum absorption at 318 nm. Skare *et al.* (105) had also characterized a biliary decarboxylated metabolite in the rat that had a maximum absorption at 308 nm. Surprisingly, the final analysis of this metabolite in this

Figure 14. The HPLC Elution Profile of Peak 2 on HPLC System C Using Partisphere C-18 .79 x 14.6 cm Column. Solvent was 25% methanol, 75% 0.01 M acetic acid at a flow rate of 1 ml/min.


DPM x 10⁻³
0
1
2
3
4
10
20
30
40
TIME (min)

study showed the metabolite to be absent. The analysis showed several discrete spectrums and the elution profile showed many peaks, none corresponding to peak 2. This aroused the suspicion of sample instability or the possibility of loosing the sample in the purification process.

To clear these suspicions, an experiment was designed to test the stability of bile retinoids.

Stability of Bile Retinoids

The decarboxylated peak previously described, when collected, was stored under nitrogen in a freezer for more than 30 days. Thus, when the experiment was devised to check the stability of bile retinoids, attention was focused on time stability.

A chick was injected with all-*trans*-[15-^{14}C]retinoic acid and the bile extract was prepared in the usual way. An aliquot of the bile extract was injected into HPLC System C immediately after the bile preparation. Materials were eluted with 25% methanol, 75% 0.01 M acetic acid for 40 minutes followed by a 1 hour 20 minute linear gradient to 100% methanol at 1 ml/min. At 24 hours and 48 hours, bile extract was run on the HPLC System C under similar conditions. Figures 15, 16, and 17 represent bile extract run immediately after collection, at 24 hours, and 48 hours, respectively.

Comparing the HPLC profile of these three runs, one could see conspicuous changes. The profiles in Figures 15, 16 and 17 followed the same general pattern seen earlier but runs at 24 hours and 48 hours showed significant peak splitting in peaks that eluted between 30 and 40 minutes as well as those that eluted between 80 and 90 minutes. Even though the areas remained approximately the same, the peaks that eluted between 70 and 80 minutes showed continual height decrease from the immediate run to the 48 hour run. To be convinced that the changes in the profile were due to bile retinoids' instability, the Partisphere C-18 column that had been used throughout the experiment was replaced with a new Partisphere C-18 column. The elution profile showed similar results. This

Figure 15. Elution Profile of Bile Metabolites Immediately After Collection on HPLC System C with Partisphere C-18 .79 x 14.6 cm Column. Materials were eluted with 25% methanol, 75% 0.01 M acetic acid for 40 min followed by 1 hr 20 min linear gradient to 100% methanol at 1 ml/min.

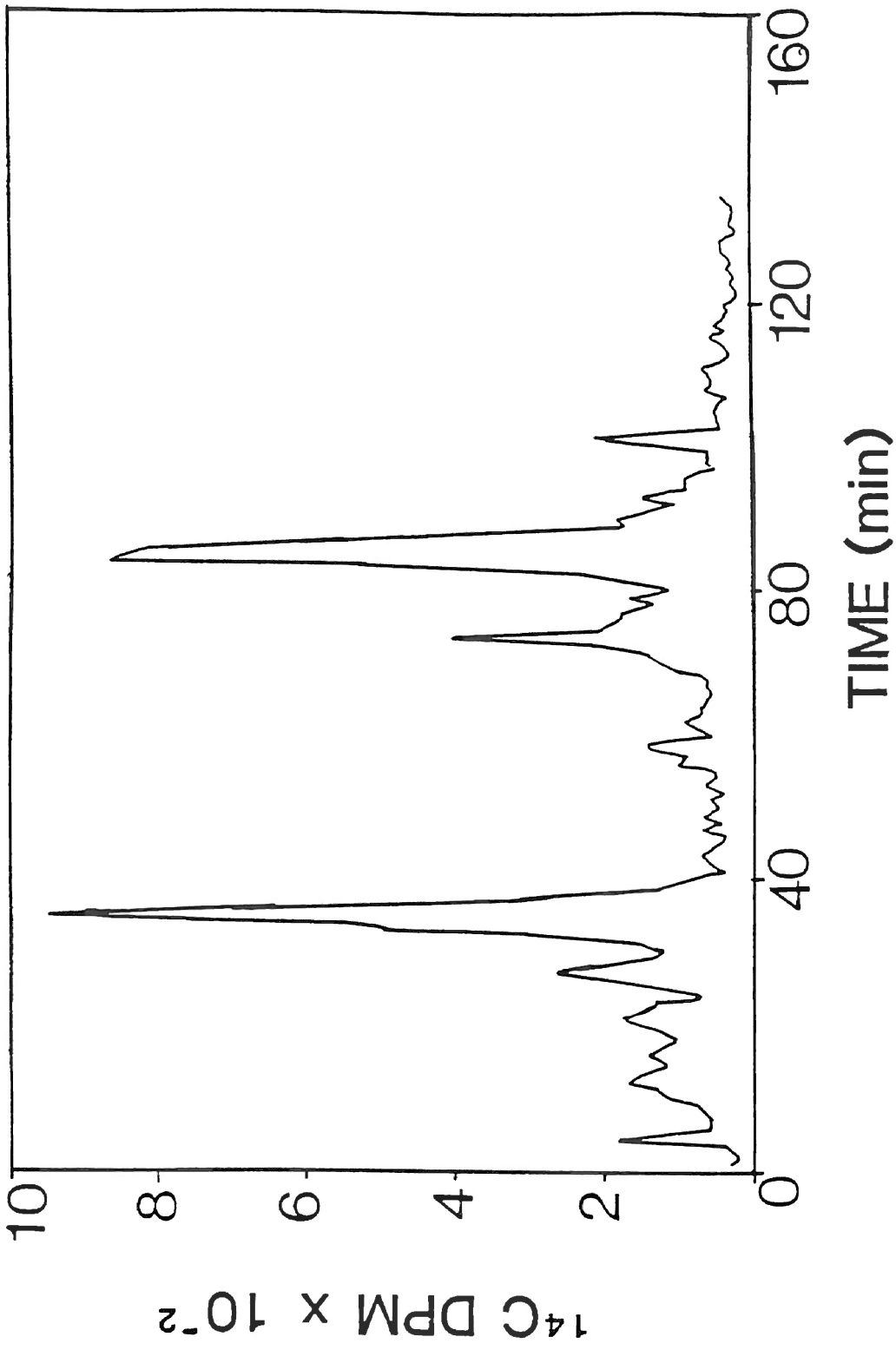

^{14}C DPM x 10^{-2}
0
2
4
6
8
10
0
40
80
120
160
TIME (min)

Figure 16. Elution Profile of Bile Metabolites 24 Hours After Collection on HPLC System C with Partisphere C-18 .79 x 14.6 cm Column. Materials were eluted with 25% methanol, 75% 0.01 M acetic acid for 40 min followed by 1 hr 20 min linear gradient to 100% methanol at 1 ml/min.

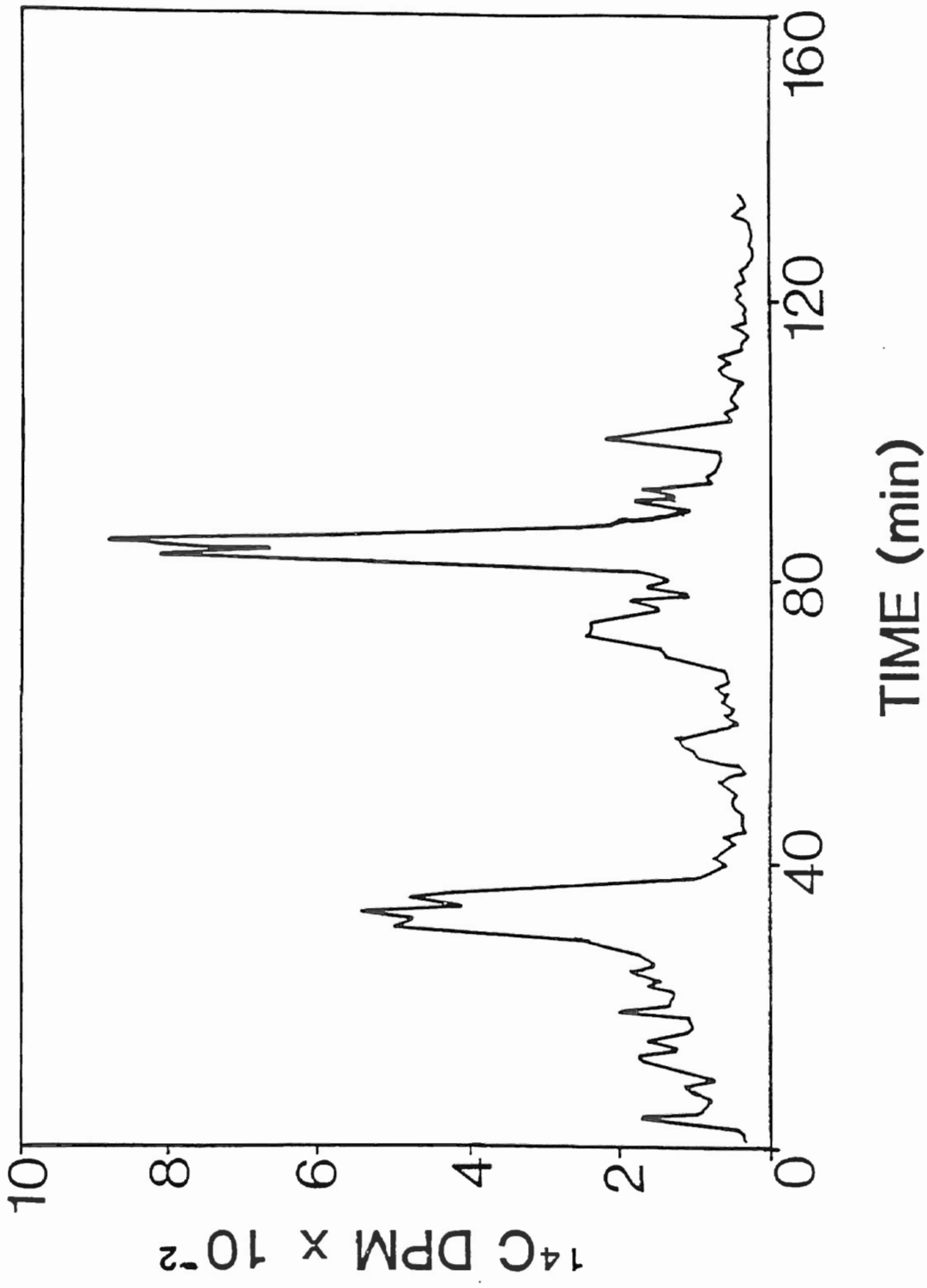
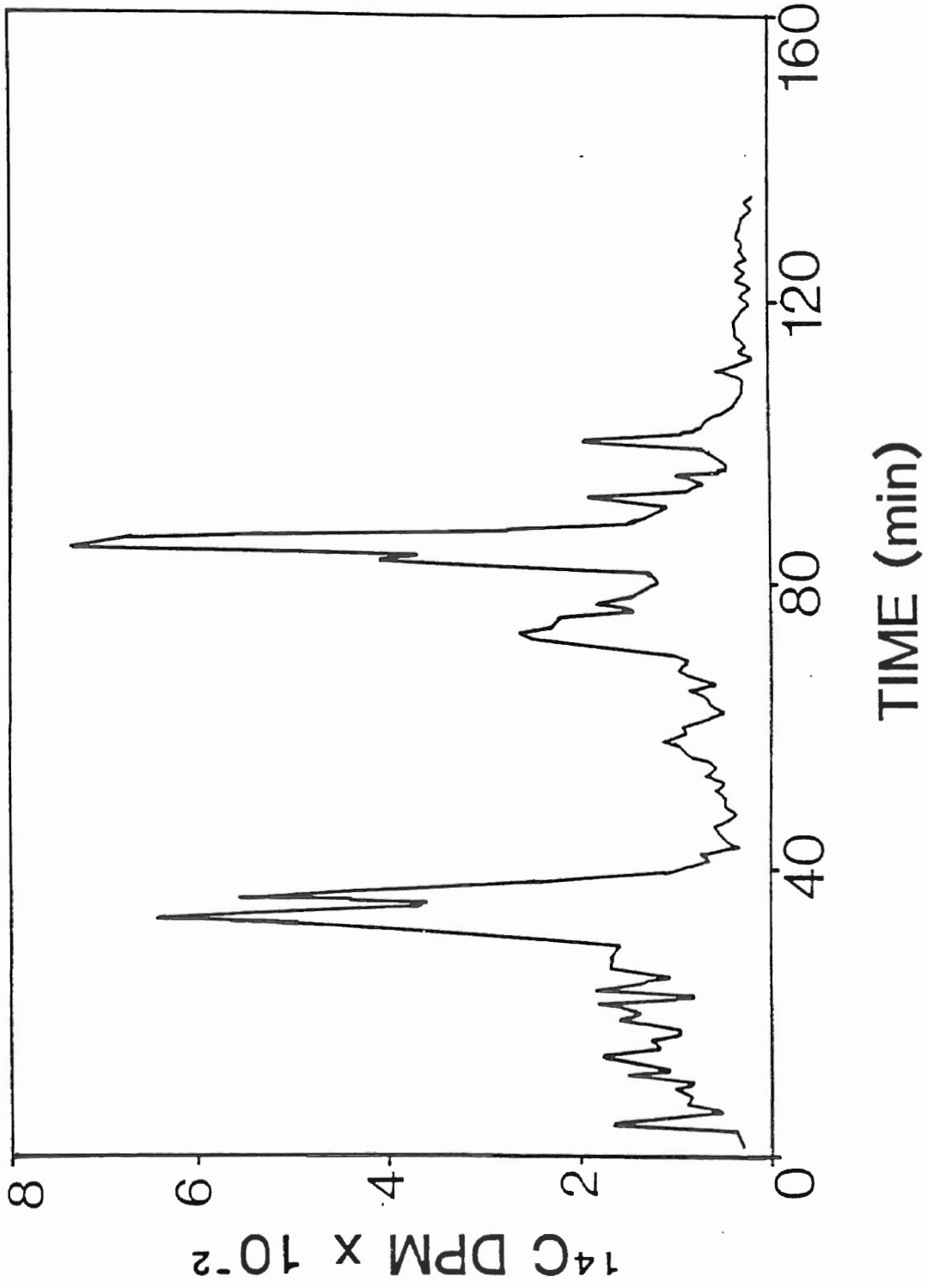

14C DPM x 10-2
0
2
4
6
8
10
0
40
80
120
160
TIME (min)

Figure 17. Elution Profile of Bile Metabolites 48 Hours After Collection on HPLC System C with Partisphere C-18 .79 x 14.6 cm Column. Materials were eluted with 25% methanol, 75% 0.01 M acetic acid for 40 min followed by 1 hr 20 min linear gradient to 100% methanol at 1 ml/min.


^{14}C DPM x 10^{-2}
0
2
4
6
8
40
80
120
160
TIME (min)

clearly showed that the biliary metabolite was unstable after a period of time.

Discussion

The results that have been presented so far showed that biliary metabolites of all-*trans*-retinoic acid ranged from polar, water soluble to unchanged all-*trans*-retinoic acid. The most polar metabolite eluted within the first 50 minutes while the least polar eluted around 110 minutes.

The appearance of a metabolite that eluted around the elution time of 13-*cis*-retinoic acid suggests the presence of a 13-*cis*-retinoic acid. Since the retinoids injected into the chicken contained virtually no 13-*cis*-retinoic acid, it led to the conclusion that if the metabolite was 13-*cis*-retinoic acid, then it originated from metabolism of all-*trans*-retinoic acid. The results of the control experiment showed no formation of 13-*cis* from all-*trans*-retinoic acid due to handling procedures. This partly supported the tentative metabolic pathway for all-*trans*-retinoic acid suggested by Zile *et al.* (110), that all-*trans*-retinoic acid is isomerized to 13-*cis*-retinoic acid *in vivo*.

In all the experiments, the most abundant metabolite was the metabolite that eluted in peak 7a which Ahern (109) had identified as a glucuronide conjugate of retinoic acid. In his study, he identified the decarboxylated peak as the major metabolite. The difference might have been due to the fact that the chicks that he used in his study were placed on retinol regulated diet before the experiments. This might have caused the animals to be vitamin A-deficient so the dose given (150 μg) never reached a toxic level. On the other hand, since the chicks used in this study were on a normal diet, they may be presumed to be vitamin A adequate. Thus, even though the dose used in both cases were comparable (200 μg on the average in this study), retinoic acid reached toxic levels in this study and was eliminated as a glucuronide conjugate. The differences may also be due to biological variations in the chicks.

The metabolite eluting in peak 2 lacked the carbon # 15 carboxylic carbon. From the

retinoids used in this investigation, one can only conclude that the side chain was intact to at least carbon # 10. It is impossible to conclude that carbon # 11 was present since the activity could be equally due to carbon # 10 or 11. All other metabolites had complete side chains which partly confirmed the finding by Dunagin *et al.* (79) that only retinoids with intact side chains were eliminated via the bile. Whether this metabolite was conjugated with another molecule or not, it could not be determined since the metabolite was not stable under the conditions stored to be purified for GC/MS analysis as anticipated. Skare *et al.* (105) characterized a decarboxylated metabolite in rat bile. He identified the metabolite to be intact to carbon # 14 and conjugated with taurine. This might be the reason why it was so polar and eluted so early in the profile. Ahern (109) identified the decarboxylated metabolite to be the major metabolite in the chick bile. Conversely, it was among the minor metabolites, 1.69% of total bile metabolites from all-*trans*-[10,11-$^{3}H_2$]retinoic acid. It could be that the organism was overdosed with retinoic acid so detoxification overshadowed metabolism of the parent compound. It could also be due to the method used to prepare the bile for HPLC analysis. The HPLC profile showed the metabolite to be very polar and soluble in water. In preparation of the bile for HPLC analysis, the bile metabolites were eluted through C-18 Sep-Pack using methanol as the eluant. Thus, the methanol might have eluted the non-polar metabolites leaving most of the polar metabolites on the Sep-Pak.

The experiment designed to investigate the stability of bile retinoids showed that the retinoids elution profile changed over the 48 hour storage period. Replacing the old Partisphere C-18 column with a new column erased the doubt that the changes were due to column malfunction. Yet still, it cannot be concluded that the changes were due to time only since there was no investigation to show the stability of the metabolites under the storage conditions.

Finally, the IR spectra obtained by Ahern (109) is identical to that of Skare *et al.* (105). Thus, peak 2 of Ahern was probably the retinotaurine.

Summary

The objective of this study was to separate metabolites of all-*trans*-retinoic acid in chick bile using HPLC as the separating tool. Also the decarboxylated peak identified by Ahern (109) was to be collected, purified and analyzed on CGC/MS. Isomerically and radiochemically pure all-*trans*-[10,11-$^{3}H_2$]- and all-*trans*-[15-^{14}C]retinoic acid was injected into chicks intraperitoneally. Chicks were sacrificed six hours after injection of the retinoids and the bile was prepared for HPLC analysis.

HPLC profile of the bile metabolites showed a spectrum of metabolites ranging from polar, water soluble metabolites that eluted within the first 50 minutes to unchanged all-*trans*-retinoic acid that eluted between 95 and 101 minutes. The major metabolite was in the peak eluting in peak 7a instead of the peak eluting in peak 2 as Ahern (109) had discovered in his study.

Comparing the radioactive profiles of bile from chicks injected with all-*trans*-[10,11-$^{3}H_2$]- and all-*trans*-[15-^{14}C]retinoic acid showed that all the metabolites had intact side chains except the peak eluting in peak 2. The decarboxylated peak eluting in peak 2 was collected and purified once for CGC/MS analysis but due to instability of the metabolite, the CGC/MS analysis could not be achieved in this study.

Analysis of the bile at different times after the bile was collected showed changes in the HPLC profile of the biliary metabolites. Peak splitting due to two or more compounds was the major change observed. Replacing the old Partisphere C-18 column with a new similar column confirmed the instability of the bile metabolites.

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