METABOLITES OF RETINOIC ACID IN BILE AND TRANSPORT OF RETINOIC ACID IN BLOOD PLASMA

By

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METABOLITES OF RETINOIC ACID IN BILE AND

TRANSPORT OF RETINOIC ACID IN

BLOOD PLASMA

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CHAPTER I

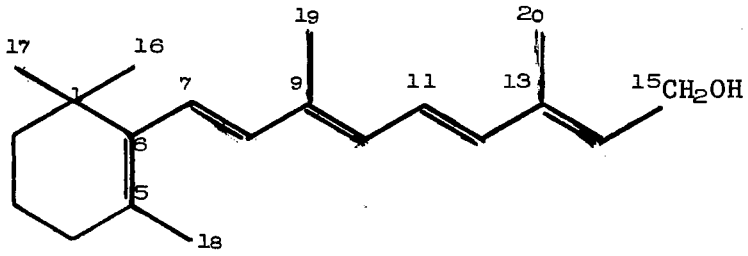
INTRODUCTION

Numerous reviews and monographs in reference to vitamin A have been published. Moore (1) has comprehensively reviewed the history, metabolism, and physiological action of vitamin A, covering information known prior to 1957, and Olson (2, 3) has recently summarized current research on vitamin A. A symposium (4) on the metabolic function of vitamin A was convened in 1968 at Massachusetts Institute of Technology to consolidate much of the newer research in the field after an earlier symposium (5) had been held in honor of Professor P. Karrer in Switzerland in 1960. Other reviews (6, 7) on the metabolism of vitamin A and related compounds have been published.

Man has been aware of nightblindness and his need for the curative agent present in animal livers for thousands of years (1). The chemical nature of this factor remained unknown until the early part of this century when specific nutritional factors, later recognized as vitamins, were discovered. One of these, "fat-soluble A", now known as vitamin A, was capable of preventing nightblindness and maintaining the integrity of epithelial tissue. The exact structure of vitamin A was elucidated by Karrer in 1931. Now that the vitamin has been chemically synthesized the annual dietary requirement for man can be purchased for about five U. S. cents (8, 9).

Nomenclature to be used when referring to vitamin A and its deriva-

tives in the present discussion is that recommended by the IUPAC-IUB Commission of Biochemical Nomenclature (10, 11, 12). Vitamin A alcohol is more properly named retinol; vitamin A aldehyde or retinene is retinal; and vitamin A acid is retinoic acid. Geometric isomers of the compounds are distinguished by prefixing the proper term to the name, i.e., all-trans retinol. The parent molecule, all-trans retinol, is numbered using the system proposed by Karrer:



The term vitamin A refers specifically to retinol, but in general practice and in the present thesis includes all related compounds that have vitamin A activity.

The mechanism of uptake of vitamin A is well known. Retinol is ingested predominantly as the palmitate ester, although it may be esterified with other long chain fatty acids (13). Uptake of these esters from the intestine is highly dependent upon dispersion with bile salts (14). Most of the esters are hydrolyzed by pancreatic retinyl palmitate hydrolase in the intestinal lumen before absorption (15, 16), and it has been found that almost all of the retinol is converted to retinyl palmitate within the intestinal mucosa (17).

β-Carotene has been known to be a source of vitamin A for several decades, but the pathway of conversion was established only recently (18). β-Carotene is cleaved in vitro into retinyl ester by intestine or perfused rat liver. With the isolation of a soluble enzyme from

liver it was possible to show that one molecule of β-carotene is cleaved into two molecules of retinal (19, 20). Molecular oxygen is required for the reaction, but a reduced cofactor is not. Tritium is not removed from the 15 and 15' positions of β-carotene and molecular oxygen is incorporated into retinal during the cleavage (21). This enzyme has been tentatively designated β-carotene 15, 15' oxygenase.

About 90% of the retinol transported in the lymph is present as retinyl ester, 3-6% as retinal, and 3-6% remains as free retinol (22, 23, 24). Nearly 80% of all vitamin A transported in the lymph is in the chylomicron fraction. Goodman and co-workers (25) 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 21,000-22,000 and binds one molecule of retinol per molecule of protein. Prealbumin (PA), which has a molecular weight of about 50,000, forms a 1:1 protein complex with plasma RBP and also binds one mole of thyroxine per mole of PA. Thyroxine binding to PA does not alter the affinity of PA for RBP nor does RBP association with PA alter the binding of thyroxine to PA. This behavior indicates that the two binding sites on PA are unique and independent (26). Addition of urea causes dissociation of the RBP-PA complex, but upon removal of urea by dialysis the complex forms again. Reduction of one disulfide bond of PA does not alter the binding of RBP and thyroxine. Reduction and alkylation of the disulfide bonds of RBP causes disruption of the retinol-RBP complex, and iodination of RBP (4.3 atoms of iodine per molecule) does not destroy the RBP-PA complex (27). It was proposed that this combination of lipid-protein and protein-protein interaction serves an important physiological function. Hence,

water-insoluble retinol is transported in an aqueous medium by a protein which also protects it from isomerization and chemical degradation. Also, the small RBP benefits from combination with PA because glomerular filtration is avoided and loss or retinol and RBP in the urine is prevented.

Retinol is stored mainly in the liver as its ester, but other sites of storage are kidney, lungs, adrenals, and the retina (1). Retinol dehydrogenase in the liver catalyzes the reversible dehydrogenation of retinol to retinal (28). Aldehyde dehydrogenase and aldehyde oxidase of liver, and xanthine oxidase of milk catalyze the oxidation of retinal to retinoic acid *in vitro*.

Vitamin A deficiency has been used as a means to investigate the function of vitamin A because the metabolic and physiological disorders which are manifested in avitaminosis A give some indications relative to its metabolic role. In vitamin A deficiency the layer of mucus secreting cells of the intestine is diminished and the incorporation of sulfate into mucopolysaccharides by the colon is inhibited (29). Urinary inorganic sulfate increases while ethereal sulfate decreases. Some experiments indicate that ATP sulfurylase is stimulated when vitamin A is supplemented (30). These data have been interpreted by some investigators to indicate an enzymic co-factor role for vitamin A, but Rogers (4) has suggested that the changes observed could have been a result of the abnormal nutritional condition of the animal rather than a dependence of the system upon vitamin A. He has investigated three of the enzymic systems which have been reported to be affected by vitamin A deficiency, i.e., sulfurylase of sulfate activation, Δ^5-3-β-hydroxysteroid dehydrogenase, and L-gulono-

δ-lactone oxidase, and his findings do not support "any possible interpretation that vitamin A is functioning in the rat as a coenzyme with any of these systems."

Symptoms of hypervitaminosis A have also disclosed some possible functions of vitamin A. It appears to function in maintaining the integrity of membrane structure and, in so doing utilizes both hydrophobic and hydrophilic portions of the molecule (31). Hypervitaminosis A results in a destabilization of the membranes of erythrocytes, bacteria, mitochondria, and lysosomes. Protease activity is liberated from preparations of cartilaginous limb bone rudiments from chick embryos and chick livers in vitro (32). In many cases these effects on membranes can be reversed by vitamin E.

Lichti and Lucy have suggested that vitamin A functions biologically as an electron donor (33, 34). They demonstrated that retinol and retinoic acid can react with 7,7',8,8'-tetracyanoquinodimethane or tetrachloro-1,4-benzoquinone in polar organic solvents to form a radical anion. Retinoic acid is a weaker electron donor than retinol, but both are capable of reducing iodine to iodide.

The only known physiological function of vitamin A was discovered by Wald and Hubbard (35) when they demonstrated that retinal is the chromophore of the visual pigment rhodopsin. In the synthesis of visual pigment retinol is oxidized to retinal. The catalytic system, composed of alcohol dehydrogenase and nicotinamide adenine dinucleotide, is found in the eye in high concentration (35, 36). Although the equilibrium of the reaction lies in the direction of the alcohol, retinal is removed from the system as it complexes with a protein (such as opsin, to form rhodopsin), and, as a result, a significant amount

of retinol is oxidized (37). The combination of opsin and 11-*cis*-retinal is spontaneous and requires neither enzymic catalysis nor energy (38). However, all-*trans*-retinal must be isomerized to 11-*cis*-retinal before synthesis of the visual pigment (39). Bleaching of the visual pigments causes all-*trans*-retinal to be released (40). A reduced derivative of rhodopsin can be formed by irradiating it in the presence of sodium borohydride. Structural studies of the derivative have shown that it contains a retinyl moiety attached to the protein *via* the ε-amino group of lysine (41, 42, 43, 44, 45). Akhtar and Hirtenstein (46) isolated a phospholipid-linked retinyl moiety which suggests that a lipid amino group is a component of the active site or rhodopsin.

Many derivatives of vitamin A and their analogues have been examined for biological activity. Several epoxides have been synthesized and characterized (47, 48, 49). Preparation, properties, and metabolism of 5,6-monoepoxyretinoic acid were reported by John *et al.* (50). All the functions of vitamin A in the rat appeared to be fulfilled by 5,6-epoxyretinal or 5,8-epoxyretinal, while 5,6-epoxyretinoate did not function in vision or reproduction (49). Anhydroretinal posseses very low biological activity (51, 52). 3-Dehydroretinol and *retro*-3-dehydroretinol accumulate in the liver after intraperitoneal administration of *retro*-3-dehydroretinol. Dehydroretinal was metabolized similiarly to retinal and had biological activity in growth tests (53, 54).

Arens and vanDorp (55, 56, 57) synthesized retinoic acid and found that its sodium salt, when administered orally in aqueous solution, was as active as retinol in restoring normal growth to retinol-deficient rats. Retinoic acid was not converted to retinol or retinal

and they concluded that it had biological activity per se. Dowling and Wald (58) showed that rats maintained on retinoic acid suffered loss of night vision and then became totally blind. They concluded that the sole function of retinol is to supply the prosthetic group of visual pigments and that all other functions, except reproduction in some species, can be fulfilled equally well by retinoic acid, or a metabolic product of it. More recently retinoic acid was shown to replace retinol in other functions, i.e., it allowed the biosynthesis of corticosterone from cholesterol (59). Nelson et al. (60) demonstrated the reduction of cerebrospinal fluid pressures of retinol deficient pigs by the acid, and showed that retinoic acid when fed either singly or in addition to retinyl palmitate spared liver stores of retinol. However, it is apparent that retinoic acid does not fulfill the functions of retinol in every instance. In addition to its inability to function in vision (58) retinoic acid does not support reproduction in the guinea pig or rat (61).

For many years workers were unable to recover retinoic acid from animal tissues, even after large doses had been administered. Jurkowitz (62) was the first to recover retinoic acid from human blood plasma and Krishnamurthy et al. (63) detected unchanged retinoic acid in tissues up to 18 hours after a massive oral dose. Nelson et al. (64) developed an extraction procedure for the quantitative recovery of retinoic acid from blood plasma and increased the precision of the Carr-Price reaction by adding acetic anhydride 2 hours prior to the addition of antimony trichloride.

With regard to the metabolism of retinoic acid, Nelson et al. (64) found that plasma retinoic acid level reached a maximum concentration

between 1.5 and 3 hours and waned until none could be detected after 8 hours. However, radioactivity remained in the blood plasma for 24 hours, which indicated that retinoic acid had been further metabolized.

Many workers have reported the isolation of metabolites of retinoic acid, retinal, and retinol. Krishnamurthy et al. (63) isolated two metabolites of retinoic acid from retinol depleted chicks which had been given large doses of retinoic acid. One was a fat-soluble compound and did not give a color reaction with antimony trichloride but did have growth promoting activity. The other compound was water-soluble but had no biological activity. Yagashita et al. (65) reported studies on a biologically active acidic metabolite of retinol and retinal, and Wolf et al. (66) characterized two water-soluble metabolites of retinol after intraperitoneal injection of retinol. A compound capable of supporting growth in vitamin A deficient animals and distinctly different from all-trans retinoic acid was isolated by Zile and DeLuca (67), but it was later shown to be 13-cis retinoic acid (68).

The only metabolite of retinoic acid that has been characterized was isolated by Dunagin et al. (69). Retinol-^{14}C and retinoic acid-^{14}C were converted to water-soluble metabolites in the liver, secreted in the bile, reabsorbed from the intestine, and again secreted in the bile (70, 71). The major metabolite resulting from intraportal injection of retinoic acid-6,7-^{14}C was purified by anion-exchange and silicic acid column chromatography. It was identified as retinoyl β-glucuronide from data such as the following: (1) the compound exhibited the ultraviolet absorption spectrum of retinoic acid, (2) retinoic acid was released by either β-glucuronidase or base catalyzed hydrolysis,

(3) the compound reacted with diazomethane, hexamethyldisilazane, and periodate, and (4) it contained 1.4 moles of glucuronic acid per mole of retinoic acid (72). Other compounds isolated in the study, such as β-glucurono-δ-lactone, cis and trans isomers of methyl retinoate, and retinoic acid, were shown to be artifacts formed during isolation (73).

The β-glucuronide of retinol is also formed in vivo. A system for biosynthesis in vitro of both retinyl and retinoyl β-glucuronides consists of washed rat liver microsomes, uridine diphosphoglucuronic acid, and either retinol or retinoic acid (74).

Retinoyl β-glucuronide has biological activity equal to retinoic acid (74). Apparently secretion of these glucuronides is a normal physiological excretory process.

Although only one metabolite of retinoic acid has been identified others must exist since retinoic acid has been shown to undergo decarboxylation in vivo and in vitro (76, 77, 78). Roberts and DeLuca (79) characterized a product resulting from the decarboxylation which was more polar than retinoic acid, had a λ_{max} at 296 nm, apparently had an aldehyde group at C-14, and was believed to lack only C-15 of the original molecule.

Lin (80) demonstrated that when retinoic acid-15-^{14}C was administered orally 4.5% of the dose was expired as $^{14}CO_2$, while only 0.4% was expired as $^{14}CO_2$ when retinoic acid-6,7-^{14}C was fed. A microsomal enzyme which catalyzes the decarboxylation was purified 292 fold from a liver homogenate and a metabolite resulting from the reaction was isolated and partially purified chromatographically.

Even though a considersble amount of research has been done on

the metabolism of retinoic acid its metabolic fate is not completely understood. Therefore, the primary objective of this research is to isolate and identify metabolites of retinoic acid present in bile and blood plasma.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF METABOLITES OF RETINOIC ACID FROM THE BILE OF CHICKS AND RATS

Dunagin et al. (72) concluded that retinoic acid was conjugated with glucuronic acid in the liver and that retinoyl β-glucuronide, along with other retinoate conjugates, was secreted in the bile. However, Lippel and Olson (74) concluded that retinoyl β-glucuronide was "the major metabolite of retinoic acid in bile, if not the sole one," and that the other conjugates isolated were formed during the purification of the glucuronide by anion-exchange chromatography.

Research in this laboratory concerning isolation and identification of metabolites of retinoic acid in chicks and rats employed techniques of isolation other than anion-exchange chromatography and indicated that retinoyl β-glucuronide was not the only conjugate of retinoate secreted in the bile. This chapter describes purification of metabolites of retinoic acid from bile by solvent extraction, gel-filtration chromatography, and thin-layer chromatography (tlc).

Experimental

Materials and Methods

Retinoic Acid

Non-radioactive all-*trans* retinoic acid was a gift from Hoffmann-

LaRoche, Inc., Nutley, New Jersey (courtesy of Drs. W. E. Scott and R. H. Bunnell). After recrystallization from 2-propanol, the melting point was 178°-180°C (cor). One spot was observed upon tlc in three solvent systems and only one peak was detected by gas-liquid partition chromatography (glpc) of methyl retinoate.

All-trans retinoic acid-15-^{14}C, also a gift from Hoffmann-LaRoche, Inc., was purified using tlc with diethyl ether-petroleum ether (90:10) as developing solvent. After purification it displayed a single radioactive spot in three tlc solvent systems.

All manipulations with retinoic acid or its metabolites were conducted in subdued light.

Reagents

Diazomethane was prepared in a generator having rubber sealed joints. A solution of 1.2 g of sodium hydroxide in 10 ml water, 25 ml carbitol (diethylene glycol monoethyl ether), and 75 ml of diethyl ether were added to a 500 ml flask and cooled to 0°C. Next, 3.55 g of EXR-101 (N,N'-dinitroso-N,N'-dimethyl terephthalamide, E. I. duPont deNemours & Co., Wilmington 98, Delaware) were added at one time. Magnetic stirring was started and the reaction mixture warmed slowly on a steam bath. The diazomethane and ether condensed as a bright yellow solution into a collection vessel packed in dry ice.

Trimethylsilyl (TMS) reagent containing hexamethyldisilazane-trimethylchlorosilane-pyridine (3:1:9) in 1 ml sealed ampoules was purchased from Applied Science Laboratories, Inc., State College, Pennsylvania, 16801. Bis-(trimethylsilyl)acetamide and trimethylchlorosilane, reagent grade, were obtained from Analabs, Inc.,

Hamden, Connecticut.

Tween 80 (polyoxyethylene sorbitan mono-oleate), bovine liver β-glucuronidase, and phenolphthalein-glucuronic acid were purchased from Sigma Chemical Co., St. Louis, Missouri.

Reagent grade absolute methanol, absolute ethanol, benzene, chloroform, anhydrous diethyl ether, glacial acetic acid, and hydrochloric acid were used without further purification.

Ninety-five percent ethanol (1500 ml) was distilled vigorously over 4-5 g $KMnO_4$ and 20 g KOH in the dark. The first and last 150 ml portions of distillate were discarded.

Petroleum ether, bp 36.0-37.5°C, was purchased from W. H. Curtin, Santa Fe Springs, California. It was purified by shaking 1500 ml vigorously 3 to 6 times with 100 ml of concentrated sulfuric acid, or until the acid layer was colorless. After washing with 100 ml of water the hydrocarbon was shaken vigorously with 100 ml of saturated aqueous permanganate made acid with 2 ml of concentrated sulfuric acid. This was followed by washing with water, shaking with 10% KOH, and washing to neutrality with water. Following filtration through fluted sharkskin paper into a 3 liter brown bottle it was stored over 1½ in. of Drierite (anhydrous $CaSO_4$), 6-8 mesh, for 12 hours. Next it was distilled over 15 g of KOH into a 5 cm X 50 cm bed of silica gel.

Scintillation solvent was prepared according to Bray (81). Reagent grade ethylene glycol, p-dioxane, sulfur-free toluene, and ethylene glycol monomethyl ether were used in its preparation.

A saturated solution of antimony trichloride was prepared by dissolving 25 g of the material in 100 ml of dry chloroform, filtering off the residual $SbCl_3$, and adding 1% (v/v) acetic anhydride.

Thin-Layer Chromatography (tlc)

Pre-coated tlc plates with a 250 μ thickness of silica gel were purchased from Quantum Industries, Fairfield, New Jersey. The sample was applied 2 cm from the bottom edge of the plate with a capillary tube. Radioactive compounds were detected using a gas flow strip counter (Actigraph III, Model 1006, Nuclear-Chicago, Des Plaines, Illinois) and unsaturated compounds were detected with iodine vapor. The areas containing the desired compound were vacuumed into a Pasteur disposable pipette plugged with glass wool at the large end and eluted with either diethyl ether or ethanol.

Silicic Acid Column Chromatography

Eighteen grams of silicic acid which had been prepared according to Hirsch and Ahrens (82) (Bio-Rad Laboratories, Berkeley, California) were poured into a glass column (1.5 (i. d.) X 25 cm, Fischer and Porter, Co., cat. #274-787) under vacuum. A circle of filter paper was placed on top of the silicic acid, chloroform applied to the column, the suction discontinued, and the column washed for 24 hr.

Gel-Filtration Chromatography

Sephadex G-25, G-10, and Blue Dextran 2000 were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey. Bio-Gel P-4 and P-2 were obtained through Bio-Rad Laboratories, Richmond, California. The material was swollen in deionized water overnight before being packed into the column. After the column was equilibrated, Blue Dextran 2000 was passed through it to determine the void volume(V_o) and to disclose any irregularities in the packing.

Gas-Liquid Partition Chromatography (glpc)

The gas chromatograph was a modified Barber-Colman Model 5000 having a hydrogen flame detector. The column used on this instrument also fits the Mass Spectrometer-Gas Chromatograph described in the next paragraph and operating conditions were established on this instrument before the Gas Chromatograph-Mass Spectrometer was used. The 0.25 in. (o. d.) X 8 ft glass column, packed with 1% OV-1 on Gas Chrom Q, (Applied Science Laboratories, State College, Pennsylvania) was modified for solid injection (83), and was furnished by Mr. Kieth Kinneberg. The column used for glpc of retinoyl β-glucuronide was identical to this one except that it was not modified for solid injection.

Mass Spectrometry

Mass spectra were obtained on the prototype of the LKB-9000 Gas Chromatograph-Mass Spectrometer (84, 85) with the total ion current recording serving as the glpc tracing. Either the direct inlet or the glpc column described in the preceding paragraph was used and the spectra were taken at 70 eV ionizing voltage, 3.5 kV accelerating voltage, ion source temp 310°C, and at a source pressure of 5×10^{-6} to 1×10^{-7} mm Hg.

Ultraviolet Absorption Spectrometry

Ultraviolet (uv) absorption spectra in 95% ethanol were taken either with a Cary Model 14 Spectrophotometer or a Beckman Model DB Spectrophotometer equipped with a Sargent Model SRL Recorder. Quartz cells having 3 ml volumes and 1 cm light paths were used in obtaining the spectra.

Hydrolytic Methods

The fraction to be hydrolyzed was dissolved in either 10% ethanolic KOH or 5% aqueous KOH and placed in a water bath maintained at 70°C. Absolute ethanol or water was added to the reaction mixture to maintain the volume, if needed, while a stream of dry nitrogen was passed over the liquid.

A 1% suspension of β-glucuronidase from bovine liver was prepared in 0.1 M sodium acetate buffer, pH 4.5. Two milliliters of enzyme suspension were incubated with the substrate and, for a contol, 2 ml of buffer were incubated with the substrate. Phenolphthalein-glucuronic acid was incubated with an aliquot of the enzyme solution with each experiment. After the incubation, the reaction mixture was diluted with four volumes of 0.4% acetic acid in acetone and the protein removed by centrifugation. The solvent was removed from the supernatant solution by evaporation and the residue taken up in a small amount of methanol before being analyzed by tlc.

Methylation and Silylation

Methyl esters were formed by reacting the compound with an ethereal solution of diazomethane. The reaction was assumed to be complete when no nitrogen was released upon addition of diazomethane.

In some cases trimethylsilyl ethers were formed by adding 1 ml of Sil-Prep (Applied Science Laboratories, State College, Pa.) to the sample and allowing it to remain at room temperature for at least one hour. Alternatively, bis-(trimethylsilyl)acetamide and trimethylchlorosilane were used according to Waller et al. (86). The sample was placed in a small vial and the solvent evaporated. Bis-(trimethyl-

silyl)acetamide (0.2 ml) was added and as much of the residue dissolved as possible before trimethylchlorosilane (0.05 ml) was added. A lid was placed on the vial and it was put into an oven and maintained at 55°C for three hours. The solvent was evaporated and the residue dissolved in acetone.

Measurement of Radioactivity

Radioactivity was counted with a Packard Tri-Carb Model 3320 Liquid Scintillation Spectrometer. Efficiency was determined by using the external standard and all data were converted to disintegrations per minute.

Experimental Animals

Male white leghorn chicks, 4-6 weeks of age, were furnished by Dr. R. H. Thayer, Poultry Science Department, Oklahoma State University, Stillwater, Oklahoma. They were maintained on a vitamin A deficient ration supplemented with retinoic acid stabilized in gelatin beadlets at a level of 2.5 mg per pound of feed. Chicks to be used were fasted 24 hr prior to the beginning of the experiment.

Male albino rats, weighing 250-400 g, were obtained from Holtzman Co., Madison, Wisconsin, and maintained on a vitamin A sufficient diet until use. They were also fasted for 24 hr prior to being used.

Administration of Retinoic Acid

Crystalline all-trans retinoic acid was weighed into #5 gelatin capsules. Radioactive retinoic acid dissolved in toluene was pipetted into another capsule and the solvent removed by evaporation. The cap-

sules were placed into the esophagus of the chick and washed down with water. In this way the dose was administered quantitatively.

For intraperitoneal injection, retinoic acid was dissolved in 0.2 ml of 95% ethanol and 0.4 ml of 3.3% Tween 80 in 0.1 M sodium phosphate buffer, pH 7.4. It was helpful to add a small amount of 0.1 N NaOH and then warm the mixture to obtain a clear dispersion.

Cannulation

The rat was anesthesized with 15 mg of sodium pentobarbital (Diamond Laboratories, Des Moines, Iowa) per pound of body weight prior to beginning the cannulation. A 1½ in. midline incision was made in the abdomen posteriorly from the sternum and the bile duct located at the point where it enters the duodenum. A small incision was made in the duct before a blunt 25 ga. needle, having an 18 in. piece of polypropylene tubing attached, was inserted. The cannula was stabilized by a thread tied on either side of the insertion before the incision was sutured. The bile was collected in a vessel cooled in ice.

Preparation of Tissues for Analysis

Blood was collected from chicks by heart puncture into oxalated tubes. It was cooled in ice for 30 minutes before the cells were centrifuged. The animal was killed by cervical dislocation and the gall bladder excised. Rats were killed by decapitation at the end of the experiment. All tissues were stored at -15°C.

Fractionation of Bile and Blood Plasma by Solvent Extraction

A modification of the extraction method of Nelson et al. (64)

was used to fractionate blood plasma and bile. A schematic diagram of the extraction procedure is shown in Fig. 1.

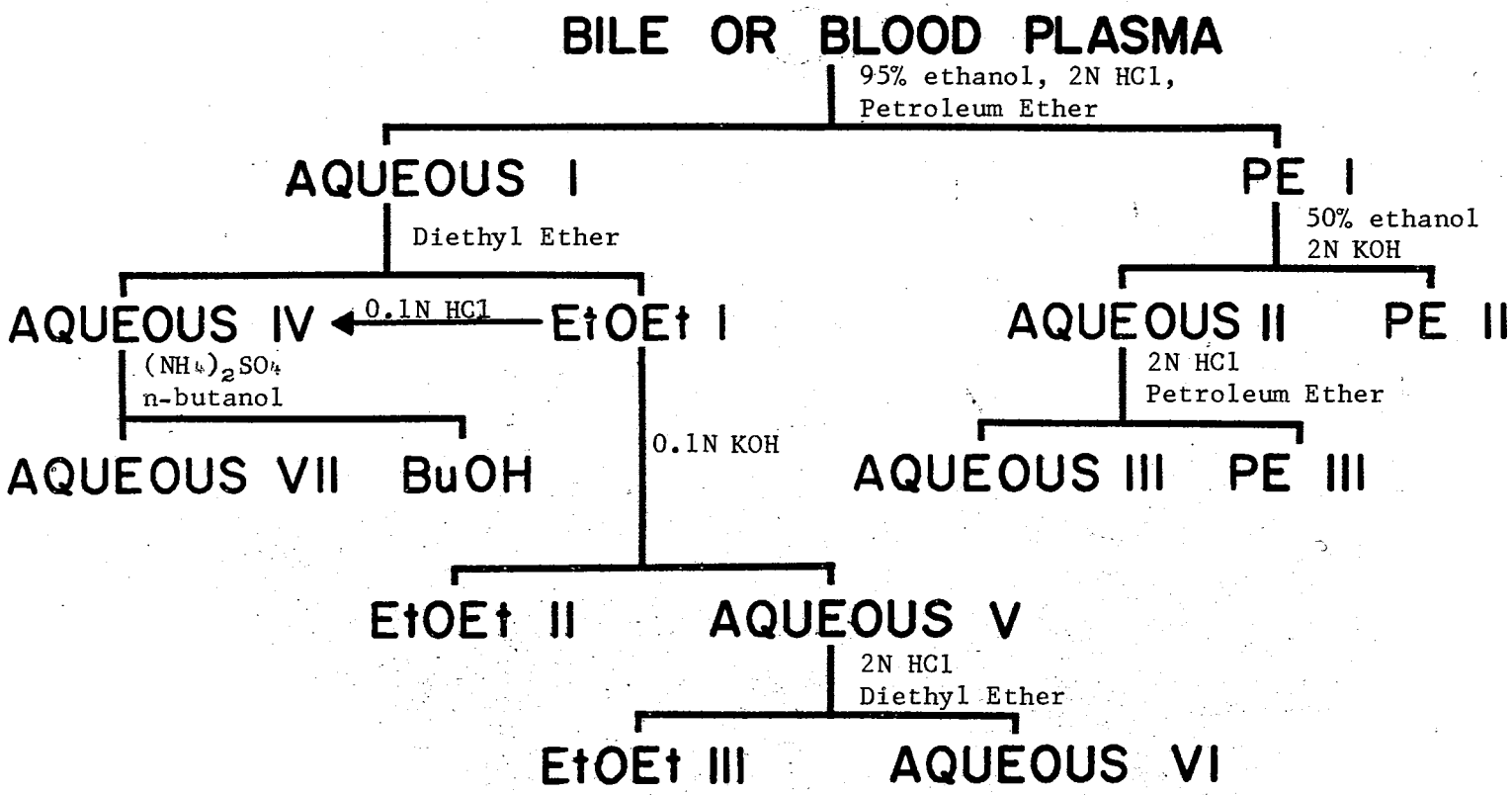
One to 3 ml of bile, or 10 ml of blood plasma, was placed in a clean 50 ml round-bottomed glass stoppered centrifuge tube. Bile was diluted to 10 ml with deionized water. To this was added 10 ml of 95% ethanol, 25 ml of petroleum ether, and 1 ml of 2 N HCl. The tube was shaken for 10 min., chilled in ice, and centrifuged five minutes. Next, the petroleum ether layer was decanted and an aliquot was removed and monitored for radioactivity. The extraction was repeated twice. The remaining aqueous-ethanol residue was labeled aqueous I and stored.

The combined petroleum ether extracts (PE I) were washed with water and dried (Na_2SO_4). The volume was reduced to 25 ml under vacuum and the extract was added to a centrifuge tube containing 20 ml of 50% ethanol and 1 ml of 2 N KOH. After the tube was shaken, cooled, and centrifuged, the petroleum ether was decanted. The extraction was repeated two times and an aliquot was taken each time for liquid scintillation counting. The combined extracts were washed with water, dried (Na_2SO_4), and reduced in volume for storage. The petroleum ether extract was labeled PE II and the remaining aqueous layer, aqueous II.

Aqueous II was acidified with 2 ml of 2 N HCl and extracted three times with 25 ml of petroleum ether. An aliquot was taken from each extract for liquid scintillation counting and all extracts were combined. They were washed with water, dried (Na_2SO_4), and reduced in volume for storage. This fraction was labeled PE III.

Aqueous I was then extracted three times with 25 ml of diethyl

Figure 1. Diagram of the Procedure Used to Fractionate Chick Bile and Blood Plasma. Animals were administered 5 μCi (5 mg) of retinoic acid-15-^{14}C orally and the tissues collected after 6 hr.


BILE OR BLOOD PLASMA
95% ethanol, 2N HCl,
Petroleum Ether
AQUEOUS I
PE I
Diethyl Ether
50% ethanol
2N KOH
AQUEOUS IV
0.1N HCl
EtOEt I
AQUEOUS II
PE II
(NH4)2SO4
n-butanol
2N HCl
Petroleum Ether
0.1N KOH
AQUEOUS VII
BuOH
AQUEOUS III
PE III
EtOEt II
AQUEOUS V
2N HCl
Diethyl Ether
EtOEt III
AQUEOUS VI

ether. An aliquot was taken for assay of radioactivity after each extraction. The combined extracts were washed once with 30 ml of 0.1 N HCl. The washings were combined with aqueous I and the fraction labeled aqueous IV.

The ether extract, EtOEt I, was dried (Na_2SO_4) and the volume reduced to 20 ml under vacuum at 30°C. It was then added to 20 ml of 0.1 N KOH and shaken. After cooling in ice and centrifugation, the ether layer was siphoned off. This was done a total of three times and the radioactivity in each extract was determined. The combined extracts were washed with deionized water, dried (Na_2SO_4), and reduced in volume for storage as fraction EtOEt II.

The alkaline aqueous solution from the diethyl ether extraction (aqueous V) was made acidic with 2 ml of 2 N HCl and extracted three times with diethyl ether. After determining the amount of radioactivity in each extract the pooled ether extract was washed, dried (Na_2SO_4), and reduced in volume for storage as EtOEt III.

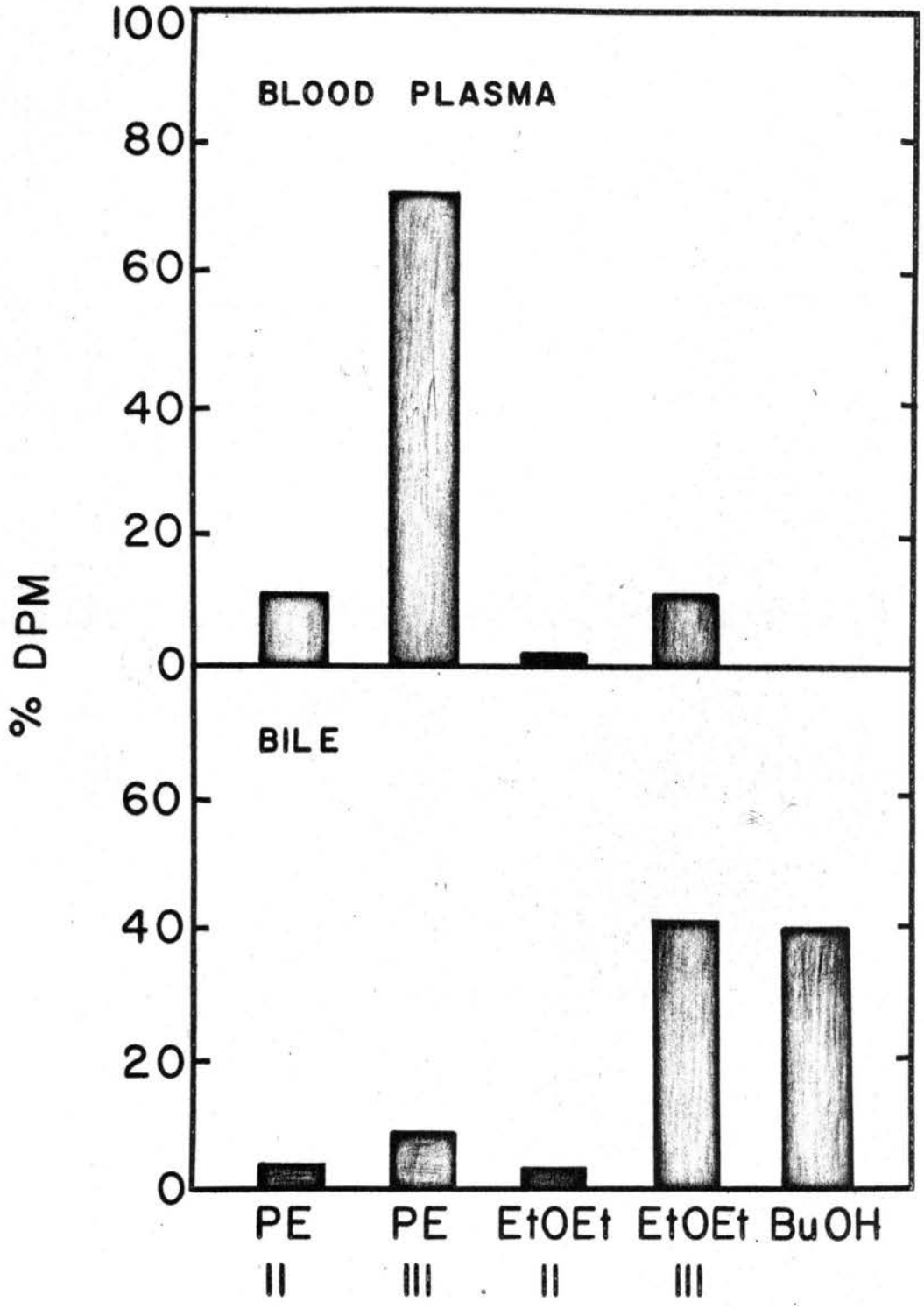
After saturation with ammonium sulfate aqueous IV was extracted with n-butanol. The radioactivity which was soluble in n-butanol was called the BuOH fraction.

Results

Distribution of Radioactivity in the Fractions

Chick bile and blood plasma were fractionated by the solvent extraction scheme shown in Fig. 1. The distribution of radioactivity among the fractions is shown in Fig. 2 for representative extractions of blood plasma and bile. The majority of the radioactivity in bile was extracted with diethyl ether and n-butanol while the major part of

Figure 2. Distribution of Radioactivity in Chick Bile and Blood Plasma after Fractionation by Solvent Extraction. The chicks were administered 5 μCi (5 mg) of retinoic acid-15-^{14}C orally and the tissues collected after 6 hr. The fractionation procedure is described in the text and diagrammed in Fig. 1. Radioactivity was assayed with a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer.


100
80
60
40
20
0
BLOOD PLASMA
% DPM
BILE
60
40
20
0
PE
II
PE
III
EtOEt
II
EtOEt
III
BuOH

the radioactivity in blood plasma was extracted with petroleum ether. This procedure extracted 85-90% of the radioactivity from the tissues.

Each fraction of bile was submitted to tlc on silica gel using benzene-chloroform-methanol (4:1:1) as the solvent system (Table 1). Each fraction displayed only one radioactive spot and each appeared to contain a different compound.

Characterization of PE II

Fraction PE II was submitted to tlc on silica gel in four solvent systems (Table 1). It was purified to the extent that there was only one radioactive spot in each of the four systems. The radioactive area was eluted from the silica gel and analyzed by glpc (Fig. 3). Three major peaks were resolved at 200°C and mass spectrometry showed the parent peaks of the compounds respective to retention time to be 284, 312, and 332. Neither the R_f value of tlc nor the elution profile from glpc was changed by treatment with diazomethane or with hexamethyldisilazane and trimethylchlorosilane (Table II). However, hydrolysis in 10% ethanolic KOH resulted in a change in the R_f from 0.66 to 0.58 in benzene-chloroform-methanol (4:1:1) and from 0.60 to 0.49 in the ethyl acetate-benzene (25:75) system (Table II). The uv absorption spectrum of the fraction had λ_{max} at 273 nm and 290 nm (Fig. 4), but after hydrolysis and purification by tlc the λ_{max} was 276 nm (Fig. 5). Fraction PE II was methylated and examined by glpc after alkaline hydrolysis and one major and five minor components were resolved (Fig. 6).

TABLE I

SILICA GEL TLC OF RADIOACTIVE[1] FRACTIONS[2] OF CHICK BILE

Solvent System (v:v)	PE II	PE III	EtOEt II	EtOEt III	BuOH	Methyl Retinoate	Retinoic Acid
					R_f		
Benzene-Chloroform-Methanol (4:1:1)	0.66	0.58	0.60	0.45	0.16	0.69	0.53
Diethyl Ether-Petroleum Ether (85:15)	0.61		0.54			0.58	0.31
Ethyl Acetate-Benzene (25:75)	0.60		0.61			0.63	0.29
Petroleum Ether-Diethyl Ether-Acetic Acid (90:10:1)	0.03					0.50	0.10
Petroleum Ether-Diethyl Ether-Acetic Acid (70:20:4)	0.80		0.77			0.75	0.44
Benzene-Chloroform-Methanol-Acetic Acid (5:5:5:1)	0.78						0.79
Chloroform-Methanol-Water (65:40:5)		0.87			0.53	0.90	0.87

[1] Radioactive areas were detected with a gas flow strip counter (Actigraph III, Model 1006, Nuclear Chicago, Des Plaines, Illinois).

[2] Nomenclature for the radioactive fractions is given in Fig. 1.

Figure 3. Glpc of Fraction PE II. The gas chromatograph was a modified Barber-Colman Model 5000 having a hydrogen flame detector. A helical glass column, 8 ft X 0.25 in. (o.d.), was modified for solid injection (83) and was packed with 1% OV-1 on Gas Chrom Q. Temperatures were 200°C (column), 215°C (injection port), and 265°C (detector). Helium flow rate was 50 ml/min.

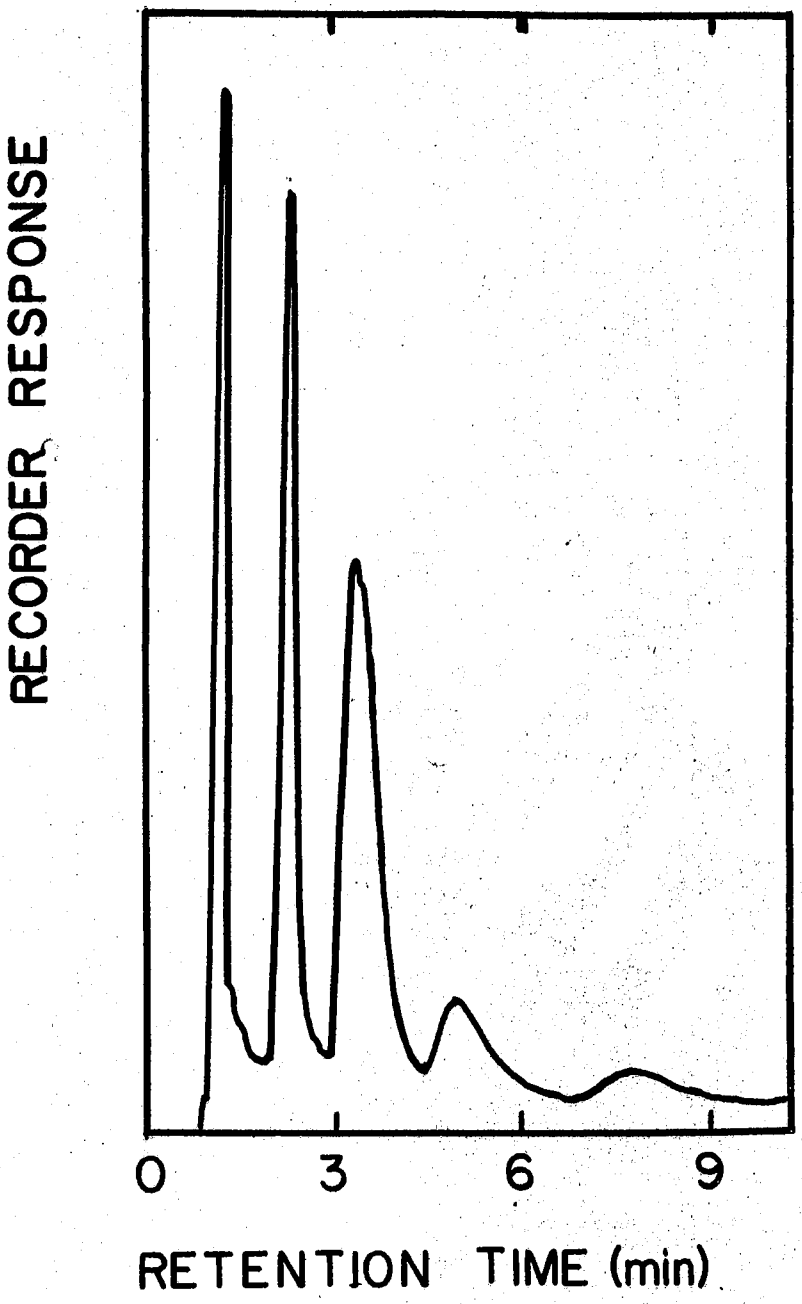

RECORDER RESPONSE
0
3
6
9
RETENTION TIME (min)

TABLE II

SILICA GEL TLC OF FRACTION[1] PE II AND ITS DERIVATIVES

Solvent System (v:v)	Treatment			
	None	TMCS[2] + HMDS[3]	CH_2N_2	Alkaline Hydrolysis
			R_f[4]	
Benzene-Chloroform-Methanol (4:1:1)	0.66	0.66	0.66	0.58
Ethyl Acetate-Benzene (25:75)	0.60			0.49

[1] Nomenclature for the fractions is given in Fig. 1.

[2] TMCS = Trimethylchlorosilane

[3] HMDS = Hexamethyldisilazane

[4] R_f of radioactive area as determined with a Nuclear Chicago Actigraph III strip counter.

Figure 4. UV Absorption Spectrum of Purified Fraction PE II. The spectrum of the sample in 95% ethanol was recorded with a Cary Model 14 Spectrophotometer in quartz cells having a 1 cm light path.

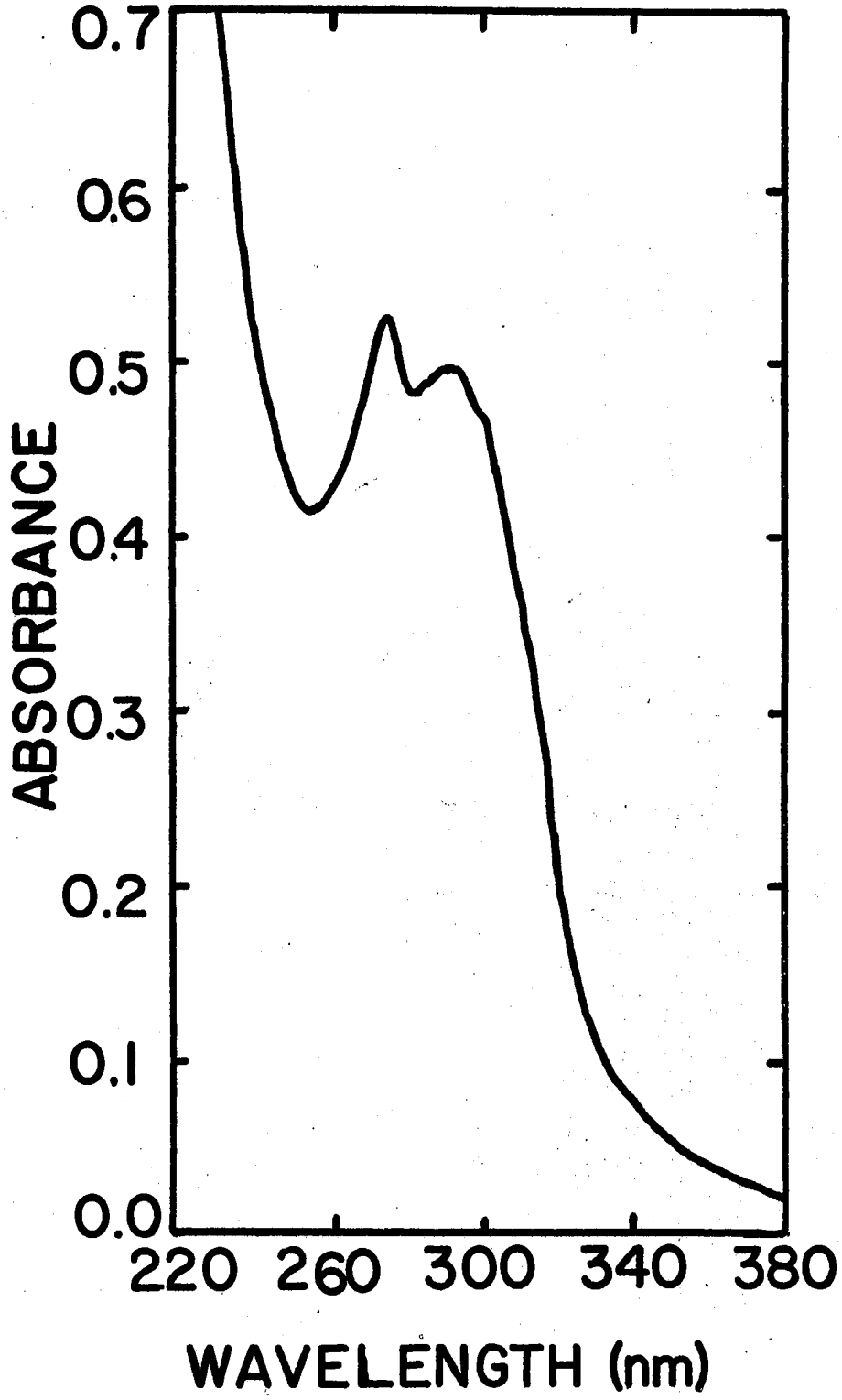

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
220
260
300
340
380
WAVELENGTH (nm)

Figure 5. UV Absorption Spectrum of Purified Fraction PE II after Alkaline Hydrolysis. The spectrum of the sample in 95% ethanol was recorded with a Cary Model 14 Spectro-Photometer in quartz cells having a 1 cm light path.

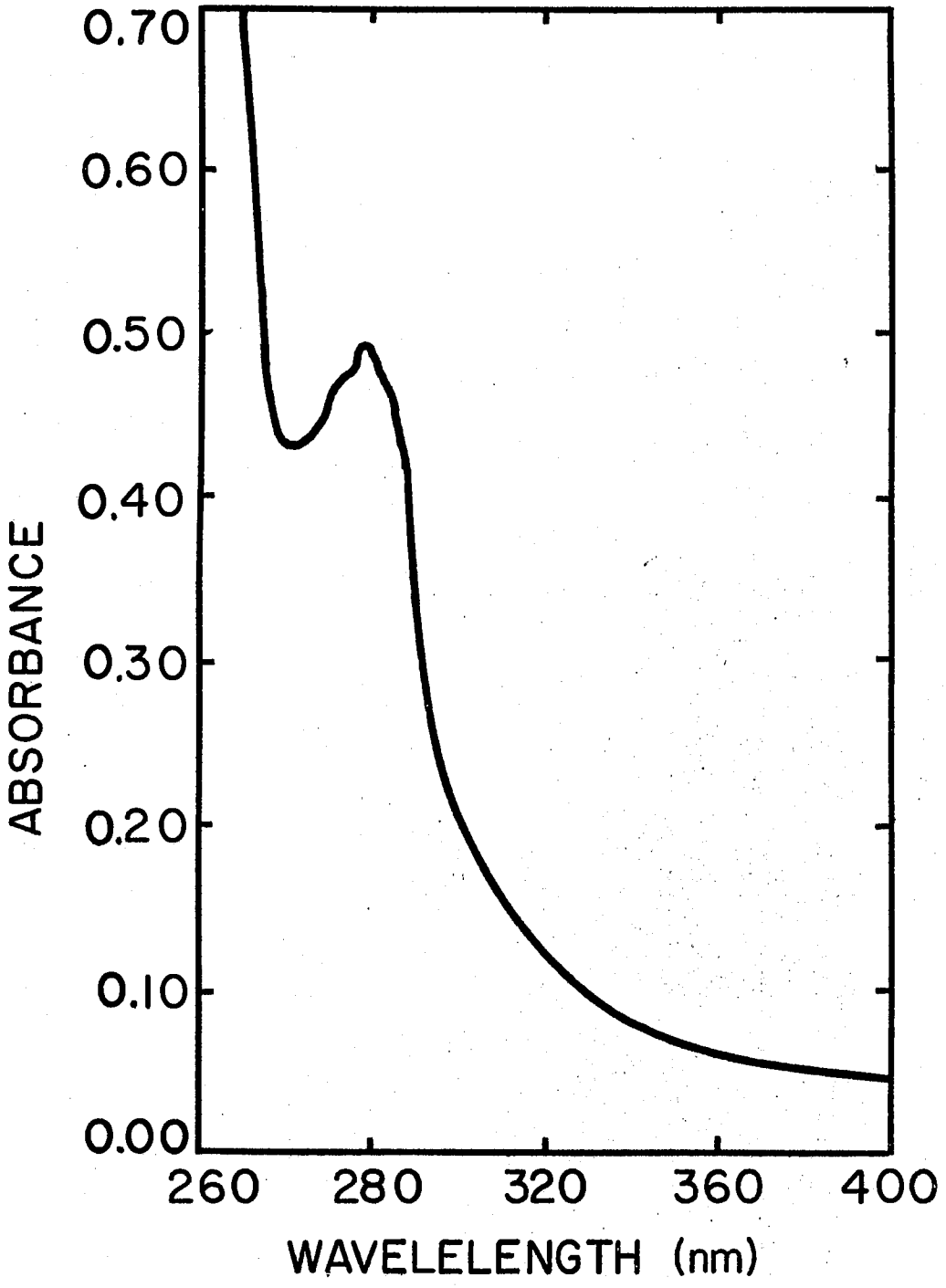
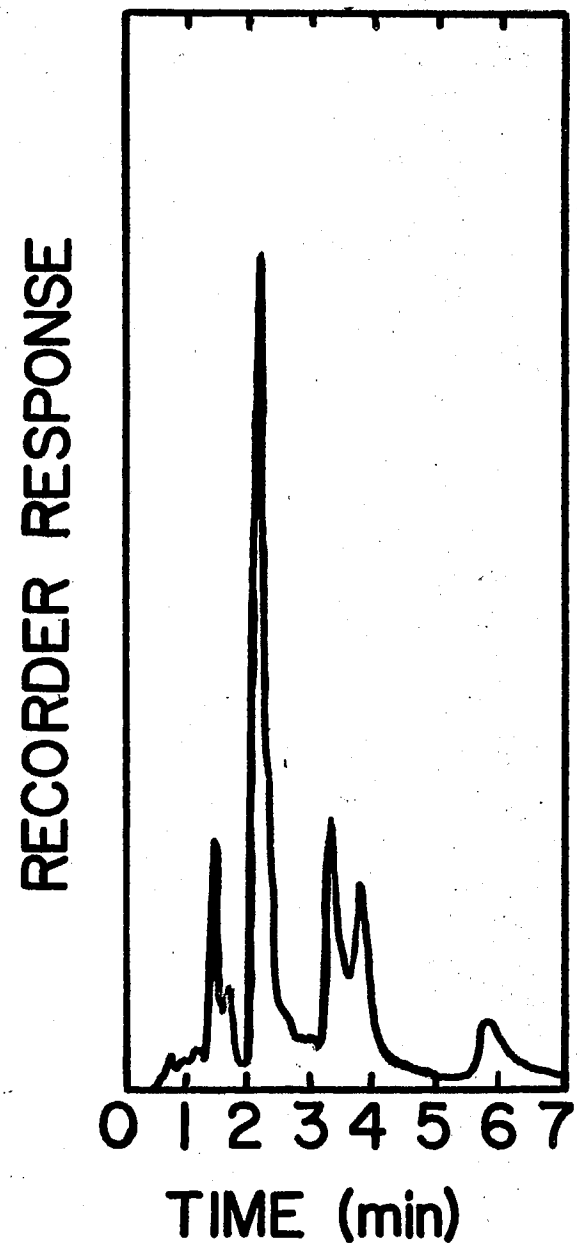

0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
ABSORBANCE
260
280
320
360
400
WAVELELENGTH (nm)

Figure 6. Glpc of Fraction PE II after Alkaline Hydrolysis and Methylation. The gas chromatograph was a modified Barber-Colman Model 5000 having a hydrogen flame detector. An 8 ft X 0.25 in. (o.d.) helical glass column, modified for solid injection (83), contained 1% OV-1 on Gas Chrom Q. Helium flow rate was 50 ml/min and temperatures were 200°C (column), 215° (injection port), and 265°C (detector). Methyl esters were formed using a freshly distilled solution of diazomethane in ether.


RECORDER RESPONSE
0 1 2 3 4 5 6 7
TIME (min)

Characterization of PE III

If retinoic acid were present in bile or blood plasma it would be extracted into fraction PE III (64). The fraction extracted from bile had R_f values similar to those of retinoic acid (Table I), and the uv absorption spectrum displayed λ_{max} at 345 nm, a characteristic of the uv absorption spectrum of retinoic acid (Fig. 7). The typical uv absorption spectrum of retinoic acid was not detected in PE III of blood plasma but R_f values of it in two solvent systems were similar to those of retinoic acid.

Characterization of EtOEt II

Fraction EtOEt II apparently is material which was not extracted into PE II because the R_f values of the two fractions are very similar (Table I) and the elution profiles of glpc were similar.

Characterization of EtOEt III

This fraction contained acidic compounds because it was soluble in dilute base but not in acid. It was more polar than retinoic acid as shown by the fact that it was not extracted into petroleum ether and it did not have the uv absorption spectrum of retinoic acid (Fig. 8). The fraction was dissolved in chloroform and subjected to silicic acid column chromatography (Fig. 9). The column was eluted stepwise with increasing concentrations of absolute ethanol in chloroform and one major radioactive peak was eluted. Stepwise elution procedures have the disadvantage of yielding false peaks (82) when the solvent is changed, and probably the smaller peaks eluted from this column are examples of false peaks.

Figure 7. UV Absorption Spectra of Purified Fraction PE III of Chick Bile and Retinoic Acid. The spectra of the samples in 95% ethanol were recorded with a Cary Model 14 Spectrophotometer in quartz cells having a 1 cm light path.

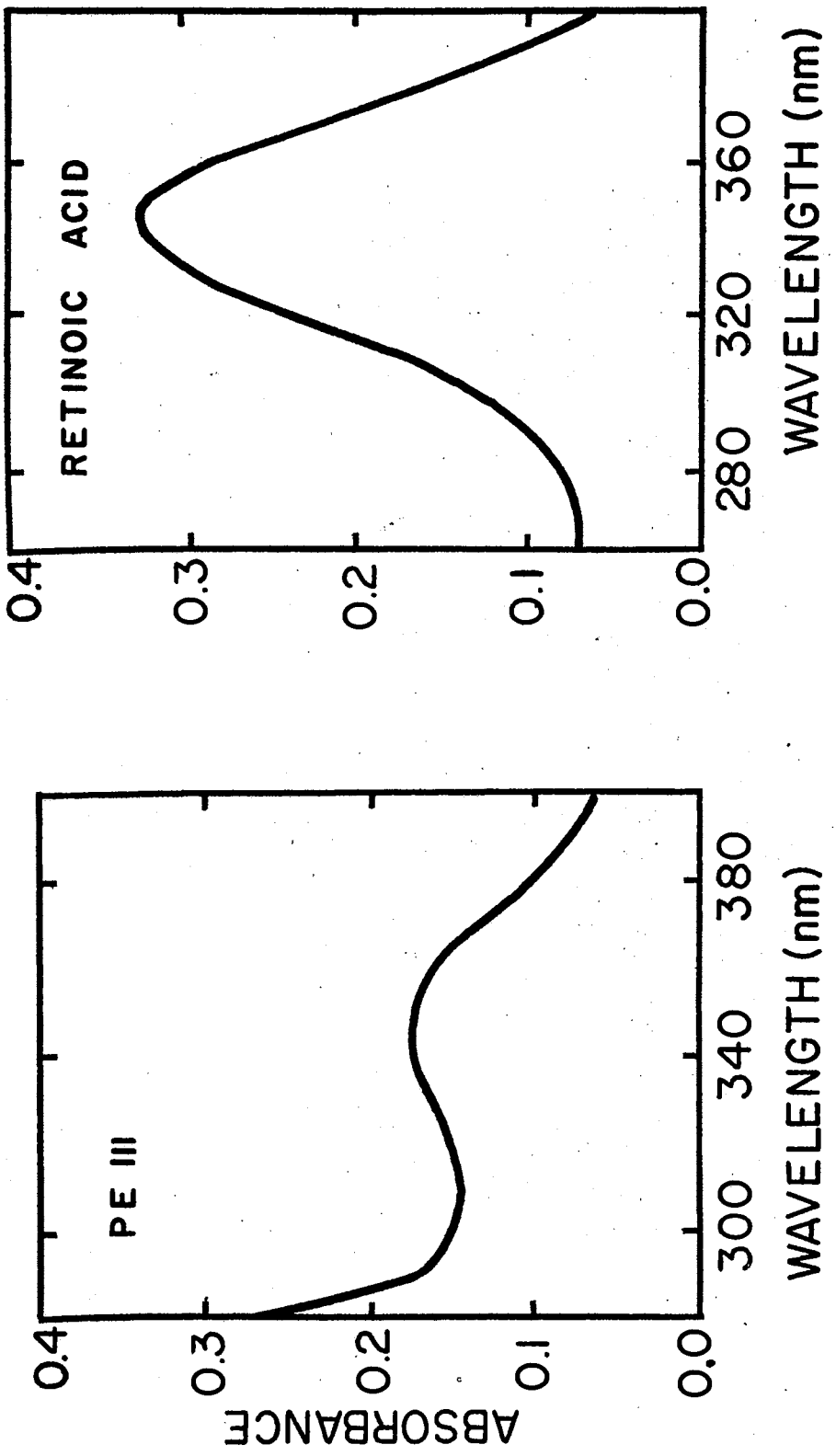

RETINOIC ACID
0.4
0.3
0.2
0.1
0.0
280
320
360
WAVELENGTH (nm)
PE III
ABSORBANCE
0.4
0.3
0.2
0.1
0.0
300
340
380
WAVELENGTH (nm)

Figure 8. UV Absorption Spectra of Purified Fractions EtOEt III and BuOH. The spectra of the samples in 95% ethanol were recorded with a Cary Model 14 Spectrophotometer in quartz cells having a 1 cm light path.

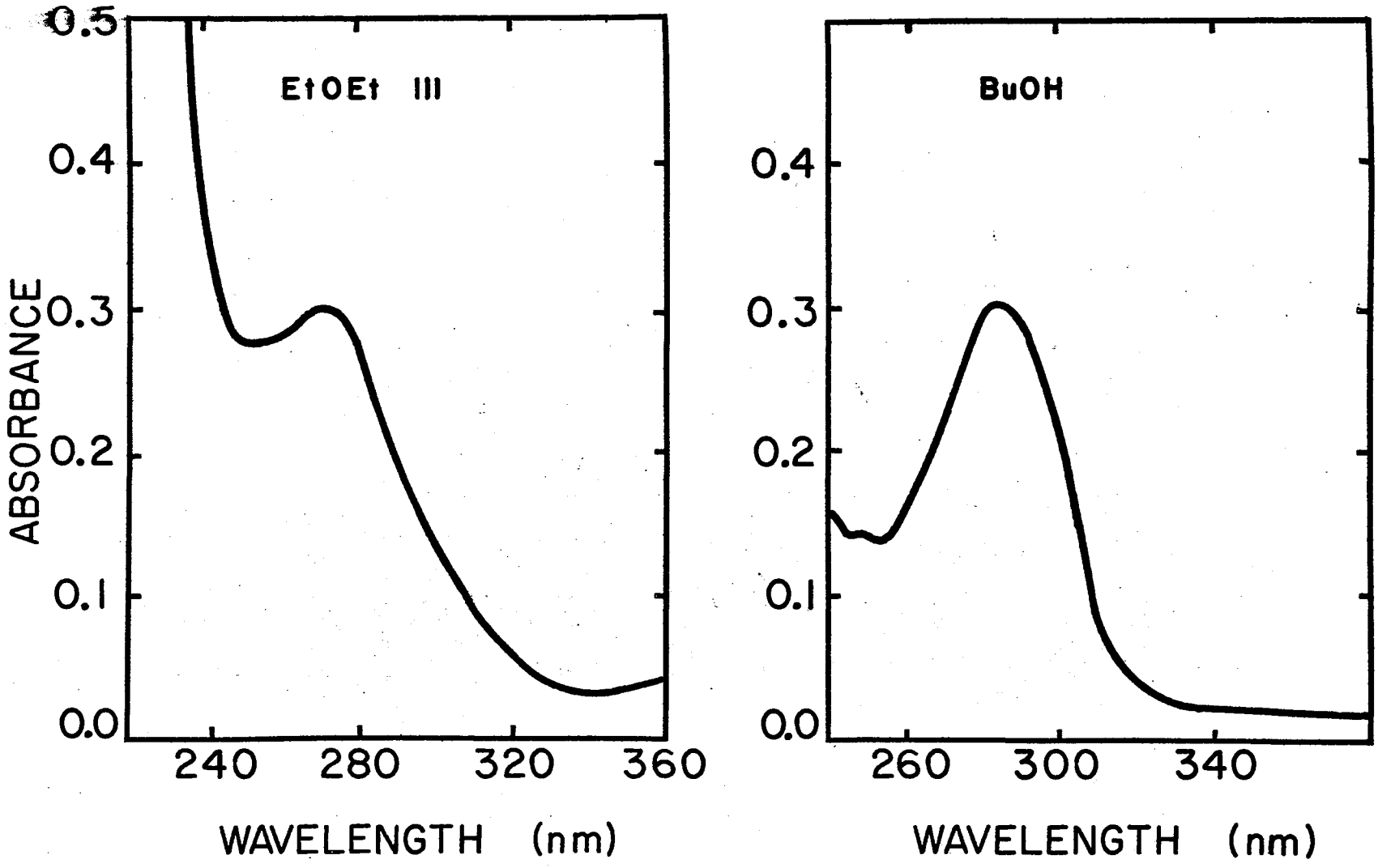
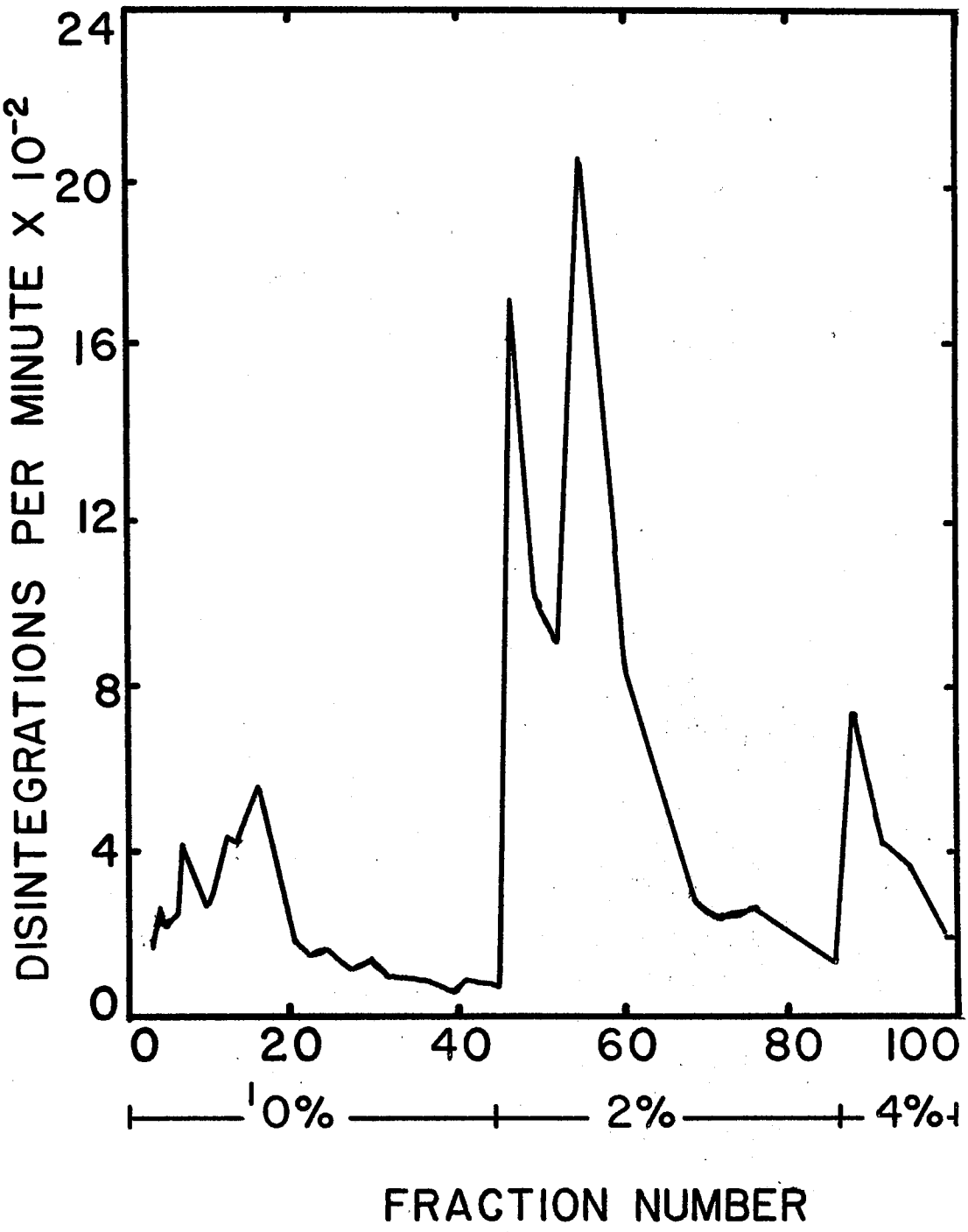

EtOEt III
BuOH
ABSORBANCE
0.5
0.4
0.3
0.2
0.1
0.0
240
280
320
360
260
300
340
WAVELENGTH (nm)
WAVELENGTH (nm)

Figure 9. Silicic Acid Column Chromatography of Fraction EtOEt III. The 1.5 cm (i. d.) column containing 18 g of silicic acid was equilibrated with chloroform before the sample in chloroform was applied. The column was eluted first with chloroform and then increasing concentrations of absolute ethanol in chloroform. Ten milliliter fractions were collected and 0.5 ml aliquots of each fraction were assayed for radioactivity in a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer System.

[1]Elution gradient = % abs ethanol in chloroform.


DISINTEGRATIONS PER MINUTE X 10^{-2}
24
20
16
12
8
4
0
0
20
40
60
80
100
10%
2%
4%
FRACTION NUMBER

Characterization of BuOH

The butanol fraction was methylated and silylated. When the fraction was allowed to react with diazomethane for 30 minutes an increase in R_f value from 0.16 to 0.24 was observed (Table III), but when the fraction was reacted with diazomethane for 12 hr the R_f increased to 0.70. Treatment with silylating reagents resulted in an increase in R_f from 0.53 to 0.73 (Table III). The uv absorption spectrum of retinoic acid was not apparent in this fraction (Fig. 8). Attempts at hydrolysis were unsuccessful when either β-glucuronidase from bovine liver or 10% ethanolic KOH was used. The fraction was eluted from a silicic acid column with increasing concentrations of absolute ethanol in chloroform and several radioactive fractions were separated (Fig. 10). However, tlc of the eluted compounds indicated that none was the same compound that was originally present in BuOH. Due to the fact that the initial fractionation was done at low pH, it was necessary to check the procedure for formation of artifacts.

Collection of Bile from Rats after Administration of Retinoic Acid-15-^{14}C

In order to obtain larger amounts of metabolite(s) for study, and to check out the extraction procedure against artifact formation, the bile duct of a rat was cannulated and the bile collected after administration of 2 mg of retinoic acid-15-^{14}C (sp act 2.5 μCi/mg). Almost 70% of the injected isotope was recovered in the bile in 12 hr (Fig. 11). The rat bile was subjected to the same extraction procedure as chick bile (Fig. 1) and the distribution of radioactivity was similar to that of chick bile, in that the majority of the radioactivity was

TABLE III

SILICIC ACID TLC OF FRACTION BuOH[1] AND ITS DERIVATIVES

Solvent System (v:v:v)	Treatment				
	None	CH_2N_2	TMCS[2] + HMDS[3]	Alkaline Hydrolysis	β-glucuronidase
				R_f[4]	
Benzene-Chloroform-Methanol (4:1:1)	0.16	0.24[5] 0.70[6]		0.16	0.16
Chloroform-Methanol-Water (65:40:5)	0.53		0.73		

[1] Nomenclature for fractions is given in Fig. 1.

[2] TMCS = trimethylchlorosilane

[3] HMDS = hexamethyldisilazane

[4] R_f of radioactive area as determined by Actigraph III strip counter

[5] Reacted with CH_2N_2 for 30 minutes.

[6] Reacted with CH_2N_2 for 12 hr.

Figure 10. Silicic Acid Column Chromatography of Fraction BuOH. The silicic acid (18 g) was packed into a 1.5 cm (i. d.) column and washed with chloroform before the sample dissolved in 10% ethanol in chloroform was applied. The column was eluted with increasing concentrations of ethanol in chloroform and 10 ml fractions were collected. Radioactivity was determined on 0.5 ml fractions with a Packard Model 3320 Tri-Carb Liquid Scintillation System.

[1]Elution gradient = % abs ethanol in chloroform.

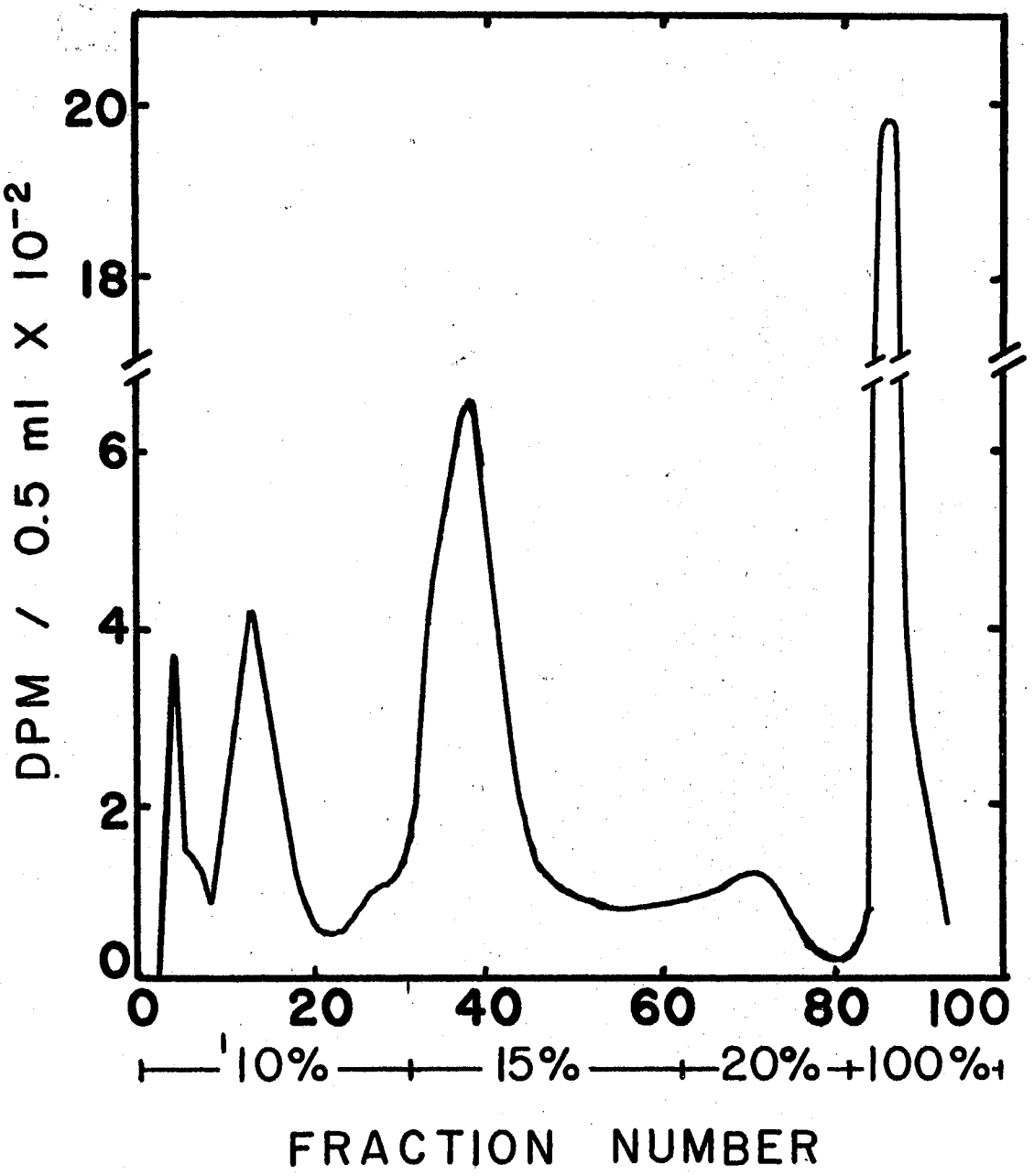
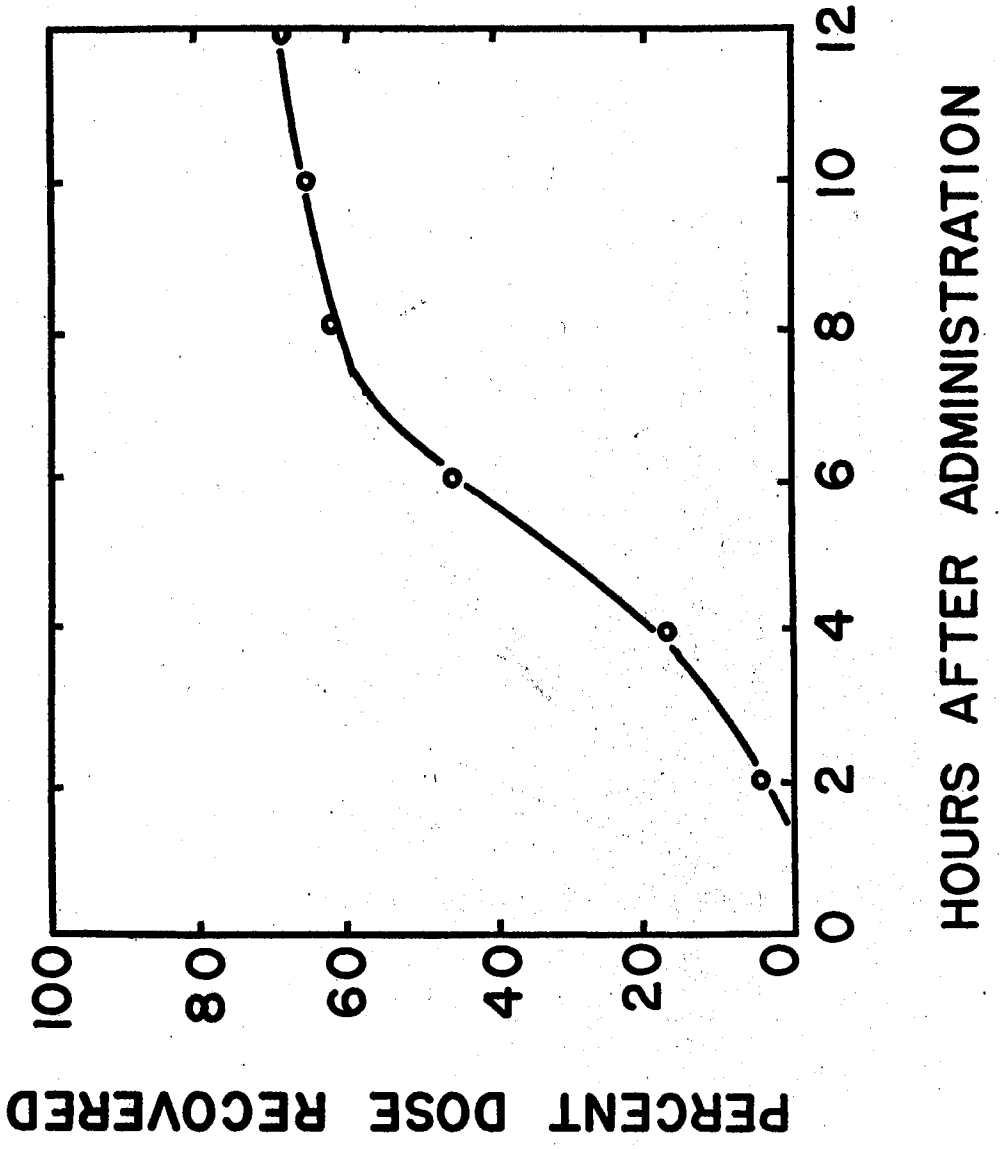

DPM / 0.5 ml X 10^{-2}
20
18
6
4
2
0
0
20
40
60
80
100
10%
15%
20%
100%
FRACTION NUMBER

Figure 11. Accumulation of Radioactivity in Rat Bile after Administration of Retinoic Acid-15-^{14}C. Two milligrams of retinoic acid-15-^{14}C (sp act 2.5 μCi/mg) in 0.1 M sodium phosphate buffer, pH 7.4, containing 3.3 % Tween 80 was administered intraperitoneally and the bile collected through a cannula in the bile duct. Radioactivity was monitored with a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer.


PERCENT DOSE RECOVERED
0
20
40
60
80
100
0
2
4
6
8
10
12
HOURS AFTER ADMINISTRATION

extracted by diethyl ether and n-butanol (Fig. 12). It was established that the two fractions had different R_f values upon tlc analysis.

A sample of unfractionated bile was chromatographed using benzene-chloroform-methanol (4:1:1) and chloroform-methanol-water (65:40:5) as tlc solvents. A single radioactive spot in each system migrated with an R_f similar to that fraction extracted into n-butanol (Fig. 13 and 14).

Incubation of Bile from Rats Administered Retinoic Acid-15-^{14}C with β-Glucuronidase

An aliquot of bile containing 100,000 DPM was incubated with β-glucuronidase and a significant amount of hydrolysis occurred (Fig. 15). The R_f value of the product of hydrolysis agreed closely with the R_f value of retinoic acid and it gave a positive color reaction for retinoic acid with $SbCl_3$. The slower moving spot also gave a positive color reaction for retinoic acid. However, 100% hydrolysis with β-glucuronidase was not achieved in any of the experiments. These data indicated that the bile contained the glucuronide of retinoic acid and also another conjugate.

Separation of Metabolites of Retinoic Acid in Rat Bile Using Gel-Filtration Chromatography

An aliquot of bile which had been collected from a rat administered 2 mg of retinoic acid-15-^{14}C (5 μCi) was chromatographed on a Bio-Gel P-2 column. Two radioactive fractions were eluted from the column; the first was in the V_o and the second in the area of smaller molecular weight compounds (Fig. 16).

Figure 12. The Distribution of Radioactivity in Rat Bile after Administration of Retinoic Acid-15-^{14}C as Fractionated by Solvent Extraction. The nomenclature of the fractions is described in Fig. 1 and the radioactivity was assayed with a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer.

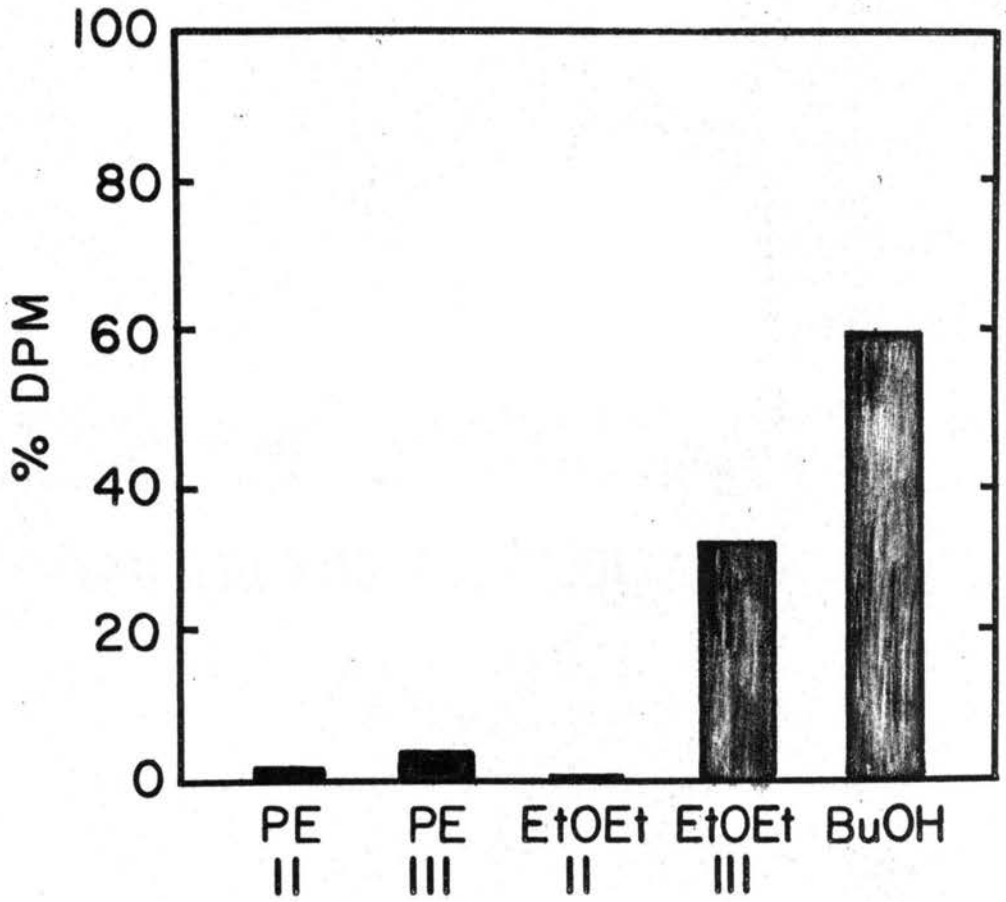

100
80
60
40
20
0
% DPM
PE II
PE III
EtOEt II
EtOEt III
BuOH

Figure 13. Distribution of Radioactivity in Rat Bile Following Silicic Acid Tlc in Benzene-Chloroform-Methanol (4:1:1). Retinoic acid-15-^{14}C, 5 μCi (sp act 2.5 μCi/mg), in 3.3% Tween 80 in 0.1 M sodium phosphate buffer, pH 7.4, was administered intraperitoneally and the bile collected through a cannula in the bile duct. An aliquot of the bile was chromatographed and the radioactive area detected with a Nuclear Chicago Actigraph III Strip Counter.

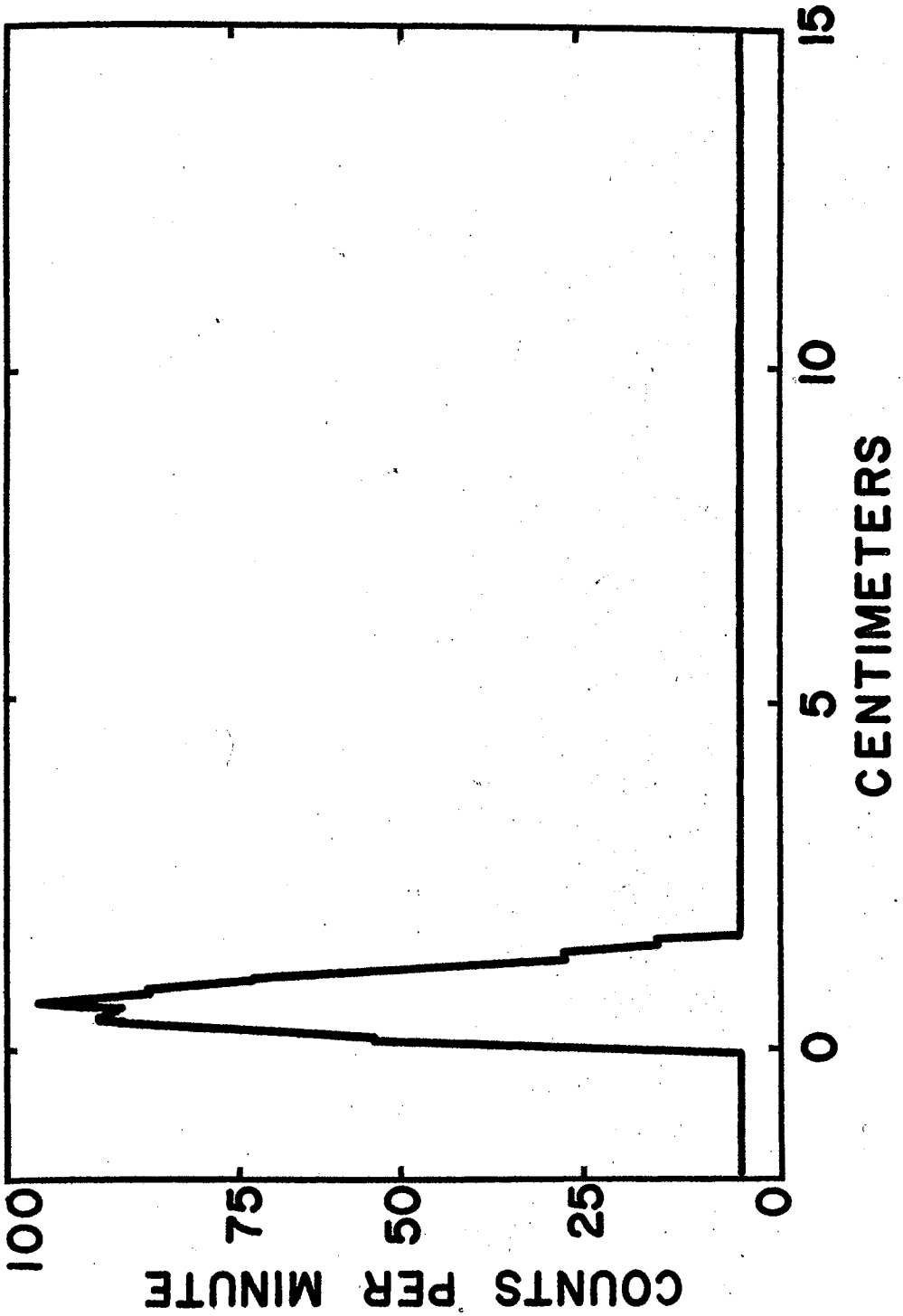

COUNTS PER MINUTE
100
75
50
25
0
0
5
10
15
CENTIMETERS

Figure 14. Distribution of Radioactivity in Rat Bile Following Silicic Acid Tlc in Chloroform-Methanol-Water (65:40:5). Retinoic acid, 5 μCi (sp act 2.5 μCi/mg), in 3.3% Tween 80 in 0.1 M sodium phosphate buffer, pH 7.4, was administered intraperitoneally and the bile collected through a cannula in the bile duct. An aliquot of bile was chromatographed and the radioactive area detected with a Nuclear Chicago Actigraph III Strip Counter.

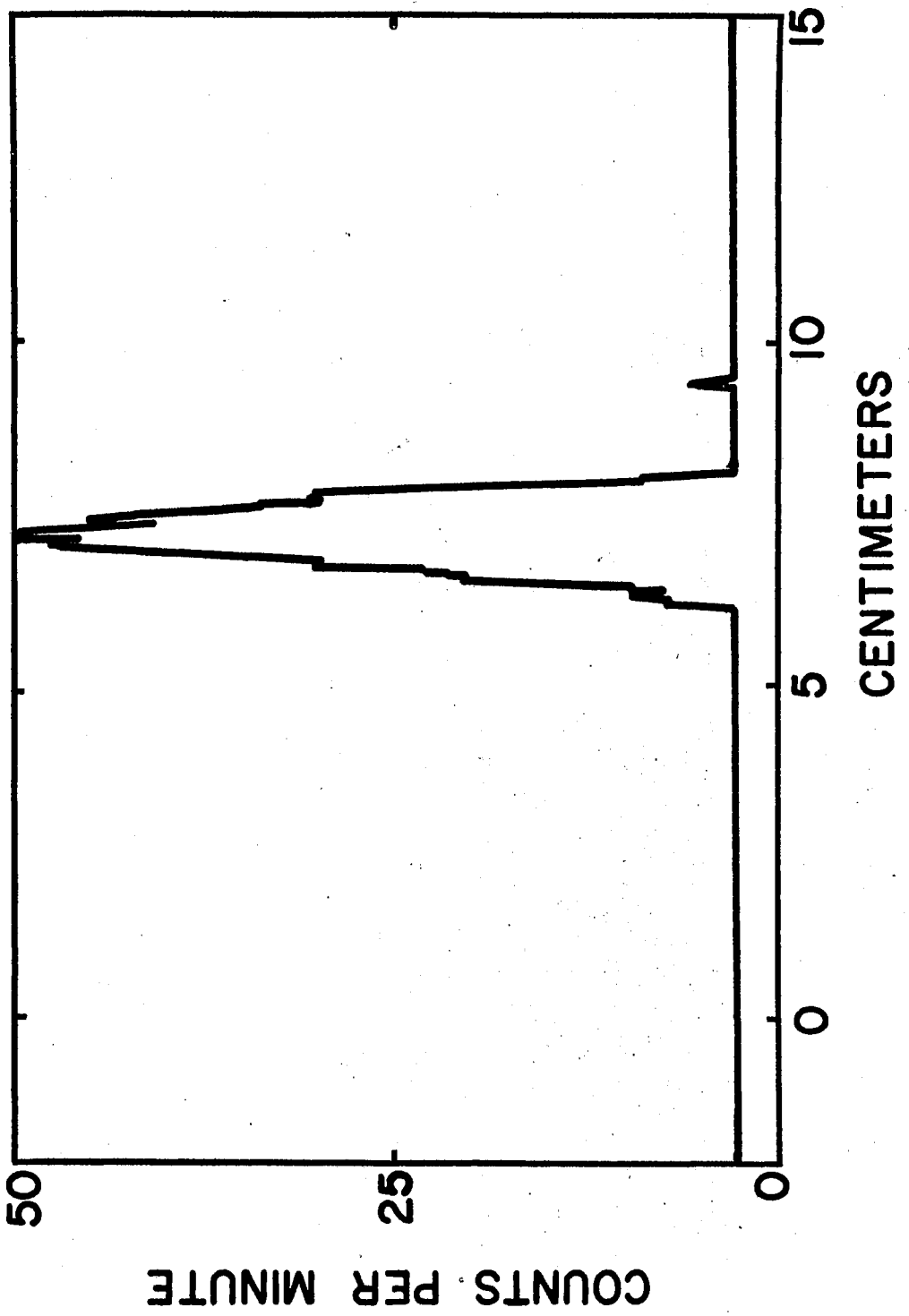

COUNTS PER MINUTE
50
25
0
0
5
10
15
CENTIMETERS

Figure 15. Distribution of Radioactivity in Rat Bile Upon Silicic Acid Tlc after Administration of Retinoic Acid-15-^{14}C and Hydrolysis with β-Glucuronidase. Retinoic Acid-15-^{14}C, 2 mg (sp act 2.5 μCi/mg), in 3.3% Tween 80 in 0.1 M sodium phosphate buffer, pH 7.4, was administered intraperitoneally and the bile collected by means of a bile duct cannula. An aliquot of bile containing about 100,000 DPM was incubated with 2 ml of a 1% suspension of β-glucuronidase in 0.1 M sodium acetate buffer, pH 4.5. For a control an identical aliquot of bile was incubated in 2 ml of buffer only. Four volumes of 0.4% acetic acid in acetone was added to each tube after 2 hr and the protein precipitated by centrifugation. The supernatant was reduced to a very small volume (less than 0.05 ml) and chromatographed in benzene-chloroform-methanol (4:1:1). Radioactive areas were detected with a Nuclear Chicago Actigraph III Strip Counter.

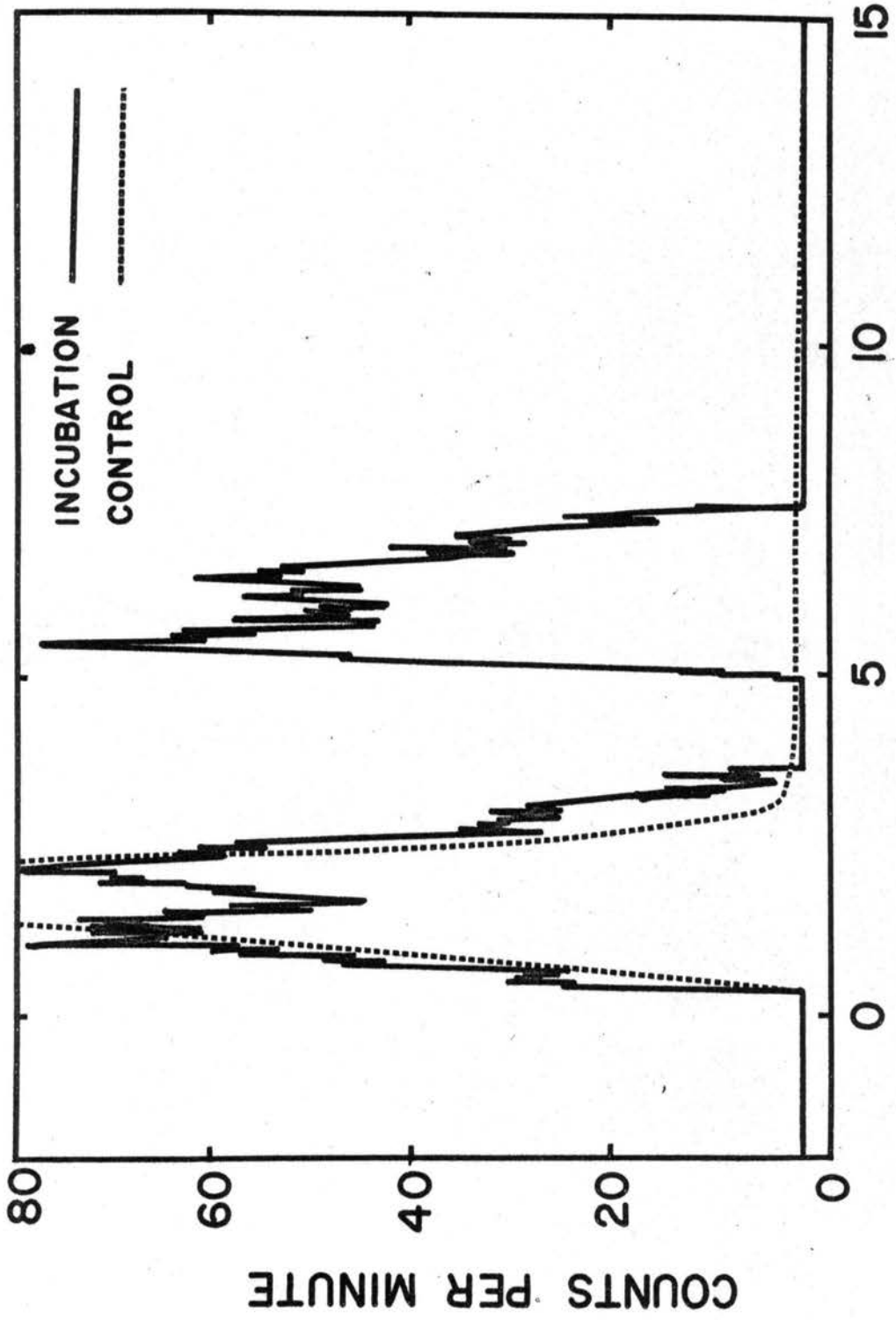
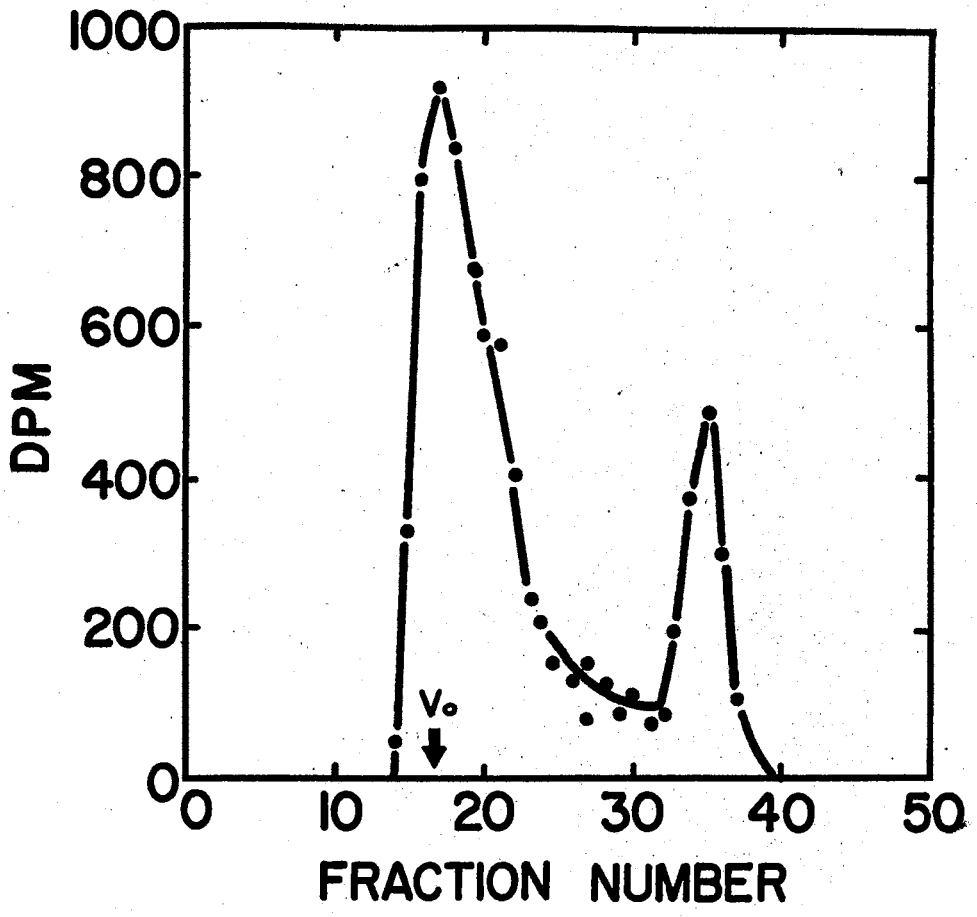

INCUBATION
CONTROL
COUNTS PER MINUTE
0
20
40
60
80
0
5
10
15
CENTIMETERS

Figure 16. Separation of Metabolites of Retinoic Acid in Rat Bile on a Bio-Gel P-2 Column. The 2.5 (i. d.) X 40 cm column was equilibrated and eluted with deionized water. Two milliliters of bile containing 750,000 DPM were chromatographed and 3 ml fractions were collected. Aliquots of 0.1 ml were assayed for radioactivity in a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer.


1000
800
600
400
200
0
DPM
V_o
0
10
20
30
40
50
FRACTION NUMBER

Elution of a sample of bile from a Sephadex G-25 column showed the major portion of the radioactivity in a fraction distinctly removed from the V_o (Fig. 17). This fraction was pooled, lyophilized, and dissolved in deionized water. It appeared that the fraction contained two peaks when it was chromatographed on a Bio-Gel P-4 column but the separation was not complete (Fig. 17). Chromatography on a Bio-Gel P-2 column completely resolved the radioactivity into two fractions (Fig. 18). The lower molecular weight fraction from the P-2 column was chromatographed on Sephadex G-10 but no greater purification was obtained (Fig. 18).

The fractions eluted from each column were independently rechromatographed on the column to insure that one peak was not formed from another during chromatography. The fractions were eluted with the same elution volumes (V_e) as previously and no new peaks were formed. Each of the fractions was lyophilized, dissolved in a small amount of methanol, and chromatographed on silica gel with benzene-chloroform-methanol (4:1:1) and chloroform-methanol-water (65:40:5). Both fractions had nearly identical R_f values in both tlc systems. It was concluded that gel filtration in deionized water did not cause artifact formation in the isolation of metabolites of retinoic acid from rat bile.

Purification and Characterization of the Lower Molecular Weight Fraction of Rat Bile From Bio-Gel P-2 Containing Metabolites of Retinoic Acid

The lower molecular weight fraction of several separations from the Bio-Gel P-2 column were pooled and lyophilized. After being dis-

Figure 17. Chromatography of Metabolites of Retinoic Acid-15-^{14}C in Rat Bile on Sephadex G-25 and Bio-Gel P-4. Both columns were equilibrated with deionized water. An aliquot of bile containing 750,000 DPM was placed upon the Sephadex G-25 column (2.0 (i. d.) X 37 cm) and the compounds eluted with deionized water. The peak containing the majority of the radioactivity was lyophilized and chromatographed on the Bio-Gel P-4 column (2.5 (i. d.) X 40 cm). Fractions of 5 ml were collected and 0.1 ml was assayed for radioactivity in a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer.

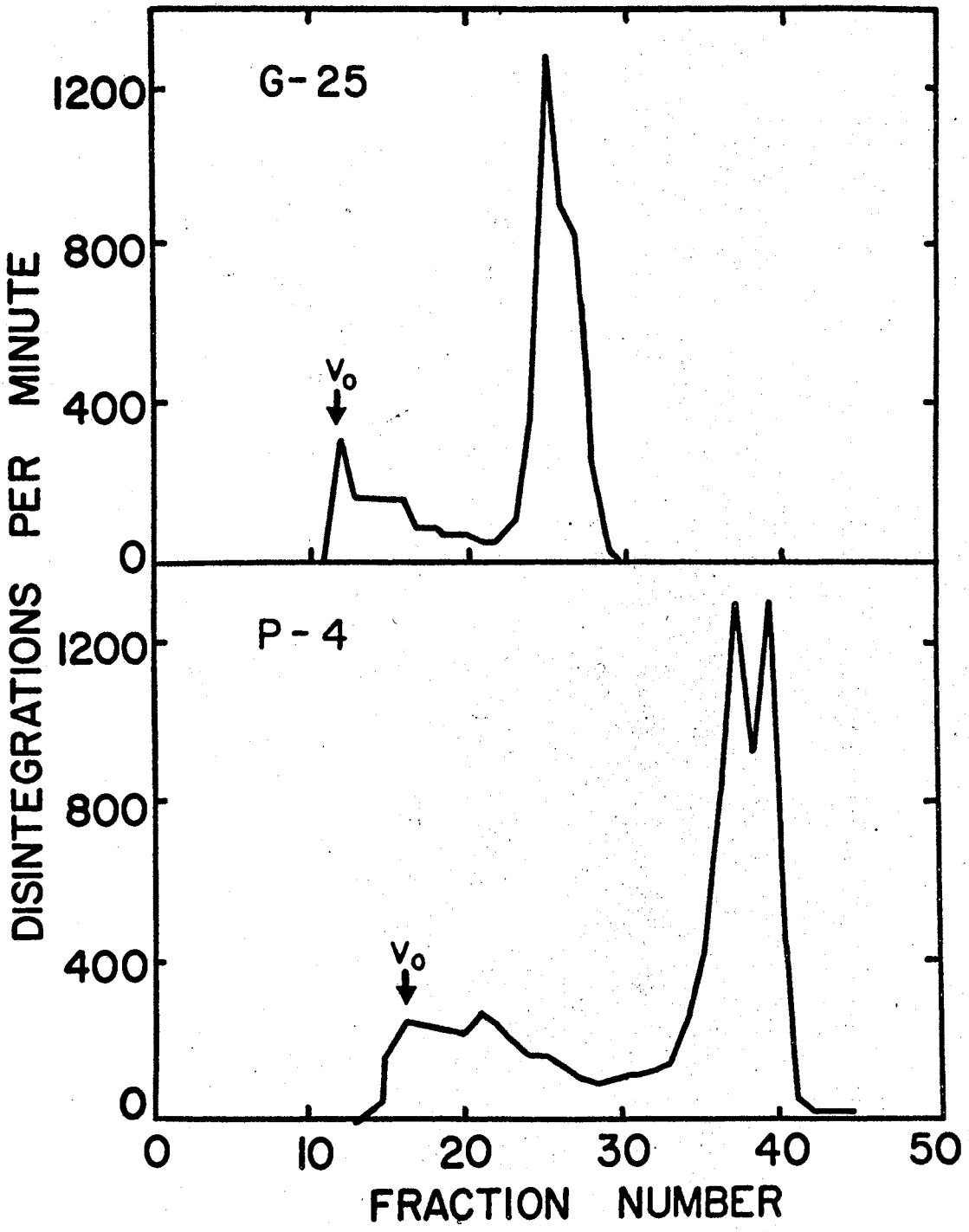
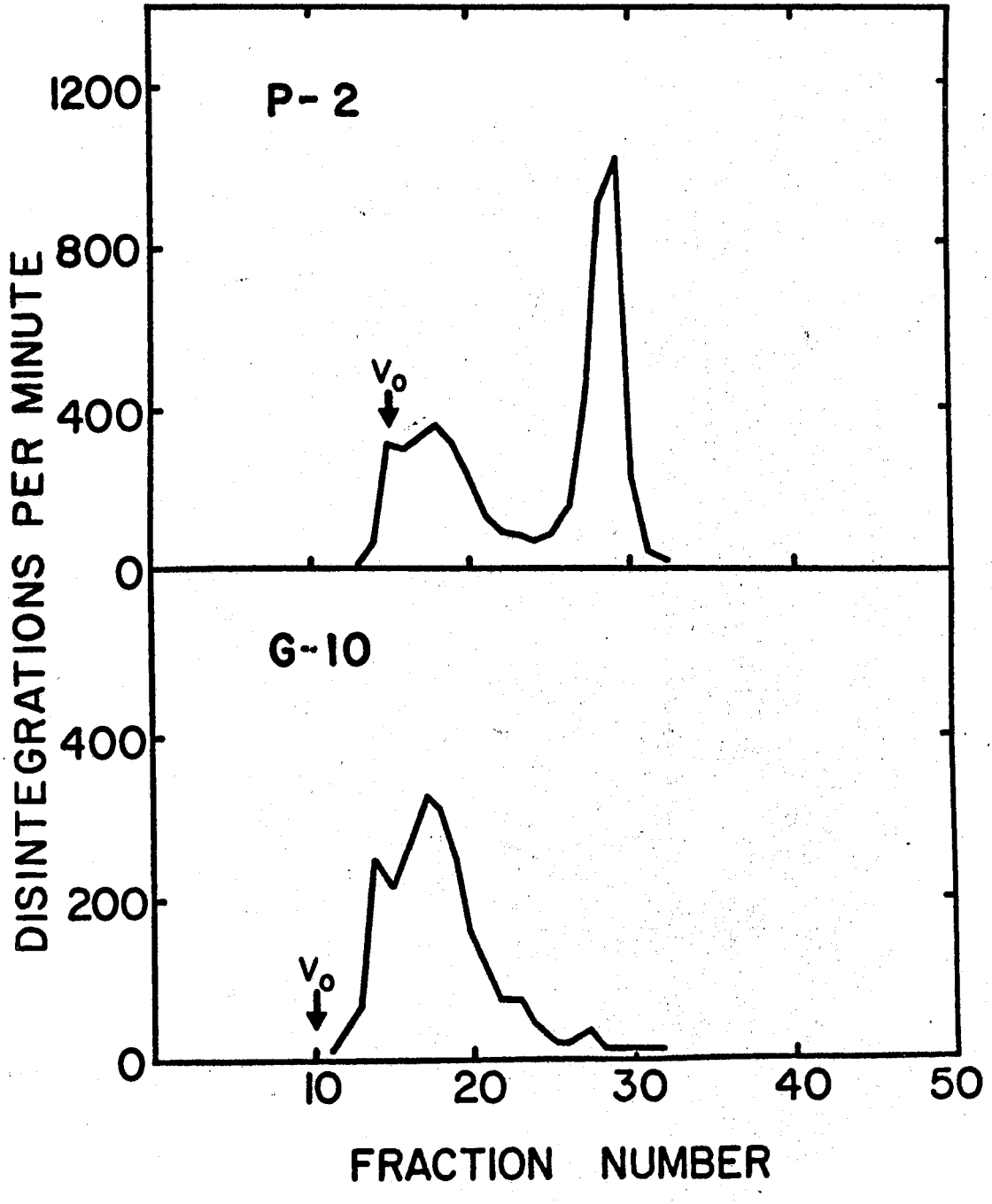

G-25
P-4
V_o
V_o
1200
800
400
0
1200
800
400
0
0
10
20
30
40
50
DISINTEGRATIONS PER MINUTE
FRACTION NUMBER

Figure 18. Chromatography of Metabolites of Retinoic Acid-15-^{14}C in Rat Bile on Bio-Gel P-2 and Sephadex G-10. The peak which contained the majority of the radioactivity from the Bio-Gel P-4 column was lyophilized and chromatographed on a Bio-Gel P-2 column. The lower molecular weight fraction from the Bio-Gel P-2 column was lyophilized and chromatographed on the G-10 column. The dimensions of the P-2 column were 2.5 (i. d.) X 40 cm, and those of the G-10 column were 2.0 (i. d.) X 30 cm. Both were equilibrated and eluted with deionized water. Five milliliter fractions were collected and 0.1 ml was assayed for radioactivity in a Packard Model 3320 Liquid Scintillation Spectrometer.


P-2
G-10
V_o
V_o
1200
800
400
0
400
200
0
10
20
30
40
50
DISINTEGRATIONS PER MINUTE
FRACTION NUMBER

solved in a small amount of methanol, the material was purified by silica gel tlc in chloroform-methanol-water (65:40:5). The radioactive area was detected with a gas flow strip counter and eluted with 95% ethanol. After the ethanol had been evaporated to less than 0.05 ml the sample was applied to another silica gel plate to be chromatographed in benzene-chloroform-methanol-acetic acid (5:5:5:1). This procedure of chromatographing and eluting the radioactive area was continued successively in benzene-chloroform-methanol (4:1:1), n-butanol-acetic acid-water (10:1:1), amyl acetate-acetic acid-n-propanol-water (40:30:20:10), and iso-amyl acetate-propionic acid-n-propanol-water (20:15:10:5). The R_f values for the radioactive area in these systems are listed in Table IV. A single radioactive peak was detected on each plate and the R_f values in the benzene-chloroform-methanol (4:1:1) and benzene-chloroform-methanol-acetic acid (5:5:5:1) systems were comparable to those of retinoyl β-glucuronide as reported by Dunagin et al. (69).

The uv absorption spectrum of the eluted fraction was recorded after each tlc separation. The λ_{max} of the spectrum was 360 nm and the λ_{max} of retinoic acid in 95% ethanol was 338 nm (Fig. 19). Although the spectra were not identical, the reaction of the metabolite on a thin layer plate with $SbCl_3$ yielded a positive reaction for retinoic acid.

A portion of the low molecular weight fraction from the Bio-Gel P-2 column was incubated with β-glucuronidase. Chromatography of the reaction mixture on silica gel in benzene-chloroform-methanol (4:1:1) gave a single radioactive peak. It had an R_f value of 0.53 which compared favorably with 0.56 for retinoic acid in the same system. The reaction product gave the same color as retinoic acid when the two

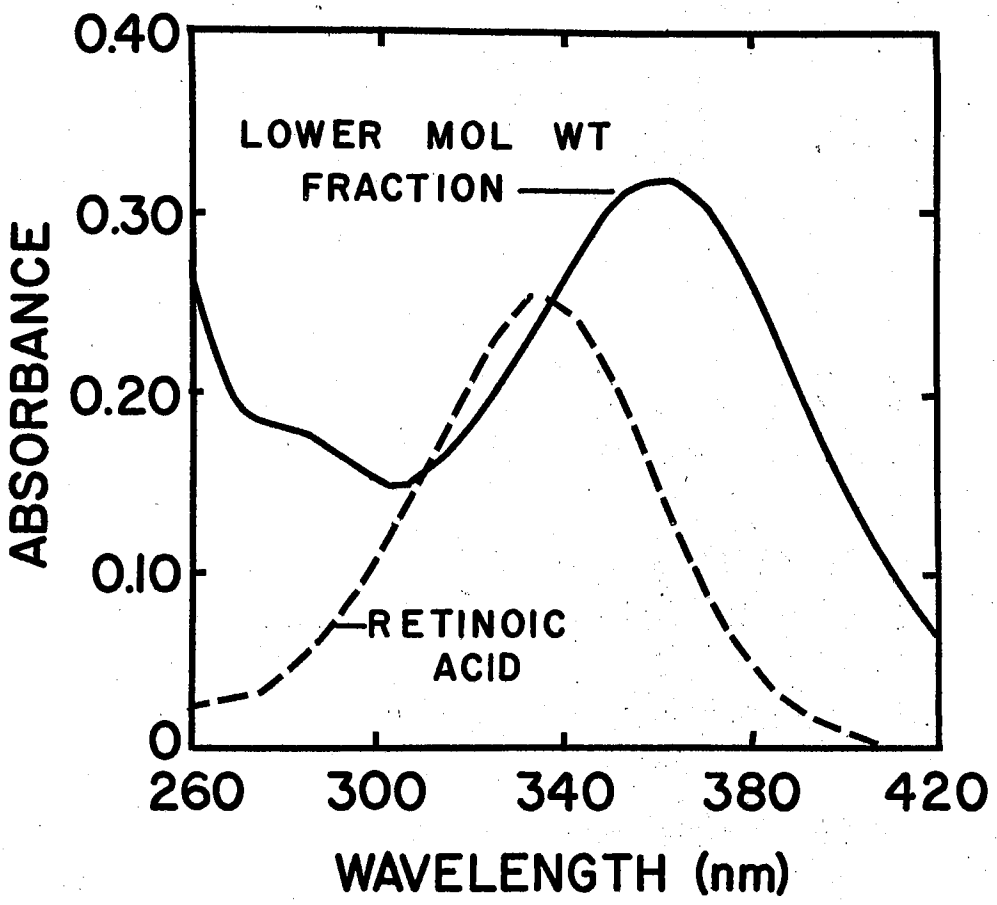
TABLE IV

SILICA GEL TLC[1] OF LOWER MOLECULAR WEIGHT FRACTION

Solvent System (v:v)	R_f
1. Chloroform-Methanol-Water (65:40:5)	0.47
2. Benzene-Chloroform-Methanol-Acetic Acid (5:5:5:1)	0.50
3. Benzene-Chloroform-Methanol (4:1:1)	0.13
4. n-Butanol-Acetic Acid-Water (10:1:1)	0.40
5. Amyl Acetate-Acetic Acid-n-Propanol-water (40:30:20:10)	0.38
6. Isoamyl Acetate-Propionic Acid-n-Propanol-Water (20:15:10:5)	0.48

[1] The lower molecular weight radioactive fraction from the Bio-Gel P-2 column was lyophilized and the dried material dissolved in methanol, before being applied to a thin layer plate of silica gel, and chromatographed in system no. 1. The radioactive area, as detected by a Nuclear Chicago Actigraph III Strip Counter, was eluted from the plate with 95% ethanol and applied to another plate to be developed in system no. 2, etc.

Figure 19. UV Absorption Spectra of Retinoic Acid and the Low Molecular Weight Metabolite of Retinoic Acid in Rat Bile. The spectra of the samples in 95% ethanol were recorded on a Cary Model 14 Spectrophotometer in quartz cells having 1 cm light paths.


0.40
0.30
0.20
0.10
0
ABSORBANCE
LOWER MOL WT
FRACTION
RETINOIC
ACID
260
300
340
380
420
WAVELENGTH (nm)

plates were treated with $SbCl_3$. These data suggested that retinoyl β-glucuronide was the radioactive metabolite present in the low molecular weight fraction from the Bio-Gel P-2 column and that it was probably the only metabolite in that fraction.

Glpc of Retinoyl β-Glucuronide

After retinoyl β-glucuronide was purified by gel-filtration chromatography and tlc an attempt was made to purify this component by glpc for analysis by mass spectrometry. The fraction was reacted with bis-(trimethylsilyl)acetamide and trimethylchlorosilane as described in the materials and methods section. It was apparent that reaction had taken place because the reaction product migrated farther on a tlc plate than did the unreacted product when both were chromatographed on silica gel in benzene-chloroform-methanol (4:1:1) (Fig. 20). A non-radioactive spot migrating behind the radioactive area was detected by iodine vapor.

The instrument and column used for glpc have been described in the materials and methods section. The temperatures (isothermal) were 250° and 300°C (column), 265° and 315°C (injector block), and 350° and 375°C (detector). No compounds were eluted from the column under these conditions.

Mass spectra of the reaction mixture were obtained using the direct inlet of the prototype of the LKB-9000 Mass Spectrometer-Gas Chromatograph. The total ion current tracing of mass spectra group no. 1887 is shown in Fig. 21 and it is apparent that the reaction mixture contained four or more compounds. All the fragment ions of mass spectra 1887-1 through 1887-5 were less than m/e 100, indicating

Figure 20. Silica Gel Tlc of the Low Molecular Weight Fraction from Bio-Gel P-2 after Reaction with Bis-(trimethylsilyl)-acetamide and Trimethylchlorosilane. Bis-(trimethylsilyl)-acetamide (0.2 ml) and trimethylchlorosilane (0.05 ml) were reacted with the fraction for 3 hr at 55°C. The liquid was evaporated from the vial and the residue dissolved in acetone to be chromatographed in benzene-chloroform-methanol (4:1:1). Radioactive areas were detected with a Nuclear Chicago Actigraph III Strip Counter.

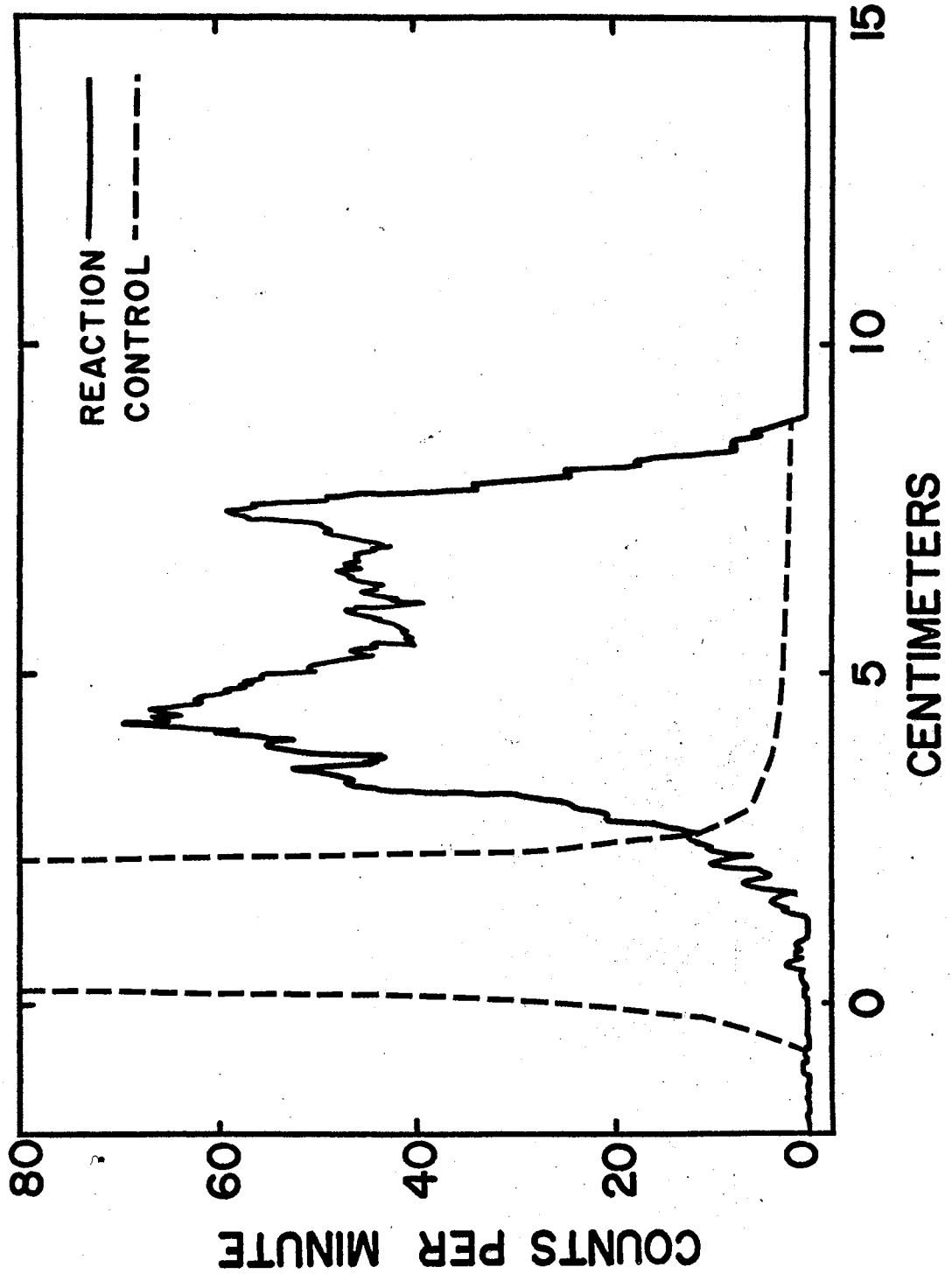

REACTION
CONTROL
COUNTS PER MINUTE
0
20
40
60
80
0
5
10
15
CENTIMETERS

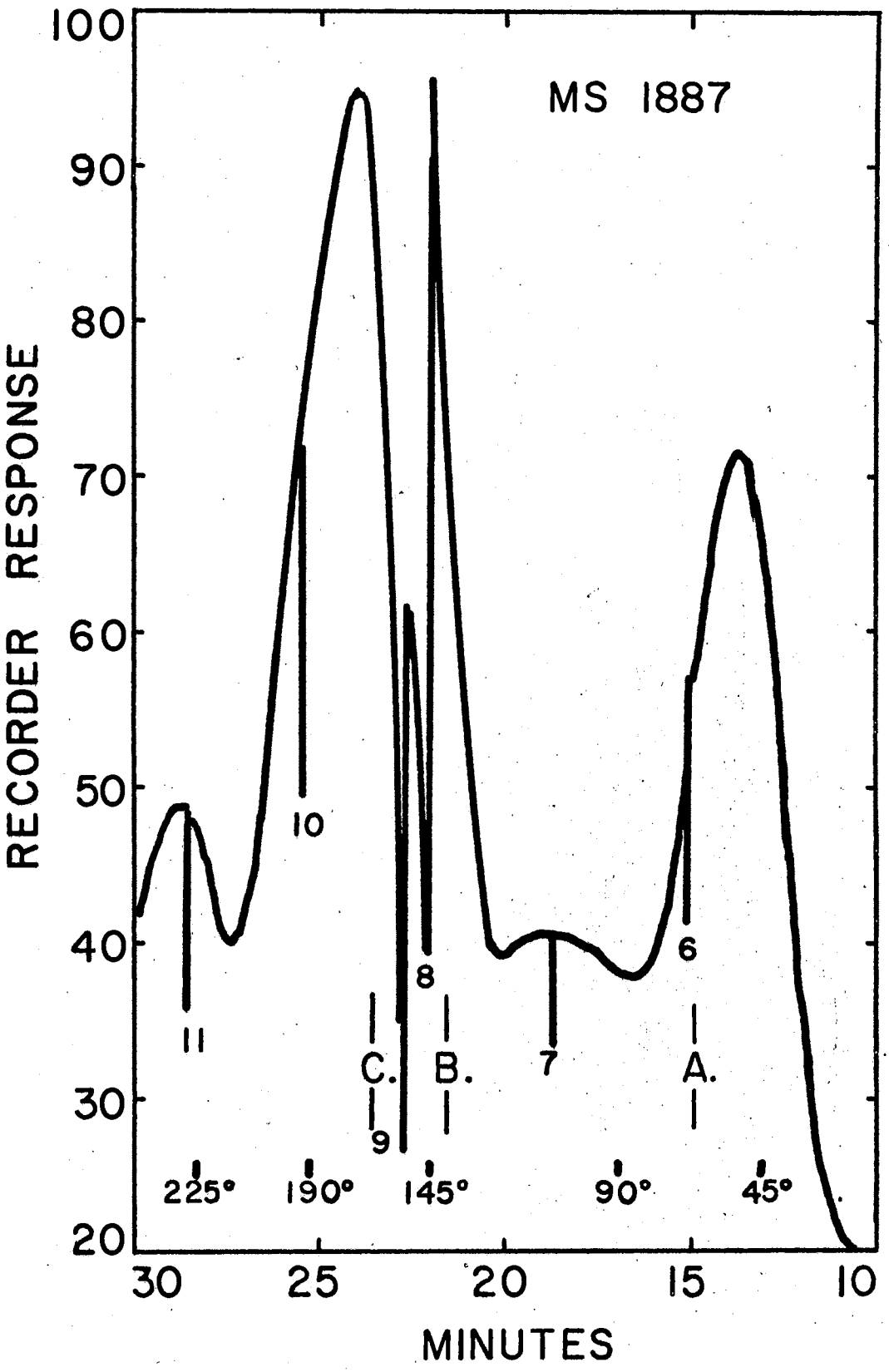
Figure 21. Total Ion Current (TIC) Tracing of the Low Molecular Weight Fraction after Silylation Using the Direct Inlet. Mass spectra were obtained on the prototype of the LKB-9000 Gas Chromatograph-Mass Spectrometer (84, 85) with the direct inlet being heated and mass spectra being taken at the numbered slashes. Those conditions which were held constant were: ionizing voltage, 70 eV; accelerating voltage, 3.5 kV; ion source 310°C; and the source pressure, 5×10^{-6} to 1×10^{-7} mm Hg. Those conditions which were varied:

-A. Trap current, 20 μA; electron multiplier HV, 1.7 kV; TIC-1.

A.-B. Trap current, 20 μA; electron multiplier HV, 1.7 kV; TIC-5.

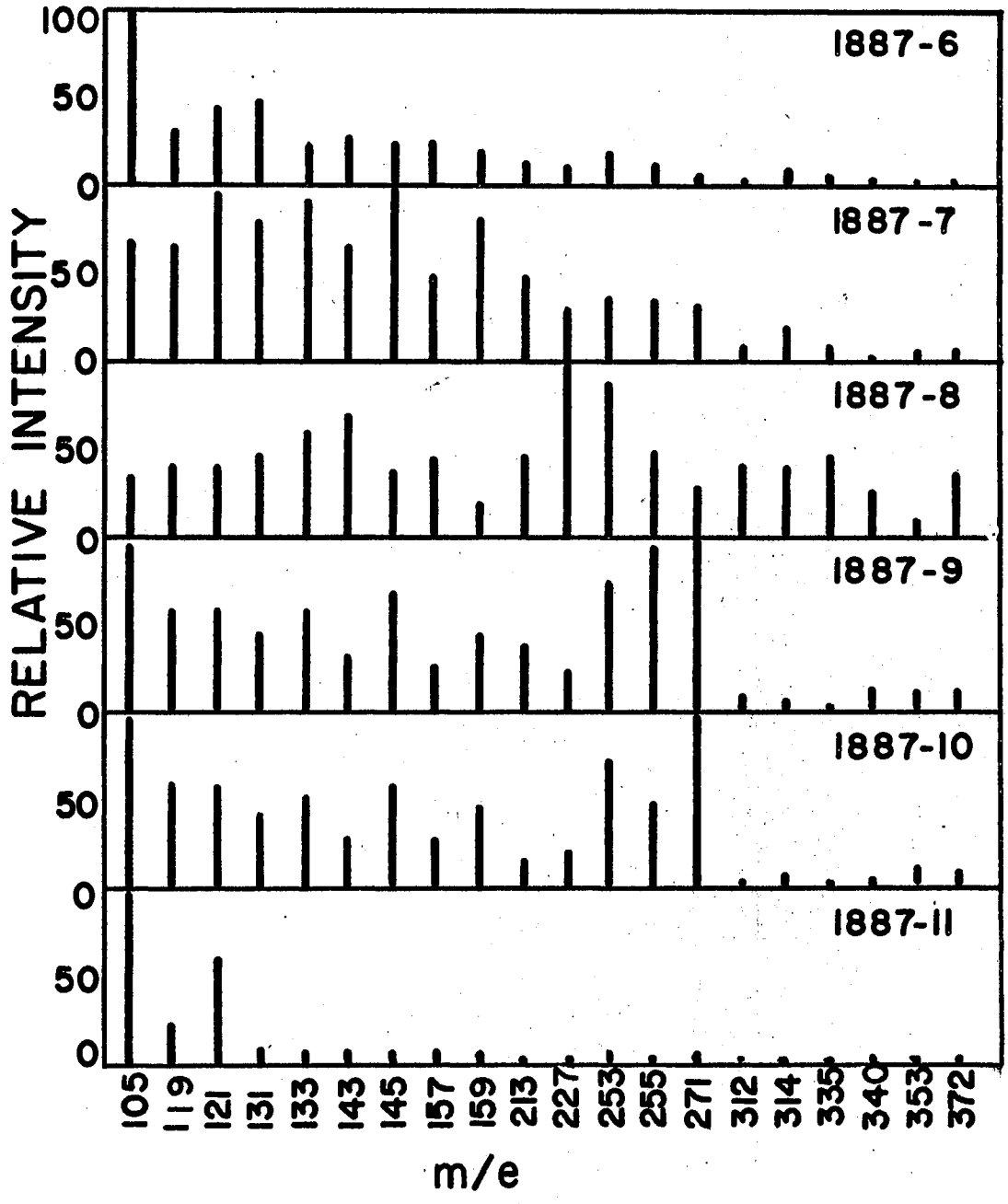
B.-C. Trap current, 65 μA; electron multiplier HV, 2.3 kV; TIC-2.

C.- Trap current, 20 μA; electron multiplier HV, 1.7 kV; TIC-2.


MS 1887
RECORDER RESPONSE
MINUTES
100
90
80
70
60
50
40
30
20
30
25
20
15
10
11
10
9
8
C.
B.
7
6
A.
225°
190°
145°
90°
45°

that they were due to solvent contamination, so they will be omitted from the discussion. However, a survey of some of the intense fragment ions from mass spectra 1887-6 through 1887-11 indicated some qualitative differences among the compounds. Some peaks which are of diagnostic value in the interpretation of the mass spectra of silyl ethers are M^+ - 90(loss of trimethylsilanol), M^+ - 72(loss of the trimethylsilyl group), M^+ - 18(loss of water), and M^+ - 15(loss of a methyl group). In the spectra under consideration all fragment ions greater than m/e 400 were of very low intensity and were not recorded under the conditions used to obtain the spectra. Among others, fragment ions m/e 105, 119, 145, 159, and 255 were chosen for comparison because they are some of the diagnostic peaks in the mass spectrum of retinoic acid (91). Fig. 22 shows that a prominent fragment ion common to all spectra was m/e 105, which represented the base peak in 1887-6 and had a relative intensity greater than 95% in 1887-9,-10, and -11. Mass spectra 1887-9 and 1887-10 had several prominent peaks of about equal intensities, i.e., m/e 105, 119, 121, 131, 133, 143, 157, 227, 253, and 271 (base peak). This result might be expected because spectra were taken on either side of the largest peak in the tracing and indicates that this peak probably represents a single compound. The small differences in relative intensities which were observed were probably the result of concentration differences and some overlapping from adjacent peaks. All the other spectra had different base peaks and were sufficiently different to indicate that they were from structurally different compounds, and not geometrical isomers. Although the reaction mixture contained several compounds the work done on it yielded important preliminary data which will be use-

Figure 22. Representative Peaks from the Mass Spectra of the Low Molecular Weight Fraction after Silylation. The mass spectra were obtained under the conditions described in Fig. 21.


RELATIVE INTENSITY
100
50
0
1887-6
1887-7
1887-8
1887-9
1887-10
1887-11
105
119
121
131
133
143
145
157
159
213
227
253
255
271
312
314
335
340
353
372
m/e

ful in mass spectral analysis of retinoyl β-glucuronide.

Characterization Studies of the Higher Molecular Weight Fraction from Bio-Gel P-2

The higher molecular weight fraction of a separation from a Bio-Gel P-2 column was lyophilized, 95% ethanol added to the dry residue, and the insoluble material was removed by centrifugation. Virtually all the radioactive material was soluble in ethanol. An uv absorption spectrum of the solution showed a λ_{max} at 338 nm (Fig. 23), which corresponds to that of retinoic acid. The ethanol was evaporated from the material and the residue was dissolved in deionized water. Upon being rechromatographed on the Bio-Gel P-2 column the radioactivity eluted in the same position as previously (Fig. 24).

The higher molecular weight fraction was hydrolyzed with 5% aqueous KOH at 70°C for 3 hr under a stream of nitrogen. Upon acidification of the solution about 40% of the radioactivity was extracted into petroleum ether. After the petroleum ether was evaporated the residue was chromatographed on silica gel in benzene-chloroform-methanol (4:1:1) and a single radioactive spot co-chromatographed with retinoic acid and gave a positive color test for retinoic acid with $SbCl_3$.

Discussion

The results of this study provide evidence that more than one conjugate of retinoic acid is secreted in rat bile. The metabolites in rat bile resulting from the administration of retinoic acid-15-^{14}C were chromatographed on a column of Bio-Gel P-2 which divided the radio-

Figure 23. UV Absorption Spectrum of the Higher Molecular Weight Fraction from Bio-Gel P-2. The spectrum of the fraction in 95% ethanol was recorded on a Beckman DB Spectrometer and a Sargent Model SRL Recorder using 3 ml quartz cells with a 1 cm light path.

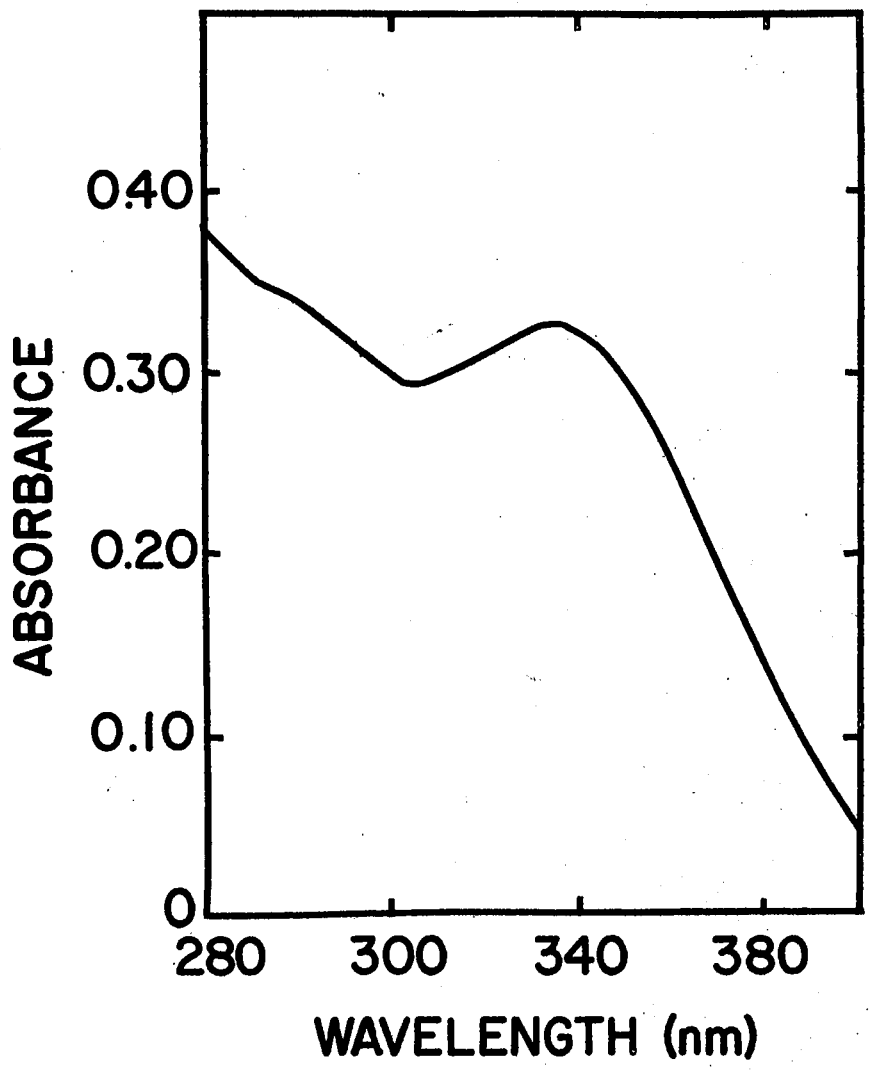
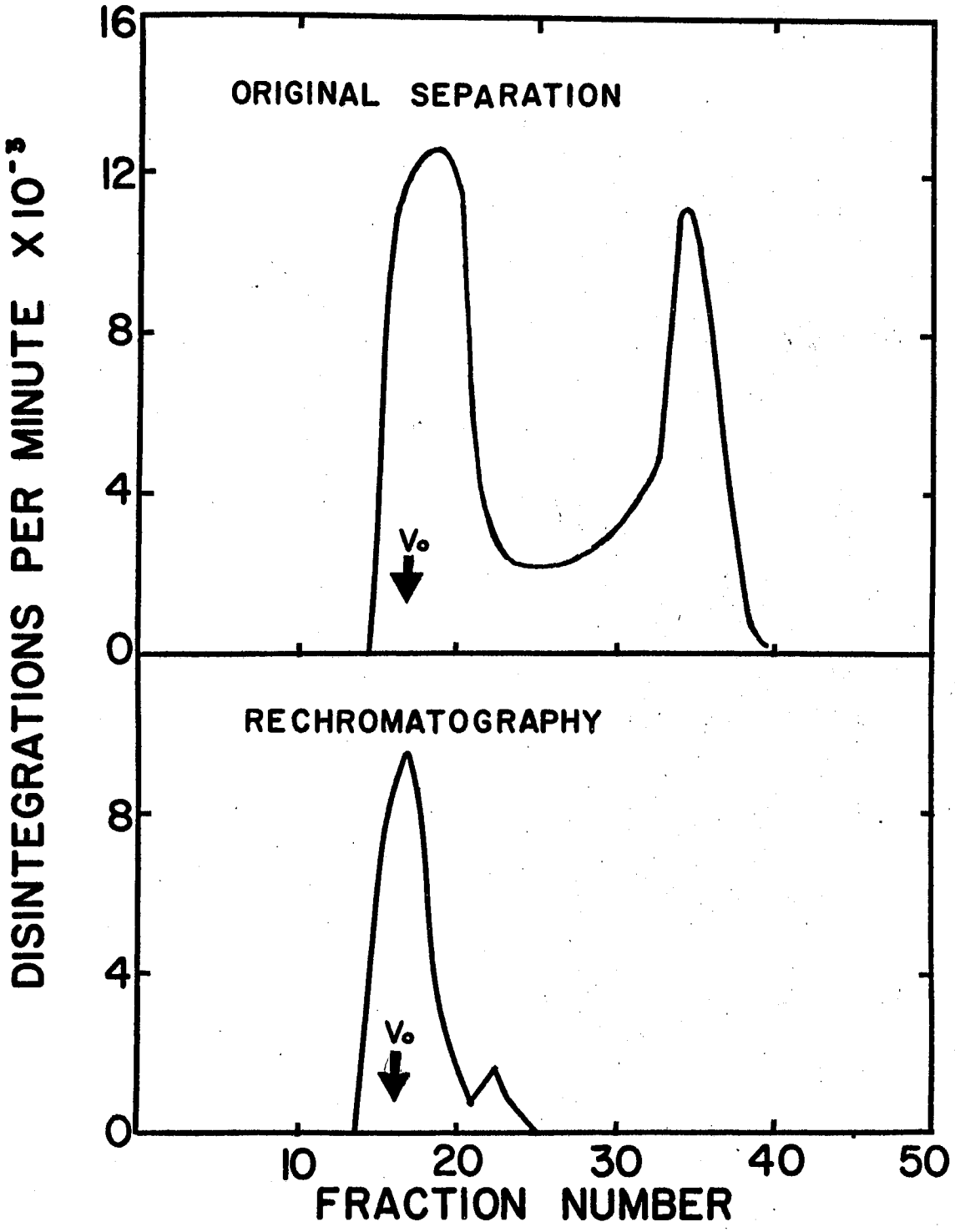

ABSORBANCE
0.40
0.30
0.20
0.10
0
280
300
340
380
WAVELENGTH (nm)

Figure 24. Separation of Metabolites of Retinoic Acid-15-^{14}C in Rat Bile and Rechromatography of the Large Molecular Weight Fraction on Bio-Gel P-2. The column, 2.5 (i. d.) X 40 cm, was equilibrated with deionized water. The large molecular weight fraction from the initial separation was s lyophilized and the dry material extracted with ethanol. The solvent was evaporated from the ethanol soluble material and the residue dissolved in 2 ml of deionized water and rechromatographed on the same column that was used for the initial separation. Fractions of 3 ml were collected and 0.1 ml was assayed for radioactivity in a Packard Model 3320 Tri-Carb Scintillation Spectrometer.


ORIGINAL SEPARATION
RECHROMATOGRAPHY
Vo
Vo
DISINTEGRATIONS PER MINUTE X 10⁻³
FRACTION NUMBER
0
4
8
12
16
10
20
30
40
50

activity into two fractions. The first fraction was eluted in the V_o and the second in the region of smaller molecular weight compounds. Although the exact molecular weight of the fraction from the V_o is not known, a certain range of molecular weights has been established by gel-filtration experiments. The exclusion limit of Sephadex G-25 is 5000 and that of Bio-Gel P-4 is 3600. The fraction was eluted from both columns in V_e much greater than the V_o which means that it is much smaller than the exclusion limit of these columns. Because the exclusion limit of Bio-Gel P-2 is 1800, the molecular weight of the fraction must be 1800 or larger but less than 3600. It is not known if there is more than one metabolite in the fraction but only small amounts of radioactivity were eluted in the V_o of the Sephadex G-25 and Bio-Gel P-4 columns, and so it is probable that a single compound contains the majority of the radioactivity. The proportion of the high molecular weight fraction to low molecular weight fraction varied from animal to animal, ranging from almost equal amounts in some to about twice as much high molecular weight material in others.

When the proteins of the fraction were precipitated with ethanol the radioactivity went into solution and the fraction retained its large molecular size (shown by gel-filtration on Bio-Gel P-2). The uv spectrum, $SbCl_3$ spot tests, and alkaline hydrolysis established that the fraction contains a conjugate of retinoic acid, and in view of its solubility in water it probably contains hydrophilic functional groups such as carboxyl and/or hydroxyl groups.

Gel-filtration chromatography was chosen for the isolation of the metabolites because it employs mild conditions and should not be as likely to cause artifact formation as solvent extraction and anion-

exchange chromatography. Also, because the technique separates compounds which differ in molecular weight it should supplement tlc on silica gel as a means of purification. Each peak was rechromatographed on the column from which it was eluted and in each case it reappeared in the same V_e with no other peaks being formed. Apparently no fraction was formed from another during isolation, or else the conversion was nearly 100% complete after the first passage through the column. The latter is probably not the case because both fractions migrated with the same R_f values upon tlc in chloroform-methanol-water (65:40:5), and this value was unchanged from that of the radioactive fraction of unfractionated bile. In short, none of the evidence indicated that artifacts were being formed on the gel-filtration columns.

The data accumulated from purification and characterization of the low molecular weight fraction indicate that it was retinoyl β-glucuronide. It was the only metabolite detected in the fraction. The λ_{max} of its uv absorption spectrum was observed to occur at a higher wavelength than that of retinoic acid and this has not been previously reported. The shape of the spectrum remained unchanged during the purification so it is believed to be that of the metabolite, although esters of organic acids generally absorb at wavelengths and intensities comparable to the parent acid (87).

Glpc would be a good method for purifying the glucuronide and the methodology necessary is probably available. Dunagin and Olson have reviewed the gas-liquid chromatography of retinol and related compounds (88). There are several reports in the literature of glpc of glucuronides and the fact that they are of high molecular weight

does not preclude their analysis but a separation temperature of 300°C is required. Ether and ester glucuronides are converted to either methyl ester-trimethylsilyl ethers or trimethylsilyl ester-ethers by reaction with diazomethane and hexamethyldisilazane-trimethylchlorosilane in pyridine or bis-(trimethylsilyl)acetamide and trimethylchlorosilane. Androstan-17-one-3α-yl-β-D-glucopyranosiduronic acid and 5-androstene-17-on-3-β-yl-β-D-glucopyranosiduronic acid were separated on SE-30 at 250° and on 2% NGS on Gas Chrom P at 240° (89). Separation of methyl ester-trimethyl ethers of steroid β-D-glucosiduronic acids from urine was accomplished by temperature programming from 150-300°C at 2° per minute on 1% SE-30 on 80-100 mesh Gas Chrom Q. Other packings tried were OV-1 and OV-17 liquid phases on acid-washed, base-washed, and silanized Gas Chrom P (90).

This investigation supports the findings of others that metabolites of retinoic acid secreted in the bile may be altered during isolation. Apparently artifacts were introduced by the solvent extraction procedure even though care was taken to avoid anion-exchange chromatography.

Lippel and Olson identified the cis- and all-trans isomers of methyl retinoate after the isolation of conjugates of retinoic acid on an anion-exchange column and presented evidence that they were formed by transesterification of retinoyl β-glucuronide on the column. A non-polar, non-ionic fraction, labeled PE II, was isolated in the present extraction procedure and, since methanol was not used, the presence of methyl retinoate would have been evidence that it is a metabolite of retinoic acid. The means of identifying methyl retinoate by glpc and mass spectrometry are available (91) so an attempt was

made to characterize the fraction. Although the compounds present in the fraction were not identified, it was established that methyl retinoate was not present. The R_f values of the fraction and methyl retinoate were similar in six different tlc solvent systems and basic hydrolysis confirmed that the fraction contained an ester, but the uv absorption spectrum of retinoic acid was not detected in the fraction and glpc-ms showed that none of the compounds was methyl retinoate. There was no evidence that the ethyl ester of retinoic acid had been formed during the extraction.

That fraction PE III contained retinoic acid was shown by tlc (Table I) and uv absorption spectrometry (Fig. 7). Retinoic acid could have been formed by the hydrolysis of retinoyl β-glucuronide in the acidic extraction media.

The material extracted into EtOEt II was probably identical to that extracted into PE II as indicated by tlc (Table I) and glpc.

Little is known of the compounds extracted into EtOEt III except that they are acidic and more polar than retinoic acid (Table I). Silicic acid chromatography was an aid in the purification of this fraction but the uv absorption spectrum of retinoic acid could not be detected.

The R_f values of fraction BuOH in two solvent systems were similar to those reported for retinoyl β-glucuronide (72) and it reacted with diazomethane and hexamethyldisilazane. Even the different behaviors of the fraction when treated with diazomethane for 30 minutes and 24 hr can be rationalized for a glucuronide, because a glucuronide could yield a methyl ester as well as a methylester-(mono-, di-, tri-) methyl ether derivative. Knaak *et al.* (92) reported difficulties in obtaining

only methyl esters of glucuronides when using diazomethane in methanol because it is a very active methylating reagent. The short time reaction product was probably the methyl ester and the long time reaction product was probably the methyl ester-methyl ether derivative. However, fraction BuOH was not hydrolyzed either in base or with β-glucuronidase and the uv absorption spectrum of retinoic acid was not detected.

Purification of fraction BuOH by silicic acid column chromatography was not successful as the elution patterns were not reproducible and tlc of the eluted compounds indicated that they were different from the compound originally in the fraction. It was also observed that the elution patterns changed as the sample was allowed to remain in storage for a longer period of time; if samples were stored two weeks in butanol before being chromatographed the radioactivity was not absorbed onto the column but was eluted in the solvent front. This is probably due to artifact formation by acid in the butanol solution. It was apparent, therefore, that the metabolites of retinoic acid secreted in chick bile are vulnerable to changes in an acidic environment.

It should be noted that the uv absorption spectrum of retinoic acid was detected in only one fraction. The concentration of retinoate conjugates in the other fractions was probably so low that their spectra were masked by the spectra of other compounds. The compounds being studied were probably conjugates of retinoic acid because they still retained the carboxyl carbon. The only well established degradation pathway of retinoic acid involves decarboxylation, and if this had occurred the metabolites could not have been detected since the

carboxyl carbon contained the radioactive label.

Additional data from studying the metabolism of retinoic acid-15-^{14}C in rats indicated that all of the fractions studied, except possibly fraction BuOH, were artifacts of the isolation procedure. Solvent extraction of radioactivity from rat bile caused it to be distributed in much the same way as with chick bile. On the other hand, tlc of unfractionated rat bile showed only a single radioactive spot, while the two major fractions from solvent extraction had different R_f values. The radioactive area of unfractionated bile migrated closely with that fraction extracted into butanol, and also with retinoyl β-glucuronide (72). The radioactive fraction of rat bile was partially hydrolyzed upon incubation with β-glucuronidase and $SbCl_3$ spot tests indicated that retinoic acid was present in both the unhydrolyzed product and the product of hydrolysis. These data, then, agree with the findings of others that retinoyl β-glucuronide is secreted in the bile (72).

Although two tlc systems showed a single radioactive spot from rat bile it was not possible to get 100% hydrolysis of the radioactive compounds with β-glucuronidase. This behavior suggested that bile contained two or more radioactive compounds which could not be resolved in the two solvent systems used.

CHAPTER III

ASSOCIATION OF RETINOIC ACID WITH SERUM ALBUMIN IN VIVO AND IN VITRO

Several investigators have demonstrated that retinoic acid manifests most of the functions of retinol. For many years the presence of retinoic acid in tissues could not be confirmed but ultimately it was recovered from blood plasma (62). Generally, lipids of blood plasma are transported bound to proteins (93) and, more specifically, free fatty acids and several other organic anions are transported by plasma albumin (94). Because of its structural similarity to fatty acids, it was hypothesized that retinoic acid was bound and transported by serum albumin; it was the purpose of this investigation to test this hypothesis.

Experimental

Materials and Methods

Retinoic Acid

Retinoic acid-15-^{14}C (sp act 59 μCi/mg) was a gift of F. Hoffmann-La Roche and Co. Ltd., Basle, Switzerland. Purity was determined by uv absorption spectroscopy and tlc. Non-radioactive crystalline retinoic acid was a gift from the same source and was purified by recrystallization from 2-propanol until the melting point was 178-180°C (cor).

The uv absorption spectrum and migration patterns on tlc indicated that it was pure.

Reagents

n-Heptane was purchased from Phillips Petroleum Co., Bartlesville, Oklahoma, redistilled and the first and final 10% of the distillate were discarded.

Bovine serum albumin, type F (fatty acid content < 0.01%), and cytochrome C were purchased from Sigma Chemical Company, St. Louis, Missouri. β-Lactoglobulin was a gift of Dr. Kurt Ebner, Dept. of Biochemistry, Oklahoma State University.

Acid-citrate-dextrose (ACD) solution was prepared by dissolving 1.46 g of anhydrous dextrose, 1.37 g of sodium citrate dihydrate, and 0.48 g of citric acid monohydrate (or 0.44 g anhydrous citric acid) in 100 ml of deionized water. One milliliter of ACD solution was used as anticoagulant for 5 ml of blood.

The buffered salt solution of Spector et al. (95) containing 0.116 M NaCl, 0.0049 M KCl, 0.0012 M $MgSO_4$, and 0.016 M sodium phosphate, pH 7.4, was used for all incubations. It is referred to as the "phosphate-buffered salt solution."

Antimony trichloride reagent was prepared as described in Chapter II.

Methods

Chromatography. Thin layer plates and procedures were as described in Chapter II.

Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, New

Jersey) was equilibrated in 0.05 M potassium dihydrogen phosphate-disodium hydrogen phosphate buffer, pH 7.6, 0.2 M in NaCl. The column was 2.6 (i. d.) X 56 cm. The proteins used for molecular weight references were cytochrome C, β-lactoglobulin, bovine serum albumin. Blue Dextran 2000 (mol wt 2 X 10^6) was eluted to determine void volume. Two milliliter fractions were collected and monitored for radioactivity and for uv absorption at 280 nm.

Preparation of blood plasma. Blood was collected in ACD solution, cooled 30 minutes and centrifuged at 1000 X g for 30 minutes. The plasma was decanted and stored at -15°C.

Incubation. Incubation flasks similar to those described by Spector et al. (95) were used. Screwcapped culture tubes (Kimax, No. 9825), 15 X 125 mm, were shortened to 6.5 cm in overall length and the end closed with a flat seal. A sampling tube, 6.25 mm (o. d.), was fitted with a slot 2.5 mm long in both sides and fused to the base of the shortened culture tube. A no. 0000 cork stopper was placed in the sampling tube before the reagents were added to the flask and, as a result, almost all the liquid was excluded from the sampling tube during incubation. After the cork stopper was removed a portion of the aqueous layer rose into the sampling tube and allowed the aqueous layer to be sampled without contamination by the organic phase. The flasks were capped with teflon-lined screw caps during the incubation.

Bovine serum albumin was dissolved in phosphate-buffered salt solution (30.3 - 606 μM) and retinoic acid-15-^{14}C, sp act 1.034 X 10^7 DPM/mg, was dissolved in heptane (67 nM to 0.33 mM). One milliliter of the serum albumin solution was placed in the incubation flask and 1 ml of retinoic acid solution was layered above this phase. The

flasks were incubated at 37°C under nitrogen for 16 hr and then samples were taken from each phase for radioactivity determinations.

Calculations. The molecular weight of bovine serum albumin used for all calculations was 66,000. Disintegrations per minute were converted to molar concentrations. A partition ratio, defined as the concentration of retinoic acid in the aqueous phase divided by its concentration in the heptane phase at equilibrium, was determined by incubating 12 flasks which contained 1 ml of phosphate buffered salt solution and 1 ml of retinoic acid solution (2.67 μM to 0.33 mM). A constant partition ratio of 0.03338 $\pm$ 0.00328 was obtained and was used to determine the concentration of unbound retinoic acid in the bovine serum albumin phase from the concentration of retinoic acid in the heptane phase. The concentration of bound retinoic acid was ascertained by subtracting the unbound from the total amount in the aqueous phase. The average number of moles of retinoic acid bound per mole of albumin ($\bar{v}$) was determined. The calculations were done using a computer program (courtesy Dr. H. Olin Spivey) which required as its input data the counts per minute, sp act of the ligand, and protein concentration.

The law of mass action concerning the reversible binding of ligands to proteins at a single site was rearranged by Scatchard (96) to give the equation

$$\bar{v}/c = k(n - \bar{v})$$

When k, the apparent association constant, is constant the plot of $\bar{v}/c$ against $\bar{v}$ is a linear relationship in which the slope is -k, the intercept on the $\bar{v}$axis is the maximum number of moles of ligand bound per mole of protein (n), and the intercept on the $\bar{v}/c$ axis is the

intrinsic association constant, nk. However, if more than one class of binding sites having different association constants exist the slope is a composite of the association constants, the intercept on $\bar{v}$ is the sum of all molecules bound at all sites, Σn_i, the intercept on $\bar{v}/c$ is a sum of the intrinsic association constants, $\Sigma n_i k_i$, and the relationship of $\bar{v}/c$ to $\bar{v}$ is non-linear.

Miscellaneous. Radioactivity was monitored with a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3320, using the scintillation solvent of Bray (81). Eluants from chromatography columns were collected with a Buchler model 3P-4002 fraction collector equipped with a volumetric dispensing head (Buchler Instruments, Inc., Fort Lee, New Jersey).

Results

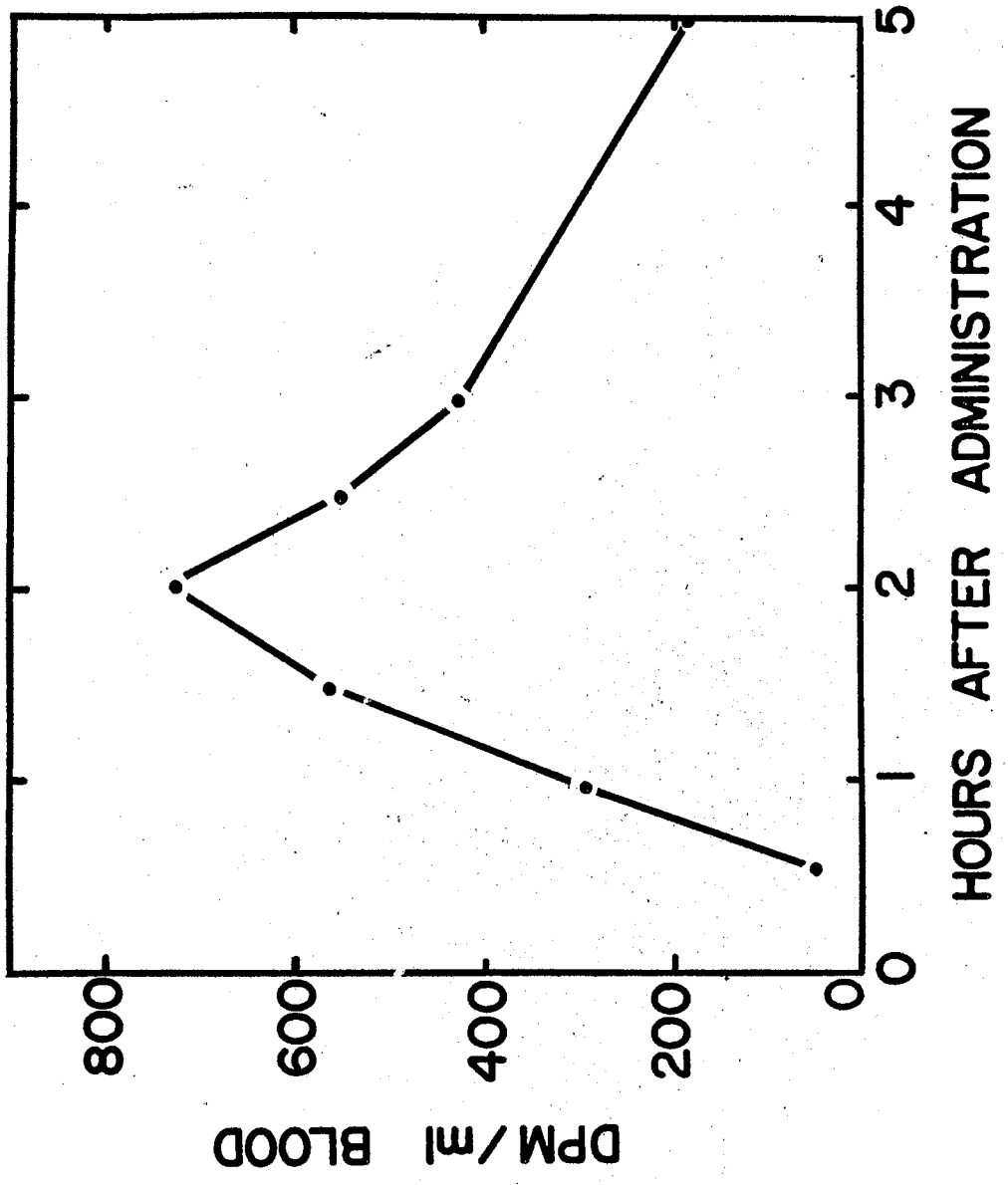
Appearance of Radioactivity in Blood After Administration of Retinoic Acid-15-^{14}C

A 250 g male albino rat was intraperitoneally administered 84 µg (5 µCi) of retinoic acid-15-^{14}C in 0.2 ml of 95% ethanol. Blood was collected from the tail at intervals and the plasma prepared for assay of the radioactivity. The maximum concentration of radioactivity appeared after about 2 hr (Fig. 25) and in subsequent experiments blood was collected 2 hr after administration of dose.

Identification of the Radioactive Component of Blood Plasma

One milliliter of blood plasma, containing 200,000 DPM, was obtained from a rat which had been given 30 µCi of retinoic acid-15-^{14}C

Figure 25. Concentration of Radioactivity in Rat Blood Plasma after Administration of Retinoic Acid-15-^{14}C. A 250 g male albino rat was intraperitoneally administered 84 µg (5 µCi) of retinoic acid-15-^{14}C in 0.2 ml of 95 % ethanol. Blood was collected from the tail into ACD solution and blood plasma was assayed for radioactivity in a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer.


DPM / ml BLOOD
800
600
400
200
0
0
1
2
3
4
5
HOURS AFTER ADMINISTRATION

in 0.2 ml of 95% ethanol intraperitoneally. An equal volume of ethanol was added and the pH lowered to 1 with 2N HCl. The solution was extracted with one volume of petroleum ether for five minutes and about 75% of the radioactivity was recovered. The petroleum ether was decanted and reduced to a very small volume before being applied to a tlc plate coated with silica gel. The plate was developed in benzene-chloroform-methanol (4:1:1) while a sample of retinoic acid was chromatographed on another plate. The radioactivity chromatographed as a single peak with the same R_f as retinoic acid and the two compounds gave identical color reactions when treated with $SbCl_3$.

Gel-Filtration Chromatography of Rat Blood Plasma Proteins

A column of Sephadex G-100 was prepared and calibrated for molecular weight determination (97) (Fig. 26). One milliliter of rat plasma containing 120,000 DPM was chromatographed on the column and the elution profiles of the uv absorbance at 280 nm and radioactivity are shown in Fig. 27. The radioactivity eluted in a V_e almost identical to that of the V_e bovine serum albumin.

Binding of Retinoic Acid-15-^{14}C to Bovine Serum Albumin

One milliliter of a solution of bovine serum albumin (606 µM) was incubated with retinoic acid-15-^{14}C in 1 ml of heptane as described by Spector et al. (95). The aqueous layer was then chromatographed on a Sephadex G-100 column and the uv absorbance at 280 nm and radioactivity were monitored. Fig. 28 shows that the retinoic acid was eluted bound to serum albumin.

Figure 26. Estimation of Molecular Weight of the Radioactive Component of Rat Blood Plasma after Administration of Retinoic Acid-15-^{14}C. Sephadex G-100 was equilibrated in 0.05 M potassium dihydrogen phosphate-disodium hydrogen phosphate buffer, pH 7.6, 0.2 M in NaCl, at 4°C and allowed to settle to a height of 56 cm in a 2.6 cm (i. d.) column. The proteins used for mol wt references were cytochrome C (1), β-lactoglobulin (2), and bovine serum albumin (3). Blue Dextran 2000 (4, mol wt 2 X 10^6) was eluted to determine V_o. One milliliter of rat blood plasma containing 120,000 DPM was chromatographed separately on the same column and the elution volume of the major radioactive peak is indicated by the arrow. The column was eluted at 4°C with the same buffer that was used for equilibration and 2 ml fractions were collected. Ultraviolet absorbance at 280 nm was monitored with a Beckman Model DB Spectrophotometer and radioactivity was assayed with a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer.

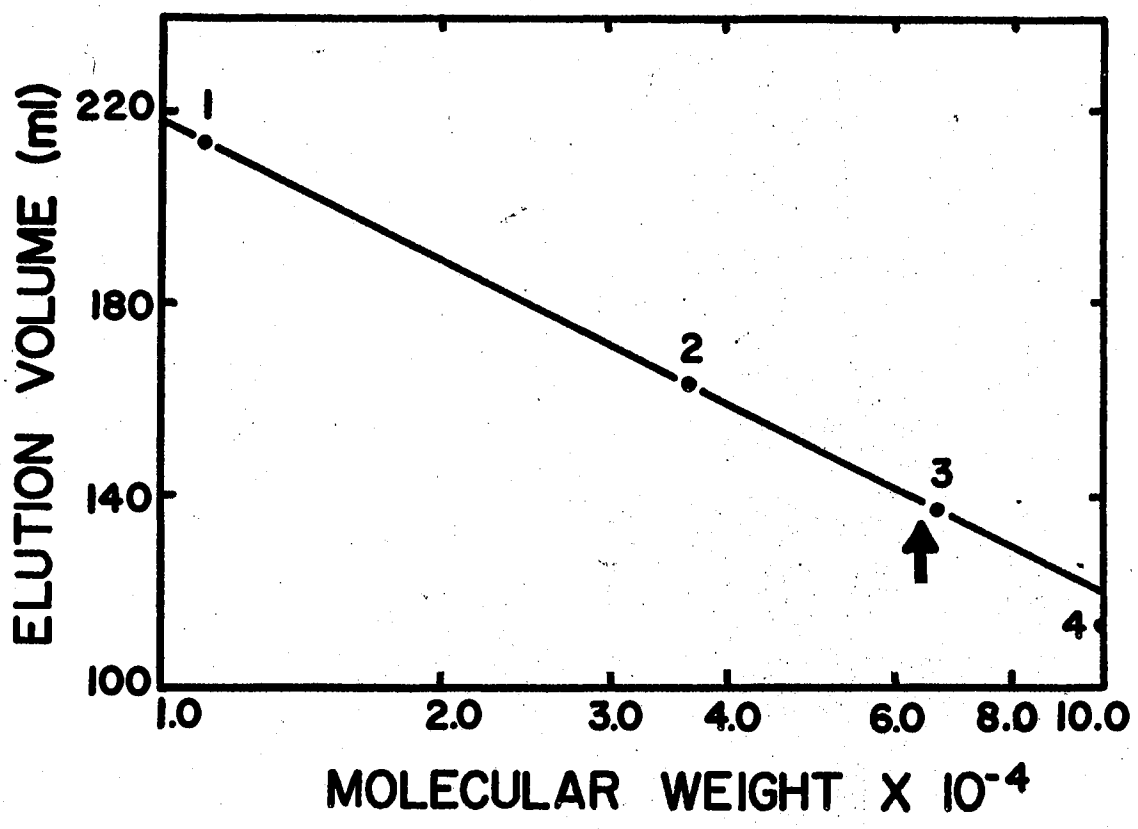

ELUTION VOLUME (ml)
220
180
140
100
1
2
3
4
1.0
2.0
3.0
4.0
6.0
8.0
10.0
MOLECULAR WEIGHT X 10^{-4}

Figure 27. Chromatography of Rat Blood Plasma on Sephadex G-100 after Administration of Retinoic Acid-15-^{14}C. The animal was administered intraperitoneally 84 μg (30 μCi) of retinoic acid-15-^{14}C in 95% ethanol. Blood was collected after 2 hr by anesthesizing the animal and withdrawing blood from the heart with a syringe. One milliliter of blood plasma, containing 120,000 DPM, was applied to the column and eluted with 0.05 M potassium dihydrogen phosphate-disodium hydrogen phosphate buffer, pH 7.6, 0.2 M in NaCl, at 4°C. Two milliliter fractions were collected and monitored for uv absorbance at 280 nm with a Beckman Model DB Spectrophotometer and radioactivity was assayed with a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer.

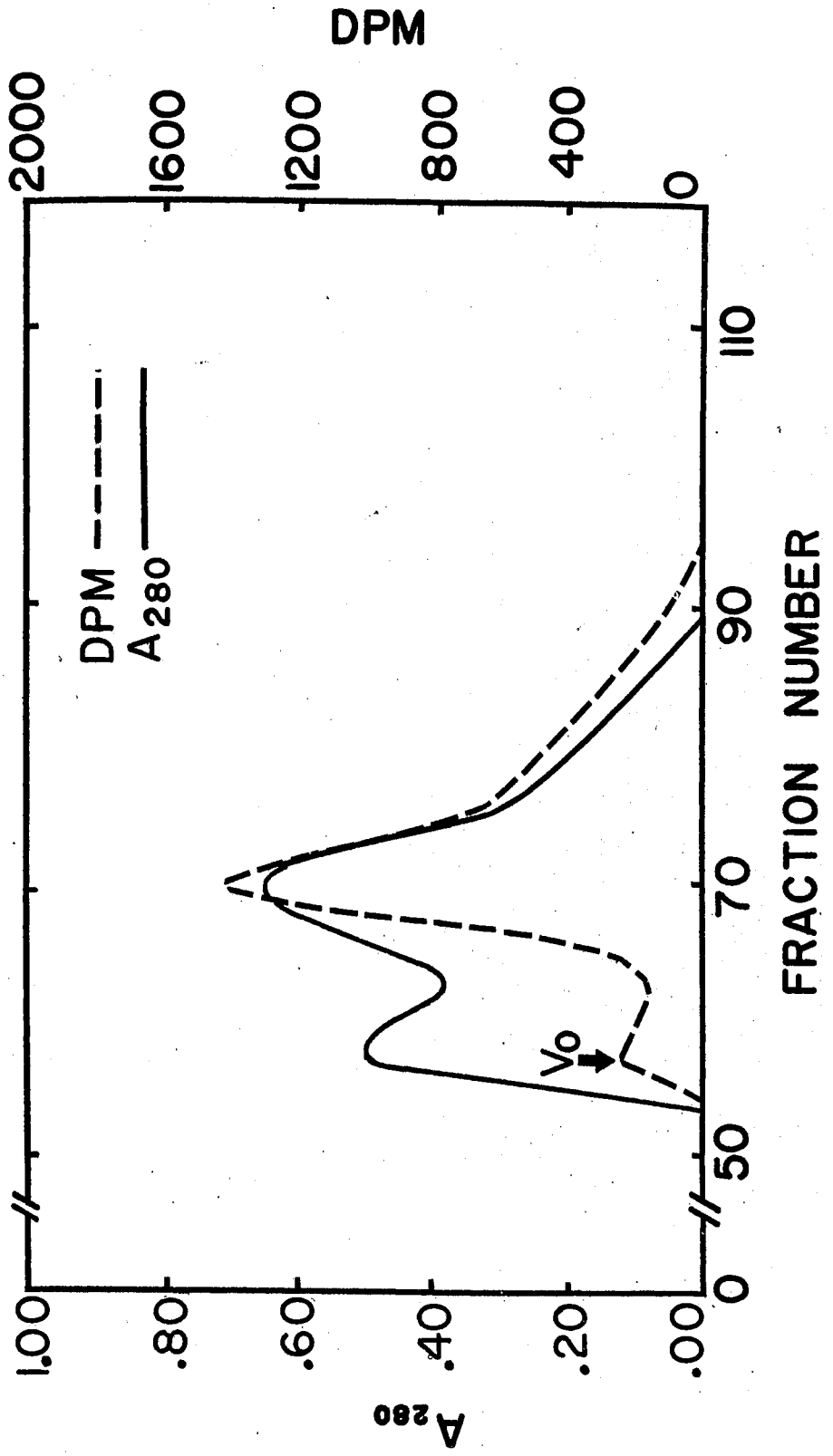

DPM
2000
1600
1200
800
400
0
110
90
70
50
0
DPM
A_{280}
V_o
FRACTION NUMBER
1.00
.80
.60
.40
.20
.00
A_{280}

Figure 28. Gel-Filtration Chromatography on Sephadex G-100 of Bovine Serum Albumin after Incubation with Retinoic Acid-15-^{14}C. Bovine serum albumin (40 mg) in 1 ml of phosphate buffered salt solution, pH 7.4, was incubated with 300 µg of retinoic acid-15-^{14}C (66,000 DPM) in 1 ml of heptane for 18 hr at 37°C as described by Spector et al. (95). The aqueous phase was chromatographed on a Sephadex G-100 column which had been equilibrated with 0.05 M potassium dihydrogen phosphate-disodium hydrogen phosphate buffer, pH 7.6, 0.2 M in NaCl, at 4°C. The column was eluted with the same buffer and 2 ml fractions were collected. Absorbance at 280 nm was monitored with a Beckman Model DB Spectrophotometer and radioactivity was assayed with a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer.

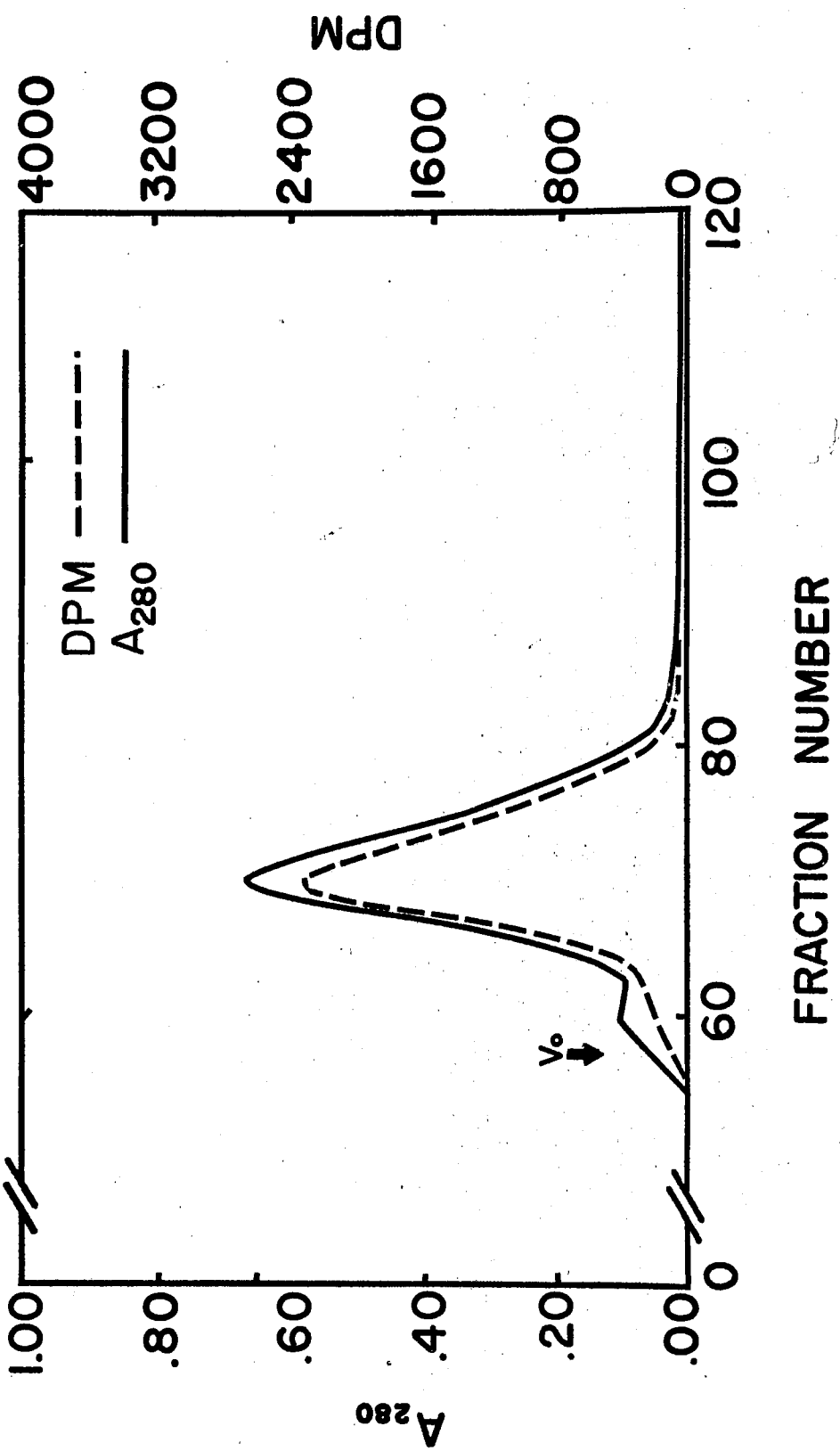

DPM
4000
3200
2400
1600
800
0
120
100
80
60
0
DPM
A_{280}
V°
FRACTION NUMBER
A_{280}
1.00
.80
.60
.40
.20
.00

Fig. 29 contains data (Table V) obtained from retinoic acid-15-^{14}C binding to bovine serum albumin in phosphate-buffered salt solution at 37°C, pH 7.4. The plot, in which $\bar{v}$ is plotted against $\bar{v}/c$, was recommended by Scatchard (96). The data were obtained using three different concentrations of bovine serum albumin (606 μM, 303 μM, 30.3 μM) but similar concentrations of retinoic acid. Although the data from the three experiments were not in good agreement they did demonstrate a non-linear relationship of $\bar{v}/c$ to $\bar{v}$, and suggest that bovine serum albumin contains more than one class of retinoate binding sites.

In analyzing the data the assumptions were made that bovine serum albumin contains two independent classes of binding sites for retinoic acid, that the number of binding sites in each class is static, and that the binding of each molecule of ligand is independent of the binding of all others. The data obtained with a protein concentration of 30.3 μM was used to estimate the number of binding sites in each class and their apparent association constants. A tangent was drawn to the curve from $\bar{v}=1$ on the abscissa and it intersected the ordinate at $\bar{v}/c=2.2 \times 10^5\ M^{-1}$, which would be an estimate of the apparant association constant for the higher energy class if it contained only one binding site. By assuming that the lower energy class contained a large number of binding sites, i.e. 10, a value of $1 \times 10^4\ M^{-1}$ for its apparent association was calculated. It was not possible to approach saturation of albumin with ligand in any of the experiments due to the limited solubility of retinoic acid in buffer, and consequently, the maximum attainable value for $\bar{v}$ was a deterrent in establishing values for the parameters.

Figure 29. Scatchard Plots of Data Obtained from Retinoic Acid Binding to Bovine Serum Albumin. Equilibrium partitioning of retinoic acid-15-^{14}C between 1 ml of heptane and 1 ml of phosphate-buffered salt solution containing bovine serum albumin was performed at 37°C and pH 7.4. Protein concentrations were 606 μM (■-■-■-■), 303 μM (•-•-•-•), and 30.3 μM (▲-▲-▲-), and retinoic acid concentrations were 67 nM to 0.33 mM. The flasks were incubated 18 hr before 0.2 ml aliquots of each phase were assayed for radioactivity with a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer. Molar concentrations were determined from DPM before unbound retinoic acid (C_f) and the average number of moles of retinoic acid bound per mole of bovine serum albumin ($\bar{v}$) were calculated.

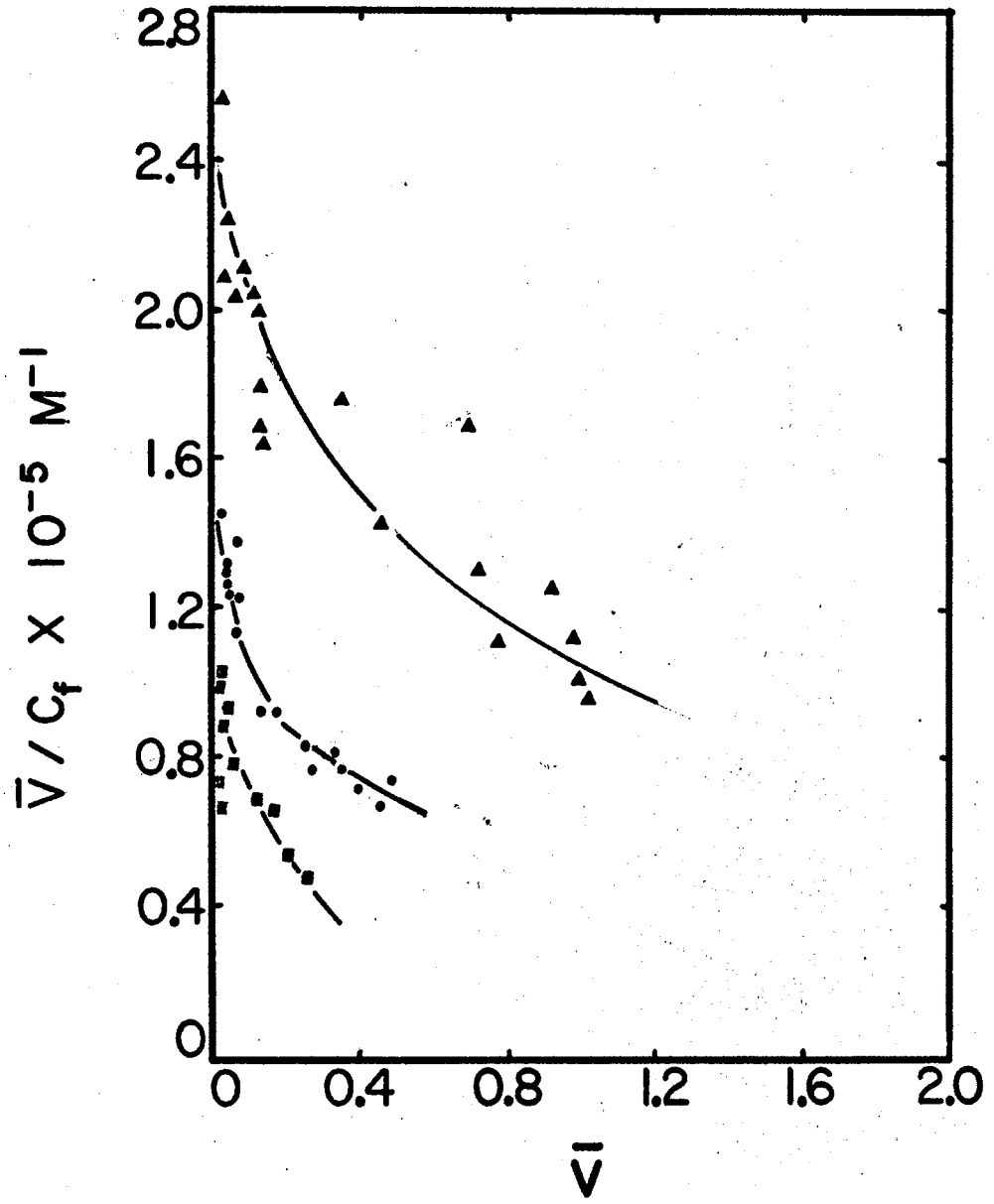

2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
0
0.4
0.8
1.2
1.6
2.0
$\bar{V} / C_f \times 10^{-5}\ M^{-1}$
$\bar{V}$

TABLE V

RETINOIC ACID BINDING TO BOVINE SERUM ALBUMIN

Equilibrium partitioning of retinoic acid-15-^{14}C between 1 ml of heptane and 1 ml of phosphate-buffered salt solution containing bovine serum albumin was performed at 37°C and pH 7.4. The flasks were incubated for 18 hr before radioactivity was determined with a Packard Tri-Carb Model 3320 Liquid Scintillation Spectrometer and DPM were converted to molar concentrations.

$\bar{v}$[1]	$S(\bar{v})$[2]	$W(\bar{v})$[3]	$\bar{v}/C_f$[4] (μM^{-1})	$S(\bar{v}/C_f)$	$W(\bar{v}/C_f)$	C_f (μM)	$S(C_f)$	C_b[5] (μM)	$S(C_b)$	C_t[6] (μM)
					30.3 μM Bovine Serum Albumin					
0.0172	0.101	0.194	0.205	0.147	0.0158	0.0661	0.109	0.377	0.101	2.26
0.0189	0.0943	0.176	0.208	0.141	0.0164	0.0736	0.108	0.428	0.0943	2.55
0.0229	0.0811	0.147	0.232	0.132	0.0154	0.0841	0.107	0.549	0.0811	2.99
0.0465	0.0535	0.0705	0.224	0.116	0.0198	0.194	0.105	1.26	0.0535	7.11
0.0649	0.0464	0.0481	0.203	0.112	0.0243	0.306	0.104	1.82	0.0464	11.1
0.0867	0.0409	0.0359	0.210	0.110	0.0238	0.402	0.104	2.48	0.0409	14.7
0.105	0.0383	0.0288	0.203	0.109	0.0257	0.511	0.103	3.04	0.0383	18.7
0.126	0.0366	0.0230	0.191	0.108	0.0288	0.657	0.103	3.68	0.0366	23.8
0.133	0.0369	0.0208	0.177	0.108	0.0328	0.751	0.103	3.89	0.0369	26.9
0.144	0.0367	0.0185	0.168	0.108	0.0358	0.858	0.103	4.24	0.0367	30.6
0.153	0.0365	0.0171	0.164	0.108	0.0376	0.936	0.103	4.49	0.0365	33.3
0.357	0.0293	0.00861	0.176	0.106	0.0343	2.05	0.103	10.6	0.0293	74.2
0.462	0.0323	0.00670	0.142	0.107	0.0502	3.33	0.103	13.8	0.0323	116
0.693	0.0276	0.00609	0.168	0.105	0.0374	4.20	0.103	20.8	0.0276	150
0.705	0.0331	0.00568	0.130	0.107	0.0587	5.57	0.103	21.2	0.0331	193
0.759	0.0375	0.00544	0.110	0.108	0.0790	7.13	0.103	22.8	0.0375	243
0.969	0.0365	0.00528	0.113	0.108	0.0762	8.92	0.103	29.2	0.0365	305
0.984	0.0400	0.00522	0.101	0.109	0.0929	10.1	0.103	29.6	0.0400	344
1.07	0.0417	0.00517	0.0961	0.109	0.102	11.7	0.103	32.3	0.0417	394

TABLE V (Continued)

$\bar{v}$ [1]	$S(\bar{v})$ [2]	$W(\bar{v})$ [3]	$\bar{v}/C_f$ [4] (μM^{-1})	$S(\bar{v}/C_f)$	$W(\bar{v}C_f)$	C_f (μM)	$S(C_f)$	C_b [5] (μM)	$S(C_b)$	C_t [6] (μM)
				303 μM Bovine Serum Albumin						
0.0108	0.0417	0.354	0.104	0.115	0.0390	0.0632	0.109	1.76	0.0417	3.65
0.0194	0.0276	0.154	0.143	0.109	0.0249	0.109	0.106	4.37	0.0276	7.59
0.0255	0.0239	0.109	0.129	0.107	0.0308	0.170	0.105	6.32	0.0239	11.3
0.0313	0.0217	0.0863	0.126	0.106	0.0325	0.222	0.104	7.99	0.02.7	14.7
0.0400	0.0195	0.0662	0.131	0.105	0.0305	0.281	0.104	10.6	0.0195	19.1
0.0431	0.0190	0.0609	0.124	0.105	0.0337	0.322	0.104	11.5	0.0190	21.3
0.0518	0.0176	0.0501	0.122	0.104	0.0349	0.402	0.104	14.2	0.0176	26.5
0.0572	0.0171	0.0448	0.113	0.104	0.0403	0.485	0.103	15.8	0.0171	30.7
0.0672	0.0159	0.0390	0.138	0.104	0.0286	0.472	0.103	18.8	0.0159	33.3
0.0701	0.0158	0.0369	0.122	0.104	0.0352	0.557	0.103	19.7	0.0158	36.8
0.125	0.0135	0.0199	0.0919	0.103	0.0613	1.38	0.103	36.4	0.0135	79.1
0.184	0.0123	0.0142	0.0927	0.103	0.0605	2.04	0.103	54.3	0.0123	117
0.245	0.0118	0.0108	0.0829	0.103	0.0754	3.09	0.103	72.9	0.0118	168
0.273	0.0119	0.00962	0.0759	0.103	0.0900	3.78	0.103	81.3	0.0119	198
0.330	0.0113	0.00875	0.0817	0.103	0.0777	4.24	0.103	98.7	0.0113	230
0.352	0.0113	0.00822	0.0773	0.103	0.0868	4.80	0.103	105	0.0113	253
0.390	0.0115	0.00750	0.0711	0.103	0.102	5.83	0.103	116	0.0115	297
0.452	0.0115	0.00683	0.0672	0.103	0.115	7.19	0.103	135	0.0115	358
0.489	0.0110	0.00686	0.0747	0.103	0.0932	6.94	0.103	146	0.0110	361

TABLE V (Continued)

$\bar{v}$[1]	$S(\bar{v})$[2]	$W(\bar{v})$[3]	$\bar{v}/C_f$[4] (μM^{-1})	$S(\bar{v}/C_f)$	$W(\bar{v}/C_f)$	C_f (μM)	$S(C_f)$	C_b[5] (μM)	$S(C_b)$	C_t[6] (μM)
				606 M Bovine Serum Albumin						
0.00564	0.0874	0.363	0.103	0.186	0.0160	0.0115	0.166	0.396	0.0874	0.598
0.00629	0.0589	0.212	0.117	0.147	0.0189	0.0165	0.136	0.791	0.0589	1.14
0.00687	0.0490	0.154	0.0730	0.124	0.0589	0.0325	0.115	1.13	0.0490	1.99
0.00733	0.0439	0.128	0.0800	0.121	0.0519	0.0360	0.114	1.41	0.0439	2.38
0.00822	0.0376	0.0965	0.0907	0.117	0.0434	0.0426	0.112	1.96	0.0376	3.12
0.0188	0.0204	0.0274	0.0988	0.106	0.0441	0.152	0.105	8.38	0.0204	12.9
0.0223	0.0189	0.0223	0.0647	0.105	0.103	0.294	0.104	10.4	0.0189	19.4
0.0328	0.0159	0.0159	0.0895	0.104	0.0549	0.334	0.104	16.9	0.0159	27.1
0.0381	0.0150	0.0142	0.0938	0.104	0.0503	0.378	0.104	20.1	0.0150	31.6
0.0664	0.0125	0.00972	0.0790	0.103	0.0712	0.835	0.103	37.2	0.0125	62.9
0.123	0.0107	0.00716	0.0694	0.103	0.0930	1.84	0.103	71.7	0.0107	128
0.172	0.0101	0.00638	0.0662	0.103	0.102	2.73	0.103	101	0.0101	185
0.213	0.0101	0.00588	0.0538	0.103	0.158	4.26	0.103	126	0.0101	258
0.246	0.0102	0.00563	0.0481	0.103	0.201	5.60	0.103	146	0.0102	319

[1] $\bar{v}$ = average no. of moles of retinoic acid bound per mole of protein.

[2] S = standard deviation propagated through the calculations due to uncertainties in the partition coefficient and determination of radioactivity.

[3] W = weighting factor.

[4] C_f = concentration of unbound retinoic acid in the aqueous phase.

[5] C_b = concentration of bound retinoic acid in the aqueous phase.

[6] C_t = concentration of bound + unbound retinoic acid in the aqueous phase.

Competition of Retinoic Acid and Palmitic Acid for Binding Sites on Bovine Serum Albumin

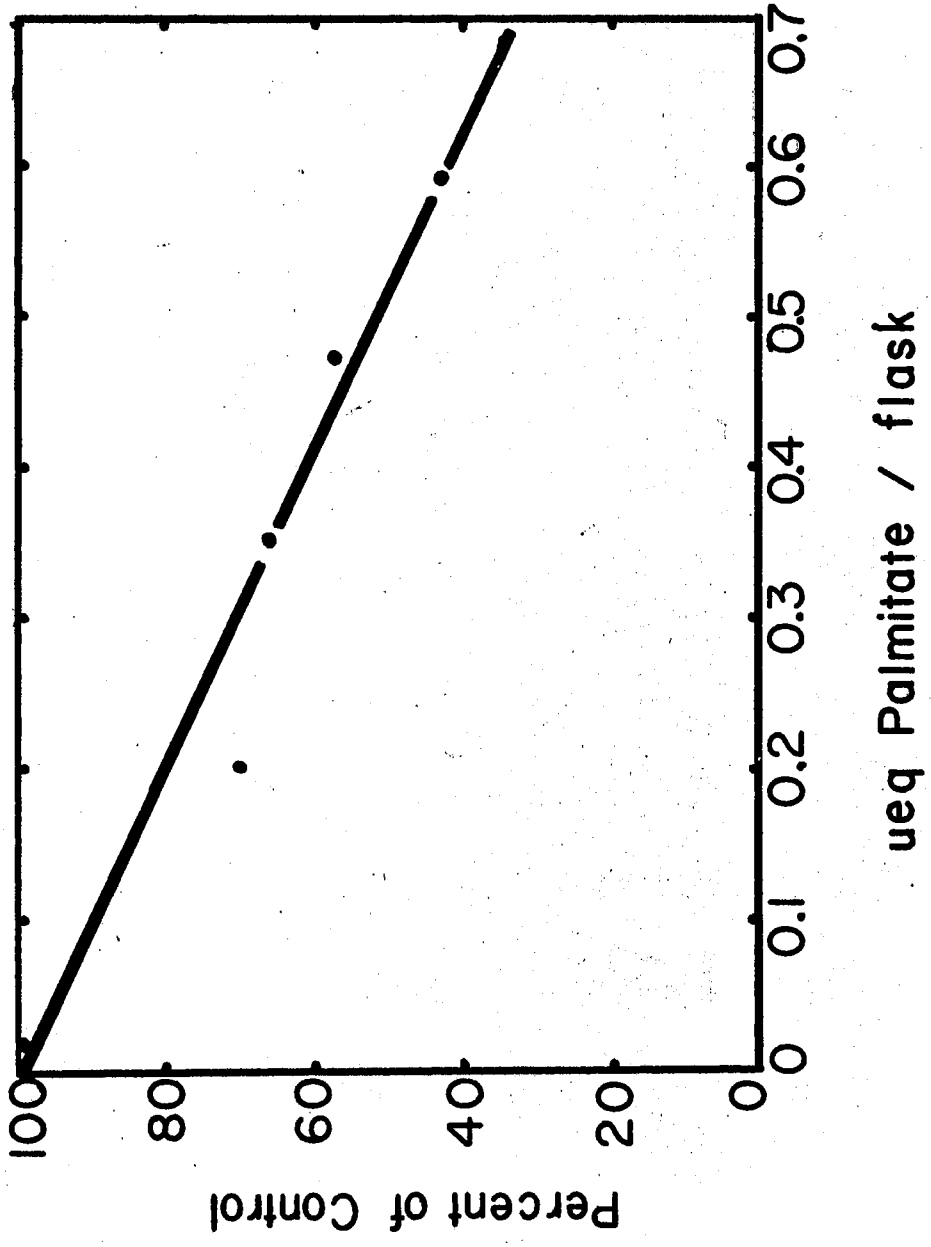
Fig. 30 shows the inhibition of retinoic acid-15-^{14}C binding to bovine serum albumin by palmitic acid. Each incubation flask contained 30.3 μM albumin, and 0.590 μM palmitic acid (0.5 ml). The flasks were incubated for 6 hr before 1.7 μM retinoic acid (0.5 ml) was added and the incubation continued for 16 hr longer. It can be seen that palmitic acid did compete with retinoic acid for binding sites on albumin, but only 43% inhibition of retinoate binding was observed with a 350 fold excess of palmitate.

Discussion

Retinoic acid was bound and transported in blood plasma by serum albumin. The radioactivity reached a maximum in blood about two hours after retinoic acid-15-^{14}C had been administered, and at this time about 75% of the radioactivity could be extracted into petroleum ether. Thin-layer chromatography and $SbCl_3$ spot tests indicated that retinoic acid was the only radioactive compound extracted.

Bovine serum albumin binds retinoic acid and the data obtained suggest that there are at least 2 classes of binding sites for retinoic acid on the protein molecule. The assumption was made that serum albumin contains only one high energy binding site for retinoic acid and the apparent association constant for the site was estimated to be 2.2×10^5 M^{-1}. A class of lower energy binding sites can bind a larger number of retinoate molecules and an apparent association constant of 1×10^4 M^{-1} was estimated for this class. It was difficult to determine the best value of n for the lower energy class of binding sites since the largest value of $\bar{v}$ that could be obtained was 1.07. Although it

Figure 30. Inhibition of Retinoic Acid Binding to Bovine Serum Albumin by Palmitic Acid. Incubations were performed at 37°C by the method of Spector et al. (95). Each flask contained 1 ml of bovine serum albumin, 30.3 μM, in phosphate-buffered salt solution. Palmitic acid in 0.5 ml of heptane was added to the flasks and they were incubated 6 hr at 37°C before 0.5 ml of retinoic acid-15-^{14}C in heptane (1.7 μM, sp act 1.034 X 10^{-7} DPM/mg) was added. The flasks were flushed with N_2 and incubated an additional 18 hr before radioactivity was determined with a Packard Model 3320 Tri-Carb Liquid Scintillation Spectrometer.


Percent of Control
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
ueq Palmitate / flask

is impossible to increase the concentration of retinoic acid in the system a greater degree of saturation could be achieved by reducing the protein concentration. The reason for the discrepancy in the three experiments using three different protein concentrations might be due to protein-protein interactions in the more concentrated solutions which could mask some of the retinoate binding sites.

A better method for analyzing the binding data would be to use a computerized curve-fitting process to determine the best values for the number of classes of sites, the number of sites within each class, and the apparent association constants for each class. Such a computer program has been described (98).

It is known that serum albumin contains several classes of binding sites for organic anions with different intrinsic association constants (99). Results of equilibrium dialysis experiments have shown that human serum albumin has a single strong binding site for thyroxine with an apparent association constant of 1.4×10^6 M^{-1} together with several sites having a lesser affinity (100). Spector et al. (95) have studied the binding of free fatty acids to bovine serum albumin and have concluded that there are six-high-energy binding sites with three sites in each of two different classes having apparent association constants of about 10^6 and 10^5 M^{-1}, and also another class containing a large number of weak binding sites.

Results obtained from the binding of retinoic acid to bovine serum albumin would indicate that it is not bound as tightly as free fatty acids. The identities of the sites which bind retinoic acid on albumin are not known but at least one of them is capable of binding palmitic acid, albeit with a lesser affinity than retinoate. Possibly

retinoate and palmitate share one class of sites while another class binds retinoate but not palmitate.

The structural components necessary on both ligand and protein for binding were not investigated. Several model compounds are available which could be used to study the groups necessary on the ligand for binding, i.e., methyl retinoate, retinol, retinal, 5,6-monoepoxy- and 5,8-monoepoxyretinoic acids. It is very likely that the carboxylate anion is a prerequisite for binding.

Likewise, the methods necessary to study the binding sites on albumin are at hand. Acetylation with acetic anhydride would eliminate the cationic ε-amino groups of lysine, and binding to the acetylated protein should be much less than to the native protein. Treatment with urea would cause hydrophobic areas to be disrupted and competition binding experiments with other organic anions such as methyl orange would provide more information about the binding loci. Probably binding of retinoic acid is very similar to association of free fatty acids to albumin, which is thought to be a result of ionic attractions between the carboxylate anion and protein cation sites, together with hydrophobic interactions between the non-polar regions of both molecules (101, 102, 103).

The finding that serum albumin binds retinoic acid has suggested a very pragmatic use for it in the laboratory. The limited solubility of retinoic acid in aqueous solutions is a limiting factor in many experiments, such as enzyme reactions, and the fact that bovine serum albumin allows the aqueous concentration of retinoic acid to be increased at least 30 fold would allow such experiments to be conducted with higher substrate concentrations. Although the binding of retin-

oic acid and serum albumin would not be unexpected *a priori* it has not yet been exploited in this manner.

SUMMARY

Bile and blood plasma were collected from chicks 6 hr after the administration of retinoic acid-15-^{14}C and fractionated by solvent extraction. The majority of the radioactivity in plasma was extracted into petroleum ether and the majority of radioactivity in bile was extracted into diethyl ether and n-butanol, with a lesser amount being extracted into petroleum ether. Tlc on silica gel indicated that all but two of the fractions contained different compounds and that each fraction contained a single radioactive compound. Retinoic acid was identified as the radioactive component of one fraction but none of the other radioactive compounds was identified. However, methyl retinoate was eliminated as being a component of any of the fractions. Further studies with bile obtained from rats administered retinoic acid-15-^{14}C indicated that all but one of the fractions from the solvent extraction could be artifacts formed in the acidic extraction medium.

Metabolites of retinoic acid were isolated and purified by gel-filtration chromatography, tlc, and glpc. Bile from rats administered retinoic acid-15-^{14}C was collected through a cannula in the bile duct and fractionated into a larger and a smaller molecular weight fraction upon a Bio-Gel P-2 column. It was established that the two fractions were originally present in the bile and not formed upon the gel-

filtration column by rechromatographing them upon the same column from which they were eluted and by showing that their tlc behavior on silica gel was not altered during the isolation procedure. The smaller molecular weight fraction contained only one metabolite of retinoic acid as demonstrated by tlc on silica gel in 6 solvent systems, and it was hydrolyzed by β-glucuronidase to yield retinoic acid. It was concluded that retinoyl β-glucuronide was the only metabolite of retinoic acid in the smaller molecular weight fraction. The larger molecular weight fraction had a molecular weight greater than 1800 but much less than 3600 as determined by gel-filtration chromatography. The uv absorption spectrum, $SbCl_3$ spot tests, and alkaline hydrolysis indicated that it was a conjugate of retinoic acid.

Retinoic acid was bound and transported by serum albumin in rat blood plasma. Scatchard plots of the data obtained from the binding of retinoic acid to bovine serum albumin in vitro show that bovine serum albumin possesses more than one class of binding sites for retinoic acid. The sites were divided into a higher and lower energy class of binding sites. If it is assumed that the higher energy class of sites binds only one molecule of retinoate an apparent association constant of 2.2×10^5 M^{-1} can be assigned. By making the assumption that the lower energy class of sites binds a larger number of molecules of retinoate an apparent association constant of 1×10^4 M^{-1} can be assigned. At least one of the retinoate binding sites on bovine serum albumin also binds palmitate, albeit with a lesser affinity.

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