METABOLISM OF RETINOIC ACID

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Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
August, 1969

METABOLISM OF RETINOIC ACID

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ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to his major adviser, Dr. Eldon C. Nelson, for his continual guidance and advice during the course of these studies and the preparation of this thesis.

Special thanks are due to the other members of the author's advisory committee, especially Dr. G. R. Waller for his advice throughout the author's graduate studies and for suggestions on the interpretation of the mass spectra, Dr. K. E. Ebner, for his advice on the enzyme section, Dr. E. J. Eisenbraun and Dr. R. H. Thayer, for their tireless efforts during the preparation of this manuscript.

To Dr. R. H. Thayer and the Poultry Science Department, the author expresses his appreciation for provision and care of the chicks.

Sincere appreciation is extended to Mrs. Marcia Mayberry, Mrs. Nancy Fancy and Mr. Rob Reid for their technical assistance. The many discussions with Dr. E. D. Mitchell, Mr. E. D. Lehman and the other staff members and students of the Department of Biochemistry were also of great help.

Acknowledgment is given to Dr. G. R. Waller and Mr. Keith Kinneberg for the Mass Spectral Data (National Science Foundation, Washington, D. C., Research Grant No. GB-7731) and to the Purdue Mass Spectrometry Center supported under U. S. Public Health Service grant FR-00354.

A very special thanks goes to the author's wife, Sung-yuan and son, Harry, Chi-tsai, for their encouragement, understanding and

patience during the years of graduate study. The concern of his parents, parents-in-law, brothers and sisters on his studies in the United States is also appreciated.

Finally, the author is indebted to the Biochemistry Department, Oklahoma State University for facilities and financial support. This study was supported in part by Public Health Service Research Grant AM-09191 from the National Institute of Arthritis and Metabolic Diseases.

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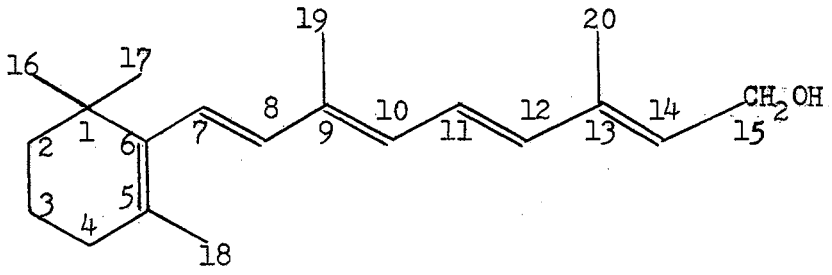
CHAPTER I

INTRODUCTION

Early research on retinol (vitamin A) was concerned with describing the physical signs of vitamin A deficiency. Nightblindness, a marked impairment of vision at low light intensity, has troubled man for thousands of years. The role of retinal in vision was discovered by Wald (1, 2) and is the only one that has been defined at the molecular level. Very little is known about the biochemical function of the vitamins A, apart from vision. Reviews on retinol and related compounds have been summarized recently by Roels (3) and by Olson (4). A comprehensive review of work reported before 1957 can be found in Thomas Moore's excellent book, Vitamin A (5). The proceedings of a symposium have been published in Vitamins and Hormones (6).

The structure of retinol (vitamin A) was elucidated by Karrer (7) in 1931. The numbering system proposed by Karrer and recommended by the International Union of Pure and Applied Chemistry (1947) is as follows:



The compound with an aldehyde group (-CHO) on the terminal carbon, C-15, is called retinal (vitamin A aldehyde). Retinoic acid (vitamin A acid) is the analogous compound with a carboxyl group (-COOH) on the terminal carbon atom (C-15).

In 1946, Arens and van Dorp (8, 9) first synthesized retinoic acid and since that time, its physiological activity has been demonstrated in the rat (8, 9, 10), chick (11) and pig (12, 13). It was reported by Dowling and Wald (10) that retinoic acid could support growth, but not vision in vitamin A deficient rats. Since in this study, no rat died of nightblindness, it was concluded that the only function retinol performs directly in the rat is to supply the prosthetic group of the visual pigments. All other functions--growth, general tissue maintenance--may be served equally well by retinoic acid.

The general metabolism of retinol may be described in the following manner. Retinol ordinarily is stored in the tissue, principally in the liver, as retinyl esters and is transported in the blood mainly as the free alcohol (14, 15). The equilibrium of the alcohol dehydrogenase system, which oxidizes it to retinal, favors reduction (16). Thus, no appreciable oxidation to retinal occurs unless the latter is removed as fast as it is formed. In the retina this is accomplished by the protein opsin trapping retinal to form the visual pigments (17).

Five alcohol dehydrogenase bands were found when soluble extracts of the liver were analyzed by starch gel electrophoresis (18). Only one of them, termed retinol dehydrogenase 3, was specific for retinol. Similarly, two enzymes, termed retinol dehydrogenase 1 and 2, were isolated from the retina and were found to be specific for retinol (18).

Retinal is readily oxidized *in vivo* and *in vitro* to retinoic acid by liver aldehyde dehydrogenase, liver oxidase, and milk xanthine oxidase. The liver enzyme which catalyzes this reaction can be readily separated from alcohol dehydrogenase by fractionation (19). The removal of retinal by oxidation to retinoic acid is irreversible.

It was reported by Arens and van Dorp in 1946 (9) that even after massive doses of retinoic acid were injected or fed orally to rats, no storage of retinol could be detected in liver. These investigators were also unable to detect retinoic acid in the body tissues of those rats (20). Recently, appreciable quantities of retinoic acid were found in the blood, liver, intestine and other tissues, shortly after the injection of several milligrams of retinal to rats. Retinoic acid reached a maximum concentration within 15 minutes, but by 180 minutes, it could not be detected in any of the examined tissues (21). Retinoic acid has also been identified as a metabolic product of retinal (22) and of β-carotene (23) in the intestine.

Nelson, *et al.* (13) developed a method for the quantitative determination of retinol and retinoic acid in blood plasma. They found that following the oral administration of retinoic acid-6,7-^{14}C to pigs, the retinoic acid concentration in blood plasma reached a maximum in 1.5 to 3 hours, then decreased until none could be detected after 8 hours. However, as plasma retinoic acid concentration decreased, the radioactivity not extractable with petroleum ether, but soluble in the plasma-ethanol residue, increased. It was concluded that retinoic acid was being converted into more polar compounds.

The biological activity of retinoic acid does not seem to involve its reduction to retinol and, as described above, there is no evidence

that the metabolic system is able to perform this reduction. Rather, retinoic acid or some derivatives obtained from it, supports the growth of the animal. It has been postulated (24) that retinoic acid may be chemically closer to the "true" active form of vitamin A and that conversion of retinol to retinoic acid may be part of the normal pathway of vitamin A metabolism.

During the past several years, considerable interest has developed in looking for degradation products and biologically active metabolites of retinol and retinoic acid. With the use of radioactive tracers in biology, investigation of the degradative metabolism of retinol and retinoic acid, as well as the search for an active form, has become more direct and feasible.

The first work was reported in 1957 by Willmer and Laughland (25), who used the biosynthetic uniformly labeled β-carotene-^{14}C. The compound was administered to rats by stomach tube, and the radioactive distribution was determined over a 28-hour period. Total radioactivity in the nonsaponifiable fractions was highest in the liver, intestine, and adrenal glands. More than 90% of the radioactivity in the liver or adrenal nonsaponifiable fraction was accounted for as retinol. Degradative metabolism of the β-carotene-^{14}C appeared to take place, as 12% of the radioactivity was found in the expired CO_2. This was thought to be derived mainly from retinol rather than carotene, since it only appeared in the later hours, when most of the conversion of carotene to retinol had taken place.

The degradative metabolism of retinol was first investigated by Wolf *et al.* in 1957 (26). These investigators synthesized retinol-14-^{14}C, and injected it into rats intraperitoneally in a colloidal

suspension. About 5% of the radioactivity was expired as CO_2 in 24 hours. The radioactive water-soluble fraction which was excreted in the urine was further investigated. It was found to consist of two distinct compounds, separable by paper chromatography. One was ether soluble and water soluble (WES, 33%) while the other was water soluble, but ether insoluble (WS, 67%) WES contained unsaturation, hydroxyl group(s), and an aldehyde carbonyl group. Upon acetylation WS became ether soluble. Therefore, it was thought to contain one or more hydroxyl groups. Dinitrophenylhydrazone derivatives could be made from WS and WES, thus, showing the presence of keto or aldehyde functional groups. Elementary analysis of WS showed it to be a ketone of the approximate composition $C_{11}H_{14}O_4$. Spectrophotometric evidence indicated the presence of a carboxyl group of an ester and a nonconjugated aldehyde.

In 1962, Krishnamurthy and Bieri (27) fed ^{14}C-labeled retinoic acid to chicks which were deficient in vitamin A and found the radioactivity mainly associated with protein in the tissues. A significant amount of the radioactivity did not extract with alcohol-ether, even after acidification of the homogenate. This indicated that retinoic acid forms a metabolite which loses its lipid properties and is bound to protein. A metabolite of retinoic acid was isolated from the stomach of rats fed retinoic acid-^{14}C by Rogers et al. (28). The compound had a spectral absorption peak at 334 nm, and stimulated the activation of sulfate to adenosine-5'-phosphosulfate and 3'-phosphoadenosine-5'-phosphosulfate by soluble enzyme systems prepared from rats which were deficient in vitamin A. Sundaresan and Wolf (29) believed that the activation of sulfate to 3'-phosphoadenosine-5'-phosphosulfate is directly influenced by an active metabolite of retinol.

Yagishita, et al. (30) fed ^{14}C-labeled retinoic acid to rats, and isolated from their intestines a radioactive fraction which was acidic in nature, but had properties different from retinoic acid. They claimed that this derivative had hydroxyl groups in the side chain and supported the growth of rats which were deficient in vitamin A. The compound had an absorption maximum at 250 nm.

The investigations of Zachman et al. (31, 32, 33, 34) and Dunagin et al. (35, 36, 37) have demonstrated the formation by the liver of water soluble metabolites of retinol, retinal, and retinoic acid which are rapidly excreted in the bile. The major metabolite of injected retinoic acid-^{14}C in rat bile was purified by ion-exchange and silicic acid chromatography. It had the spectrum of methyl retinoate, released retinoic acid upon basic hydrolysis or by treatment with β-glucuronidase, and contained glucuronic acid. The metabolite was characterized by treatment with diazomethane followed by hexamethyldisilazan or with periodate followed by semicarbazide, and the products were chromatographed. The metabolite has been tentatively identified as retinoyl β-glucuronide. Some of these metabolites have been characterized as esters of retinoic acid, but the alcohol portion of the ester remains unknown. Furthermore, it was found that retinoic acid was formed to some extent from retinal but was rapidly excreted in the bile. It is the opinion of these investigators that the excretion of metabolites of retinol in the bile is a normal physiological event.

Recently Lippel and Olson (38) reported that after retinoic acid-15-^{14}C had been injected intraperitoneally into rats, all-*trans* methyl retinoate, a *cis* isomer of methyl retinoate, retinoyl β-glucurono-γ-lactone, retinoic acid, and retinoyl β-glucuronide were isolated.

Methanol extracts of rat bile were chromatographed on an anion-exchange resin and silicic acid column. The radioactive compounds were characterized on thin-layer plates of Silica Gel G. On the other hand, when bile was extracted with n-butanol or analyzed directly by thin-layer chromatography, only retinoyl β-glucuronide and a very small amount of retinoic acid could be detected. When retinoyl β-glucuronide was incubated with an anion-exchange resin in the presence of methanol, several nonpolar products appeared. Apparently methyl retinoate, retinoyl β-glucurono-γ-lactone, and most of the retinoic acid previously found in bile after retinoic acid administration were produced from retinoyl β-glucuronide during the isolation procedure. Both retinoyl β-glucuronide and retinyl β-glucosiduronate were synthesized *in vitro* by rat liver and kidney microsome and the particulate fractions of intestinal mucosa, in the presence of uridine diphosphoglucuronic acid (UDPGA) and either retinoic acid or retinol, respectively (39).

A derivative of retinoic acid, which is capable of supporting growth in vitamin A deficient rats was isolated from the liver of rats fed retinoic acid (40). Zile and DeLuca (40) reported that the derivative was distinct from known vitamin A compounds and was more potent than retinoic acid itself. However, it was found later to be 13-*cis*-retinoic acid (41) which has biological activity equal to all-*trans* retinoic acid (42).

Epoxides of retinyl acetate, retinol and retinal have been prepared and found to have biological activity (43, 44). Morgan and Thompson (45) in 1966 reported that they also prepared methyl 5,6-epoxyretinoate which has been found to have biological activity. In 1968, John *et al.* (46) reported that retro-3-dehydroretinyl acetate

possesses 2.6% of the biological activity of all-*trans* retinyl acetate.

The terminal decarboxylation of retinoic acid has been observed *in vitro* using rat liver and kidney slices (47) and microsomes of rat liver and kidney (48). The reaction required NADPH and Fe^{++}. It was not inhibited by Krebs cycle or electron transport inhibitors but was strongly inhibited by N,N'-diphenylphenylene diamine (DPPD), phenazine methosulfate and cyanide. The system resembled lipid peroxidation in both its cofactor requirements and its response to inhibitors (48). It was concluded that retinoic acid and retinol are metabolized by either the same or at least similar pathways and that retinol becomes oxidized to retinoic acid before any degradation of the side chain occurs. The probable mechanism for oxidative reactions of retinoic acid may be analogous to the metabolism of either α-hydroxy fatty acids or of α-keto isovaleric acid (47). Little was reported on the products of this decarboxylation reaction except that the product had a UV absorption maximum at 295 nm and seemed to have an aldehyde group at C-14 (48).

Three possible pathways have been proposed for the metabolism of retinoic acid (49). The first pathway, including urinary and fecal products which still contained all the carbon atoms, accounts for 66% of the administered dose. The other two are oxidative pathways, one accounting for the terminal decarboxylation and the other for the elimination of the last two carbon atoms of the isoprenoid chain as CO_2.

All the details of the isolation of biologically active metabolites of retinoic acid have not been reported and the metabolites in question have not been chemically defined. The only chemically defined metabolite of retinoic acid is retinoyl β-glucuronide.

The biological importance of retinol has been recognized for many, many years, but the biochemical mode of action of retinol in its systemic effects is still not understood. Recently, the interactions of retinol and retinoic acid with electron acceptors, 7,7,8,8-tetracyanoquinodimethane (TCNQ) and tetrachloro-1,4-benzoquinone (chloranil) and also with iodine were studied by Lucy and Lichti (49, 50). Their findings suggest that the ability of retinol and retinoic acid to donate electrons may be an important aspect of its biochemical mode of action.

Thus, the identification of the degradative products and metabolites of retinoic acid and a measure of their biological activity will be required before the active forms and their mode of action can be determined. The objective of this study is to isolate and identify some of the products and metabolites of retinoic acid formed <u>in vitro</u>.

CHAPTER II

THE MASS SPECTRA OF RETINOL AND RELATED COMPOUNDS

Many biologically active compounds have been examined by mass spectrometry, but very little information has been reported on retinol (vitamin A) and related molecules. Research concerning the metabolism of the vitamins A has provided the impetus for a study using mass spectrometry as a method to identify metabolites. This chapter reports the mass spectral analyses of the all-*trans*-isomers of retinol, anhydroretinol, retinyl acetate, retinal, retinoic acid, methyl retinoate and the 9-*cis* isomer of retinal.

Experimental

Materials

The compounds investigated were obtained from the following sources: all-*trans*-retinol, all-*trans*-retinyl acetate, 9-*cis*-retinal from Sigma Chemical Co., St. Louis, Missouri; all-*trans*-retinal from Distillation Products Industry, Division of Eastman Kodak Co., Rochester, New York; all-*trans*-retinoic acid was a gift from Dr. R. H. Bunnell of Hoffmann-LaRoche, Inc., Nutley, New Jersey. The purity of these compounds was checked by thin-layer chromatography using benzene : chloroform : methanol (4 : 1 : 1) as the solvent system just prior to analyses and only one spot was observed.

Methods

Conventional mass spectra were obtained on approximately 1 μg of compound using the direct inlet of a prototype of the LKB-9000 combination mass spectrometer - gas chromatograph (LKB Instruments, Inc., 1221 Parklawn Drive, Rockville, Maryland)(51, 52). The direct inlet and ion source temperature were 50°C and 270°C, respectively. Spectra were taken at 70 electron volts using an accelerating voltage of 3.5 kv. The source pressure was 5×10^{-6} to 1×10^{-7} mm Hg. A mass spectrum of anhydroretinol was obtained following the introduction of retinyl acetate onto a 1% SE-30 glass chromatography column $\frac{1}{4}$ inch x 4 feet in length. The column temperature was 210°C with a flow rate of 25 ml/min. The formation of anhydroretinol using this technique has been described by Dunagin and Olson (53). All mass spectra were computer plotted from tabular intensity data. A CalComp 565 Plotter driven by an IBM 1620 Computer required about 3 minutes of plotting time and 4 minutes of computer time (54).

High resolution mass spectra were obtained on a CEC 21-110-B mass spectrometer (Consolidated Electrodynamics Corp., Pasadena, California) by Dr. R. D. Grigsby, Continental Oil Company, Ponca City, Oklahoma. The direct inlet and source temperature were 120°C. Perfluorokerosene was used for peak-matching.

Results and Discussion

The mass spectra of all-*trans*-retinol, all-*trans*-retinyl acetate, all-*trans*-retinal, all-*trans*-retinoic acid and methyl retinoate are shown in Figures 1, 2, 3, 4 and 5 respectively. The molecular ion of each compound was found to be the base peak except for retinol and

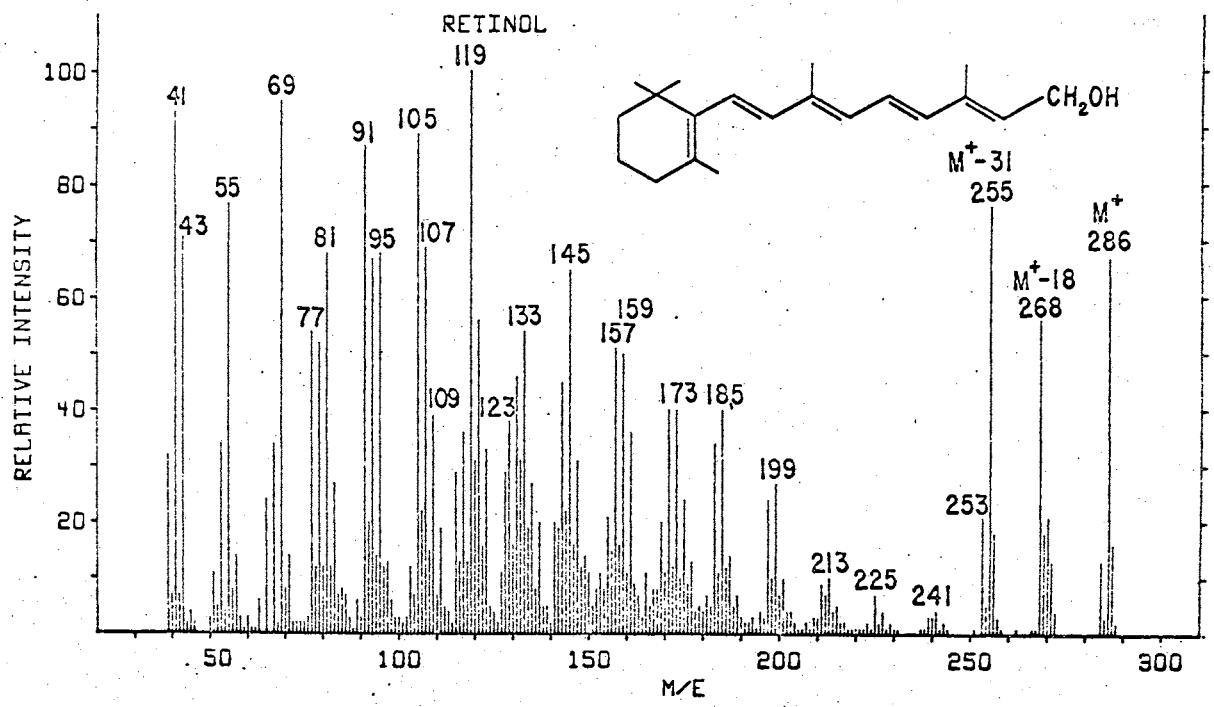


Figure 1. The Mass Spectrum of All-trans-Retinol

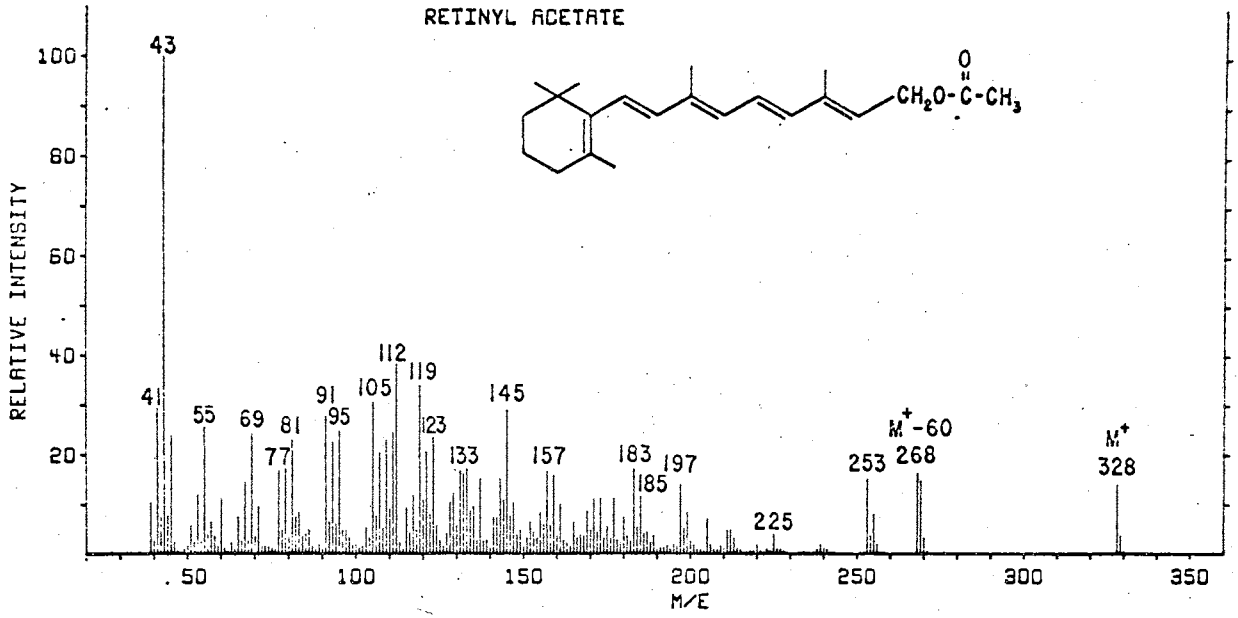


Figure 2. The Mass Spectrum of All-trans-Retinyl Acetate

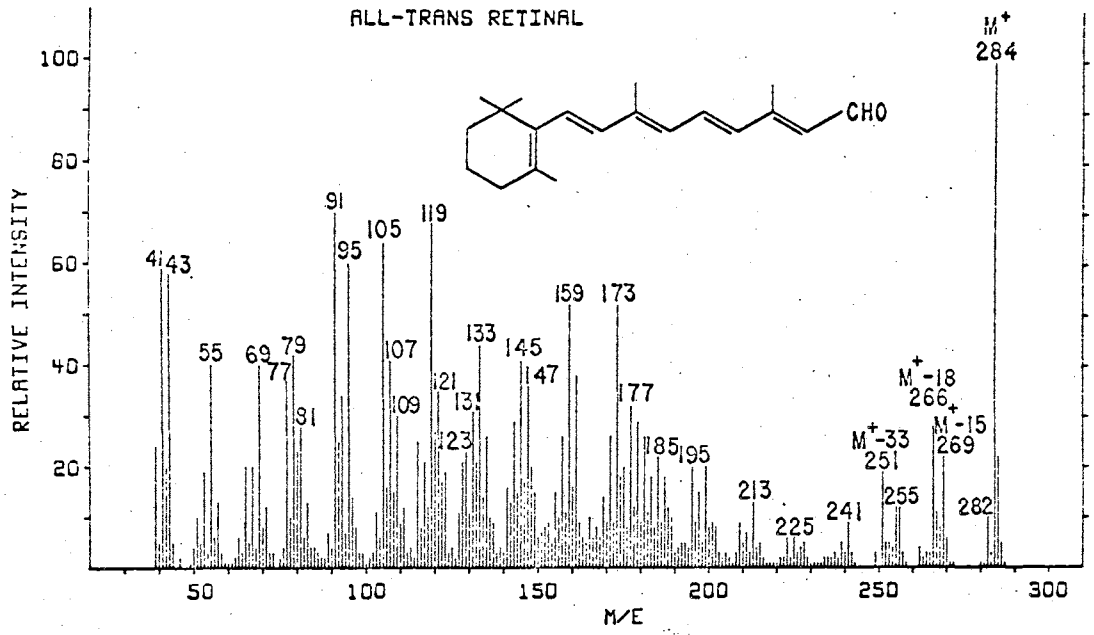


Figure 3. The Mass Spectrum of All-<u>trans</u>-Retinal

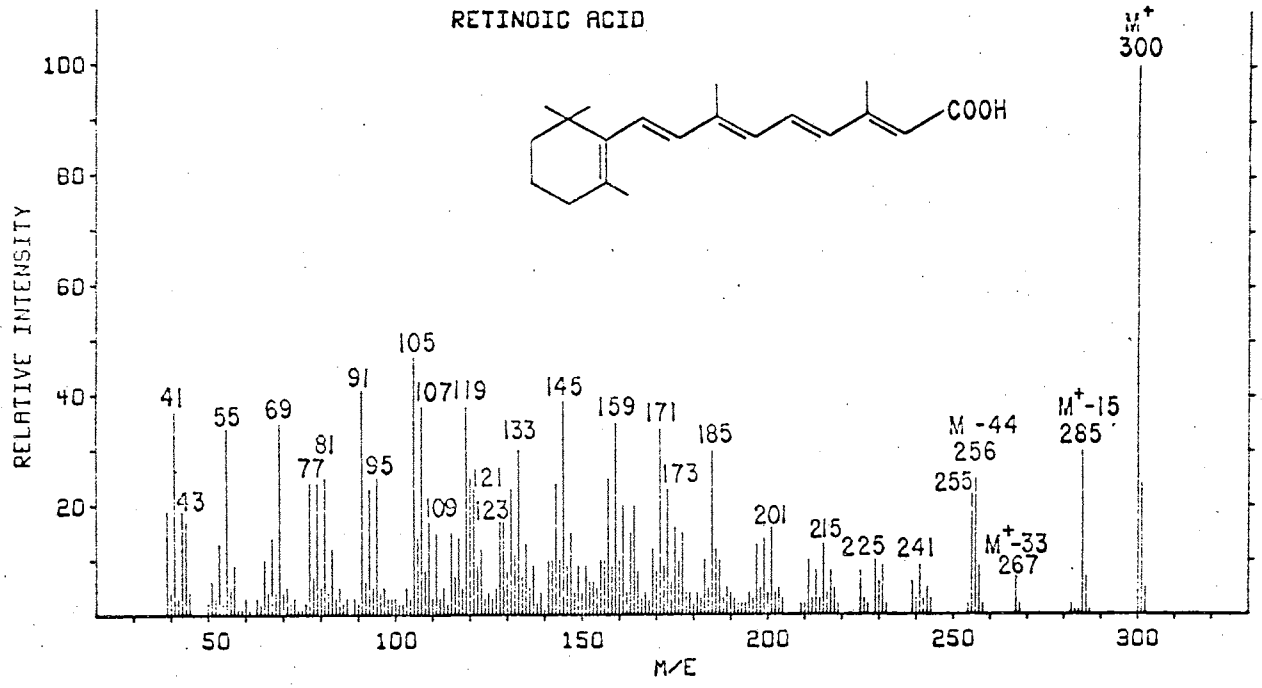


Figure 4. The Mass Spectrum of All-trans-Retinoic Acid

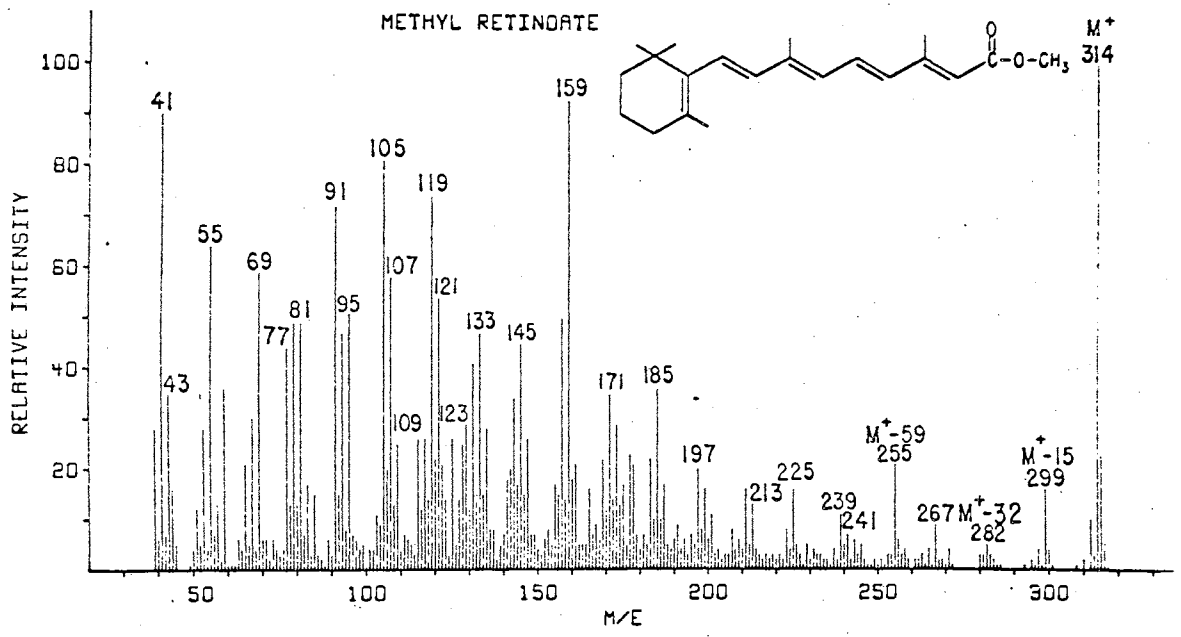


Figure 5. The Mass Spectrum of Methyl Retinoate

retinyl acetate. The primary fragment ion formed from the loss of a functional group was of additional help in the identification of these compounds; i. e., retinol, m/e 268 [M^+ - 18 (H_2O)]; retinyl acetate, m/e 268 [M^+ - 60 (CH_3COOH)]; retinal, m/e 255 [M^+ - 29 (CHO)]; retinoic acid, m/e 256 [M^+ - 44 (CO_2)]; and methyl retinoate m/e 255 [M^+ - 59 (CH_3COO)]. The relatively abundant molecular ion present for retinal, retinoic acid and methyl retinoate may be due to the conjugation of the carbonyl group with the double bond in the acyclic chain (55, 56).

Retinal, retinoic acid and methyl retinoate lose a methyl group [M^+ - 15 (CH_3)] i. e., m/e 269, m/e 285 and m/e 299 respectively whereas retinol and retinyl acetate did not. The elimination of a methyl group may have occurred after loss of the functional group, i. e., retinol, m/e 253 (M^+ - H_2O - CH_3); retinyl acetate, m/e 253 (M - CH_3COOH - CH_3); retinal, m/e 241 (M^+ - CO - CH_3); and retinoic acid, m/e 241 (M^+ - CO_2 - CH_3). It was first suggested by Biemann (57) that this elimination of a methyl group is due to the loss of one of the nuclear methyl groups leading to the formation of a stable tertiary allylic carbonium ion, since a quaternary carbon atom allylic to the double bond exists in each ring. Recently it was shown by the mass spectrum of hexadeuterated (on gem-dimethyl) β-ionone that it arises 90% from the loss of CH_3 attached on the double bond in the ring (58).

For ease in identification, these characteristic peaks and the ten most intense peaks of retinol and related compounds are shown in Table I. Note that the peak resulting from the loss of the functional group was not always one of the ten most intense peaks but that it is very important in establishing identity of the compound. Hence, based

TABLE I

CERTAIN CHARACTERISTIC PEAKS AND THE TEN MOST INTENSIVE PEAKS OF RETINOL AND RELATED COMPOUNDS

Compound	Molecular Ion	Interpretive Peaks	Ten Most Intensive Peaks
Retinol	286	268 $\{M-H_2O\ (18)\}^+$	119 69 41 105 91
		255 $\{M-CH_2OH\ (31)\}^+$	55 255 43 107 95
		253 $\{M-H_2O\ (18)-CH_3(15)\}^+$	
Retinyl Acetate	328	268 $\{M-CH_3COOH\ (60)\}^+$	43 112 119 105 41
		255 $\{M-CH_2O-\overset{O}{\overset{\|}{C}}-CH_3(73)\}^+$	145 91 55 95 69
		253 $\{M-CH_3COOH\ (60)-CH_3(15)\}^+$	
Retinal	284	269 $\{M-CH_3(15)\}^+$	284 91 119 105 95
		266 $\{M-H_2O\ (18)\}^+$	41 43 173 159 133
		255 $\{M-CHO\ (29)\}^+$	
		251 $\{M-H_2O\ (18)-CH_3\ (15)\}^+$	
Retinoic Acid	300	285 $\{M-CH_3\ (15)\}^+$	300 105 91 145 119
		267 $\{M-CH_3\ (15)-H_2O\ (18)\}^+$	107 41 159 69 55
		256 $\{M-CO_2\ (44)\}^+$	
		255 $\{M-COOH\ (45)\}^+$	
		241 $\{M-CO_2\ (44)-CH_3\ (15)\}^+$	
Methyl Retinoate	314	299 $\{M-CH_3\ (15)\}^+$	314 159 41 105 119
		282 $\{M-CH_3OH\ (32)\}^+$	91 55 69 107 121
		267 $\{M-CH_3\ (15)-CH_3OH\ (32)\}^+$	

on a rather limited number of interpretive peaks, it is possible to quickly identify each of these compounds by the appearance of their respective molecular ions and the fragment ions corresponding to the loss of their functional and methyl groups. The loss of a methyl group was also supported by observed metastable peaks which are listed in Table II with the transitions denoted by them. Decomposition reactions that might account for at least some of the prominent features could have been predicted from experience with simpler molecules containing the same functional groups; i. e., primary loss of H_2O, CH_3COOH, CH_3COO^-, CHO, CO_2, CH_3 and H (56, 59, 60, 61).

The effect of a change in probe temperature (while holding the ion source temperature constant) was studied for retinal (20° - 60°C) and retinoic acid (70° - 115°C) and the results are shown in Table III and IV. No significant differences were observed. Three isomers of retinal (all-trans, 9-cis, 13-cis), gave spectra which were similar to that obtained for all-trans-retinal. The spectrum of the 9-cis-isomer is shown in Figure 6. The major differences in the fragmentation of the retinal isomers was the very small M^+ - 18 peak in the cis isomers (Figure 6). This difference is of considerable help in distinguishing between the cis and trans isomers of retinal (Figures 3 and 6). The ion intensities of the ten highest peaks found in these isomers are presented in Table V.

Since these compounds possess similar hydrocarbon skeletons, additional information on the fragmentation pattern of the hydrocarbon skeletons due to electron impact can be obtained by comparison with the spectra of anhydroretinol (Figure 7), terpenes (55, 56, 60) and β-ionone (61, 62, 63). The molecular ion of anhydroretinol m/e 268

TABLE II

METASTABLE PEAKS IN THE MASS SPECTRUM OF RETINOL AND RELATED COMPOUNDS

Apparent Mass	Transition Denoted	Probable Neutral Product	Retinoic Acid	Retinal	Retinol	Retinyl Acetate
36.6	$(123^+) \longrightarrow (67^+) + 56$	C_4H_8		x	x	
37.1	$(41^+) \longrightarrow (39^+) + 2$	2H	x	x	x	x
39.2	$(43^+) \longrightarrow (41^+) + 2$	2H	x	x	x	x
46.6	$(91^+) \longrightarrow (65^+) + 26$	$CH{\equiv}CH_2$	x	x	x	
63.0	$(67^+) \longrightarrow (65^+) + 2$	2H	x	x	x	x
75.1	$(79^+) \longrightarrow (77^+) + 2$	2H	x	x	x	x
77.1	$(81^+) \longrightarrow (79^+) + 2$	2H	x	x	x	x
89.2	$(93^+) \longrightarrow (91^+) + 2$	2H	x	x	x	x
101.2	$(105^+) \longrightarrow (103^+) + 2$	2H		x		x
103.3	$(107^+) \longrightarrow (105^+) + 2$	2H	x	x		
113.3	$(117^+) \longrightarrow (115^+) + 2$	2H		x	x	
115.2	$(119^+) \longrightarrow (117^+) + 2$	2H	x	x	x	
227.4	$(286^+) \longrightarrow (255^+) + 31$	CH_3O			x	
254.8	$(284^+) \longrightarrow (269^+) + 15$	CH_3		x		
270.7	$(300^+) \longrightarrow (285^+) + 15$	CH_3	x			

x - Indicates presence of metastable ions.

TABLE III

THE EFFECT OF PROBE TEMPERATURE ON THE TEN MOST INTENSE IONS OF RETINAL

Probe Temperature	m/e	41	43	69	91	95	105	119	133	173	284
°C		Relative Intensity									
20		59	58	43	70	60	64	68	44	52	100
30		57	62	48	68	63	68	64	49	58	100
40		63	59	57	73	59	72	67	50	55	100
50		61	62	52	76	65	73	71	46	64	100
60		63	65	50	73	67	70	69	48	60	100

TABLE IV

THE EFFECT OF PROBE TEMPERATURE ON THE TEN MOST INTENSE IONS OF RETINOIC ACID

Probe Temperature	m/e	41	55	69	91	105	107	119	145	159	300
°C		Relative Intensity									
70		37	34	35	41	47	38	38	39	35	100
80		36	37	39	45	49	42	41	46	34	100
90		39	42	41	43	50	43	40	44	39	100
100		41	38	40	42	47	40	39	43	44	100
115		43	41	43	51	50	45	42	52	46	100

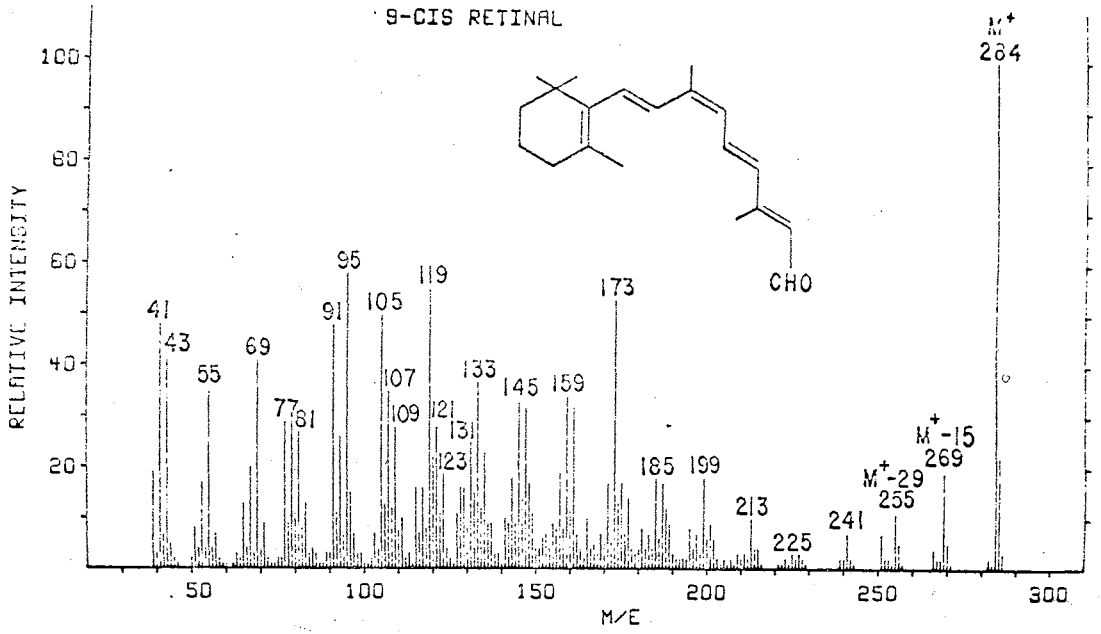


Figure 6. The Mass Spectrum of 9-cis-Retinal

TABLE V

THE TEN MOST INTENSE IONS OF RETINAL ISOMERS

Isomer	m/e 41	43	69	91	95	105	119	133	173	284
All-trans	59	58	43	70	60	64	68	44	52	100
9-cis	48	41	41	48	58	50	55	37	53	100
13-cis	52	42	43	67	57	56	64	43	53	100

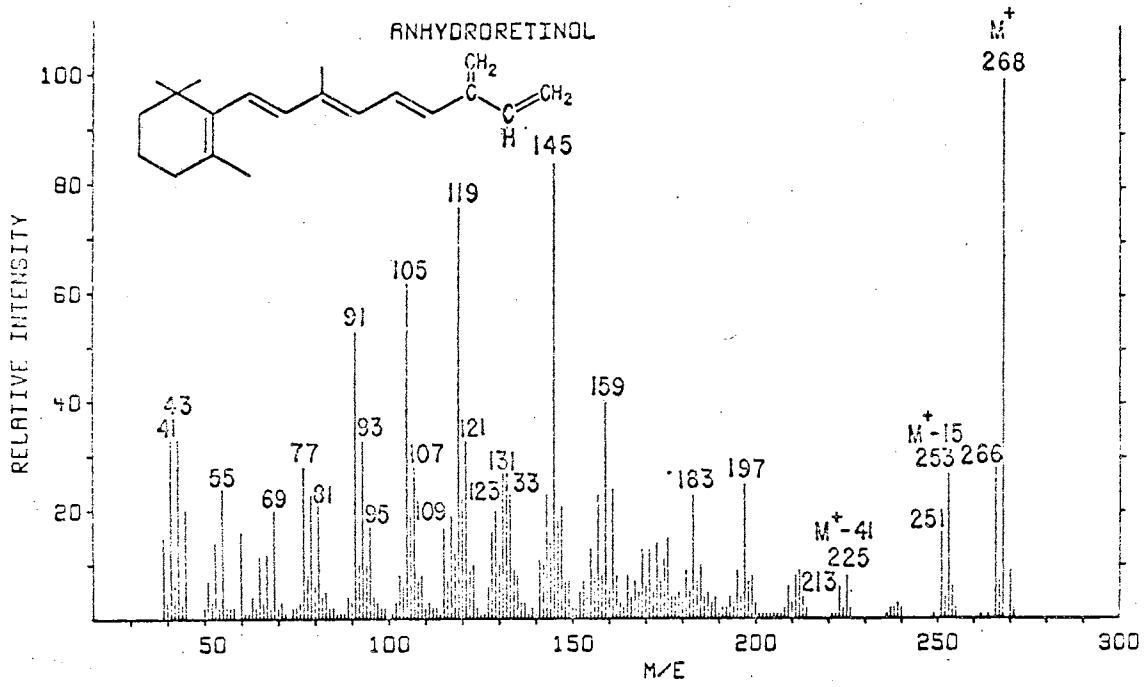


Figure 7. The Mass Spectrum of Anhydroretinol

corresponded to the fragmentation peak, m/e 268, common to retinol and retinyl acetate and could have been formed by the cleavage of their functional groups via a McLafferty type of rearrangement (64) (Figure 8). Therefore, the remainder of the spectrum of retinol and retinyl acetate are quite similar and are analogous to that of anhydroretinol. Certain pertinent features of these spectra that aid in their interpretation were α-cleavage of the β-ionone ring that resulted in the formation of an intense ion of mass m/e 145 which was the acyclic portion. However, a much less intense ion at mass m/e 123 which corresponds to the β-ionone ring was also observed (Figure 9). In all of the compounds studied the fragment at m/e 123 was observed.

The acyclic hydrocarbon fragment m/e 145 may lose 26 mass units (HC ≡ CH) to yield the ion of m/e 119 which could again lose 26 mass units to yield the ion m/e 93. The fragment ion m/e 105 is not easily explained but it might arise from the loss of 14 mass units from the ion m/e 119 or from the loss of C_3H_4 from the hydrocarbon with m/e 145. Alternatively the ion of mass 105 may result from the desaturation of the cyclohexene nucleus which has lost some of its methyl groups (see discussion on the formation of ions from the β-ionone nucleus below).

The hydrocarbon ions m/e 255 and m/e 241 probably arise from the cleavage of retinal via the proposed pattern shown in Figure 10. An additional interpretive peak, which could arise from α-cleavage at the β-ionone ring, is the ion of mass 161. Subsequently a loss of 28 mass units (CO) may result in a hydrocarbon ion of mass 133.

A similar type of cleavage occurred with retinoic acid (Figure 11) and methyl retinoate (Figure 12). An exception was the formation of

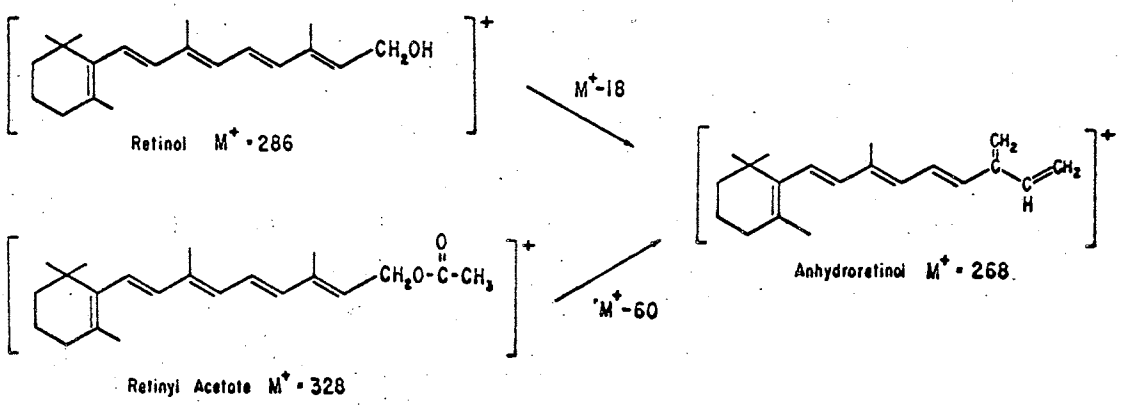


Figure 8. The Formation of Anhydroretinol from Retinol and Retinyl Acetate

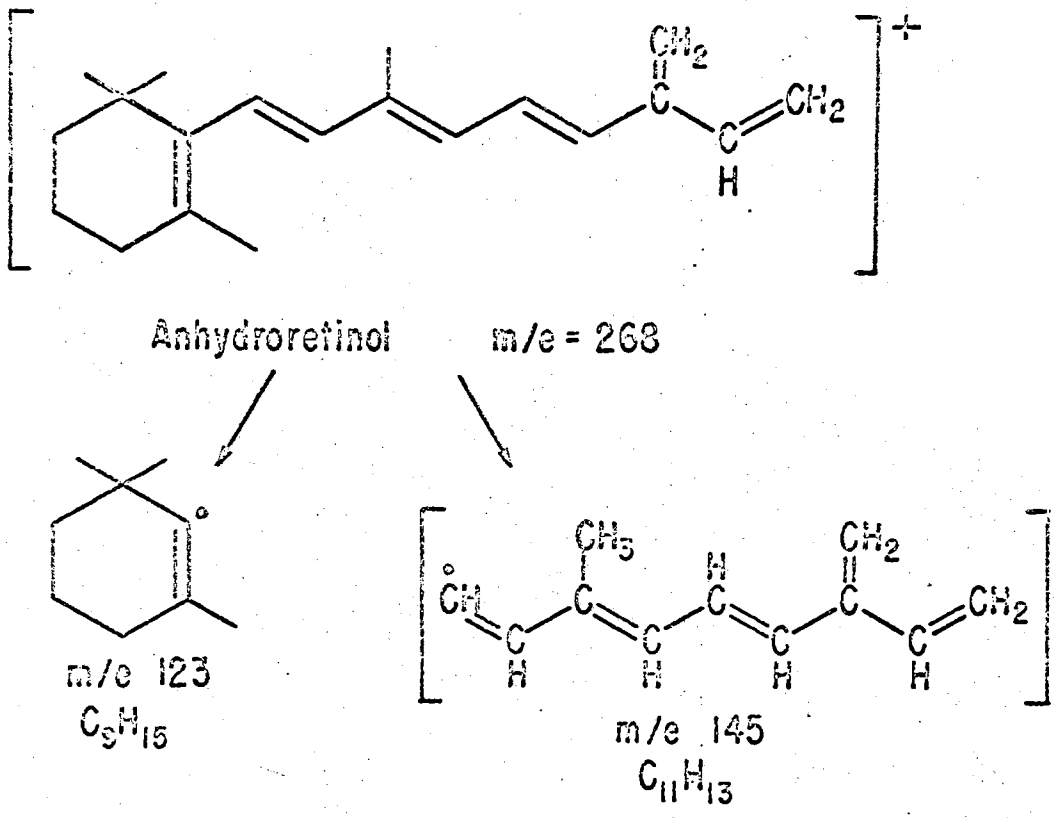


Figure 9. The α-Cleavage of Anhydroretinol

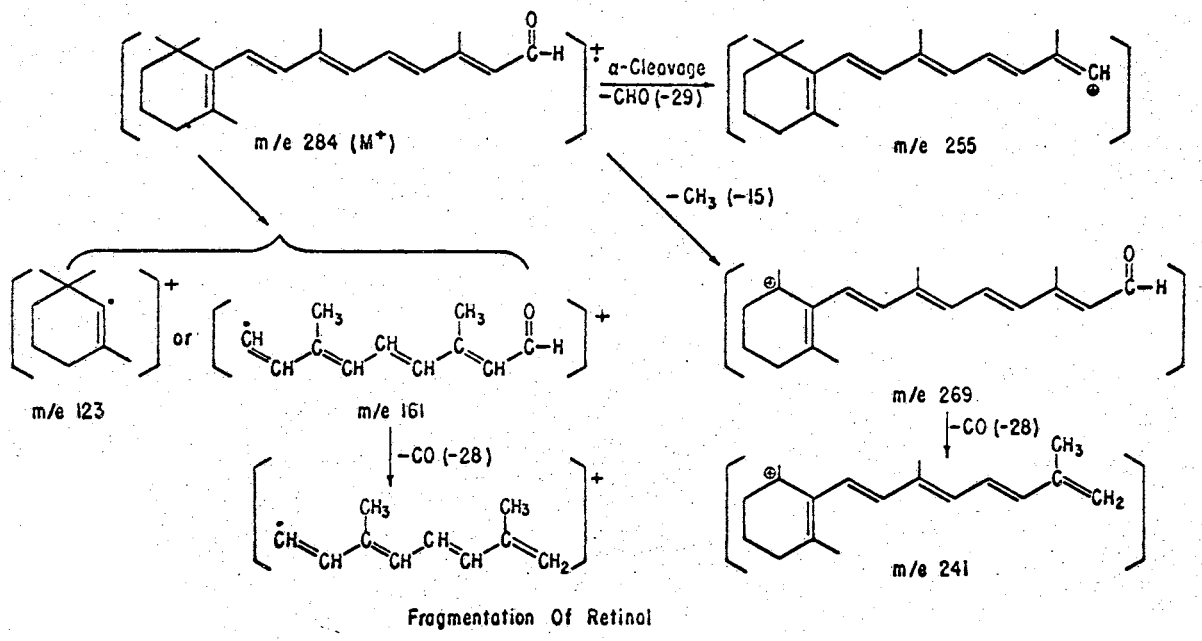


Figure 10. Proposed Partial Fragmentation Pattern of Retinal

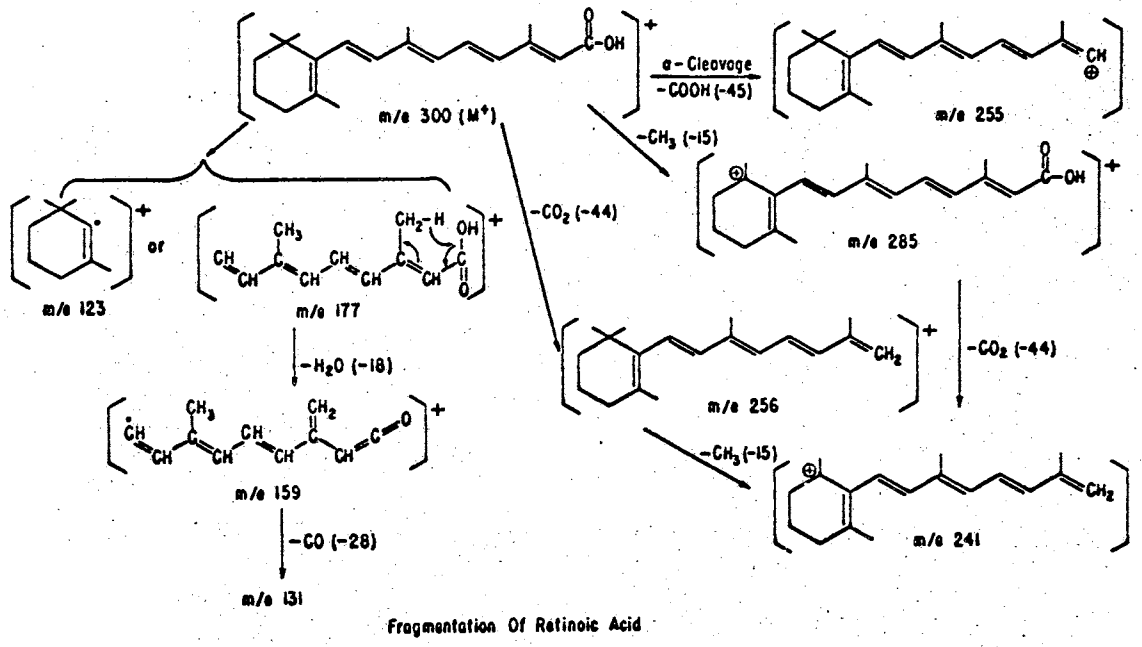


Figure 11. Proposed Partial Fragmentation Pattern of Retinoic Acid

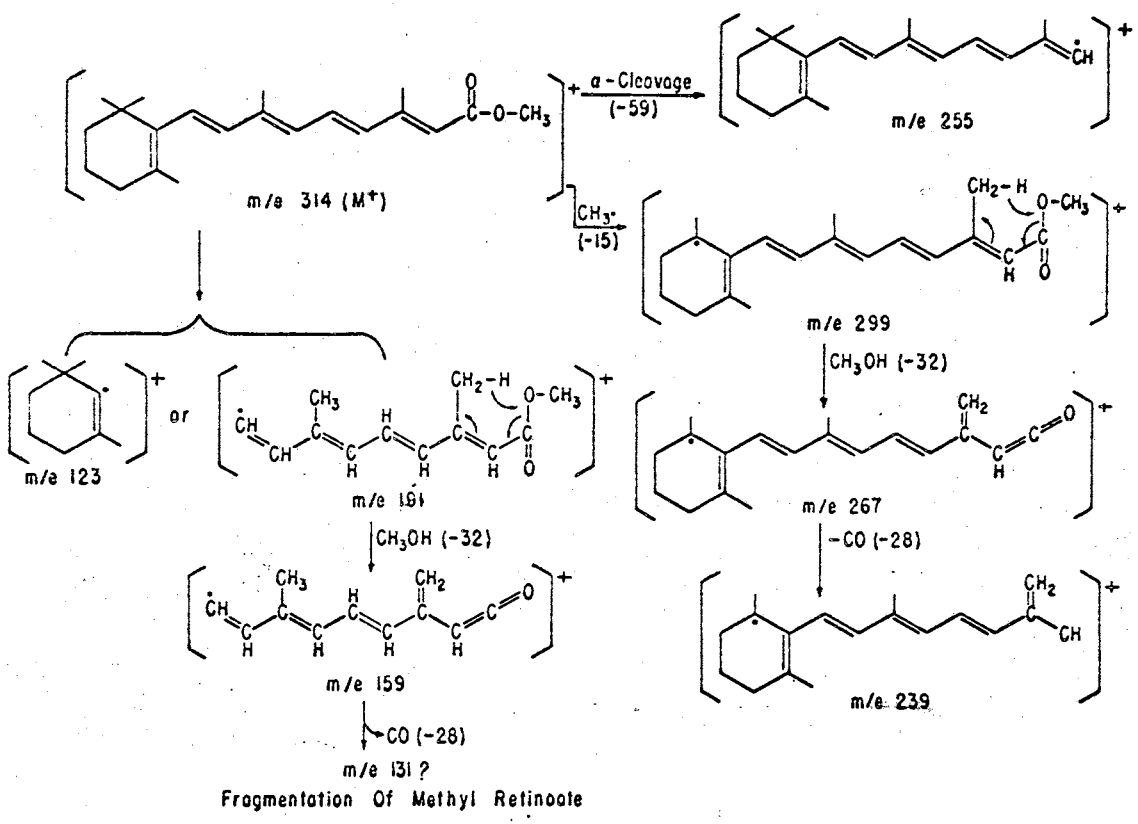


Figure 12. Proposed Partial Fragmentation Pattern of Methyl Retinoate

the hydrocarbon fragment m/e 256 (corresponding to the loss of CO_2) instead of m/e 255 which was more intense in retinal and methyl retinoate. Triplet groups of ions (m/e 109, 107, 105; 95, 93, 91 and 81, 79, 77) were observed in the spectrum of retinol and related compounds. The formation of such ions is probably due to the loss of molecular hydrogen in two successive steps from the ring nucleus (Figure 13)(65, 66). It is of particular interest that in the series in which 2 methyl groups were lost to form the ion m/e 95 the sequential loss of 2 hydrogen atoms led to the formation of an ion with m/e 91. The fragment ion m/e 91 was probably the tropylium ion (67) and would represent another example of the breakdown via common tropylium ion intermediates (68). These sequential losses of molecular hydrogen were also confirmed by the observed metastable peaks (Table II).

It is also possible that the mass spectrometric equivalent of a ring that has undergone retro-Diels-Alder (RDA) reactions (69) occurs, which would be expected to result in the corresponding loss of ethylene to give: m/e 123 $\longrightarrow$ m/e 95; m/e 109 $\longrightarrow$ m/e 81; m/e 95 $\longrightarrow$ m/e 67; m/e 81 $\longrightarrow$ m/e 53; etc. The loss of ethylene is very common in the breakdown of olefins; however, it is not known if this occurs through cyclic or aliphatic species. The similarity between these spectra and those from the alkyl benzenes with respect to the m/e series 91^+, 105^+, 119^+, 133^+, 145^+, 159^+, 171^+, 185^+ should be noted but it is not readily apparent how alkyl benzenes might be derived from retinol and related compounds.

It is of interest that the base peak in retinyl acetate was m/e 43. The question arises whether the m/e 43 ion from retinyl acetate and related compounds was derived from ($C_3H_7^+$) or from ($CH_3C{=}O^+$).

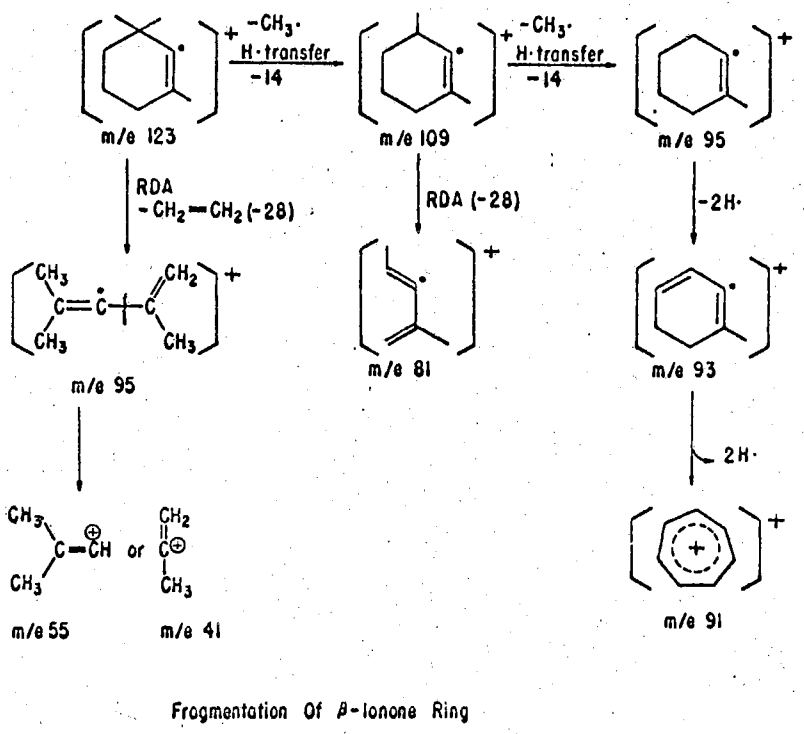


Figure 13. Proposed Partial Fragmentation Pattern of the β-ionone Nucleus

The spectrum of anhydrovitamin A (Figure 7) shows a fragmentation pattern similar to that of retinyl acetate, but it does not show the m/e 43 ion as the base peak. Moreover, the spectra of 3,3,5-trimethylcyclohexane (70) which has a similar nucleus, showed only a small abundance of the m/e 43 ion. Analysis of retinyl acetate, retinoic acid, retinal and retinol by high resolution mass spectrometry showed the ratios of m/e 43.0186 ($CH_3C{=}O^+$) to m/e 43.0549 ($C_3H_7^+$) of 9.1, 0.6, 10.0 and 1.1, respectively (Table VI). The predominance of the CH_3CO^+ ion in retinyl acetate (~90%) and in retinal (~50%) agree with what could have been predicted based on the known cleavage pattern of esters and aldehydes.

The decomposition reactions described are plausible explanations of the change occurring in these molecules under electron impact. Rearrangements such as double bond shifts cannot be completely ruled out; however, the double bonds in the acyclic portions of these molecules are essentially "locked in" by the tertiary carbon atoms tending to lend support for the reactions proposed. Confirmation of these proposed transitions will have to come from isotope labeling studies.

TABLE VI

THE INTENSITY RATIO OF M/E 43.0186 ($CH_3C{=}O^+$) TO M/E 43.0549 ($C_3H_7^+$) FOR RETINOL AND RELATED COMPOUNDS

Compound	Ratio
Retinol	1.06
Retinyl Acetate	9.09
Retinal	10.02
Retinoic Acid	0.55

CHAPTER III

PARTIAL PURIFICATION AND PROPERTIES OF A RETINOIC ACID DECARBOXYLATING ENZYME FROM CHICKEN LIVER

In the past it has been shown that retinol is capable of undergoing oxidative metabolism in the rat resulting in the release of at least the last two carbon atoms of the isoprenoid side chain as CO_2 (26, 71). Recently, these oxidative reactions have been demonstrated for retinoic acid as well (72, 73). These reports concerning the production of CO_2 from retinoic acid were the result of *in vivo* experiments. In order to simplify the reaction systems for isolating the reaction products, a direct approach for studying these enzymatic decarboxylation reactions *in vitro* has been taken. Recently, studies of these decarboxylation reactions in tissue slices (47) and microsomes (48) from rat liver and kidney were reported.

Experimental

Materials

Radioactive Retinoic Acid

The all-*trans* retinoic acid-6,7-^{14}C and all-*trans* retinoic acid-15-^{14}C used in this study were gifts from Hoffmann-LaRoche, Inc., Nutley, New Jersey. These radioactive compounds were purified by recrystallization from methanol using pure non-radioactive retinoic acid as carriers. The radioactive purity was confirmed by gas flow

strip counter scan of the thin-layer chromatogram and only one radioactive spot was found.

Solvents and Chemical Reagents

Solvents and chemical reagents were of analytical reagent grade unless otherwise noted. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced nicotinamide adenine dinucleotide (NADH), adenosine-5'-triphosphate (ATP), coenzyme A and cytochrome C were obtained from Sigma Chemical Co., St. Louis, Missouri. L-cysteine and glutathione were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Sodium glycocholate and sodium taurocholate were obtained from Calbiochem, Los Angeles, California.

Carbon dioxide free NaOH solution was prepared as follows: deionized water was boiled vigorously for 10 minutes and cooled while stoppered. Air was allowed to enter the cooling flask through a drying tube containing sodium hydrate asbestos absorbent (Ascarite, Arthur H. Thomas Company, Philadelphia, Pa.). Sodium hydroxide was added and the flask was restoppered. A toluene-ethylene glycol monomethyl ether mixture for counting $^{14}CO_2$ was prepared by the method of Jeffax and Alvarez (74).

Animals

Chicks, 2-8 weeks of age, were obtained from Dr. R. H. Thayer, Poultry Science Department, Oklahoma State University, Stillwater, Oklahoma. All chicks, from the time of hatching, had been fed a vitamin A deficient ration supplemented with retinoic acid stabilized in gelatin beadlets (courtesy of Dr. R. H. Bunnell, Hoffmann-LaRoche, Inc., Nutley, New Jersey) at a level of 2.5 mg per pound of feed.

Methods

Procedures for $^{14}CO_2$ Collection *in vivo*

All-*trans* retinoic acid-15-^{14}C and all-*trans* retinoic acid-6,7-^{14}C were administered orally to chicks which had been maintained previously on retinoic acid. The chicks were placed in a metabolic chamber and the respiratory $^{14}CO_2$ was collected at desired intervals using 1 N CO_2 free NaOH solution as trap. The trapped sample (in 1 N NaOH solution) was treated with perchloric acid to release $^{14}CO_2$ into an ionization chamber. It was then counted by using a Cary Vibrating Reed Electrometer (Model 31, Applied Physics Corporation, Pasadena, California).

Homogenization and Fractionation of Chicken Liver

Liver was removed immediately after the chick was sacrificed by cervical dislocation. The liver was chilled over cracked ice and partially homogenized by expulsion through a 1 mm stainless steel screen by means of a screw press (Harvard Apparatus, Inc., Cambridge, Massachusetts) to remove connective tissue. Two grams of the liver pulp were homogenized in a Potter-Elvejhem glass tissue grinder with a motor driven, grooved teflon pestle in 18 ml of ice-cold isotonic 0.25 M sucrose.

Two hundred milliliters of homogenate were fractionated in accordance with the procedure of Schneider and Hogeboom (75), (Figure 14). The particulate fractions were washed once with ice-cold 0.25 M sucrose and these washings were added to the next fraction. The washed fractions were resuspended in 100 ml of 0.1 M sodium phosphate buffer, pH 6.4.

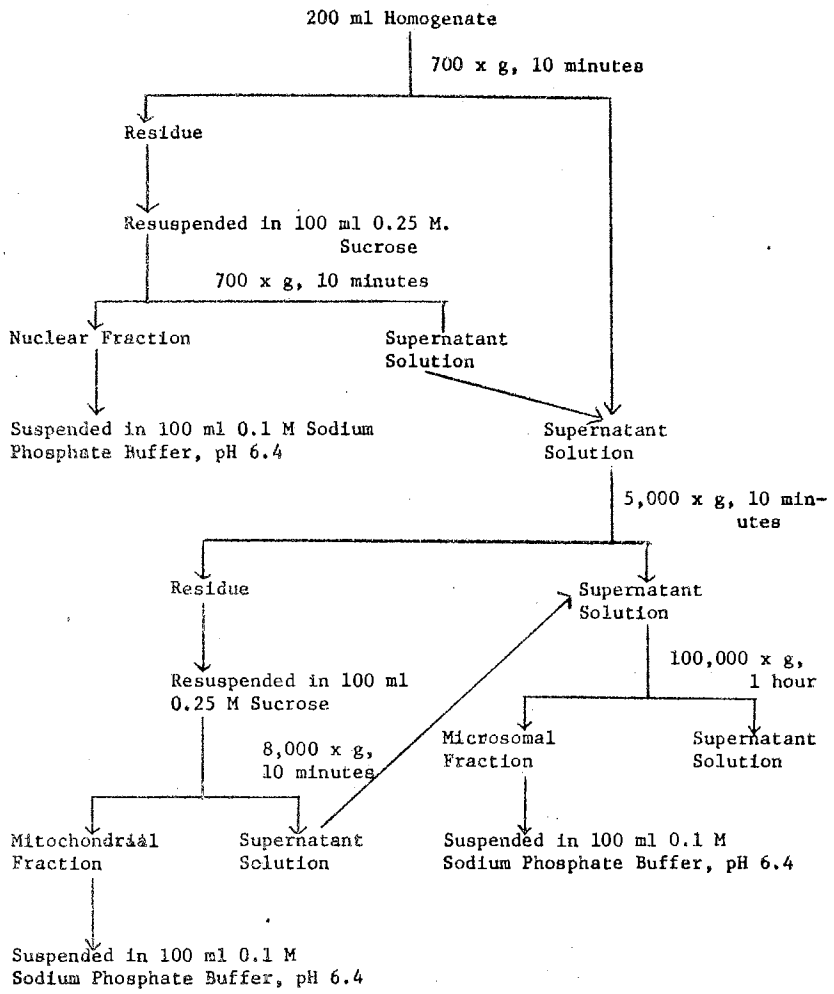


Figure 14. Scheme for the Subcellular Fractionation of Chicken Liver

Partial Purification Procedures

The enzyme was partially purified from the microsomal fraction by the following methods:

Step I. Solubilization of microsomes--The microsomes were solubilized according to the method of Ziegler et al. (76). Microsomal suspensions in 0.1 M sodium phosphate buffer, pH 6.4 in an ice-bath were subjected to sonic vibration with a Bronson 20 KC sonifier, model No. 5125 set at position 8, for 5 minutes. The suspension was centrifuged at 105,000 X g for 2 hours.

Step II. Fractionation with ammonium sulfate--The sonified supernatant solution from step I was brought to approximately 30% saturation by adding 17 grams of ammonium sulfate to each 100 ml of the supernatant solution. The percent saturation at 25°C was based on the table in reference 77. The solid ammonium sulfate was added over a 30 minute period and the solution was stirred for 20 minutes in an ice-bath. The cloudy solution was centrifuged at 12,000 X g for 20 minutes, and the precipitate was discarded. The supernatant solution was brought up to 55% saturation by adding 16 grams ammonium sulfate to every 100 ml of the 30% supernatant solution over a period of 30 minutes. The solution was stirred for another 20 minutes and centrifuged at 12,000 X g for 20 minutes. The supernatant solution was discarded and the precipitate was dissolved in 0.1 M sodium phosphate buffer, pH 6.4, to a final volume of 50 ml. This second ammonium sulfate fraction (30-55% ammonium sulfate fraction) was used for studying the properties of the enzyme.

Protein Determination

Protein was determined by the method of Lowry et al. (78).

Enzymatic Incubation Procedures

The substrate, retinoic acid-15-^{14}C, was dissolved in 95% ethanol and all incubations were carried out in 25-ml Erlenmeyer flasks, each containing 0.05 ml substrate, 0.5 ml of enzyme and 0.1 ml cofactor. The total volume was made up to 1.0 or 2.0 ml with 0.1 M sodium phosphate buffer, pH 6.4. Unless otherwise stated, the final concentration of retinoic acid was 20 μM and other components were 10^{-3} M. The incubations were carried out at 37°C in a shaker water bath (Gyrotory Shaker, Model G-76, New Brunswick Scientific Co., New Brunswick, New Jersey) at a shaker speed setting of 2.5 for 20 minutes or for the time stated. Carbon dioxide was collected using a small glass vial which contained the trapping solution (ethanolamine:ethylene glycol monomethyl ether v/v 1:2). The whole trapping solution of each incubation, at the end of the incubation period, was transferred into a counting vial and the trapping vial was rinsed with 10 ml of a toluene-ethylene glycol monomethyl ether mixture. Samples were then counted in a Packard Tri-Carb Model 314 EX-2 scintillation counter. Blanks, control using boiled enzymes and the complete system without enzymes were included in every assay. The degree of reaction is expressed as the radioactivity (cpm) in $^{14}CO_2$ released and also as the percentage of retinoic acid decarboxylated.

Results

Decarboxylation of Retinoic Acid in vivo

The study of the decarboxylation of retinoic acid in the chick *in vivo* was conducted as described under "Experimental." The results are shown in Figure 15. The radioactive $^{14}CO_2$ expired by chicks within

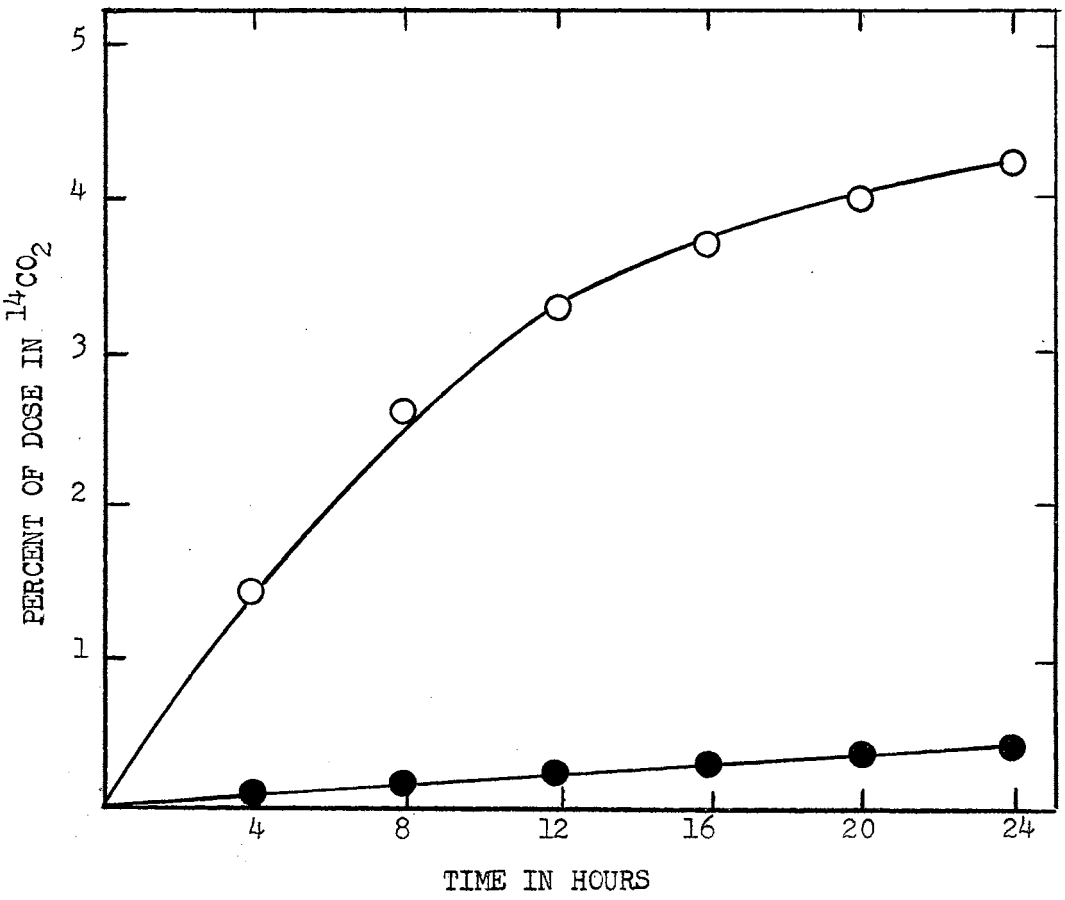


Figure 15. Decarboxylation of Retinoic Acid *in vivo*

The open circle (-O-) represents $^{14}CO_2$ from retinoic acid-15-^{14}C and the closed circle (-●-) $^{14}CO_2$ from retinoic acid-6,7-^{14}C.

24 hours is much higher in the group fed retinoic acid-15-^{14}C than in the group fed retinoic acid-6,7-^{14}C. The total radioactivity expired as $^{14}CO_2$ within 24 hours reached 4.5% of the dose in chicks fed retinoic acid-15-^{14}C, but was much lower (0.4%) in the case of retinoic acid-6,7-^{14}C.

Subcellular Distribution of the Retinoic Acid Decarboxylating Enzyme

Table VII shows the subcellular distribution of this enzyme in chicken liver when the fractions were prepared as described under "Experimental." It can be seen that the specific activity and the total activity of liver tissue was maximum in the microsomal fraction. However, enzymatic activity was also present in the mitochondrial fraction. Preliminary data from recent experiments using the fractionation scheme described by Mahler and Cordes in the textbook "Biological Chemistry" (79) indicates that the decarboxylating enzyme is present only in the microsomal fraction.

Temperature Stability of the Retinoic Acid Decarboxylating Enzyme

The stability of this enzyme is shown in Table VIII. The enzyme, boiled for 10 minutes, loses almost all activity, and the enzyme which set for 12 hours at room temperature lost about 85% of its activity. On the other hand, the frozen enzyme retains full activity and can be stored at -20°C for at least 3 weeks without any loss of activity.

TABLE VII

SUBCELLULAR DISTRIBUTION OF THE RETINOIC ACID DECARBOXYLATING ENZYME IN CHICKEN LIVER

Fraction	Total Volume	Enzymatic Activity[a]	Substrate Decarboxylated	Total Activity	Total Protein	Specific Activity	Distribution in Liver
	ml	cpm	percentage	cpm	mg	cpm/mg protein	percentage
Nuclear	100	23	1	2300	103	22	2
Mitochondrial	100	428	14	42800	43	995	44
Microsomal	100	514	18	51400	36	1428	54
Supernatant	400	0	0	0	184	0	0

[a]Each assay mixture, containing 0.5 ml of 0.1 M sodium phosphate buffer, pH 6.4, 0.5 ml of each fraction and 18 pM (approximately 2950 cpm) retinoic acid-15-^{14}C as substrate, was incubated at 37°C for 20 minutes. The enzymatic activity is expressed as the radioactivity (cpm) in $^{14}CO_2$ and also as the percentage of retinoic acid decarboxylated.

TABLE VIII

TEMPERATURE STABILITY OF THE RETINOIC ACID DECARBOXYLATING ENZYME

Fraction	Enzymatic Activity[a]	Substrate Decarboxylated
	cpm	percentage
Mitochondrial	468	16
Microsomal	524	18
Heated Mitochondrial[b]	41	1
Heated Microsomal[b]	62	2
Set Mitochondrial[c]	75	3
Set Microsomal[c]	75	3
Frozen Mitochondrial[d]	529	18
Frozen Microsomal[d]	538	18

[a]Assay conditions were the same as described in Table VII.

[b]Enzyme was kept in a boiling water bath for 10 minutes, cooled and added to the assay system.

[c]Enzyme was held 12 hours at 23°C.

[d]Enzyme was frozen 3 weeks at -20°C.

Effect of Addition of Supernatant Fraction To Particulate Fractions on the Activity of the Retinoic Acid Decarboxylating Enzyme

The supernatant fraction, which has no activity, as shown in Table VII was added to the assay system which contained either the mitochondrial and/or the microsomal fraction. The results are shown in Table IX. The addition of supernate fraction almost doubled the enzymatic activity of both the mitochondrial and microsomal fractions. It was concluded that there must be some cofactors present in the supernatant solution which can increase the enzymatic activity.

Partial Purification of the Retinoic Acid Decarboxylating Enzyme

Since the purpose of this investigation was chiefly to isolate the metabolites resulting from the decarboxylation reaction of retinoic acid, the enzyme was only partially purified. Table X shows a summary of purification steps. No attempts were made to purify the enzyme further. The enzyme from chicken liver was purified 13 fold from microsomes or 292 fold from liver homogenate, with an increased yield. The second ammonium sulfate (30 - 55% saturation) precipitate was used to study the properties of this enzyme.

Effect of Metal Ions on the Enzymatic Activity

At a concentration of 10^{-3} M, and under the conditions of this assay most of the compounds listed in Table XI had no measureable effect on enzymatic activity. Mn^{++} and Co^{++} inhibited the enzyme and

TABLE IX

EFFECT OF ADDITION OF SUPERNATANT FRACTION TO PARTICULATE FRACTIONS ON THE ACTIVITY OF THE RETINOIC ACID DECARBOXYLATING ENZYME

Fraction	Enzymatic Activity [a]	Substrate Decarboxylated
	cpm	percentage
Mitochondrial	412	14
Microsomal	476	16
Mitochondrial + Supernatant	812	27
Microsomal + Supernatant	884	30
Mitochondrial + Microsomal + Supernatant	982	32

[a] Assay conditions were the same as described in Table VII except the total volume was made up to 2 ml with 0.1 M sodium phosphate buffer.

TABLE X

PARTIAL PURIFICATION OF THE RETINOIC ACID DECARBOXYLATING ENZYME

Fraction	Total Volume	Total Protein	Total Activity[a]	Yield	Specific Activity	Fold Purification
	ml	mg	cpm	percentage	cpm/mg protein	
Homogenate	200	388	24000	---	62	--- (1)[b]
Microsomal	100	36	51400	100	1430	1
Sonified Supernatant	90	12	48780	94	4065	3
2nd Ammonium Sulfate ppt.	50	4	72300	140	18075	13 (292)[b]

[a]Activity is expressed as the radioactivity (cpm) in $^{14}CO_2$. Assay system was the same as described in Table VII.

[b]The number in parenthesis represents the fold purification with the homogenate being one.

TABLE XI

EFFECT OF METAL IONS ON ENZYMATIC ACTIVITY

Compound	Enzymatic Activity [a]	Percentage of Control
	cpm	
Control	614	100
$MgCl_2$	584	95
KCl	548	89
$CaCl_2$	556	91
$MnCl_2$	136	22
$ZnCl_2$	533	87
$HgCl_2$	846	141
$CoCl_2$	208	34
$FeCl_3$	722	118
$BaCl_2$	538	88
$FeCl_2$	4283	698

[a]Each assay mixture, containing 1 ml of 0.1 M sodium phosphate buffer, pH 6.4, 40 µg of enzyme protein (2nd ammonium sulfate precipitate), 20 µM (approximately 8000 cpm) retinoic acid-15-^{14}C and 10^{-3} M of the compound tested, was incubated at 37°C for 20 minutes. The control contained only enzyme and substrate. Enzymatic activity is expressed as radioactivity in $^{14}CO_2$ and as percentage of control.

Hg^{++} slightly increased the enzymatic activity. In sharp contrast to the other metal ions tested, Fe^{++} caused a 7 fold activation of the enzyme. The enzyme did not seem to be sensitive to Fe^{+++}.

Effect of Reducing Agents on the Enzymatic Activity

Table XII shows the effect of certain reducing agents on the enzymatic activity under the stated assay conditions. At 10^{-3} M concentration, all the test compounds, except glutathione, activated the enzyme. L-Cysteine and NADPH increased the enzymatic activity 5 fold. Among all the compounds tested, addition of Fe^{++} again resulted in the highest activation of the enzyme. NADH showed a lower activating effect on the enzyme than NADPH.

Effect of Combining Reducing Agents on the Enzymatic Activity

The effect of combining certain reducing agents on the enzymatic activity is shown in Table XIII. All tested compounds were at a concentration of 10^{-3} M. Although all combinations did increase the enzymatic activity, their activating effects were much lower than the effect of Fe^{++} alone. Upon the addition of NADPH, the activating effect of Fe^{++} on the enzyme was depressed 42%. The addition of L-cysteine reduced the activating effect of Fe^{++} 72% and the addition of both NADPH and L-cysteine resulted in a depression of 78%.

Effect of Bile Salts on the Enzymatic Activity

Since it was known that bile salts increase the activity of the enzymes which act upon vitamin A compounds (80, 81), two of the most common bile salts, sodium glycocholate and sodium taurocholate, were

TABLE XII

EFFECT OF REDUCING AGENTS ON ENZYMATIC ACTIVITY

Compound	Enzymatic Activity[a]	Percentage of Control
	cpm	
Control	623	100
Fe^{++}	4307	691 (100)[b]
NADPH	3319	533 (77)
NADH	1175	189 (27)
L-Ascorbic Acid	1160	186 (27)
L-Cysteine	3123	501 (73)
Glutathione	516	83 (12)

[a]Assay conditions were the same as described under Figure XI with all the test compounds at a concentration of 10^{-3} M. The control contained only enzyme and substrate. Enzymatic activity is expressed as radioactivity in $^{14}CO_2$ and as a percentage of control.

[b]The number in parenthesis represents the enzymatic activity as a percentage of the activity of the incubation mixture containing Fe^{++}.

TABLE XIII

EFFECT OF COMBINATION OF REDUCING AGENTS ON ENZYMATIC ACTIVITY

Compound	Enzymatic Activity[a]	Percentage of Control
	cpm	
Control	628	100
Fe^{++}	4295	684 (100)[b]
Fe^{++} + NADPH	2513	400 (58)
Fe^{++} + L-Cysteine	1208	192 (28)
Fe^{++} + NADPH + Cysteine	947	151 (22)

[a]Assay conditions were the same as described under Figure XI with all the test compounds at a concentration of 10^{-3} M. The control contained only enzyme and substrate. The enzymatic activity is expressed as radioactivity (cpm) in $^{14}CO_2$.

[b]The number in parenthesis represents the enzymatic activity as a percentage of the activity of the incubation mixture containing Fe^{++}.

added to the incubation system. The effects are shown in Table XIV and XV. Five milligrams of sodium glycocholate showed a slight activation of the enzyme, however, increasing concentrations of the bile salt to 50 mg per reaction mixture decreased the activity. Sodium taurocholate had the same effect.

Effect of Other Compounds on the Enzymatic Activity

The compounds tested were cytochrome C, citrate, ATP, and coenzyme A. The results are shown in Table XVI. All the tested compounds, except citrate which had no effect, increased the activity slightly. Among them, ATP was the most effective one. However, when compared with the activating effect of Fe^{++}, the effects of these compounds were quite low. The fact that there was no effect from citrate and a slight effect from ATP and the combination of Mg^{++}, ATP, and Coenzyme A, seems to eliminate the possibility that this oxidation resembles β-oxidation of fatty acids.

Effect of Concentration of Fe^{++} on the Enzymatic Activity

Figure 16, which illustrates the dependence of the reaction on the concentration of added Fe^{++}, shows that a concentration of 10^{-3} M Fe^{++} gave the maximum activity in which approximately 50% of added retinoic acid-15-^{14}C was recovered as $^{14}CO_2$. Increasing the concentration of Fe^{++} beyond 10^{-3} M did not increase the activity. Therefore in the assay system for studying the properties of the enzyme, 10^{-3} M Fe^{++} was used.

TABLE XIV

EFFECT OF SODIUM GLYCOCHOLATE CONCENTRATION ON ENZYMATIC ACTIVITY

Sodium Glycocholate	Enzymatic Activity[a]	Percentage of Control
mg	cpm	
0 (Control)	614	100
5	951	155
15	351	57
25	316	51
35	298	48
50	202	33

[a]Assay conditions were the same as described under Table XI except various amounts of sodium glycocholate were added to the assay system. The control contained only enzyme and substrate. The enzyme activity is expressed as radioactivity (cpm) in $^{14}CO_2$ and as a percentage of control.

TABLE XV

EFFECT OF SODIUM TAUROCHOLATE CONCENTRATION ON ENZYMATIC ACTIVITY

Sodium Taurocholate	Enzymatic Activity[a]	Percentage of Control
mg	cpm	
0 (Control)	614	100
5	1169	190
15	396	64
25	356	58
35	300	49
50	196	32

[a]Assay conditions were the same as described under Table XI except various amounts of sodium taurocholate were added to the assay system. The control contained only enzyme and substrate. The enzymatic activity is expressed as radioactivity (cpm) in $^{14}CO_2$.

TABLE XVI

EFFECT OF OTHER COMPOUNDS ON ENZYMATIC ACTIVITY

Compound	Enzymatic Activity[a]	Percentage of Control
	cpm	
Control	627	100
Fe^{++}	4254	678 (100)[b]
Cytochrome C	1072	171 (25)
Citrate	653	104 (15)
ATP	1308	209 (31)
Co A	824	131 (19)
Mg^{++} + ATP + Co A	1026	163 (24)

[a]Assay conditions were the same as described under Table XI with all test compounds at a concentration of 10^{-3} M. The control contained only enzyme and substrate. Enzymatic activity is expressed as radioactivity (cpm) in $^{14}CO_2$.

[b]The number in parenthesis represents the enzymatic activity as a percentage of the activity of the incubation mixture containing Fe^{++}.

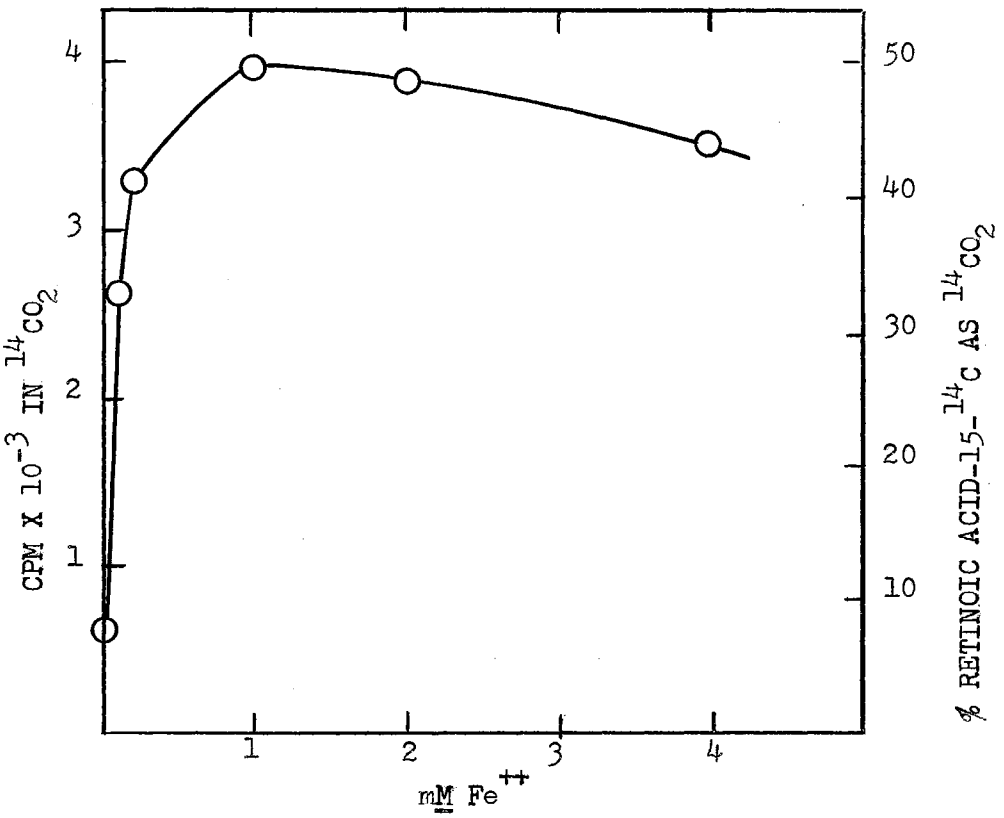


Figure 16. Effect of Concentration of Fe^{++} on Enzymatic Activity

Each assay mixture, containing 1.0 ml of 0.1 M sodium phosphate buffer, pH 6.4, 40 µg of enzyme protein, 20 µM retinoic acid-15-^{14}C approximately 8000 cpm) and various concentrations of Fe^{++}, was incubated at 37°C for 20 minutes. Enzymatic activity is expressed as radioactivity (cpm) in $^{14}CO_2$ and as the percentage of retinoic acid-15-^{14}C decarboxylated.

Requirement for Oxygen

Table XVII shows that the enzyme required oxygen for activity. Even in the presence of 10^{-3} M Fe^{++}, the enzyme had very low activity if nitrogen was used for the gas phase. The substitution of oxygen for air did not increase the activity. Oxygen in air seems to fulfill the requirement for enzymatic activity. Therefore, air was used for the gas phase in the assay of enzymatic activity.

Enzymatic Activity Versus Protein Concentration

The assay mixture was incubated with various levels of enzyme protein, partially purified through the second ammonium sulfate (30 - 50% saturation) precipitation step, for 20 minutes at 37°C. The results are shown in Figure 17. The reaction is linear with protein concentration up to about 40 µg of protein per 1.0 ml total volume. Since maximum decarboxylation was desired assays contained enough enzyme to get 40 - 50% of the substrate decarboxylated.

Time Course of Reaction

The reaction mixture was incubated with 40 µg of enzyme protein, partially purified through the second ammonium sulfate precipitation step, at 37°C for the times listed. The results are shown in Figure 18. The reaction reached a maximum by 20 minutes. Therefore, tc obtain maximal decarboxylation reaction mixtures were incubated for 20 minutes.

TABLE XVII

REQUIREMENT OF OXYGEN FOR ENZYMATIC ACTIVITY

Gas Phase	Enzymatic Activity [a]	Percentage of Substrate
	cpm	
Air	4161	51
Nitrogen	753	9
Oxygen	4218	52

[a]Each assay mixture, containing 1.0 ml of 0.1 M sodium phosphate buffer, pH 6.4, 10^{-3} M Fe^{++}, and 20 µM retinoic acid-15-^{14}C (approximately 8000 cpm), was incubated at 37°C for 20 minutes under the gas phases listed. Enzymatic activity is expressed as radioactivity (cpm) in $^{14}CO_2$ and as percentage of retinoic acid-15-^{14}C decarboxylated.

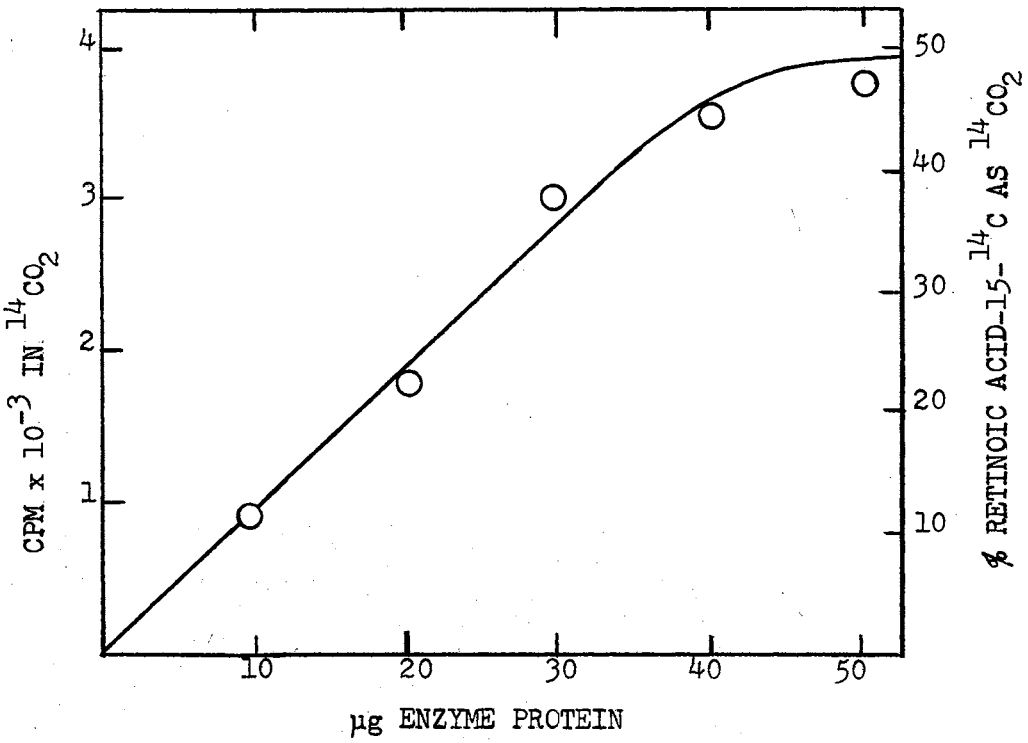


Figure 17. Enzymatic Activity Versus Protein Concentration

Each assay mixture, containing 20 µM retinoic acid-15-^{14}C (approximately 8000 cpm), 10^{-3} M Fe^{++} and indicated amount of enzyme protein in 1.0 ml 0.1 M sodium phosphate buffer, pH 6.4, was incubated at 37°C for 20 minutes. Enzymatic activity is expressed as radioactivity (cpm) in $^{14}CO_2$ and as the percentage of retinoic acid-15-^{14}C decarboxylated.

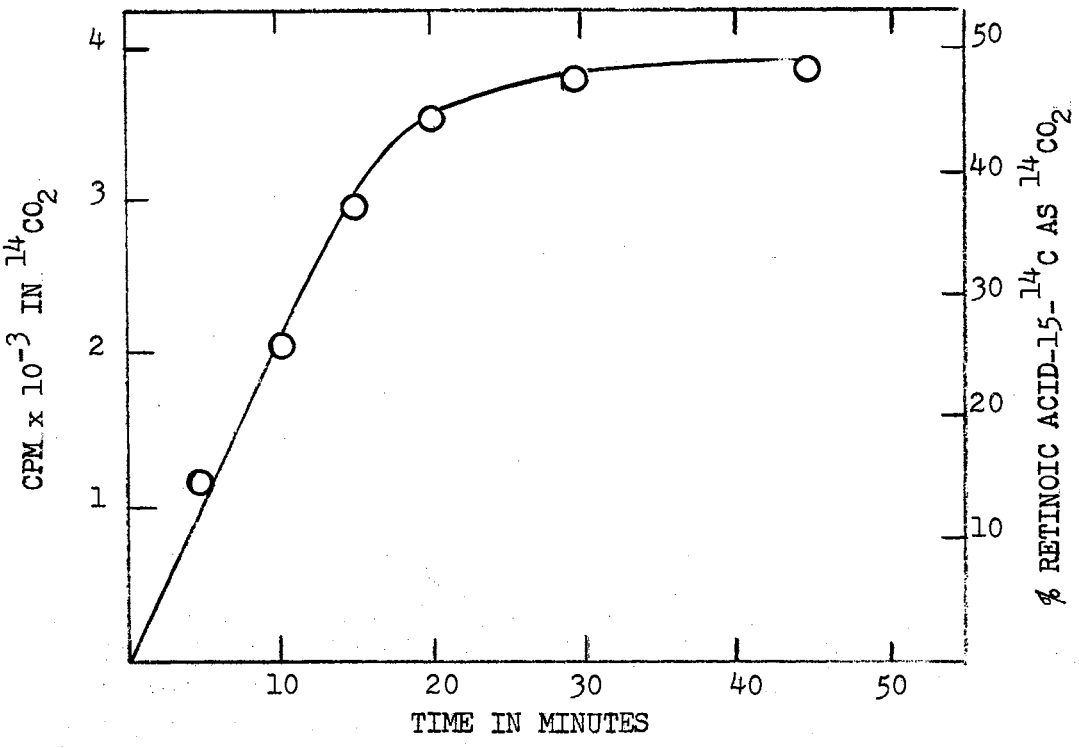


Figure 18. Time Course of the Enzymatic Reaction

Each assay mixture, containing 20 μM retinoic acid-15-^{14}C (approximately 8000 cpm), 10^{-3} M Fe^{++} and 40 μg of enzyme protein in 1.0 ml 0.1 M sodium phosphate buffer, pH 6.4, was incubated at 37°C for various times. The enzymatic activity is expressed as radioactivity (cpm) in $^{14}CO_2$ and as the percentage of retinoic acid-15-^{14}C decarboxylated.

Effect of Temperature on the Enzymatic Activity

Forty μg of enzyme protein, partially purified through the second ammonium sulfate precipitation step, were incubated at indicated temperatures for 20 minutes. The results are shown in Figure 19. Even at $0^{\circ}C$, the enzyme showed some activity. However, the optimum temperature was found to be $37^{\circ}C$ and the enzyme still had high activity at temperatures to $45^{\circ}C$.

Effect of pH on the Enzymatic Activity

The partially purified enzyme had a pH optimum at 6.4 as shown in Figure 20. The enzyme was active between pH 5 and 7. On the other hand, the activity was very low below pH 4 or beyond pH 10. Therefore, routine incubations were conducted with 0.1 M sodium phosphate buffer at pH 6.4.

Effect of Substrate Concentration on the Enzymatic Activity

Figure 21 shows that the amount of $^{14}CO_2$ produced at the completion of the reaction was directly proportional to the concentration of retinoic acid in the range of 0 - 40 μM. A graphical determination of the Michaelis constant for retinoic acid by a Lineweaver-Burk plot (Figure 22) gave a K_m value of 1.0×10^{-4} M under the condition of this assay.

Effect of Inhibitors on the Enzymatic Activity

Incubation mixture, containing 20 μM retinoic acid-15-^{14}C, 10^{-3} M Fe^{++}, and 40 μg of enzyme protein in 1.0 ml sodium phosphate buffer,

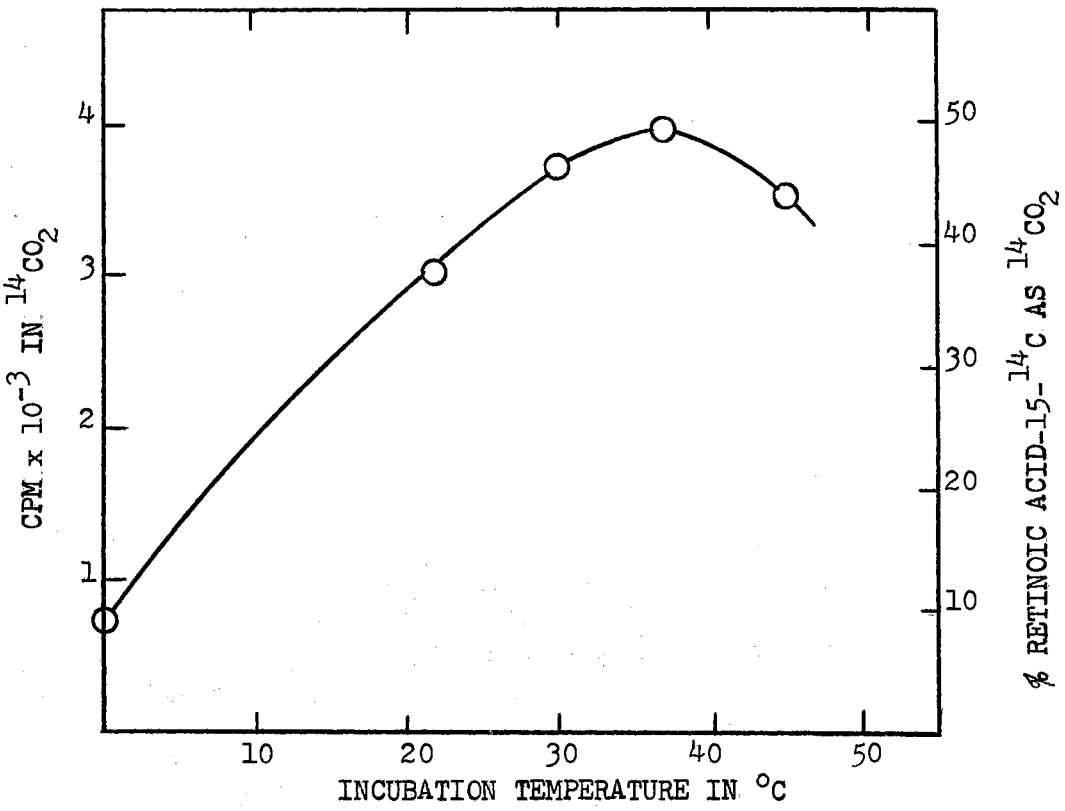


Figure 19. Effect of Temperature on the Enzymatic Activity

Forty µg of the enzyme protein was incubated at various temperatures for 20 minutes in 1.0 ml 0.1 M sodium phosphate buffer, pH 6.4, containing 20 µM retinoic acid-15-^{14}C (approximately 8000 cpm) and 1 mM Fe^{++}. The enzymatic activity is expressed as radioactivity (cpm) in $^{14}CO_2$ and as the percentage of retinoic acid-15-^{14}C decarboxylated.

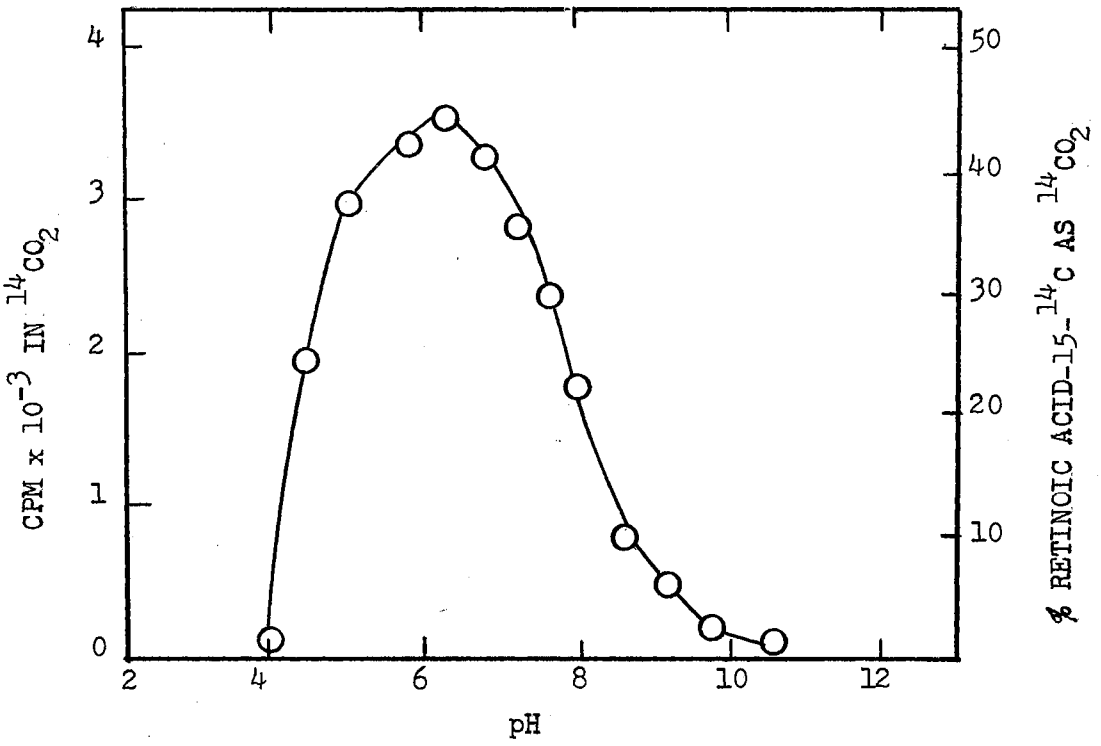


Figure 20. Effect of pH on the Enzymatic Activity

Each assay mixture containing 20 μM retinoic acid-15-^{14}C (approximately 8000 cpm), 10^{-3} M Fe^{++} and 40 μg of enzyme protein in 1.0 ml 0.1 M buffer tested was incubated at 37°C for 20 minutes. Sodium acetate buffer was used for pH 4-5, sodium phosphate buffer for pH 5.8-8.0 and glycine buffer for pH 8.6-10.6. The enzymatic activity is expressed as radioactivity (cpm) in $^{14}CO_2$ and as the percentage of retinoic acid-15-^{14}C decarboxylated.

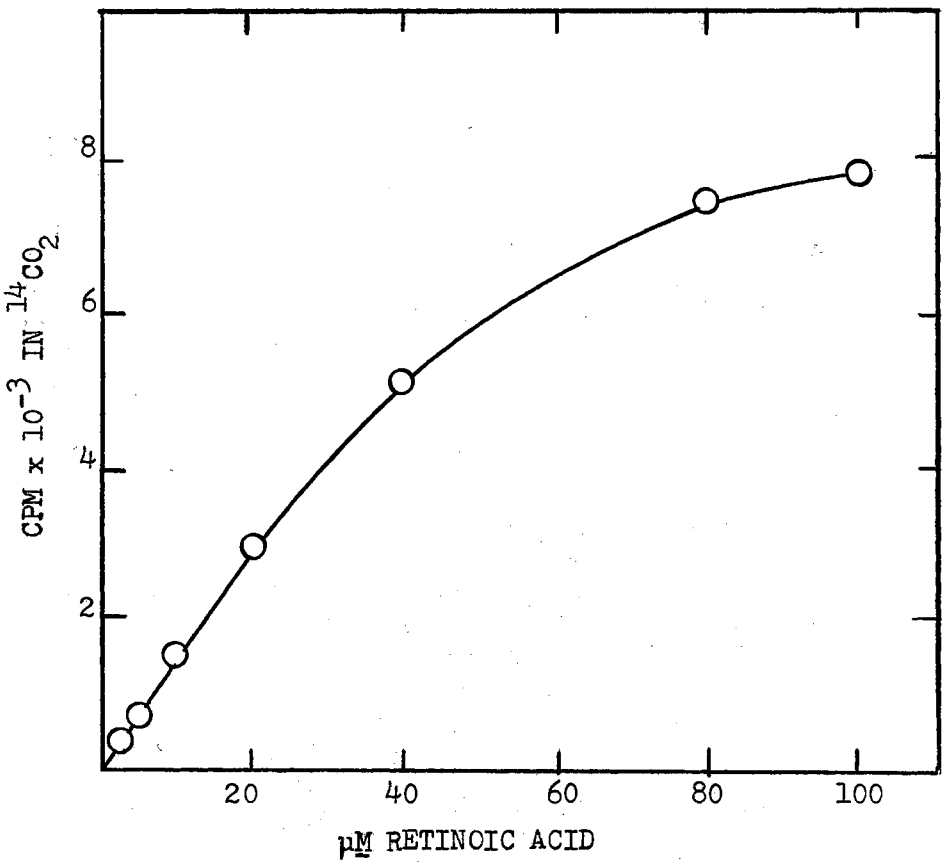


Figure 21 Effect of Retinoic Acid Concentration on the Enzymatic Activity

Thirty μg of enzyme protein was incubated at 37°C for 20 minutes with 10^{-3} M Fe^{++} and indicated concentration of retinoic acid-15-^{14}C (approximately 4000 cpm/10 μM) in 1.0 ml 0.1 M sodium phosphate buffer. The enzymatic activity is expressed as radioactivity (cpm) in $^{14}CO_2$ and as the percentage of retinoic acid-15-^{14}C decarboxylated.

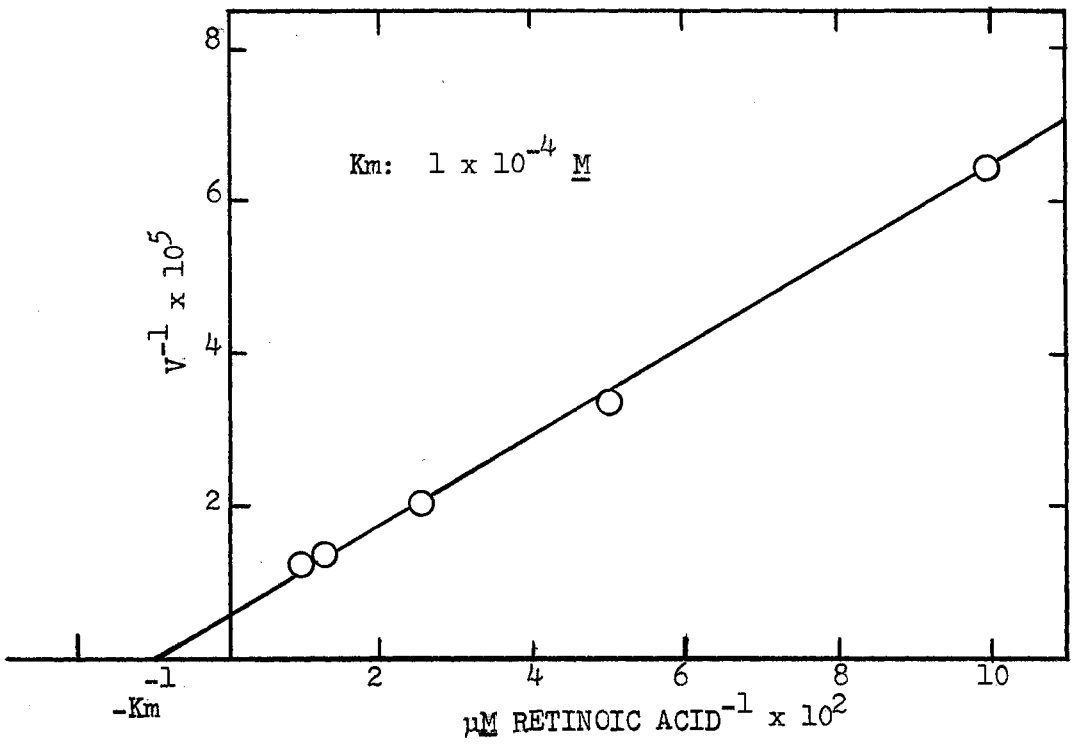


Figure 22. Lineweaver-Burk Plot of Figure 21

A Lineweaver-Burk plot, reciprocal velocity versus reciprocal substrate concentration, was made from the data of Figure 21 and the Km was determined by extrapolating to the base line.

pH 6.4 was incubated at 37°C for 20 minutes. Certain inhibitors were added to the mixture before incubation and their effects on the enzyme activity were compared to the mixture without inhibitor. The results are shown in Table XVIII. Most of the inhibitors tested inhibited the reaction under the condition of this assay. N,N'-diphenyl-p-phenylene diamine (DPPD) phenazine methosulfate and probably cyanide completely inhibited the enzyme activity. o-Phenanthroline and EDTA also strongly inhibited the reaction, however, azide only gave a slight inhibition. p-Chloromercuribenzoate had about 50% inhibition.

Discussion

The results presented in this chapter indicate that retinoic acid is capable of undergoing decarboxylation in chicks *in vivo* and *in vitro*. These findings agree with the *in vivo* observations in rats by Roberts and DeLuca (72) and Sundaresan and Therriault (73), and also with the *in vitro* observations in rat liver and kidney slices (47) and microsomes (48) by Roberts and DeLuca. The *in vivo* study with chicks shows that decarboxylation within 24 hours was about 4.5% of the oral dose, which is lower than the findings of 9% and 14% in rats by Roberts and DeLuca (72) and Sundaresan and Therriault (73), respectively.

By the fractionation method of Schneider and Hogeboom (75), this enzyme was found to be maximum in the microsomes and was also present in mitochondria. A decarboxylating enzyme in rat liver and kidney microsomes, which were prepared according to the method of Ernster, *et al.* (82), was reported by Roberts and DeLuca (48). However, they did not report the enzymatic activity of other fractions. Recent experiments in our laboratory using another fractionation method (79)

TABLE XVIII

EFFECT OF INHIBITORS ON ENZYMATIC ACTIVITY

Inhibitor	Enzymatic Activity[a]	Percentage of Control
	cpm	
Control (Fe^{++})	4137	100
NaN_3	3308	80
NaCN	290	7
o-Phenanthroline	508	12
Phenazine methosulfate	53	1
DPPD	0	0
EDTA	974	23
p-Chloromercuribenzoate	2181	52

[a]Each assay mixture contained 1.0 ml of 0.1 M sodium phosphate buffer, pH 6.4, 40 µg of enzyme protein, 20 µM retinoic acid-15-^{14}C (approximately 8000 cpm), 10^{-3} M Fe^{++} and 10^{-3} M of the compound tested. The enzymatic activity is expressed by the radioactivity (cpm) in $^{14}CO_2$ and as the percentage of control.

showed that this enzyme is a microsomal enzyme. Therefore, the activity in the mitochondria obtained by the fractionation method of Schneider and Hogeboom was probably due to contamination by microsomes.

The maximum of approximately 50% decarboxylation achieved in vitro is in complete agreement with the results reported by Roberts and DeLuca (48). The maximum of 50% decarboxylation achieved must reflect the enzymatic destruction of retinoic acid. It does not reflect a nonenzymatic reaction, for this proceeds at too low a rate to account for 50% decarboxylation in the short time in which a mixture of retinoic acid and Fe^{++} were incubated. However, the reason why no more than 50% decarboxylation has been achieved is perplexing and awaits further study.

The experiments reported here have defined many of the properties of the enzyme which is responsible for the decarboxylation of retinoic acid in chicken liver. The enzyme was partially purified by precipitation with ammonium sulfate between 30 to 55% saturation. It can be stored at -20°C for 3 weeks without any loss of activity, but loses most of the activity if set at room temperature for 12 hours. Similar stability of an enzyme in rat liver and kidney microsomes was reported by Roberts and DeLuca (48). They found that a microsomal suspension of rat liver and kidney could be stored at -17°C for periods of up to 3 weeks, however, it lost about 40% activity after aging at 4°C for 48 hours.

Bile salts, which were reported to have a strong activating effect on enzymes acting upon vitamin A compounds such as retinol palmitate hydrolase (80) and β-carotene cleavage enzyme (81), were found to have a slight activating effect on the decarboxylating enzyme at a

concentration of 5 mg per incubation mixture, but decreased the enzymatic activity at higher concentration. The similarity in effect possessed by both sodium glycocholate and sodium taurocholate indicates the non-specificity of the bile salts to this decarboxylating enzyme. Therefore, it is less likely that these bile salts activate the enzyme directly.

The partially purified enzyme required only O_2 and Fe^{++} to achieve the highest rate of approximately 50% decarboxylation, although other biological reducing agents such as NADPH, L-cysteine, and ascorbic acid also enhanced the enzymatic activity. Any combination of these reducing agents with Fe^{++} did not increase the enzymatic activity. These results are somewhat different from the observations of Roberts and DeLuca (48). They reported that a complete system for liver and kidney microsomes require NADPH in addition to O_2 and Fe^{++} for full enzymatic activity of 50% decarboxylation. However, NADPH also gave a very high activating effect on this partially purified enzyme. In contrast to NADPH, NADH only had a slight activating effect on this enzyme.

The enzyme was inhibited by DPPD, phenazine methosulfate, cyanide and o-phenanthroline. The inhibition produced by the chelating agents suggests the involvement, in the reaction, of a metal ion bound to the enzyme. The participation of a metal ion (specially Fe^{++}) in this reaction might be similar to the mechanism of dioxygenase reactions postulated by Hayaishi for metapyrocatechase and pyrocatechase (83). However, Roberts and DeLuca (48) claimed this decarboxylation reaction mechanism resembles the mechanism of peroxidation. Direct evidence for the mechanism cannot be obtained until the products and intermediates

in this reaction are positively identified.

The metal ions, Mn^{++} and Co^{++}, which activate the α-carboxylase from both yeast (84) and wheat germ (85) were found to inhibit this partially purified enzyme. Oxygen, which had no effect on the activity of acetoacetate decarboxylase (86), appears to be absolutely required for this decarboxylation reaction. It seems, therefore, that the mechanism of this decarboxylation reaction may be quite different from those of carboxylases and decarboxylase.

The decarboxylation reaction, as studied in vitro, has a fairly wide optimal pH range (5.0 to 7.0), and optimal temperature range (30° to $45^{\circ}C$). This partially purified enzyme displays a Michaelis constant, apparent K_m, of 1.0×10^{-4} M under the experimental condition described in the text.

Since the main purpose of this investigation was to obtain maximal decarboxylation of retinoic acid for the isolation of products, all the properties of this enzyme, which were defined in this chapter, were obtained under conditions which would result in maximum decarboxylation. Therefore, more proper conditions should be used to study the actual kinetic parameters of this enzyme. The question, whether or not this oxidative decarboxylation of retinoic acid is significant in vivo, remains unknown.

CHAPTER IV

ISOLATION AND CHARACTERIZATION OF A METABOLITE RESULTING FROM THE DECARBOXYLATION OF RETINOIC ACID *IN VITRO*

The metabolic fate of retinoic acid following its formation from retinal remains largely unknown. Numerous metabolites of radioactive retinoic acid have been isolated from the liver, small intestine and bile of rats, but these metabolites have not been completely characterized (28, 30, 31, 40, 87, 88). Retinoyl β-glucuronide, isolated from rat bile, was the first retinoic acid metabolite to be identified (36, 89). However, this metabolite is not a degradation product of retinoic acid, but is a conjugate of retinoic acid and β-glucuronic acid.

As shown in the previous chapter, retinoic acid is capable of undergoing rapid decarboxylation *in vitro* by a partially purified enzyme from chicken liver. The purpose of the present investigation was to isolate some of the products resulting from the decarboxylation using the incubation system described in Chapter III.

Experimental

Materials

Retinoic Acid and Methyl Retinoate

Radioactive retinoic acid was purified as described under

"Experimental" in Chapter III. Non-radioactive retinoic acid was obtained from Hoffmann-LaRoche, Inc., Nutley, New Jersey. It was purified by recrystallization several times from methanol until the melting point and the ultraviolet spectrum agreed very closely with the reported values (90, 91). All-trans methyl retinoate was synthesized by treating all-trans retinoic acid with diazomethane (92) prepared from N-methyl-N'-nitroso, p-toluene sulfonamide (Aldrich Chemical Co., Milwaukee, Wisconsin) by treatment with sodium hydroxide and was codistilled with ethyl ether (93). Methyl retinoate was recrystallized from methanol:water (5:1) until the melting point and ultraviolet spectrum agreed very closely with the reported values (94). The purity was also checked by gas-liquid chromatography (GLC) and mass spectrometry and only one compound was detected.

Solvents and Chemicals

Reagent grade methanol, 95% ethanol, benzene, chloroform, isopropanol, diethyl ether, n-hexane, petroleum ether, glacial acetic acid, ammonium hydroxide and hydrochloric acid were used without further purification for column and thin-layer chromatography (TLC). Solvents used for spectral measurements were purified by distillation. Reagent grade methanol, ethylene glycol, p-dioxane, sulfur free toluene, and ethylene glycol monomethyl ether were used without further purification for the preparation of scintillation counting fluid.

Methods

Isolation of Reaction Products

At least 40 individual incubation mixtures, containing 40 μg of partially purified enzyme protein, 20 μM retinoic acid and 10^{-3} M Fe^{++}

were incubated at 37°C for 20 minutes. Retinoic acid-6,7-^{14}C, retinoic acid-15-^{14}C or a mixture of retinoic acid-15-^{14}C and retinoic acid-6,7-^{14}C was used as substrate. After the reaction was allowed to proceed for the desired time, all samples were pooled and lyopholized for 12 hours. The lyopholized samples were extracted with a mixture of 100 ml of methanol and chloroform (1:1) in the cold under nitrogen according to the procedure of Bligh and Dyer (95). The extract was evaporated to dryness under vacuum and the residue was dissolved in either 1 ml of methanol or 95% ethanol. The methanol or ethanol solution was applied to an anion exchange resin column or DEAE-cellulose column, respectively. The radioactive fractions were reduced to very small volume (<0.5 ml) under vacuum and were chromatographed on thin-layer plates several times using different solvent systems. Finally, the purified sample was submitted to UV, GLC, and mass spectrometric analysis.

Column Chromatography

An analytical column (1.5 x 8 cm) containing 200-400 mesh Bio-Rad AG2-x8 anion exchange resin in the acetate form (Bio-Rad Laboratories, Richmond, California) was used for chromatographing the methanol extract. Samples were eluted with 100 ml methanol, 100 ml of 1% acetic acid in methanol and a gradient elution of 1% to 30% acetic acid in methanol.

The ethanol extract of the reaction mixture was applied to a DEAE-cellulose column. A column (1.5 x 9 cm) was prepared from a slurry of DEAE-cellulose (medium mesh, Sigma Chemical Company, St. Louis, Missouri) in 95% ethanol, converted to the hydroxyl form by passing through 30 ml of ammoniacal ethanol (95% ethanol, 240 ml;

concentrated NH_3, 10 ml), and washed with 30 ml of 95% ethanol. The sample was transferred to the column, and 100 ml of 95% ethanol was passed through the column. It was then eluted with 100 ml of acidified ethanol (95% ethanol, 300 ml; concentrated HCl, 1 ml). The effluent from the column was collected in 10 ml fractions by an automatic fraction collector (Buchler Instrument Co., Fort Lee, New Jersey). A 0.5 ml aliquot of each fraction was transferred to a counting vial, 15 ml of dioxane-naphthalene scintillation fluid was added (96), and the radioactivity was measured in a Packard Tri-Carb Model 314 EX-2 scintillation counter. The absorbance of each fraction was measured at 350 nm in a Beckman DB spectrophotometer.

Thin-Layer Chromatography (TLC)

Samples were applied on pre-coated analytical TLC plates (Q1, Silica Gel, 5 cm x 20 cm; layer thickness, 250 u ± 4%) which were purchased from Quantum Industries, Fairfield, New Jersey. Plates were pre-developed in the solvent system used, before spotting. Glass capillaries and 100 µl Hamilton syringes were used to apply the samples to the plates. After sample application, the plates were developed. The solvent systems , v/v, used were as follows:

(a). Isopropanol : 30% ammonium hydroxide (4:1)

(b). Benzene : chloroform : methanol (4:1:1)

(c). Benzene

(d). Petroleum ether : ether : acetic acid (25:25:1)

(e). Petroleum ether : ether : acetic acid (90:10:1)

(f). Benzene : chloroform : methanol : acetic acid (5:5:5:1)

Separated components were detected by fluorescence under ultraviolet light at 366 nm, and by iodine vapor. The radioactive spots were

located by a gas flow strip counter (Actigraph III, Model 1006, Nuclear-Chicago, Des Plaines, Illinois) using radioactive ink as marker.

Gas Liquid Chromatography (GLC)

A modified Barber-Colman Gas Chromatograph (Barber-Colman Company, Rockford, Illinois) Model 5000 equipped with hydrogen flame detector (51) was used for all gas chromatography. The column used is interchangeable between this instrument and the mass spectrometer-gas chromatograph. Suitable conditions for separating compounds of interest are developed on this instrument prior to using the mass spectrometer-gas chromatograph.

A $\frac{1}{4}$ in. X 8 ft. helical glass column packed with 1% OV-1 on Gas Chrom Q (Applied Science Laboratories, State College, Pennsylvania) and equipped with a solid injection system (97) was used. In this technique, a sample in chloroform or ether was transferred to a Dixon gauze on a teflon plate and set to dryness. The dry sample on the gauze was introduced to the GLC column by a small magnet.

The DEAE-cellulose column eluates were analyzed without further treatment. The TLC purified metabolite was methylated by the addition of etheral diazomethane until the persistence of yellow color. The reaction mixture was kept for two hours at room temperature and concentrated to a small volume (< 0.5 ml) under nitrogen gas and then applied to the GLC column by the solid injection technique.

The metabolite was also converted to the more volatile trimethyl silyl ethers (TMS-derivative) by the use of bis-(trimethyl-silyl) acetamide (BSA)(98) and trimethylchlorosilane (TMCS) silylating reagents without using pyridine as a solvent (99). Recently McCloskey et al. (100) have also reported this technique on the use of BSA-d_{18}

and TMCS-d_9 in the preparation of groups of compounds. BAS and TMCS are added to the sample and was kept at 55°C for 3 hours, excess reagent was removed by nitrogen stream. The residue (in chloroform) was analyzed by the GLC under the conditions: column: 225°C, injector: 255°C, detector: 265°C and gas flow rate: 50 ml/min.

Ultraviolet Spectrophotometry (UV)

Ultraviolet spectra for the unknown were obtained on a Spectronic 505 ultraviolet recording spectrophotometer (Bauch and Lomb, Inc., Rochester, New York), and retinoic acid on a Beckman DB spectrophotometer. For all compounds, standard 3 ml cells with 1 cm light paths were employed. Column eluates were monitored at 350 nm on a Beckman DB spectrophotometer.

Infrared Spectrometry (IR)

Infrared spectra were determined using a Perkin-Elmer 457 Grating Infrared Spectrophotometer in KBr pellets prepared with a microsampling kit.

Hydrolysis of Metabolite

The TLC purified metabolite was treated with 30% alcoholic KOH and heated in a water bath at 60°C under a nitrogen stream for 2 hours. After neutralizating the hydrolysate with concentrated HCl and evaporating to dryness, the residue was suspended in 2 to 3 ml of ethanol and was again evaporated to dryness. The anhydrous residue was then extracted with methanol. The sample was applied to TLC and GLC. Acid hydrolysis was carried out in 6 N HCl under the same conditions.

Mass Spectrometry

Samples were analyzed on a LKB-9000 Mass Spectrometer-Gas Chromatograph (51, 52) using the direct probe or through the gas chromatograph as described in Chapter II "Experimental". The purified metabolite was also submitted to analysis by CEC 21-110B high resolution Mass Spectrometer at Purdue University, Lafayette, Indiana.

Results

Preliminary Thin-layer Chromatography of Chloroform: Methanol Extract of Incubation Mixtures

Forty incubation mixtures were pooled after incubation as described under "Experimental" and lyopholized for 12 hours. The chloroform : methanol (1:1 v/v) extract of the lyopholized sample was reduced to very small volume (<0.5 ml) and spotted onto analytical thin-layer plates. The plates were developed in the solvent system of isopropanol : 30% ammonium hydroxide (4:1 v/v).

Three different incubation groups were studied. The first group contained retinoic acid-6,7-^{14}C as substrate. From the TLC distribution of radioactivity shown in Figure 23 and 24, it can be seen that two major radioactive peaks were present in both cases. When compared to all-*trans* retinoic acid and all-*trans* methyl retinoate, the compound with the lower R_f value was found to be unchanged retinoic acid and the compound with the higher R_f was neither retinoic acid nor methyl retinoate. The percent of decarboxylation was determined by measuring the amount of $^{14}CO_2$ produced when retinoic acid-15-^{14}C was used as substrate in another incubation mixture assayed concurrently. The ratio of the two radioactive peaks was 1:1 and 3:1 when the decarboxylation

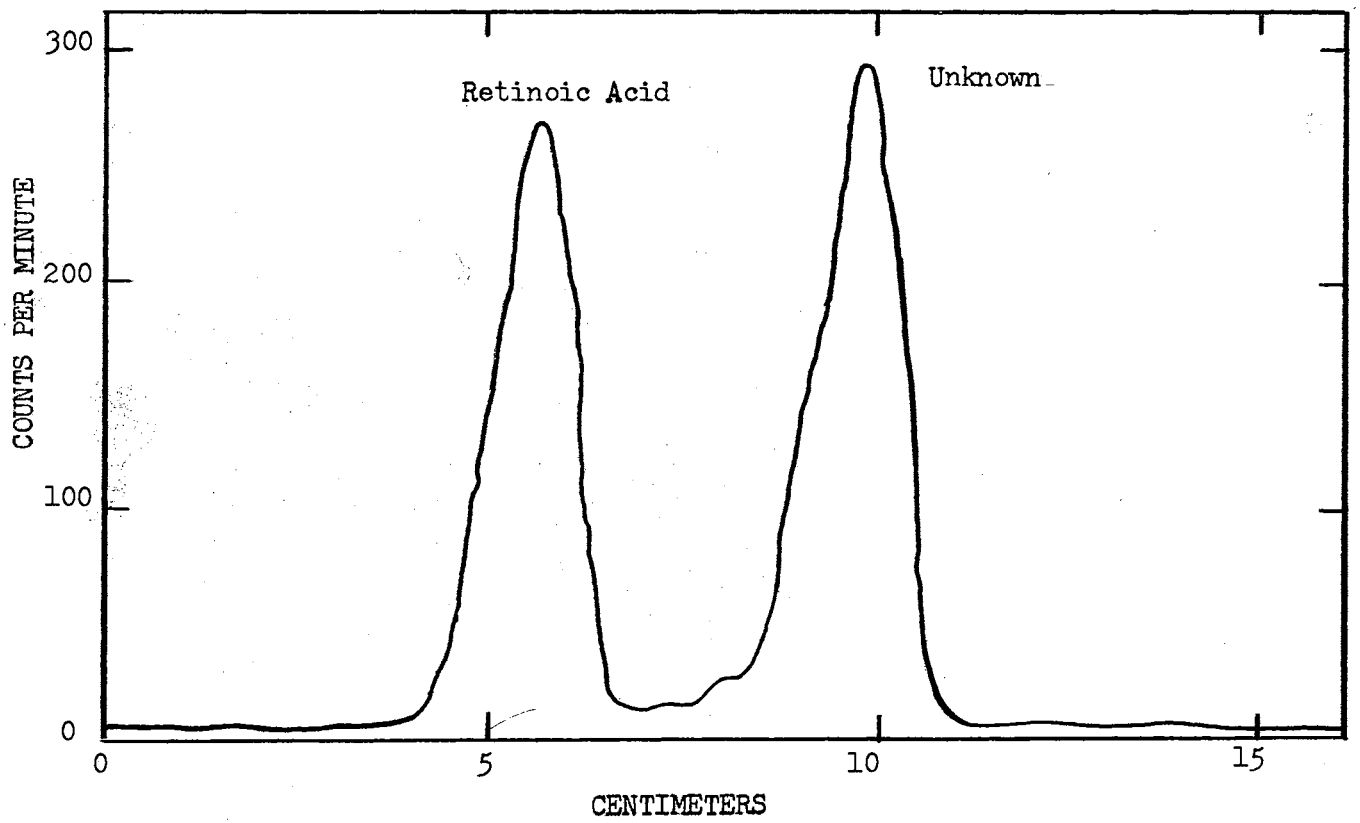


Figure 23. TLC Distribution of Radioactivity of an Extract of the Incubation Mixture using Retinoic Acid-6,7-^{14}C as Substrate with approximately 50% decarboxylation. Solvent system was Isopropanol: 30% ammonium hydroxide (4:1).

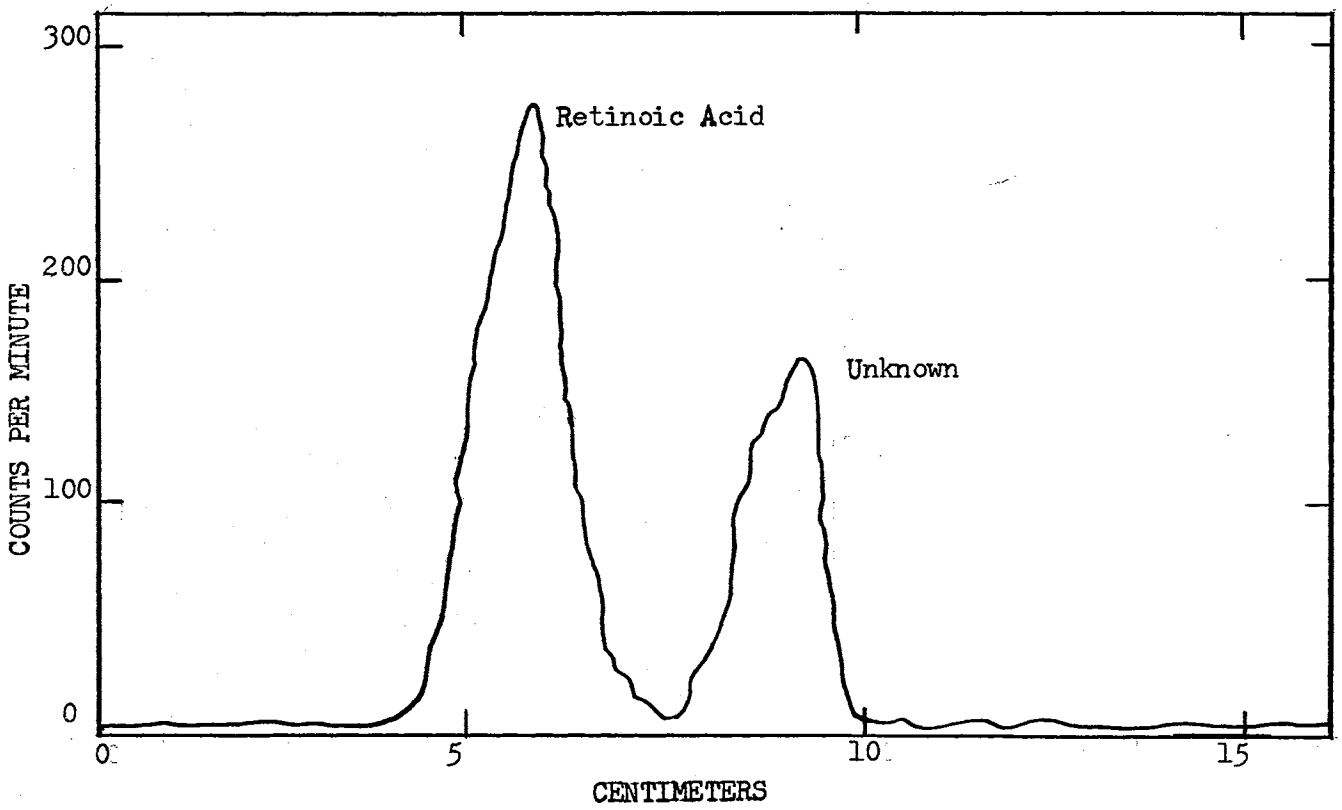


Figure 24. TLC Distribution of Radioactivity of an Extract of the Incubation Mixture using Retinoic Acid-6,7-^{14}C as Substrate with with approximately 35% decarboxylation. Solvent system was isopropanol : 30% ammonium hydroxide (4:1).

was 50 and 35% respectively. These results indicate that the amount of the unknown was proportional to the amount of retinoic acid decarboxylation. Therefore, the compound with the higher R_f value seems to be the major product of this enzymatic reaction. In addition a second group, using only retinoic acid-15-^{14}C as substrate, was studied. Figure 25 shows the TLC distribution of radioactivity for this group. In this case, only one peak, retinoic acid, was obtained, indicating that the unknown product is the decarboxylation product itself or a compound containing the decarboxylation product.

In order to show that the unknown compound was the product of an enzymatic reaction, a third group, the control, containing only retinoic acid-6,7-^{14}C, buffer and Fe^{++} without enzyme was carried through the procedure. The results (Figure 26) show only one peak, retinoic acid thus providing additional evidence that the unknown product was the result of an enzymatic decarboxylation.

Column Chromatography of Chloroform:Methanol Extract of Incubation Mixtures

The chloroform:methanol extract of the incubation mixture, as described under "experimental", was evaporated to dryness under reduced pressure and the residue was dissolved in 1.0 ml 95% ethanol. The ethanol solution of the extract was applied to a DEAE-cellulose column, prepared as described under "Experimental." Again, three different incubation groups (as described in the TLC section) were studied and the results are shown in Figures 27, 28, and 29. When retinoic acid-6,7-^{14}C was used as substrate, there were two major radioactive peaks (Figure 27), one of which was eluted with 95% ethanol and the other

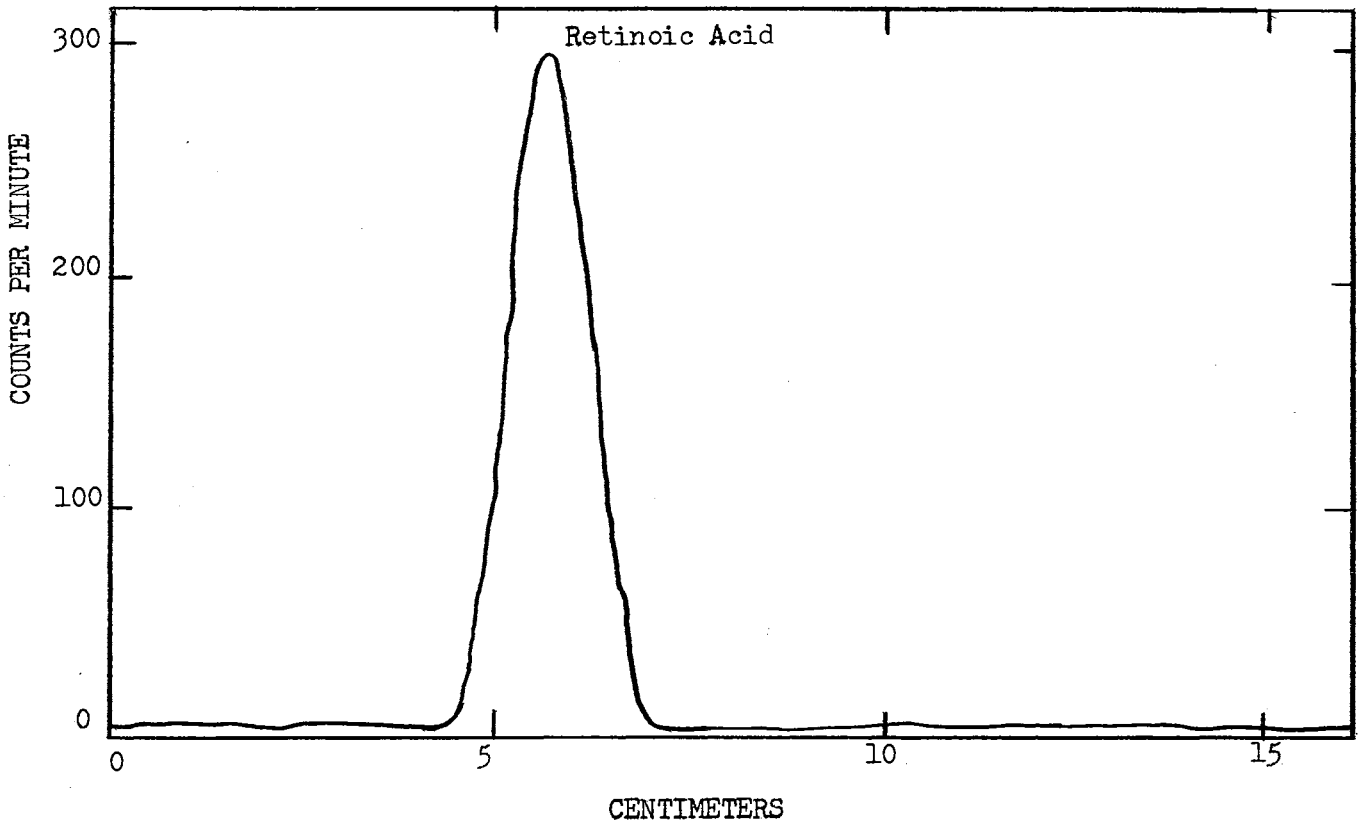


Figure 25. TLC Distribution of Radioactivity of an Extract of the Incubation Mixtures with Retinoic Acid-15-^{14}C as substrate. Solvent system was isopropanol : 30% ammonium hydroxide (4:1).

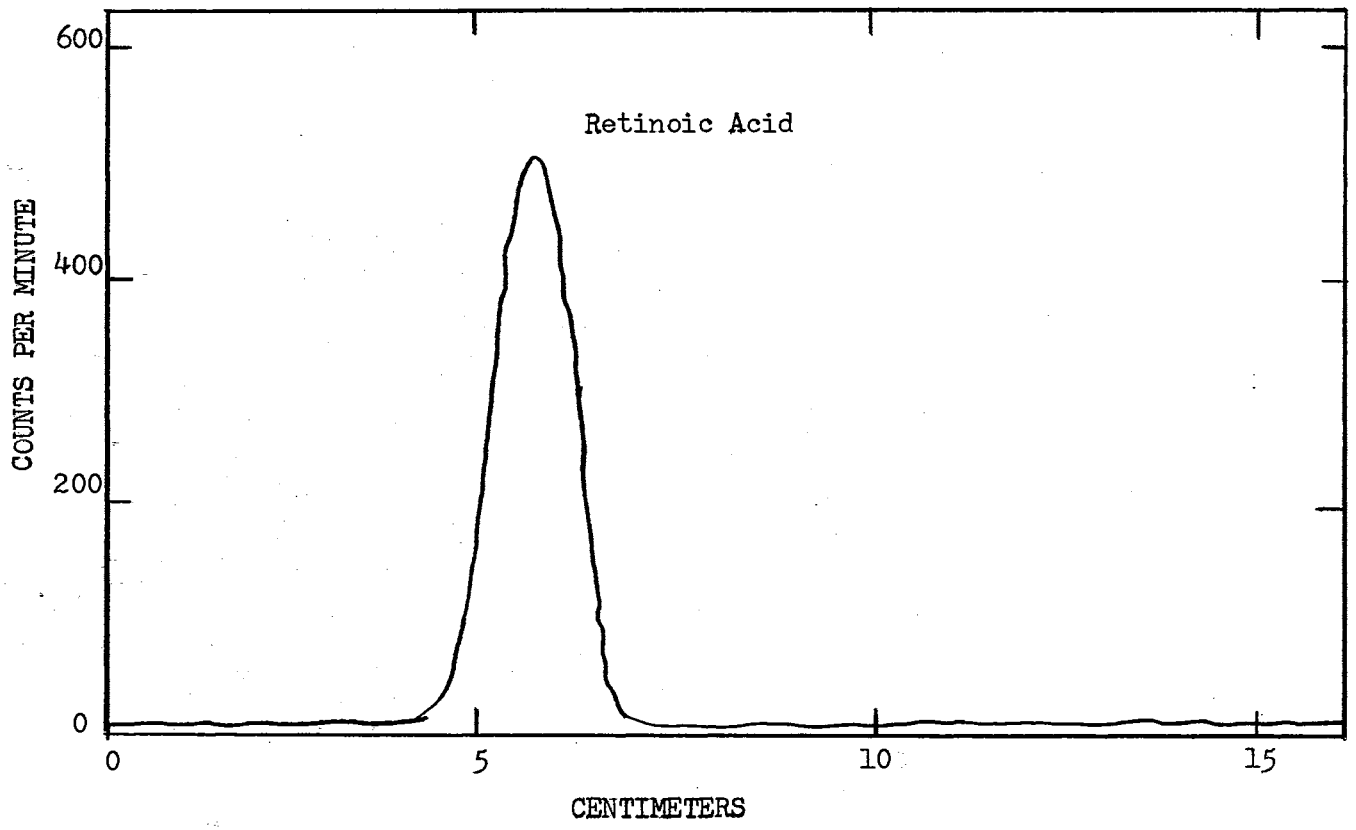


Figure 26. TLC Distribution of Radioactivity of an Extract of Control Incubation containing Retinoic Acid-6,7-^{14}C without Enzyme. Solvent system was isopropanol : 30% ammonium hydroxide (4:1).

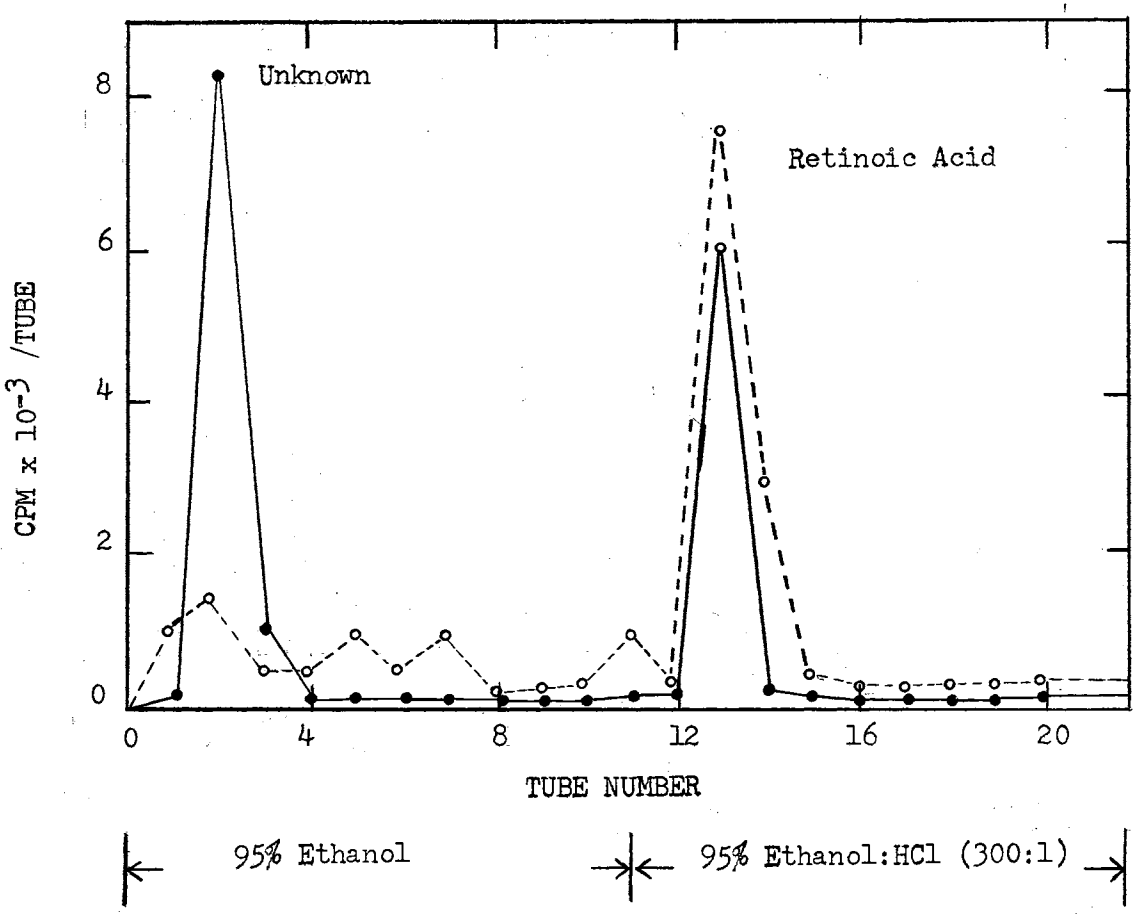


Figure 27. DEAE-cellulose Column Chromatography of a Chloroform:Methanol Extract of Incubation Mixtures with Retinoic Acid-6,7-^{14}C as Substrate. The solid line represents cpm/tube; the broken line represents absorbance at 350 nm.

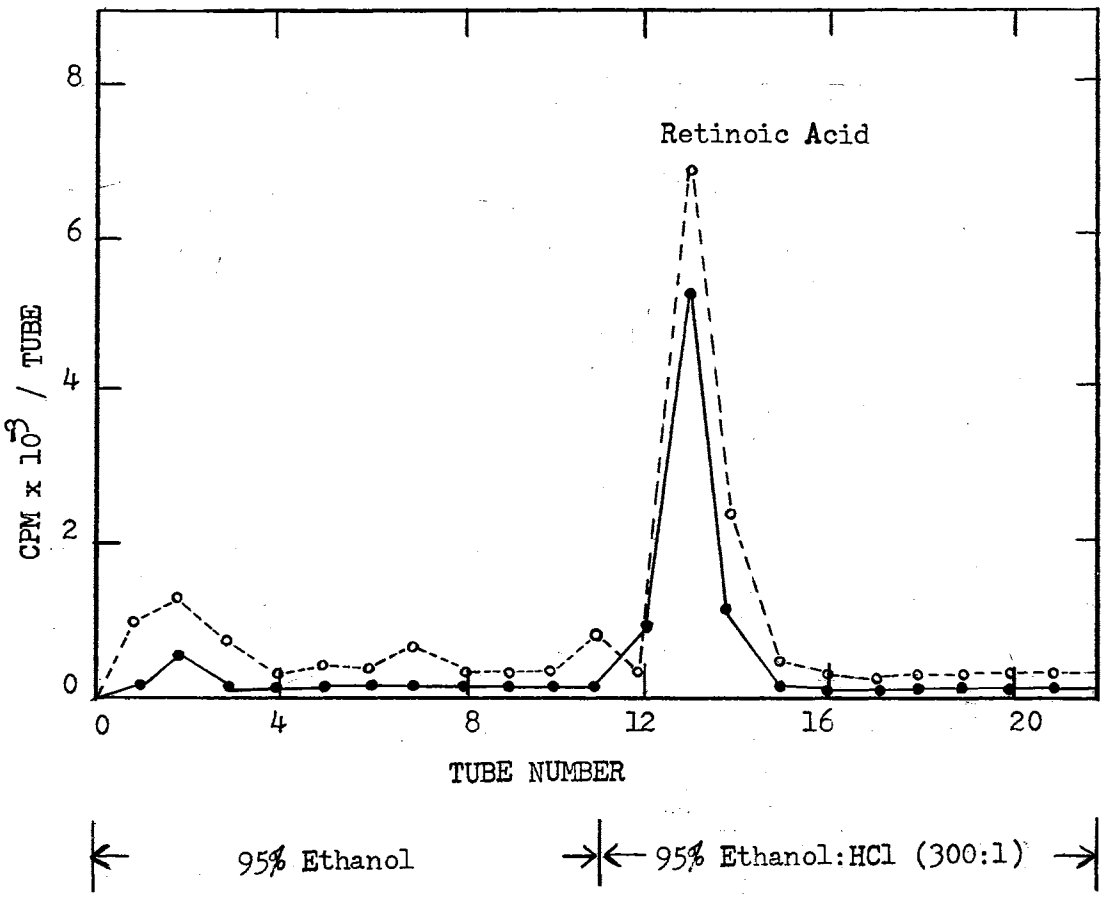


Figure 28. DEAE-cellulose Column Chromatography of a Chloroform:Methanol Extract of Incubation Mixture using Retinoic Acid-15-^{14}C as Substrate. The solid line represents cpm/tube; the broken line represents absorbance at 350 nm.

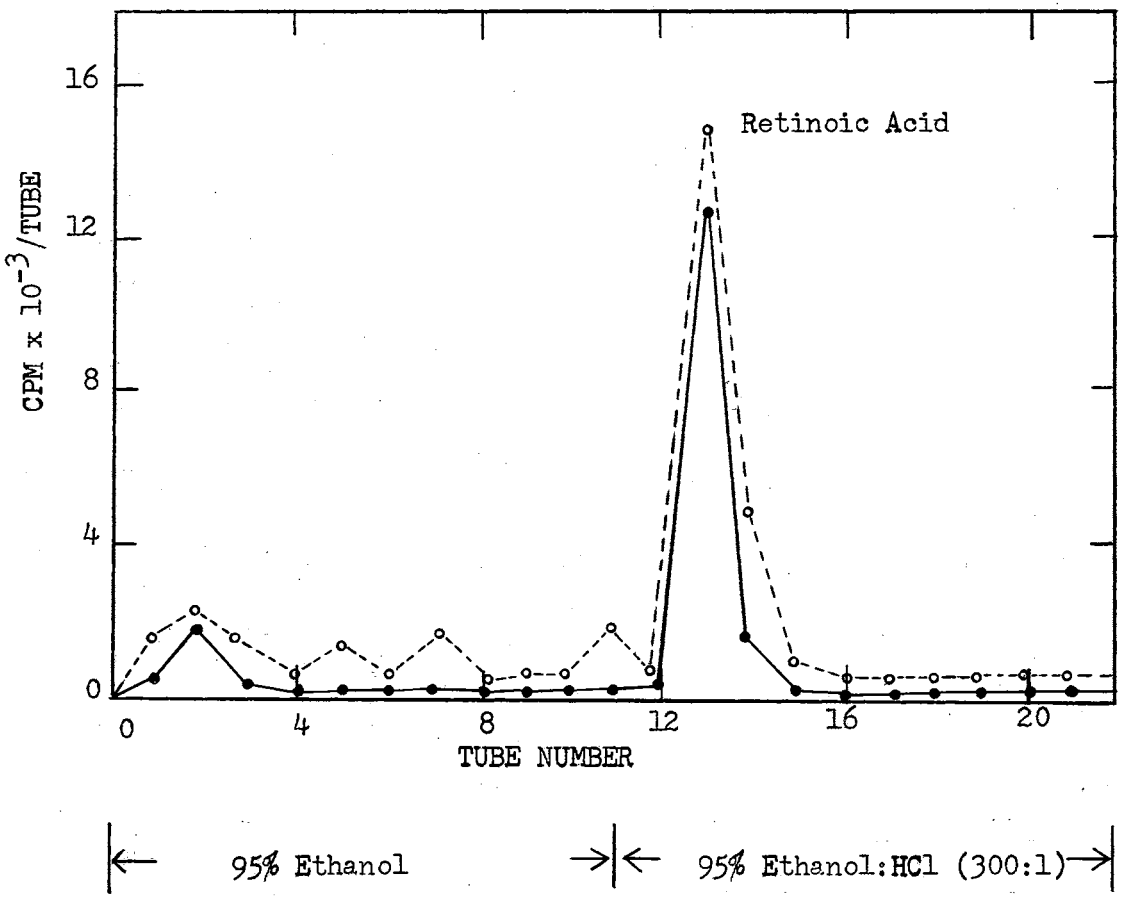


Figure 29. DEAE-cellulose Column Chromatography of the Chloroform:Methanol Extract of Control Incubation containing Retinoic Acid-6,7-^{14}C, buffer and Fe^{++} without Enzyme. The solid line represents the cpm/tube; the broken line represents the absorbance at 350 nm.

with acidic ethanol. The absorbance at 350 nm of the non-acidic radioactive fraction was much lower than that of the acidic radioactive fraction. With radioactive retinoic acid as marker, the acidic radioactive fraction was found to be retinoic acid. The unknown product was no longer acidic and also had lost its maximum absorbancy at 350 nm.

The second group containing retinoic acid-15-^{14}C as substrate showed only one major peak, retinoic acid, which was eluted with acidic ethanol, and a small radioactive peak in the same non-acidic fraction as group one (Figure 28). The 350 nm absorbance pattern was quite similar to the first group. Therefore, the small absorbance at 350 nm in the non-acidic fraction was contributed by a small amount of radioactive compounds which were present in both group 1 and 2. These results also indicate that the major unknown product lost its 350 nm absorbance. Group 3 was the control which contained retinoic acid-6,7-^{14}C, buffer and Fe^{++}, but no enzyme. Figure 29 shows only one major radioactive peak, unchanged retinoic acid and a very small radioactive peak in the non-acidic fraction. The pattern of absorbance at 350 nm was quite similar to that of group 1 and 2. It can be concluded that the small non-acidic radioactive peak present in all groups comes from both retinoic acid-15-^{14}C and retinoic acid-6,7-^{14}C by non-enzymatic reaction and the absorbance at 350 nm in this fraction was completely contributed by this compound, not by the major unknown compound.

An extract of an incubation mixture containing retinoic acid-6,7-^{14}C as substrate (same as group 1) was also applied to an anion exchange column. The chromatogram is shown in Figure 30. The results are in accordance with those from DEAE-cellulose column chromatography

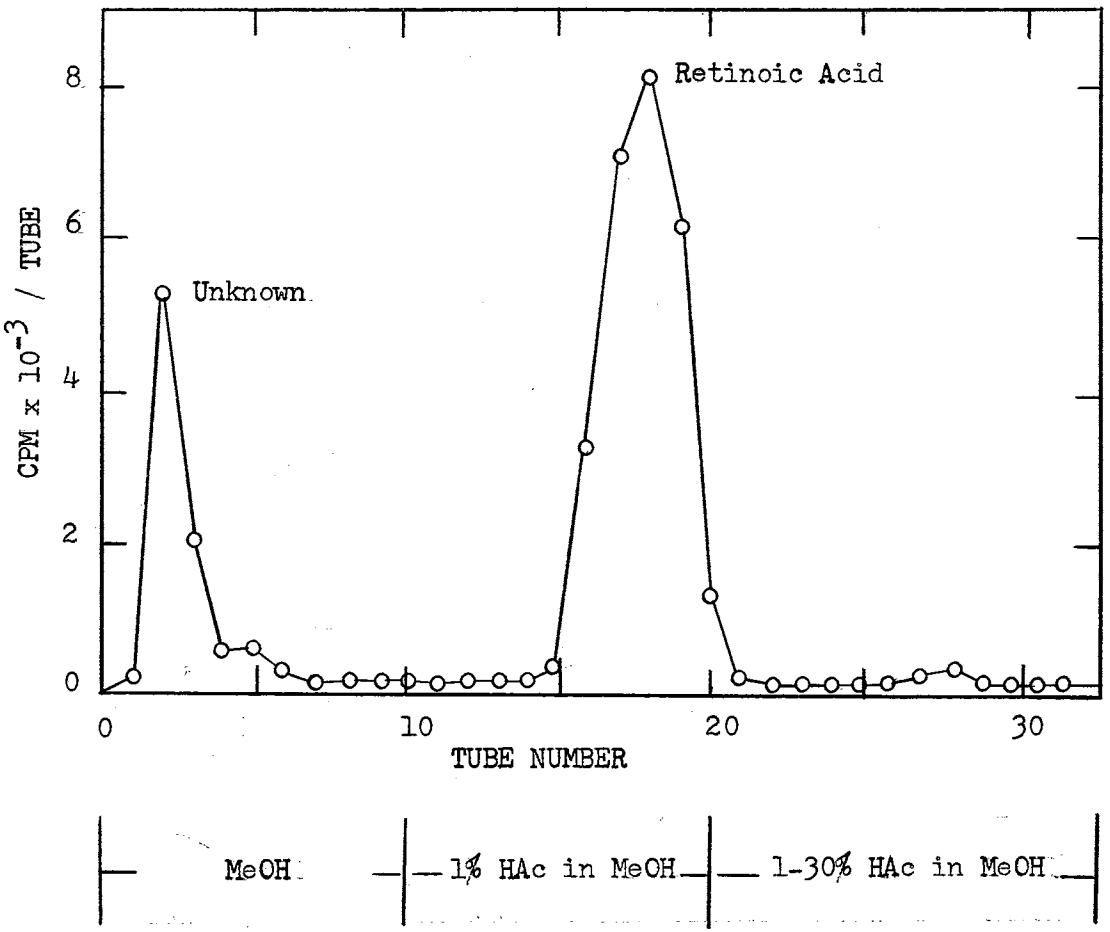


Figure 30. Anion Exchange Column Chromatography of a Chloroform:Methanol Extract of Incubation Mixtures with Retinoic Acid-6,7-^{14}C as Substrate.

(Figure 27). Two major radioactive peaks were found, one in the non-acidic fraction eluted with pure methanol and the other in the acidic fraction, eluted with 1% acetic acid in methanol. The acidic peak was shown to be unchanged retinoic acid and the non-acidic peak was the unknown product.

Thin-layer Chromatography of the Non-acidic Radioactive Fraction Obtained by DEAE-Cellulose Column Chromatography

To further purify the unknown product, the non-acidic radioactive fraction (Fraction II) obtained by DEAE-cellulose column chromatography (Figure 27) was reduced to a very small volume (< 0.5 ml) and was applied to analytical thin-layer plates. The plates were developed in a solvent system of petroleum ether:ether:acetic acid (25:25:1). The results are shown in Figure 31. Only one major radioactive peak was found. Again, in order to prove this major compound was the metabolite of the enzymatic decarboxylation reaction, Fraction II obtained from the retinoic acid-15-^{14}C incubation (Figure 28) and from the control incubation (Figure 29) was also chromatographed using the same solvent system. Figures 32 and 33 show the results. Although these two chromatography patterns were different, the major peak present in Figure 31 was absent in both cases, confirming that the major peak found was an enzymatic decarboxylation reaction product. Furthermore, the radioactive peak at the origin was found in all three cases, indicating that the small radioactivity in Fraction II in Figures 32 and 33 was probably contributed by this compound.

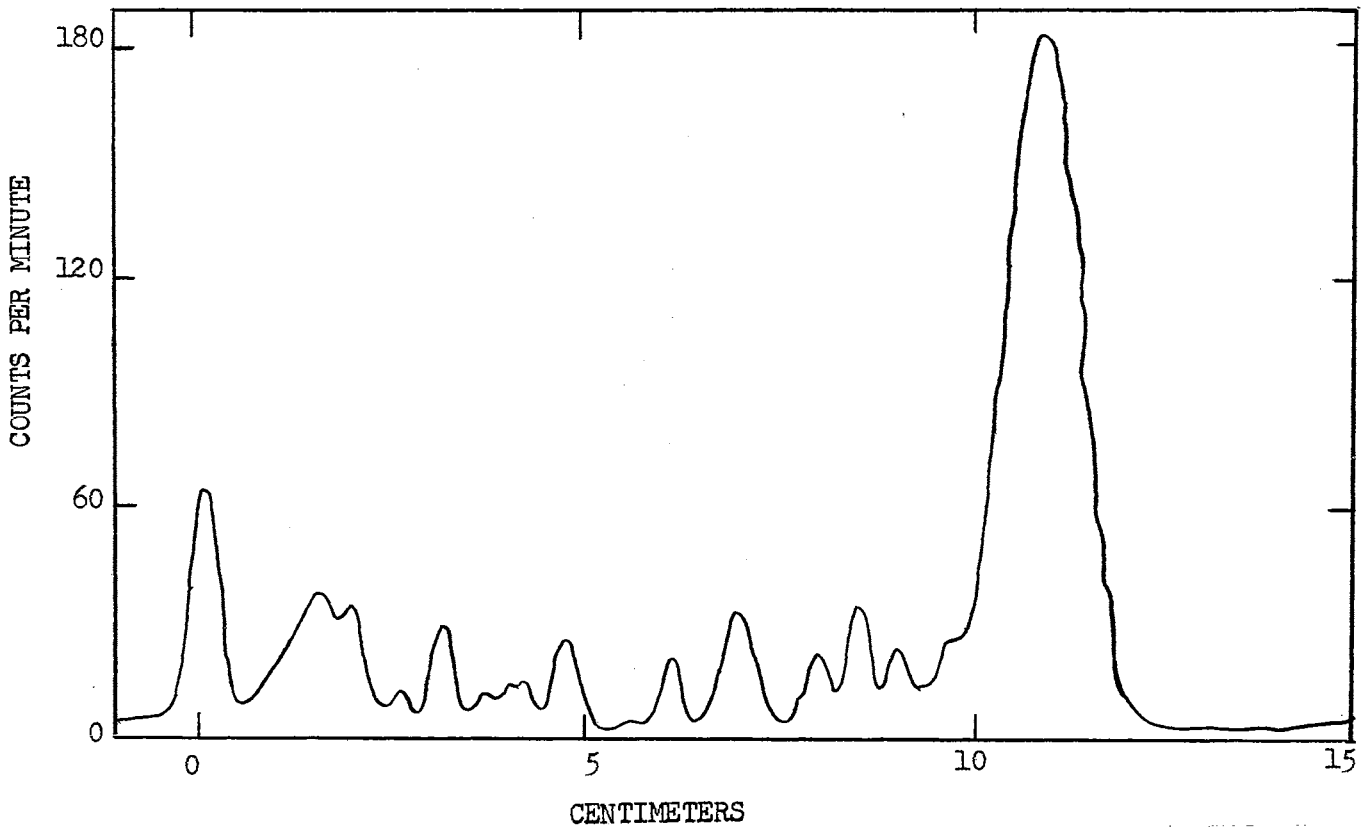


Figure 31. TLC Distribution of Radioactivity of Fraction II obtained by DEAE-Cellulose Column Chromatography of Incubation Mixtures with Retinoic Acid-6,7-^{14}C as Substrate. Solvent system used was petroleum ether : ether : acetic acid (25:25:1).

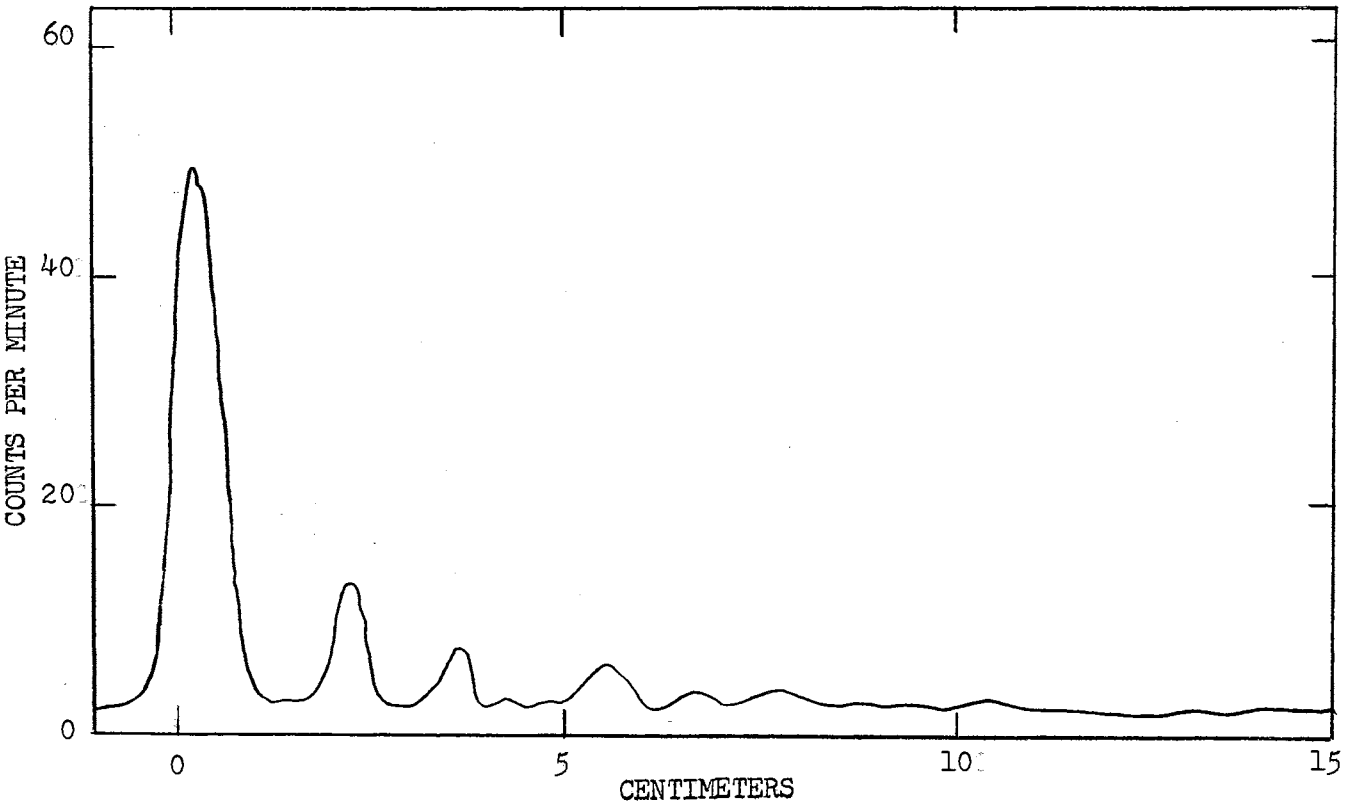


Figure 32. TLC Distribution of Radioactivity of Fraction II obtained by DEAE-Cellulose Column Chromatography of Incubation Mixtures with Retinoic Acid-15-^{14}C as Substrate. Solvent system used was petroleum ether : ether : acetic acid (25:25:1).

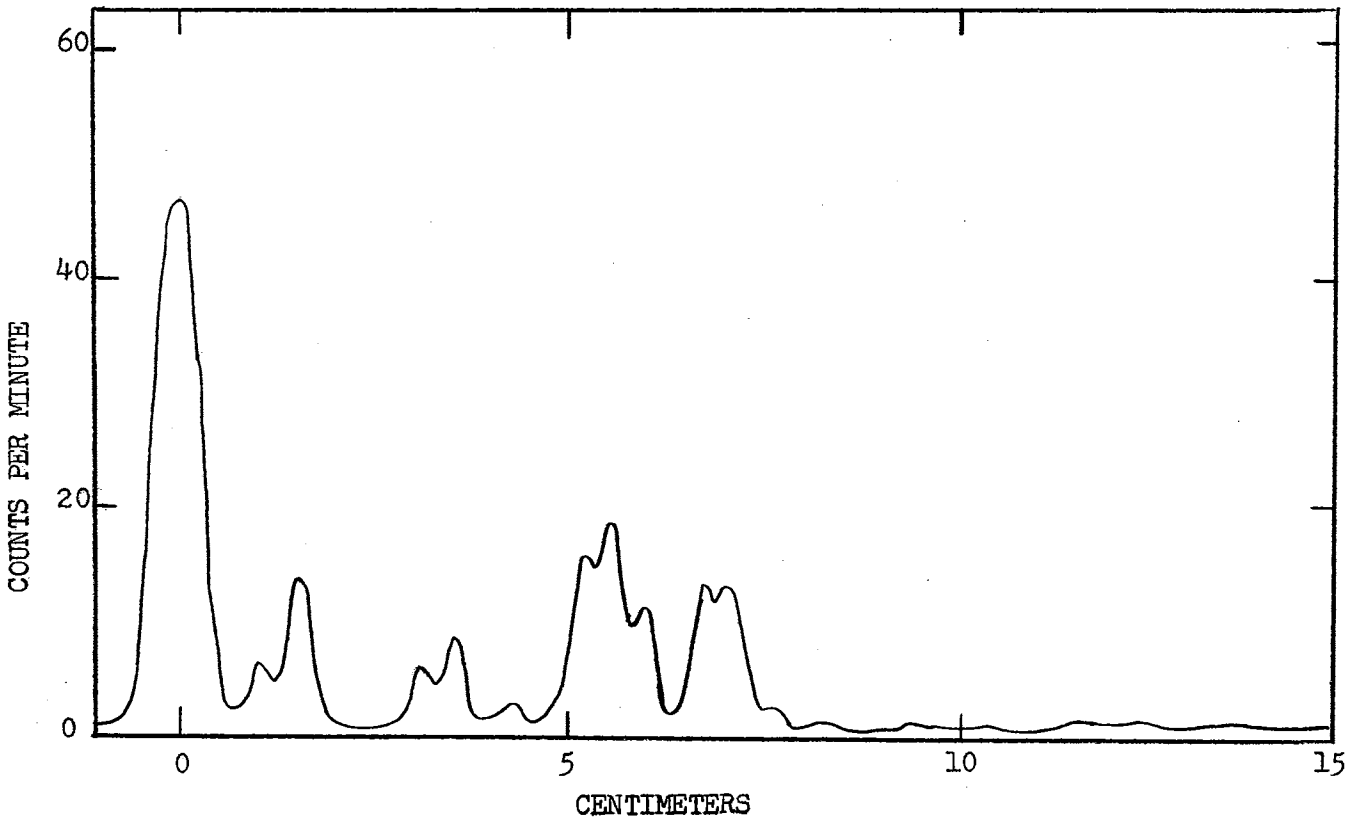


Figure 33. TLC Distribution of Radioactivity of Fraction II obtained by DEAE-Cellulose Column Chromatography of Control Incubations containing Retinoic Acid-6,7-^{14}C and Fe^{++} without Enzyme. Solvent system used was petroleum ether : ether : acetic acid (25:25:1).

Further Purification of the Metabolite

Numerous attempts were made to further purify the metabolite. Finally, the following methods were selected. The major peak obtained by the first TLC of Fraction II was removed from the plates and extracted with a chloroform:methanol (1:1) mixture. The extract was filtered, reduced in volume under vacuum and was further purified with TLC using benzene as the solvent system. The distribution of radioactivity is shown in Figure 34. Only one radioactive peak was detected at the origin. Following elution and reapplication to a new plate a solvent system (isopropanol:30% ammonium hydroxide, 4:1) was used to develop the TLC. The results are shown in Figure 35. The radioactive portion was again extracted and further purified on a TLC plate. The plate was developed in a solvent system of benzene:chloroform:methanol (4:1:1). Figure 36 shows the distribution of radioactivity. Again only one radioactive peak was detected. This radioactive metabolite, after the fourth TLC, was found to be at least 95% pure. The purity was checked by mass spectrometry and gas chromatography (see below).

Thin-layer Chromatographic Behaviors of Purified Metabolite

The purified metabolite (fourth TLC step) was spotted onto a TLC plates with all-*trans* retinoic acid and all-*trans* methyl retinoate and the plates were developed in several different solvent systems. Retinoic acid and methyl retinoate were detected by iodine vapor and the metabolite by radioactivity using a gas flow strip counter. Figure 37 and 38 shows the TLC of these compounds. The R_f values of these compounds in several solvent systems are summarized in Table XIX.

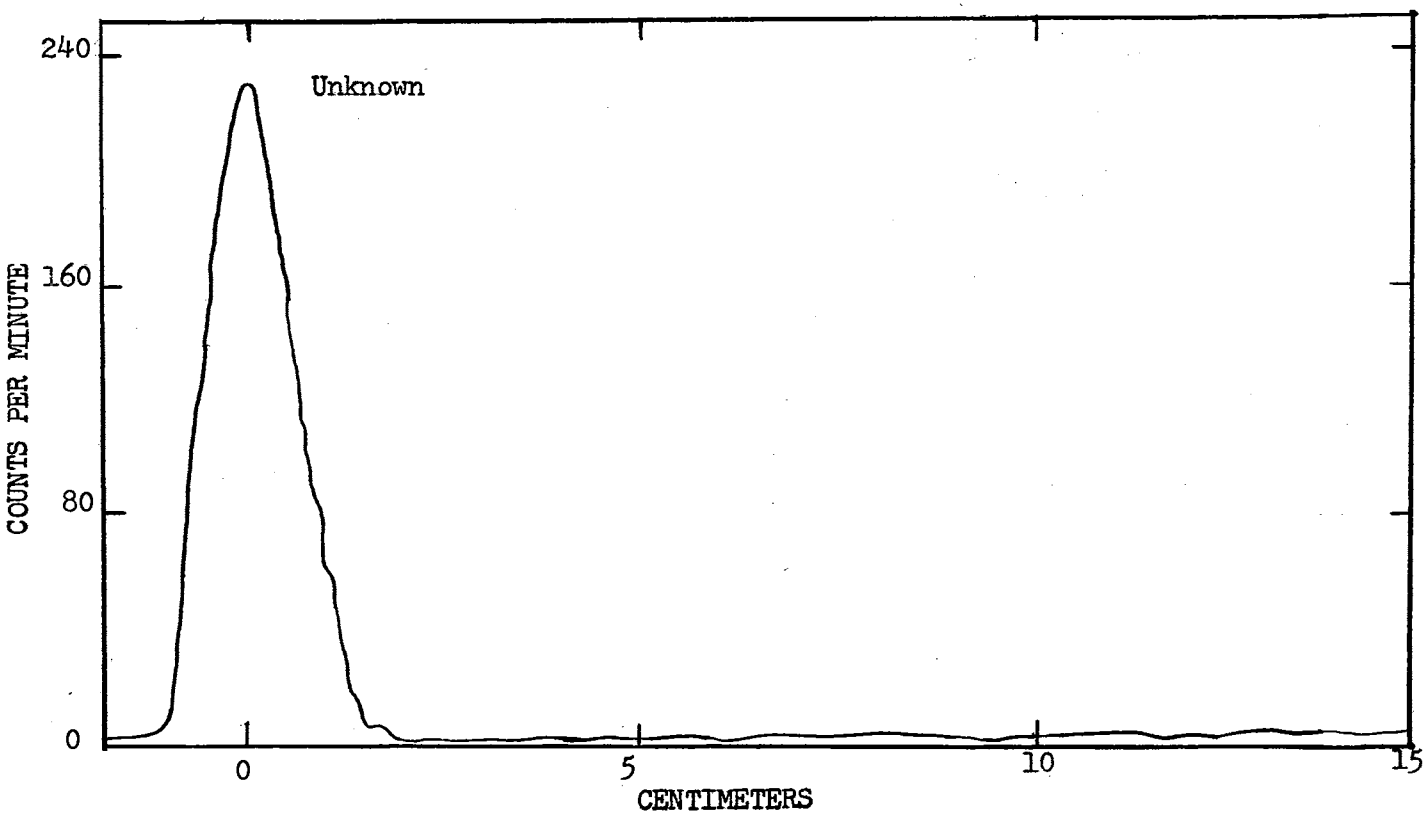


Figure 34. Second TLC Distribution of Radioactivity of the Major Peak (Unknown) obtained from first TLC (Figure 31). Solvent was Benzene.

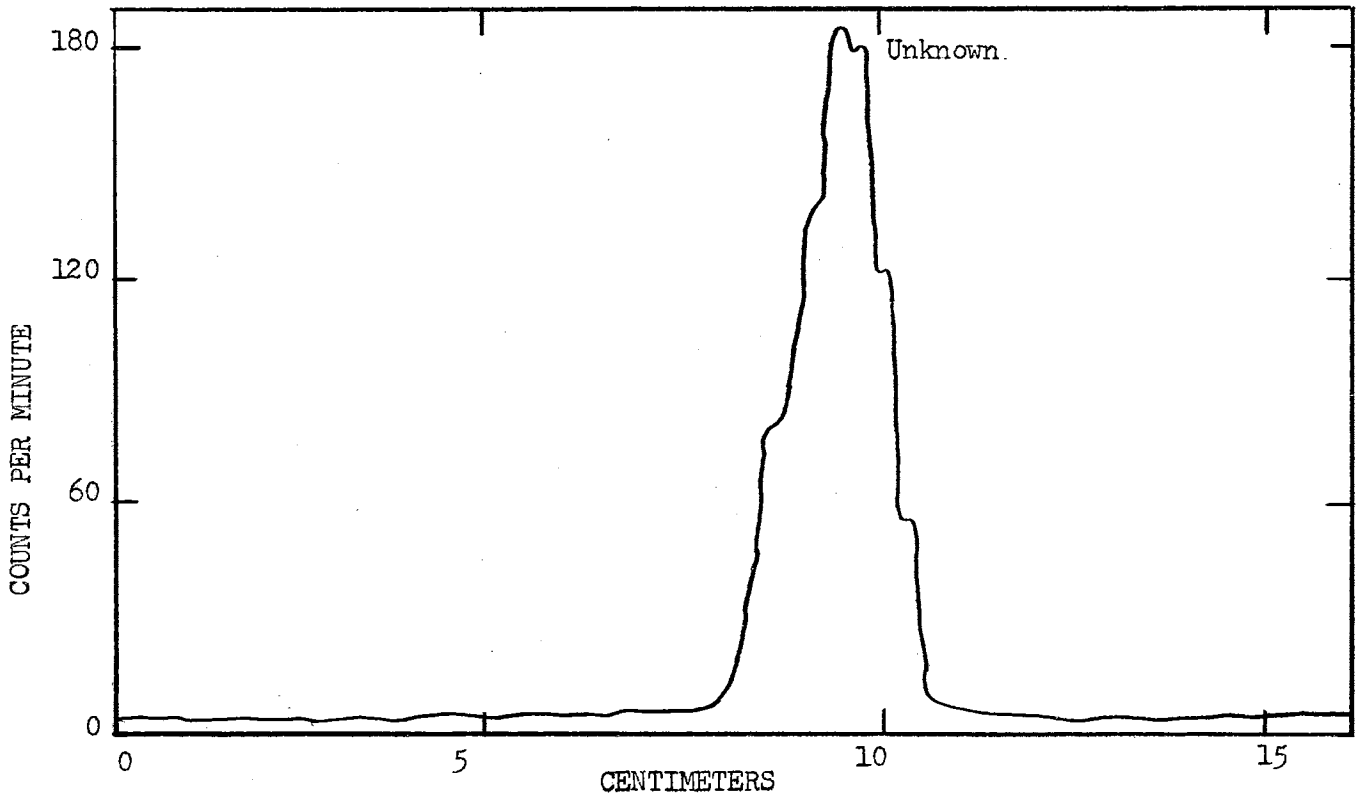


Figure 35. Third TLC Distribution of Radioactivity of the Major Peak (Unknown) obtained from the Second TLC (Figure 34). Solvent system was Isopropanol:30% ammonium hydroxide (4:1).

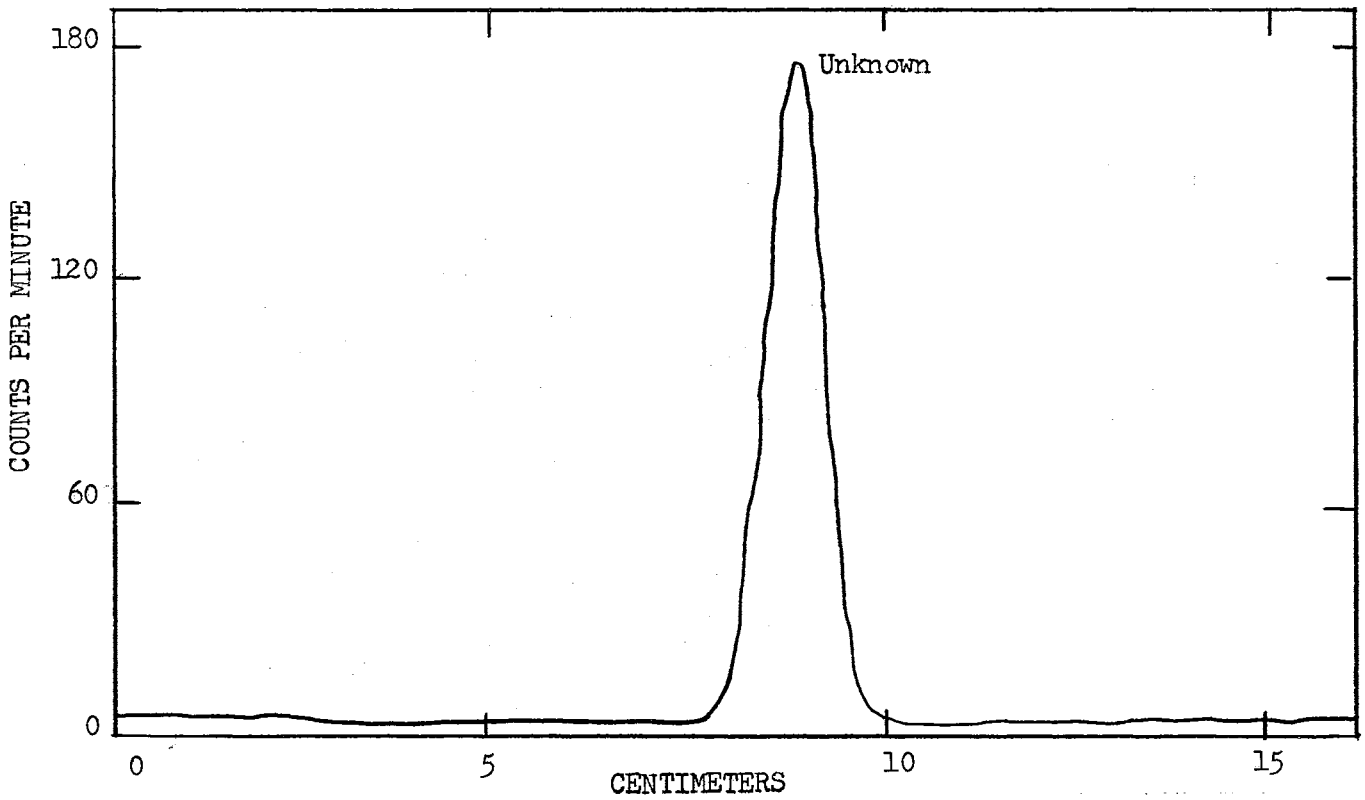


Figure 36. Fourth TLC Distribution of Radioactivity of the Major Peak (Unknown) obtained from the Third TLC (Figure 35). Solvent system used was Benzene : chloroform : methanol (4:1:1).

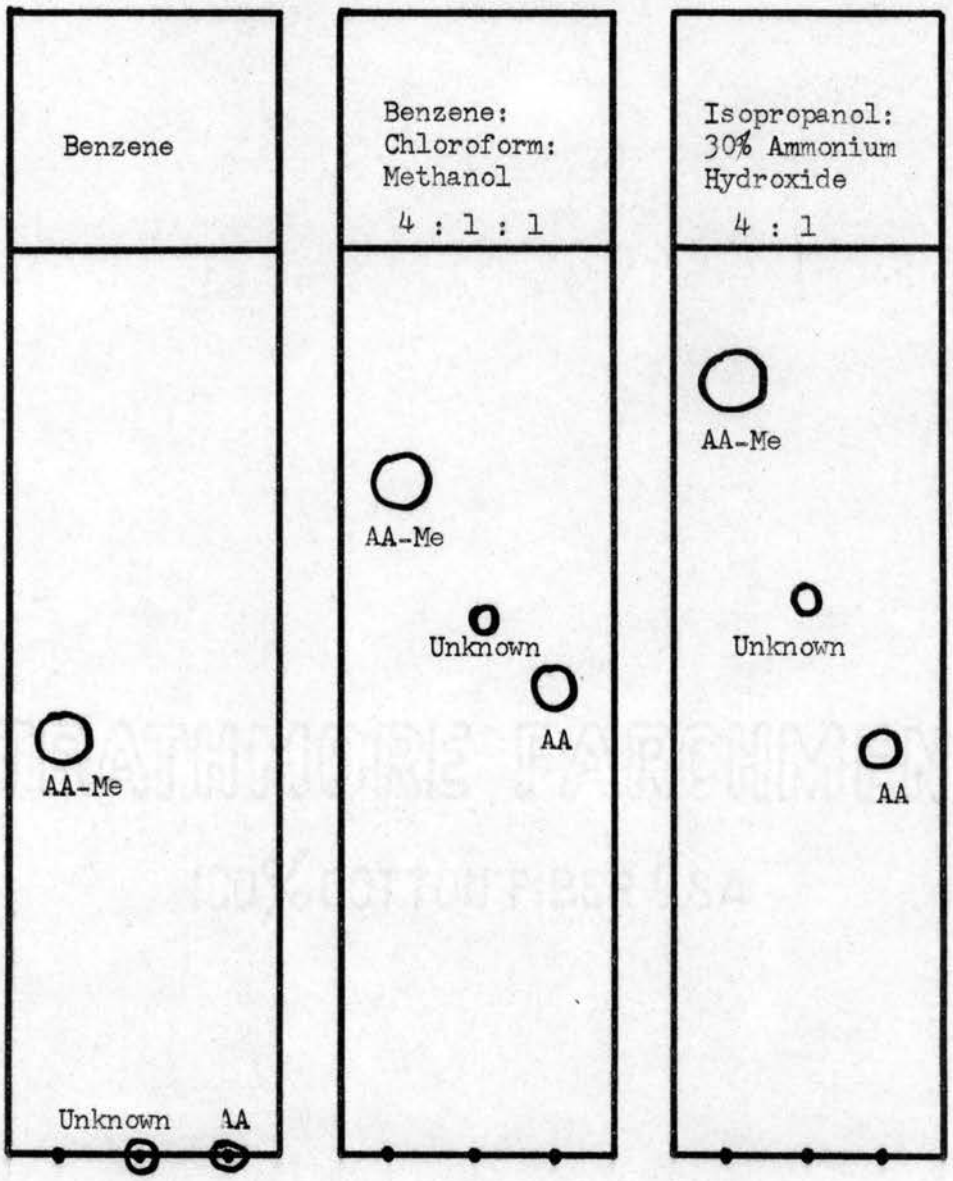


Figure 37. TLC of the Purified Metabolite, All-trans Retinoic Acid and All-trans Methyl Retinoate in Alkaline or Neutral Solvent Systems. AA-Me: Methyl Retinoate; AA: Retinoic Acid; Unknown: Purified Metabolite.

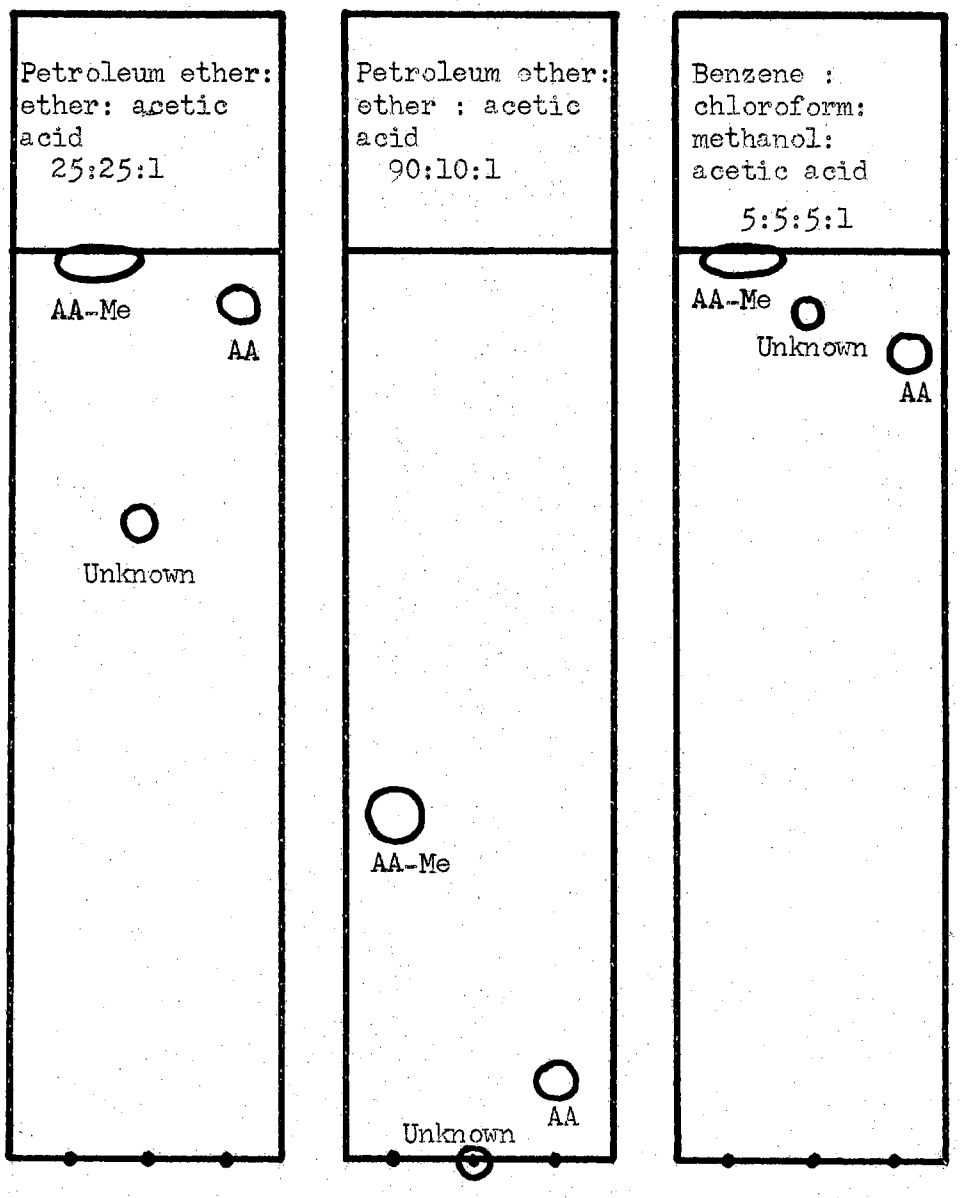


Figure 38. TLC of the Purified Metabolite, All-trans Retinoic Acid and All-trans Methyl Retinoate in Acidic Solvent Systems. AA-Me: All-trans Methyl Retinoate, AA: All-trans Retinoic Acid, Unknown: Metabolite.

TABLE XIX

R_f VALUES OF METABOLITE, RETINOIC ACID, AND METHYL RETINOATE

Solvent System	Proportion of Solvent by Volume	Metabolite	All-trans Retinoic Acid	All-trans Methyl Retinoate
Isopropanol : 30% ammonium hydroxide	4 : 1	0.64	0.39	0.85
Benzene : chloroform : methanol	4 : 1 : 1	0.54	0.48	0.74
Benzene	---	0.00	0.00	0.45
Petroleum ether : ether : acetic acid	25 : 25 : 1	0.71	0.95	1.00
Petroleum ether : ether : acetic acid	90 : 10 : 1	0.00	0.09	0.38
Benzene : chloroform : methanol : acetic acid	5 : 5 : 5 : 1	0.95	0.92	1.00

These results show that the metabolite is different from retinoic acid and methyl retinoate. The R_f values of the metabolite were lower than that of methyl retinoate in all tested solvent systems and were higher than that of retinoic acid in all solvent systems except petroleum ether:ether:acetic acid (25:25:1) and petroleum ether:ether:acetic acid (90:10:1). Retinoic acid, containing a free carboxyl group, and strongly affected by H^+ or OH^-, showed, as expected, extremely different TLC behaviors in acidic and alkaline solvent systems. On the other hand, the polarity of the metabolite and methyl retinoate was not affected by H^+ or OH^- and their R_f values, therefore, were determinted only by the polarity of the solvent system. These results indicate that the metabolite does not contain a free carboxyl group or basic group. The R_f values of the metabolite when compared to the R_f of methyl retinoate indicates that the metabolite contains more polar functional groups or possess a higher molecular weight.

Ultraviolet Absorption Spectrum of the Metabolite

The ultraviolet absorption spectrum of the purified metabolite was obtained in an ethanolic solution and was found to be different from that of retinoic acid as shown in Figure 39. The metabolite gave a maximum absorption at 285 nm instead of 350 nm which is the maximum absorption of retinoic acid (Figure 39). This suggests that the metabolite lost some conjugated double bonds, becoming more saturated than retinoic acid. Probably only three or four conjugated double bonds remained in the molecule.

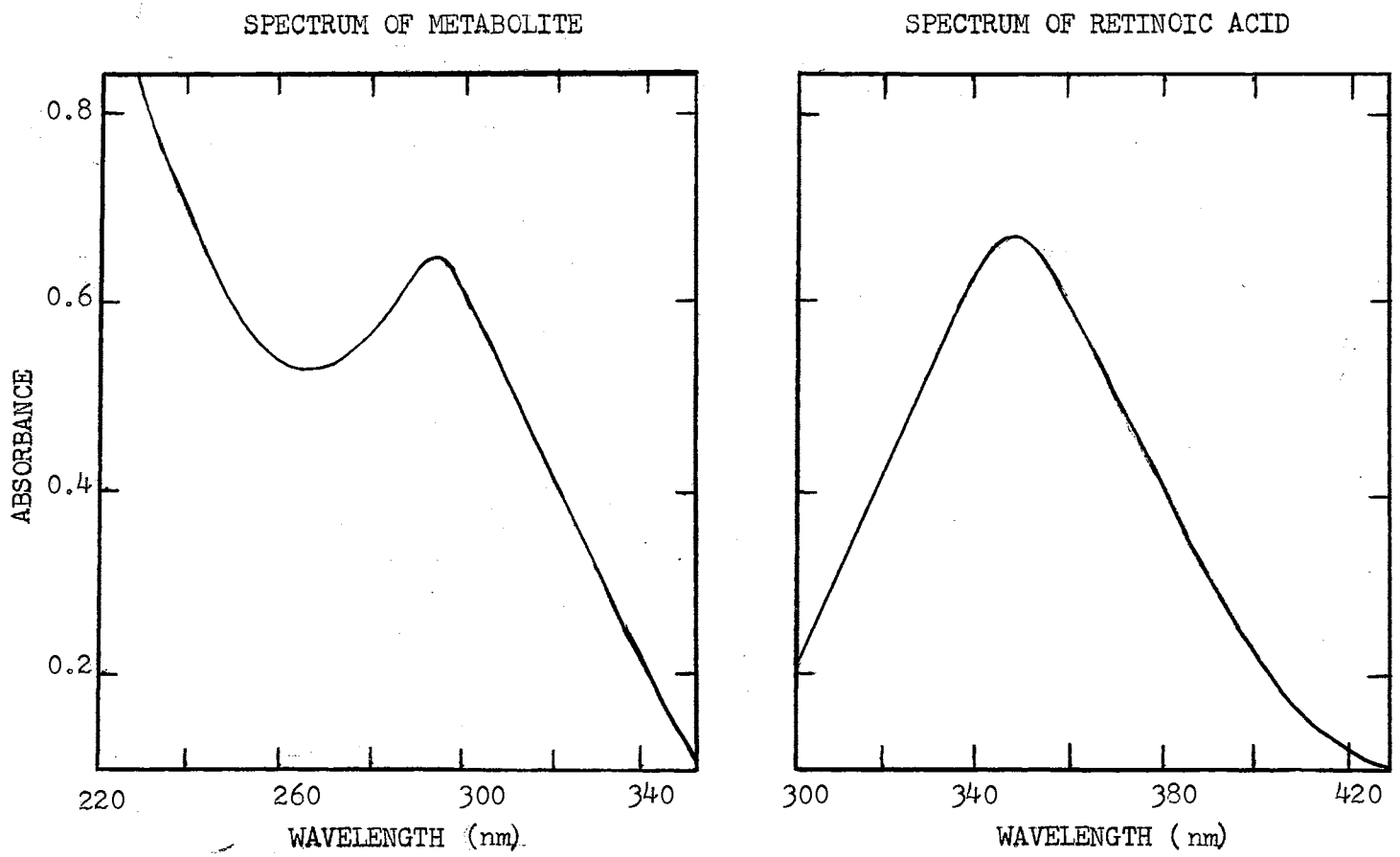


Figure 39. Ultraviolet Absorption Spectrum of the Metabolite and Retinoic Acid

Infrared Spectra of the Metabolite

The infrared spectra of the metabolite is shown in Figure 40. It shows a broad absorption band between 3,550 and 3,450 cm^{-1} indicating a OH-group and at 2,942 and 2,870 cm^{-1} indicating CH_3 and CH_2. The metabolite therefore, contains hydroxyl, CH_3 and CH_2 groups.

Hydrolysis of Purified Metabolite

The purified metabolite was heated with 30% alcoholic KOH as described under "Experimental." A methanolic extract was spotted onto a TLC plate and the plate was developed in a solvent system of petroleum:ether:acetic acid (25:25:1). The results are shown in Figure 41. The major peak obtained after hydrolysis was found to be the unchanged original compound. This was also confirmed by gas chromatography, indicating that the metabolite is not subject to basic hydrolysis, and is therefore, not an ester. Acid hydrolysis of the metabolite was also unsuccessful.

Gas Chromatography of the Metabolite

Several columns containing 1% SE-30, 5% SE-52, 1% OV-1, 3% OV-1, and 4% OV-17 were tried to chromatograph the metabolite and 1% OV-1 was found to be the best for this compound. The conditions developed are described under "Experimental." The purified metabolite was applied to a 1% OV-1 column at a column temperature 225°C and its retention time was 13.8 minutes (Figure 42). Only one peak was obtained. Figure 43 shows a gas chromatogram of a mixture of the metabolite and all-*trans* methyl retinoate. The metabolite has longer retention time than methyl retinoate, which as retention time of 3.8 minutes under the

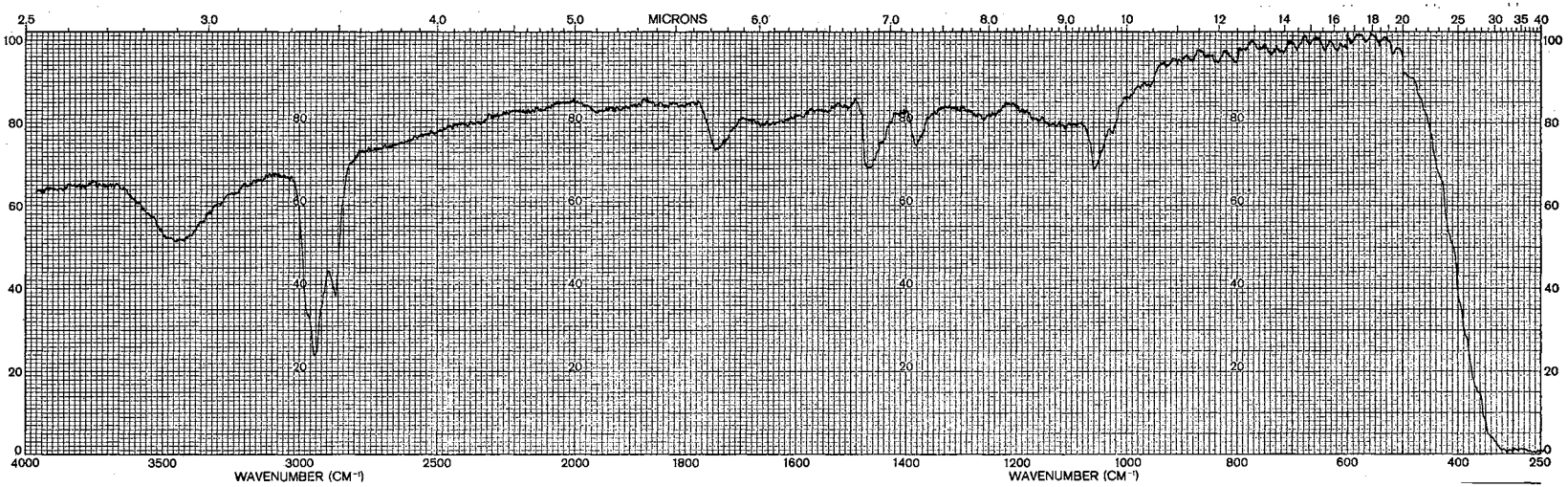


Figure 40. Infrared Spectrum of the Metabolite

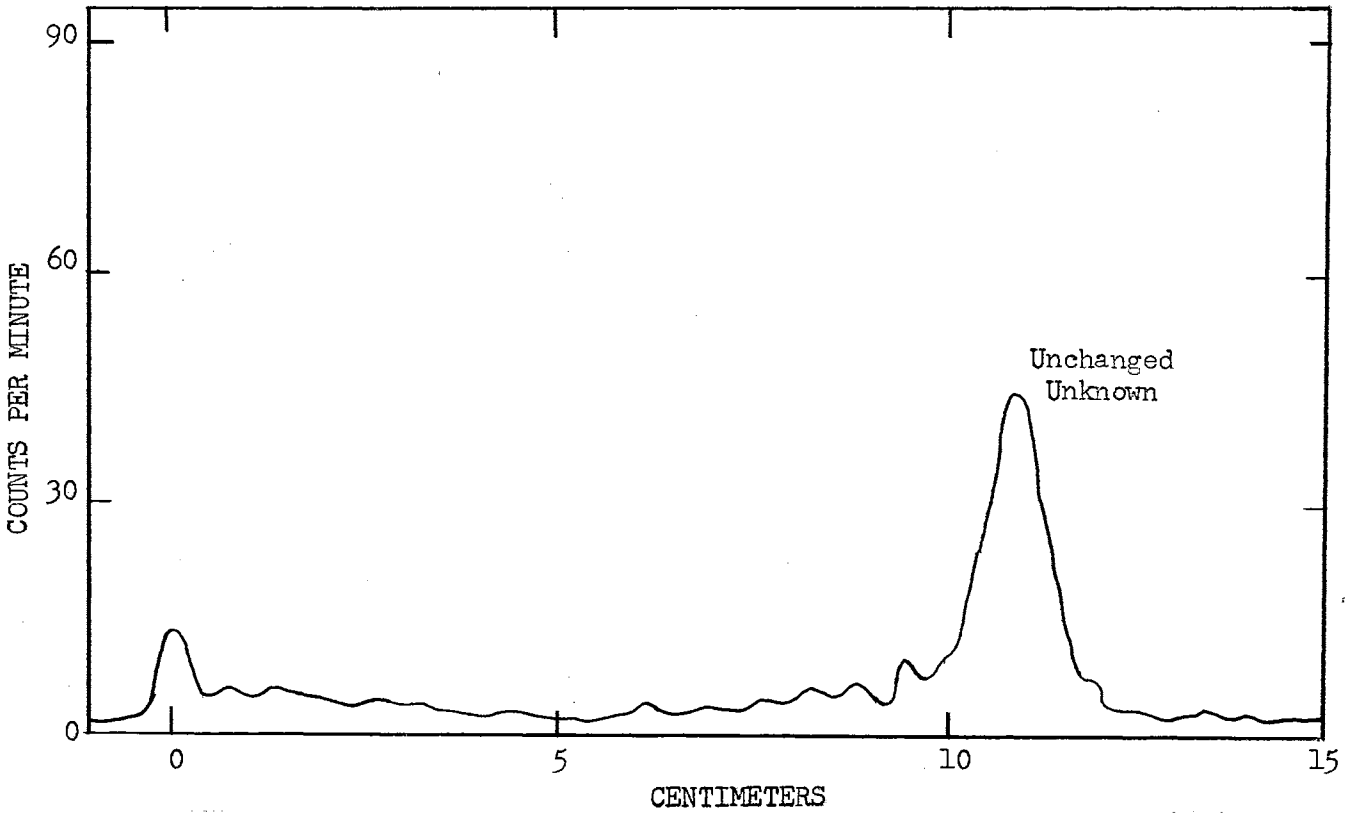


Figure 41. TLC Distribution of Radioactivity of the Methanol Extract obtained from Alkaline Hydrolysis of the Metabolite. Solvent system: petroleum ether : ether : acetic acid (25:25:1).

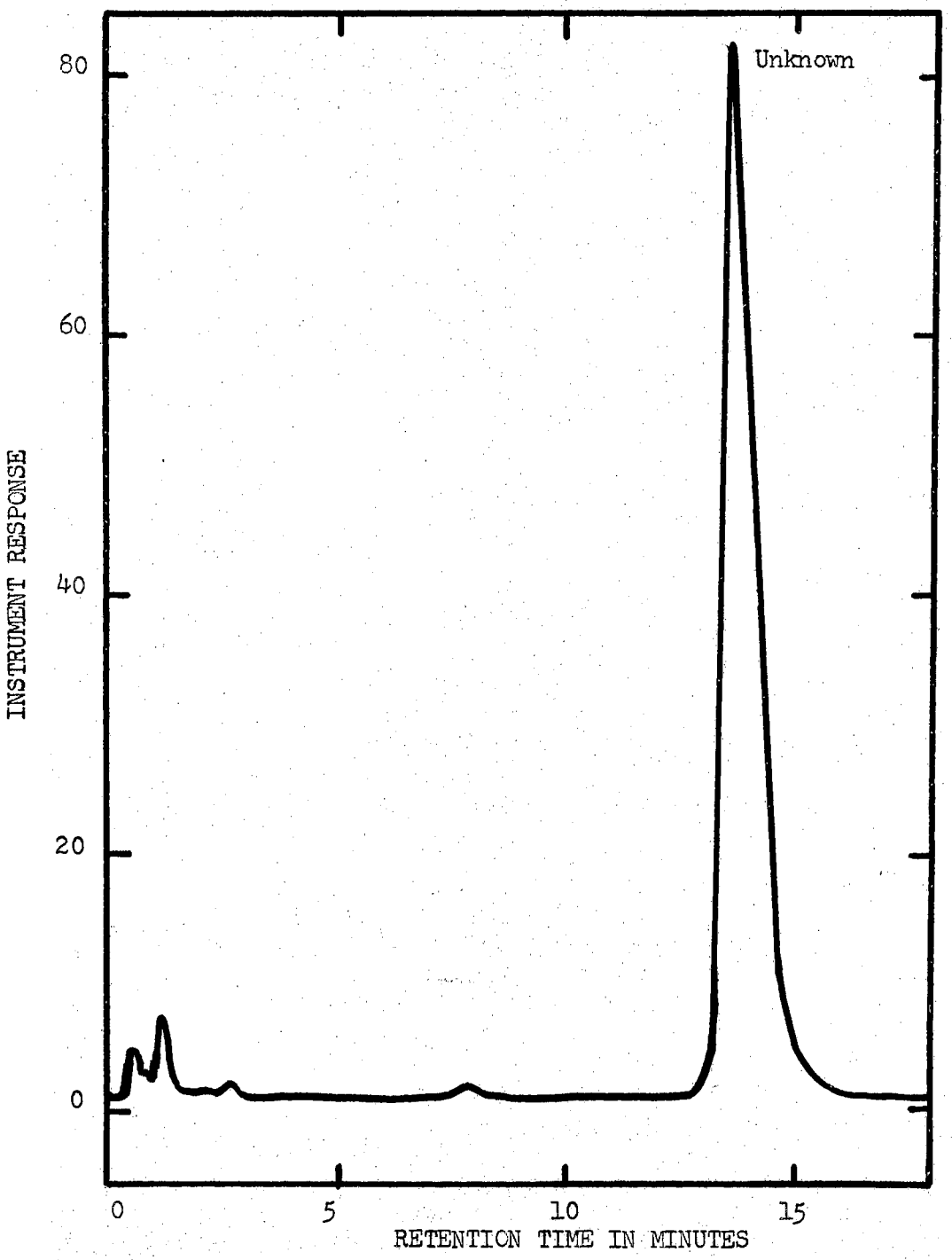


Figure 42. Gas Chromatography of the Purified Metabolite. Column: 1/4 inch x 8 feet 1% OV-1 on Gas Chrom Q helical glass column. Conditions: Column Temperature 225°C, Injector 255°C, Detector 265°C, Gas flow rate: 50 ml/minute.

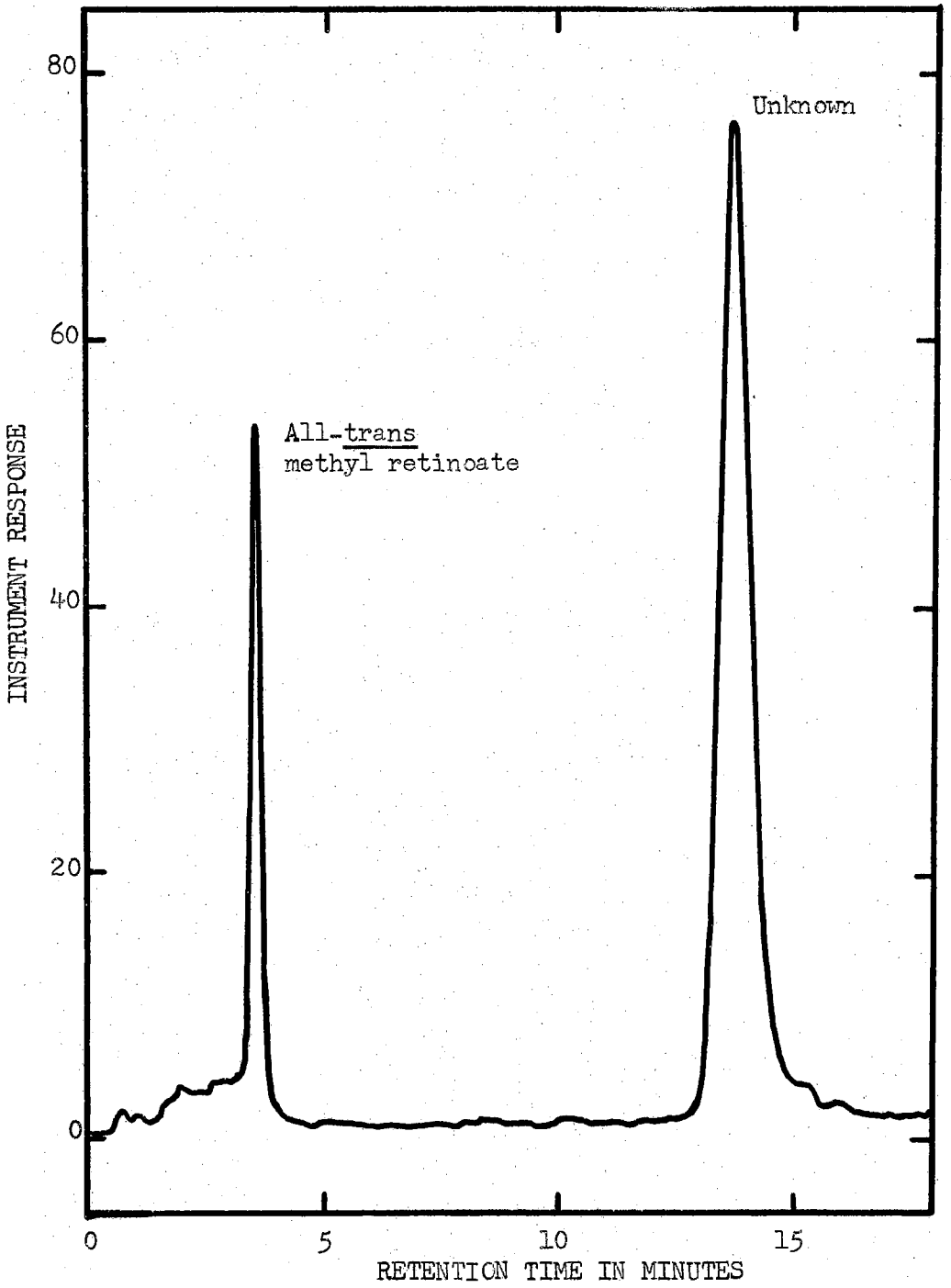


Figure 43. Gas Chromatography of a Mixture of Purified Metabolite and All-trans Methyl Retinoate. Column and conditions are the same as described under Figure 42.

same conditions. In order to compare the metabolite with available vitamin A compounds all of these compounds with the metabolite were applied to 1% OV-1 column at two different column temperatures, 180°C 225°C. Table XX summarizes the results. All tested compounds had a much shorter retention time than the metabolite.

The conclusion that the compound with a retention time of 13.8 minutes was the major product of the decarboxylation reaction was subsequently verified by the finding that this peak was not present in a blank incubation, containing only enzyme without substrate (Figure 44) and control incubation, containing substrate and retinoic acid-6,7-^{14}C, but without enzyme (Figure 45). It was also present in the incubation mixture, when retinoic acid-15-^{14}C was the substrate (Figure 46) although it had not been detected in the TLC studies by measuring radioactivity.

The metabolite treated with diazomethane gave only the unchanged original compound on GLC, indicating that there is no free carboxyl group present in the metabolite. This result coincides with the TLC behaviors of the metabolite. After reaction with hexamethyldisilazane, the metabolite yielded less polar compounds, as shown in Figure 47. The major compound with retention time of 8.2 minutes was found to be the TMS-derivative of the metabolite. Its molecular weight was 458 (386 + 72), (see below), confirming that only one free hydroxyl group was present in the metabolite.

Mass Spectrometry of the Metabolite

An attempt was made to characterize the metabolite by mass spectrometry. The mass spectrum of the purified metabolite was

TABLE XX

RETENTION TIMES OF METABOLITE, ALL-TRANS METHYL RETINOATE AND RELATED COMPOUNDS

Compound	Column Temperature (°C)	Retention Time (minutes)
Metabolite	180	113.6
	225	13.8
All-trans methyl retinoate	180	20.5
	225	3.8
All-trans retinal	180	16.6
	225	2.0
All-trans retinyl palmitate	180	5.6
	225	1.6
All-trans retinol and retinyl acetate	180	1.1
	225	1

Column: 1/4 in. x 8 ft. 1% OV-1 Gas Chrom Q helical glass column.

Flow Rate: 50 ml/minute

Injector temperature: 210° and 255°C at column temperature 180° and 225°C respectively.

Detector: 265°C at both column temperatures.

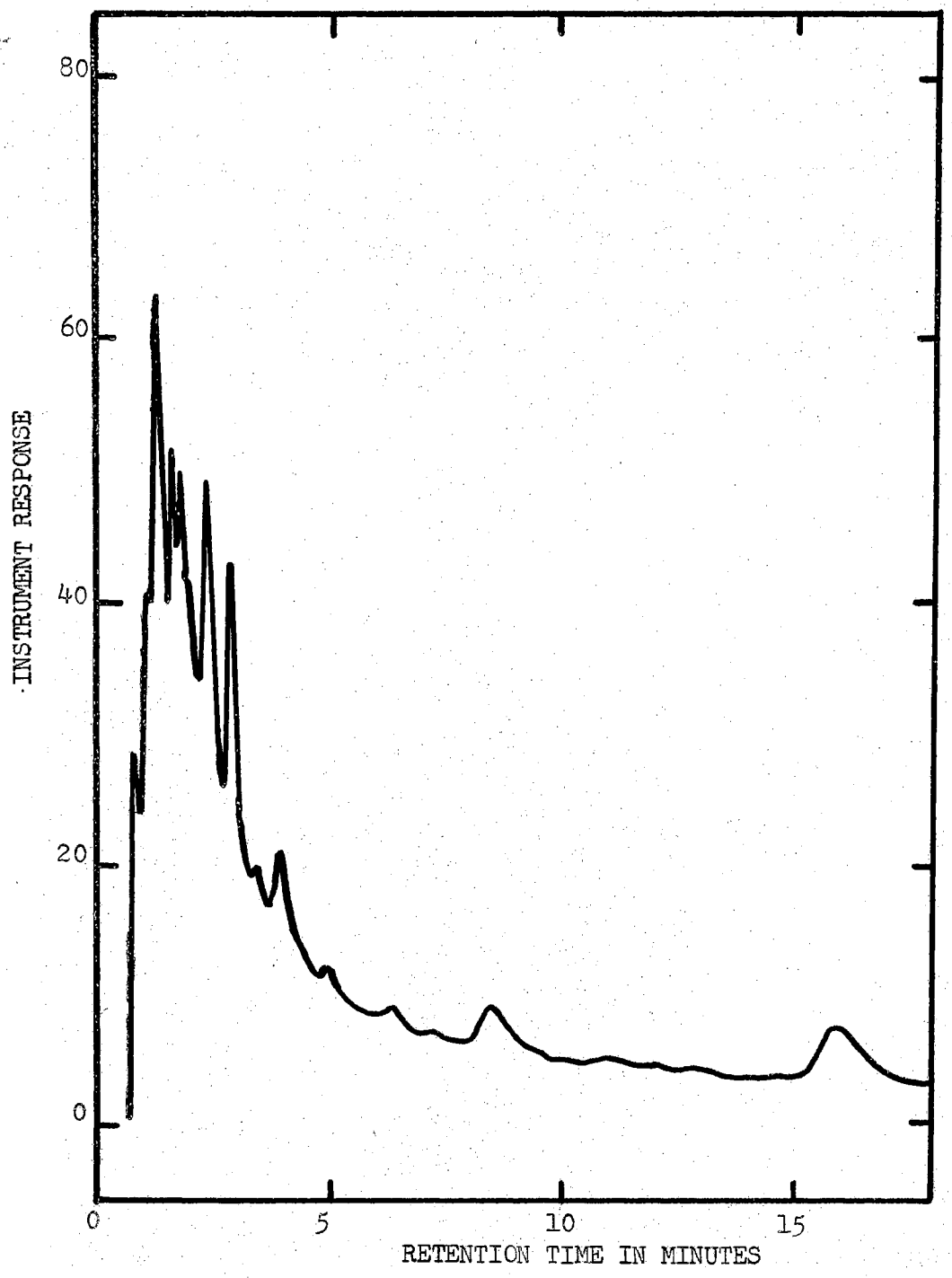


Figure 44. Gas Chromatography of the Extract of the Incubation Mixture, containing Enzyme without Substrate. Column and conditions are the same as described under Figure 42.

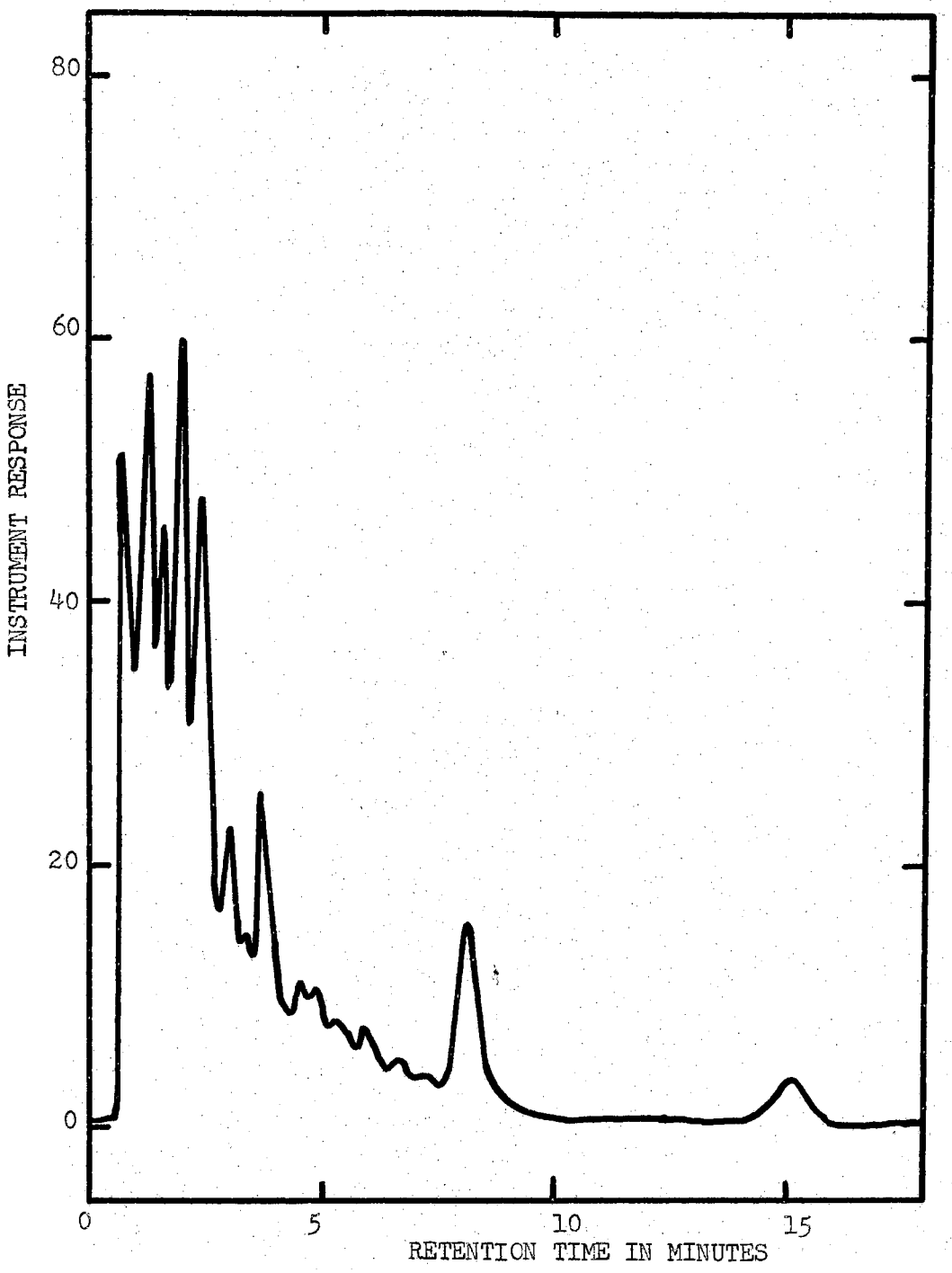


Figure 45. Gas Chromatography of an Extract of the Incubation Mixture, Containing Retinoic Acid-6,7-^{14}C without Enzyme. Column and conditions are the same as described under Figure 42.

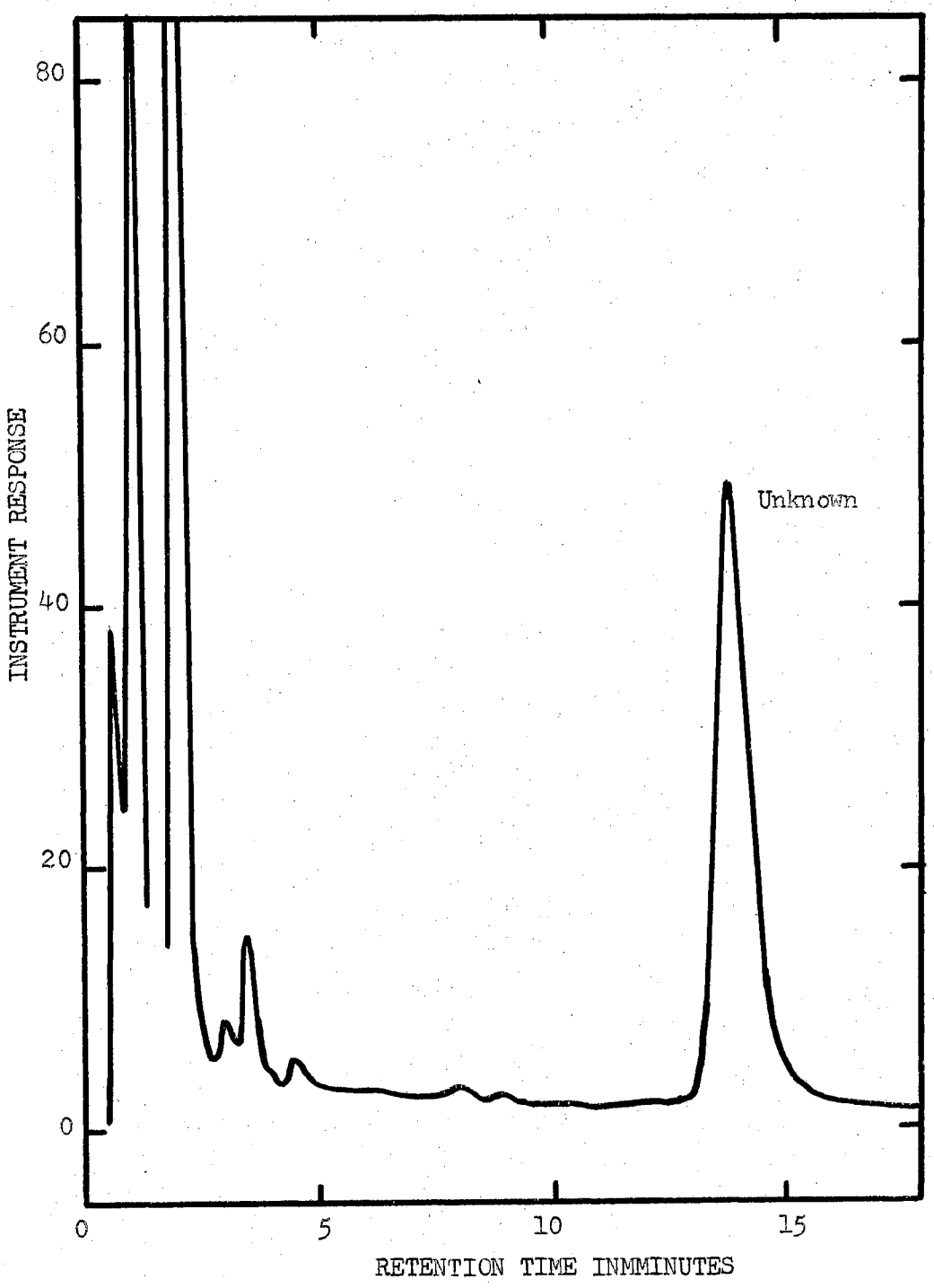


Figure 46. Gas Chromatography of 1st TLC of Fraction II Obtained by DEAE-Cellulose Column Chromatography of the Incubation Mixture, containing Retinoic Acid-15-^{14}C as Substrate. Column and conditions are the same as described under Figure 42.

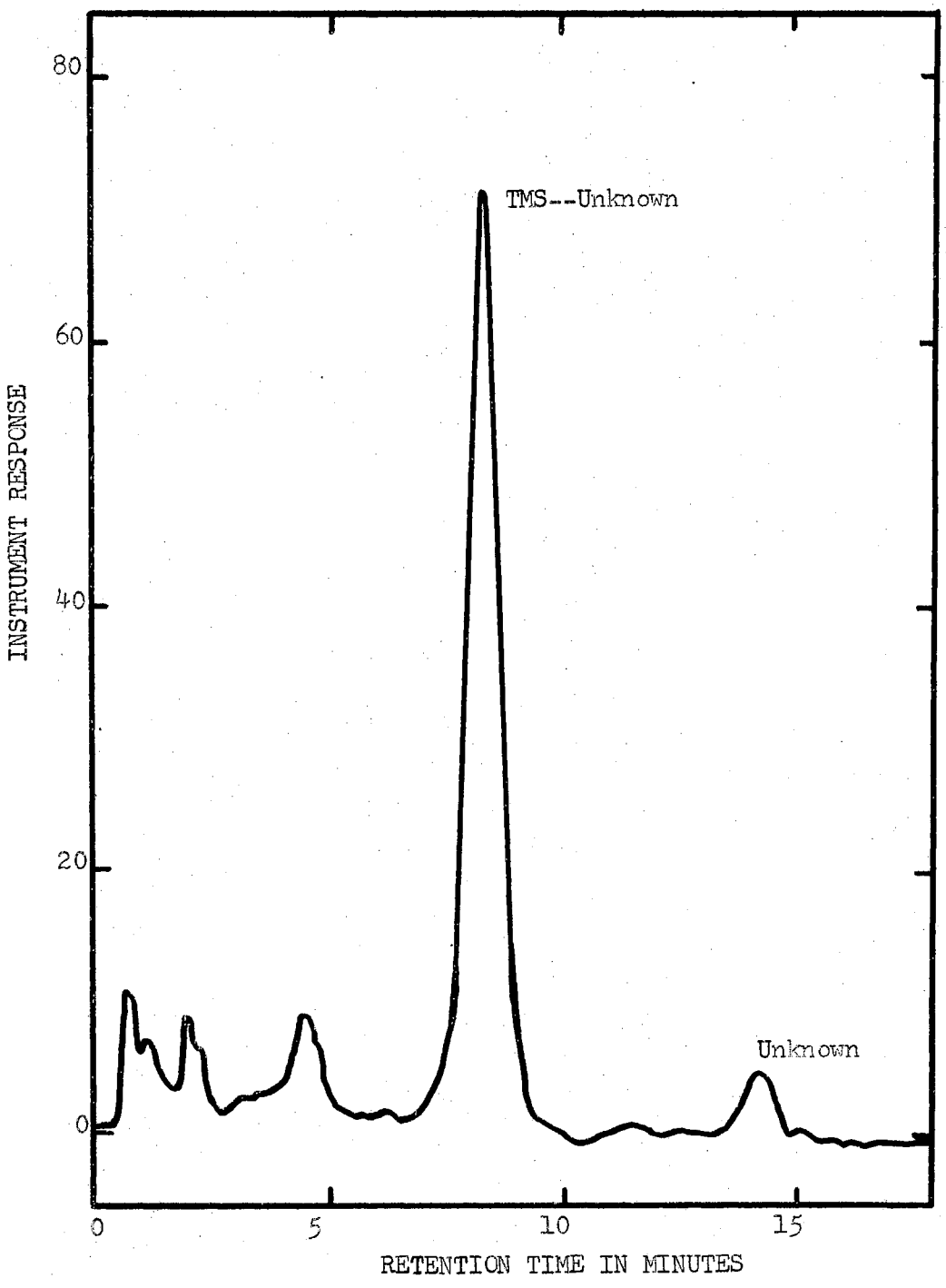


Figure 47. Gas Chromatography of TMS-derivative of the Purified Metabolite. Column and conditions are the same as described under Figure 42.

obtained by introducing it directly and through the gas chromatograph inlet systems as described in "Experimental."

Using the direct introduction technique, the probe was heated from 20° to 300°C at an ion source temperature of 310°C and a pressure of 10^{-7} Torr. The metabolite peak appeared at 30°C, and had been pumped out five minutes later at a temperature of about 100°C. The total ion current tracing (TIC-tracing) is shown in Figure 48. Only a very small amount of impurity was found at a probe temperature of 135°C, indicating that the purity of the metabolite was very high. This was also confirmed by gas chromatography of the metabolite (Figure 42).

The mass spectrum of the metabolite obtained by direct probe at TIC-tracing position 4 is shown in Figure 49. The mass spectrum was also obtained through gas chromatography, and Figure 50 shows the total ion current tracing of the metabolite through GLC. The mass spectrum obtained by GLC at TIC-tracing position 3 (Figure 50) is almost the same as the mass spectrum obtained by the direct probe (Figure 49). The metastable ions appearing in the spectra are listed in Table XXI. Since the metabolite was a decarboxylation product of retinoic acid it would be reasonable to assume that the molecular weight would be less than the parent compound, i.e., 300. However as can be seen from the mass spectrum (Figure 49) the metabolite had a molecular weight of 386. Therefore, it was necessary to determine the composites and structure of the metabolite to predict the source of the extra mass units.

The high resolution mass spectrum of the metabolite was obtained and is shown in Tables XXII and XXIII and in Figure 51 as an element map as devised by Burlingame and Smith (101, 102). The high

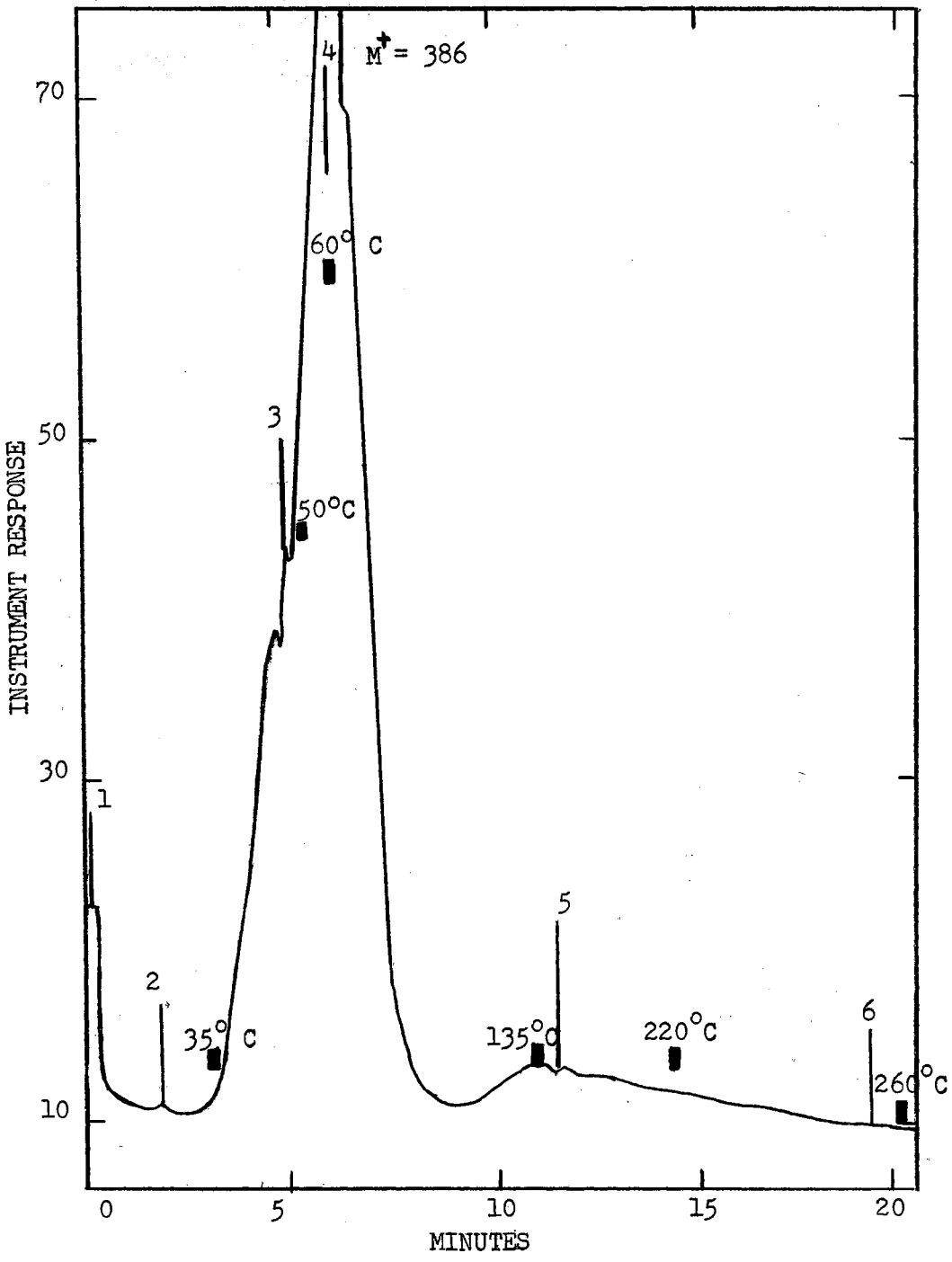


Figure 48. Total Ion Current—Tracing of the Metabolite in Direct Probe Method. Numbers correspond to places where mass spectra were taken.

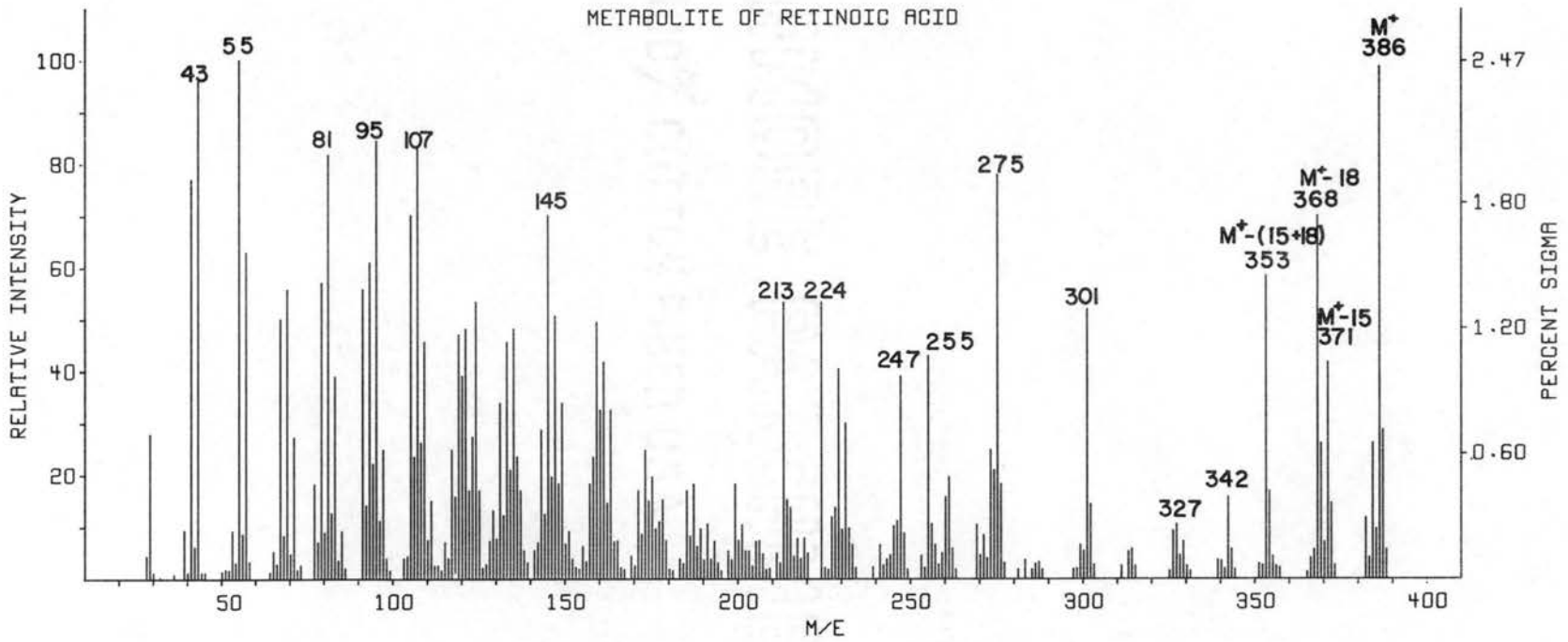


Figure 49. Mass Spectrum of the Metabolite.

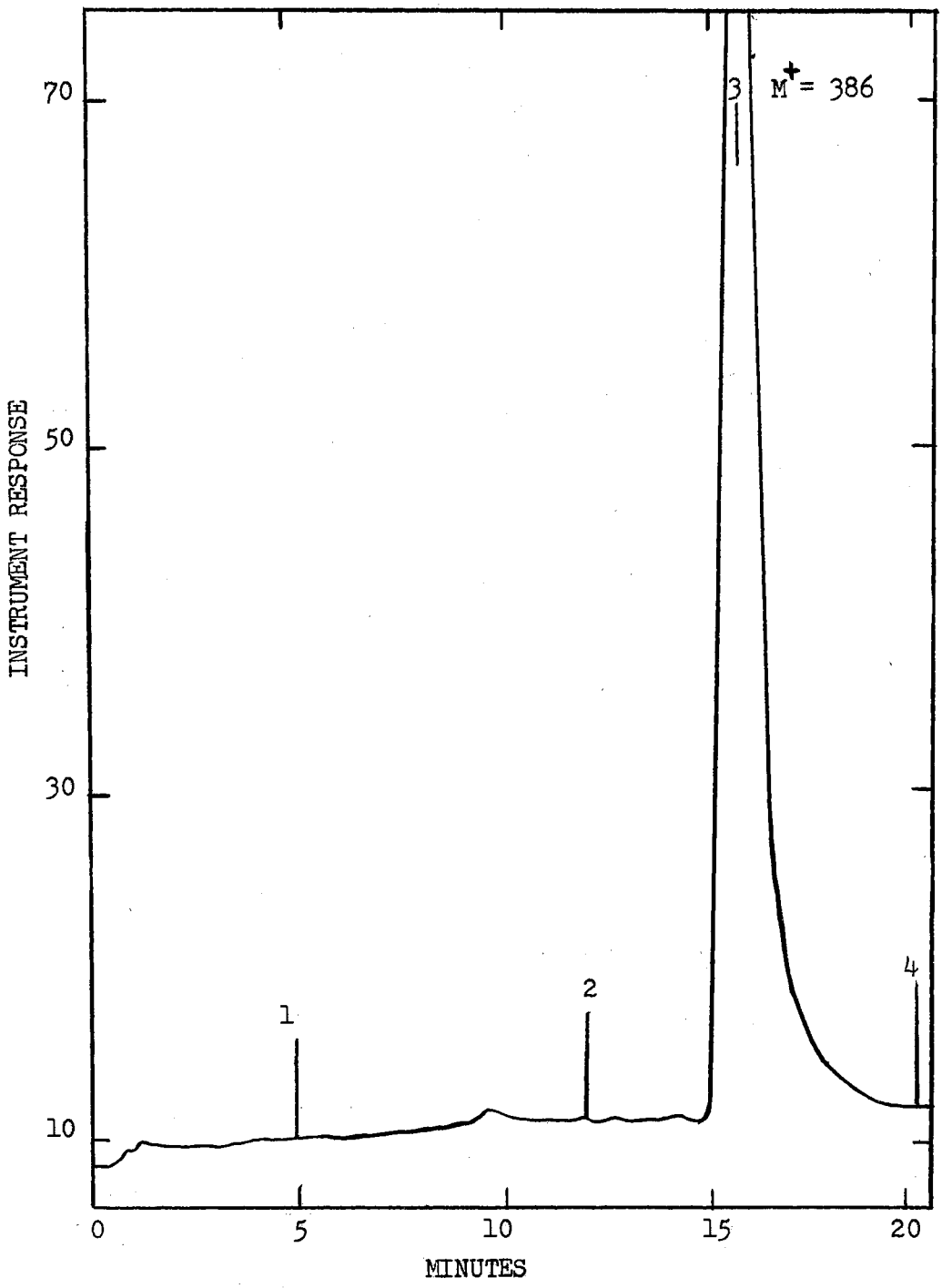


Figure 50. Total Ion Current—Tracing of the Metabolite Using the Gas Chromatography Inlet Method. Numbers correspond to places where mass spectra were taken.

TABLE XXI

METASTABLE PEAKS IN MASS SPECTRUM OF THE METABOLITE

Apparent Mass	Transition Denoted	Probable Neutral Product
356.58	$(386^+) \longrightarrow (371^+) + 15$	CH_3
350.84	$(386^+) \longrightarrow (368^+) + 18$	H_2O
338.61	$(371^+) \longrightarrow (353^+) + 18$	H_2O
335.87	$(368^+) \longrightarrow (353^+) + 15$	CH_3
195.92	$(386^+) \longrightarrow (275^+) + 111$	$C_7H_{11}O$

TABLE XXII

HIGH RESOLUTION MASS SPECTRUM OF THE METABOLITE: OXYGEN-CONTAINING FRAGMENTS

m/e	Density	Composition (CHO)
71	5.8	4/7/1
81	3.4	5/5/1
83	6.0	5/7/1
85	4.1	5/9/1
95	4.4	6/7/1
96	3.3	6/8/1
97	5.0	6/9/1
107	3.4	7/7/1
108	2.4	7/8/1
109	4.9	7/9/1
110	4.2	7/10/1
111	4.5	7/11/1
121	3.5	8/9/1
122	3.2	8/10/1
123	5.0	8/11/1
125	6.1	8/13/1
127	2.5	9/3/1
135	2.8	9/11/1
136	2.7	9/12/1
137	4.0	9/13/1
138	4.5	9/14/1
150	3.7	10/14/1
152	3.0	10/16/1
163	5.2	11/15/1
165	3.1	11/17/1
175	3.0	12/15/1
176	3.4	12/16/1
177	3.9	12/17/1
178	6.5	12/18/1
217	2.5	15/21/1
231	6.1	16/23/1
246	3.4	17/26/1
271	3.2	19/27/1
272	2.8	19/28/1
273	6.3	19/29/1
371	7.0	26/43/1
386	10.7	27/46/1

TABLE XXIII

HIGH RESOLUTION MASS SPECTRUM OF THE METABOLITE: HYDROCARBON FRAGMENTS

m/e	Density	Composition (CH)	m/e	Density	Composition (CH)
70	10.5	5/10	137	5.2	10/17
71	13.2	5/11	141	4.9	11/9
77	11/1	6/5	142	5.2	11/10
78	9.3	6/6	143	8.1	11/11
80	10.7	6/8	155	3.9	12/11
81	15.5	6/9	156	3.5	12/12
82	10.0	6/10	157	6.7	12/13
83	10.3	6/11	158	7.1	12/14
84	6.3	6/12	159	9.3	12/15
85	6.4	6/13	160	8.2	12/16
89	2.6	7/5	162	6.2	12/18
91	14.0	7/7	165	3.3	12/21
92	10.5	7/8	169	3.5	13/13
93	12.8	7/9	171	6.0	13/15
94	10.2	7/10	173	6.9	13/17
95	13.0	7/11	177	3.4	13/21
96	8.4	7/12	183	3.0	14/15
97	8.1	7/13	185	5.7	14/17
103	5.9	8/7	186	4.5	14/18
104	6.3	8/8	187	5.6	14/19
105	12.2	8/9	188	3.5	14/20
106	9.7	8/10	189	4.0	14/21
107	12.7	8/11	191	3.3	14/23
108	9.1	8/12	193	3.6	14/25
109	10.0	8/13	197	3.3	15/17
110	6.4	8/14	199	5.8	15/19
111	4.5	7/11	200	3.9	15/20
111	6.4	8/15	201	4.5	15/21
112	2.9	8/16	205	3.3	15/25
113	2.9	8/17	206	3.1	15/26
115	6.5	9/7	207	2.7	15/27
116	6.1	9/8	213	7.4	16/21
117	8.6	9/9	219	2.8	16/27
118	7.9	9/10	220	3.0	16/28
119	10.4	9/11	227	3.7	17/23
120	10.2	9/12	228	4.5	17/24
121	10.9	9/13	229	4.5	17/25
122	7.3	9/14	241	2.9	18/25
123	7.1	9/15	247	5.9	18/31
125	3.6	9/17	248	3.2	18/32
127	3.4	10/7	255	6.5	19/27
128	6.2	10/8	256	3.3	19/28
129	7.3	10/9	260	2.4	19/32
130	6.7	10/10	261	3.1	19/33
131	9.3	10/11	275	8.5	20/35
132	7.6	10/12	301	7.7	22/37
133	9.9	10/13	326	3.2	24/38
134	8.2	10/14	353	6.9	26/41
135	9.2	10/15	368	7.5	27/44
136	6.4	10/16			

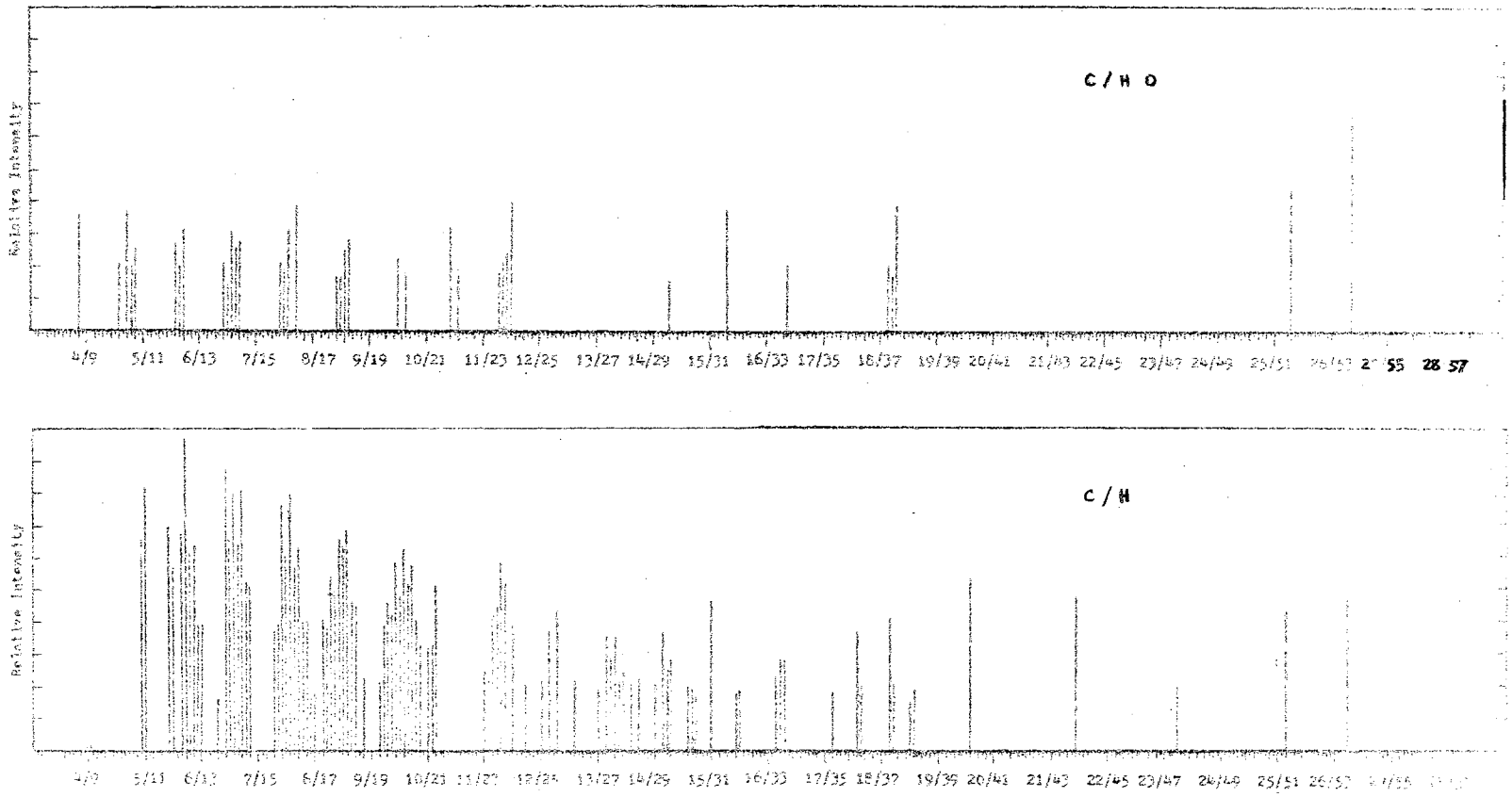


Figure 51. Element Map of High Resolution Mass Spectrum of the Metabolite. Each plot gives only ions with a specific heteroatom content. Each major division on the X-axis represents a CH_2 unit and is divided into 14 sub-divisions, each corresponding to one hydrogen or one mass unit.

resolution mass spectrum showed that the metabolite has the emperical formula of $C_{27}H_{46}O$ and that oxygen is present as a free hydroxyl group. Although the base peak for the metabolite is m/e 55 (Figure 49), the molecular ion, m/e 386, is almost as intense as the base peak. A distinct and prominent peak was found at $M^+ - 18$ from loss of water and $M^+ - 15$ peak, a characteristic peak found in all vitamin A compounds (Chapter II), representing loss of one methyl group. The spectrum showed a fairly strong peak at $M^+ - 33$ resulting from loss of CH_3 and H_2O. A strong peak at $M^+ - 111$ due to the loss of $C_7H_{11}O$ was also found. All these transitions were supported by the appearance of the metastable ions in the spectrum (Table XXI) and the high resolution mass spectrum (Table XXII and XXIII). The spectrum for the hydrocarbon portion below m/e 213 of the metabolite was quite similar to the substrate, retinoic acid and related compounds (Figures in Chapter II). For instance, the peak at m/e 213 $C_{16}H_{21}$ (Table XXIII) and m/e 255, $C_{19}H_{27}$ (Table XXIII), and the loss of a methyl group from the β-ionone ring, provide strong supporting evidence that β-ionone ring and the isoprenoid side chain carbon atoms, except C-15, remained in the metabolite.

The trimethylsilyl derivative (TMS-derivative) of the metabolite was obtained as described under "Experimental" and was analyzed on the mass spectrometer-gas chromatograph. Figure 52 shows the total ion currents tracing map of the TMS-treated metabolite and the mass spectra of the TMS-derivative of the metabolite obtained at position 5 in the TIC-tracing (Figure 52) is shown in Figure 53. From the molecular ion, m/e 458 (386 + 72), it can clearly be seen that there is only one free hydroxyl group present in the metabolite. This is also confirmed

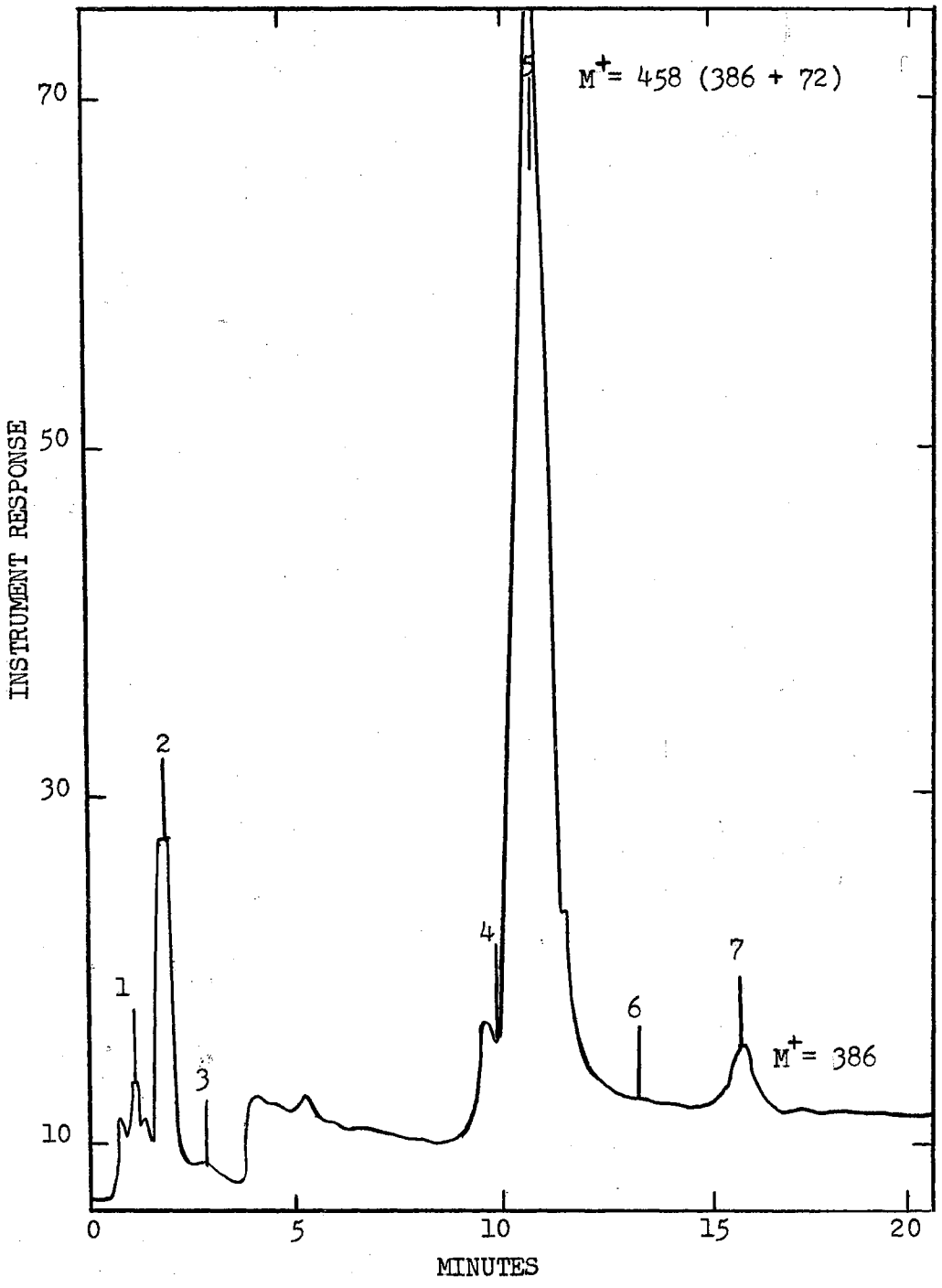


Figure 52. Total Ion Current—Tracing of the TMS-treated Metabolite in Gas Chromatography Inlet Method. Numbers correspond to places where mass spectra were taken.

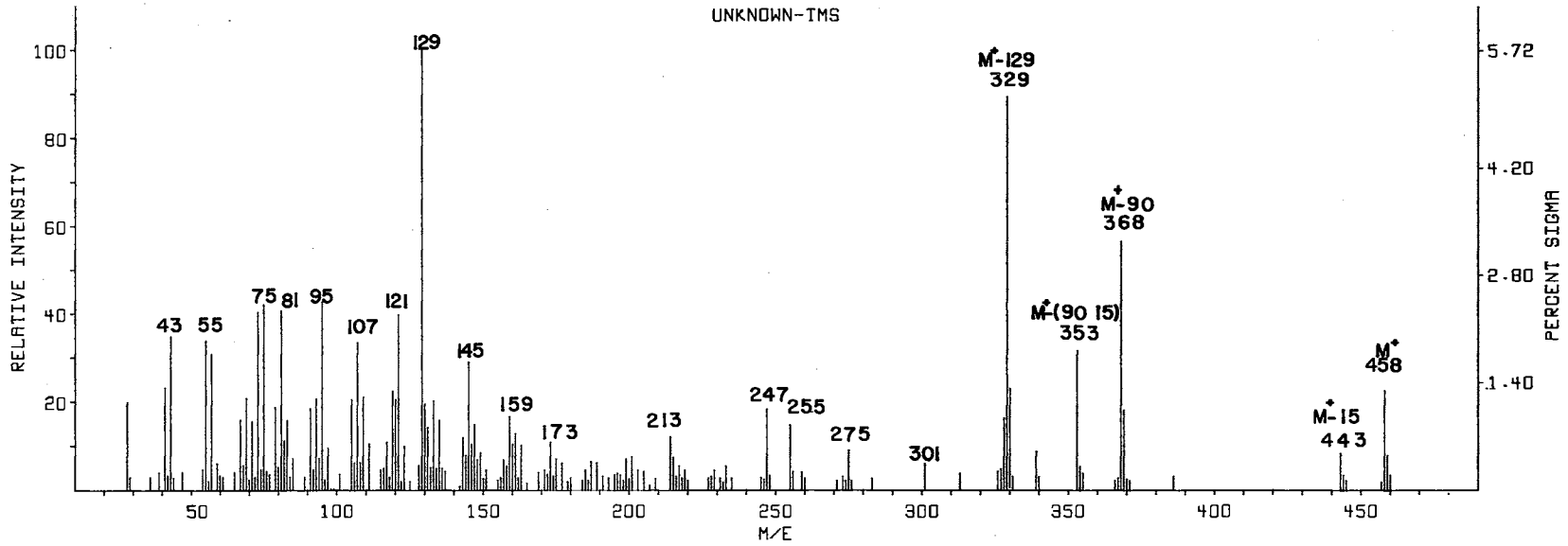


Figure 53. Mass Spectrum of TMS-derivative of the Metabolite

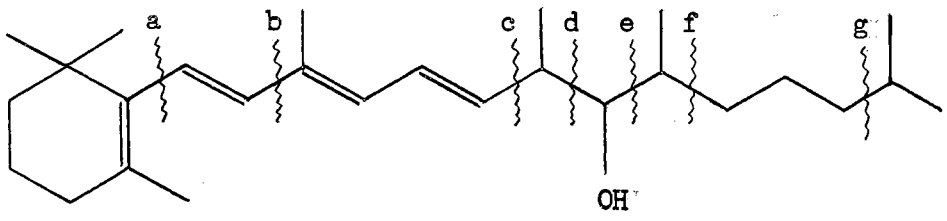
by the presence of a fairly strong peak at $M^+ - 90$ resulting from loss of a HO-Si-$(CH_3)_3$ group. Loss of one methyl group from M^+ and $M^+ - 90$ were again found at m/e 443 and 353 respectively. Furthermore, the base peak, m/e 129, and the very intense peak, m/e 329 ($M^+ - 129$), clearly showed that the TMS-derivative of the metabolite readily lost a group of mass 129, which may contain the trimethylsilyl group as most of the normal TMS-derivatives from alcohols do (103). Therefore, m/e 129 is believed to be due to R-O-Si-$(CH_3)_3$ in which R could be $CH_3-\underset{|}{C}=CH-$ or $CH_2=\underset{|}{C}-CH-$.

Discussion

In the present experiment, retinoic acid was decarboxylated *in vitro* by a partially purified enzyme from chicken liver and a major metabolite of retinoic acid was isolated and purified. The metabolite was radioactive when retinoic acid-6,7-^{14}C was the substrate but non-radioactive when retinoic acid-15-^{14}C was the substrate. This clearly showed that the isolated metabolite was a decarboxylation product of retinoic acid. It is an enzymatic reaction product since the control incubations, containing only retinoic acid-6,7-^{14}C without enzyme, and the blank incubations, containing only enzyme, never resulted in the formation of such a compound. Upon anion exchange and DEAE-cellulose column chromatography, this metabolite was found in the non-acidic fraction. It had a lower UV absorption maximum (285 nm) than the starting material, retinoic acid, which has UV maximum absorption at 350 nm. The lower absorption maximum of the metabolite indicates that the conjugated system has been shortened. The IR spectra of the metabolite shows a broad absorption band of a hydroxyl group and CH_3

and CH_2 peaks. Furthermore, a single TMS-derivative can be formed from the metabolite, indicating that the metabolite contains a hydroxyl group.

High resolution mass spectra of the metabolite showed that the composition of the metabolite is $C_{27}H_{46}O$. That a single hydroxyl group was present in the metabolite was also shown by the mass spectrum of the TMS-derivative of the metabolite. The possibility of being a primary alcohol is ruled out since the mass spectrum did not show a strong peak at m/e 31 and $M^+ - 31$ which are diagnostic for a primary alcohol (104, 105, 106, 107). The strong intensity of the $M^+ - 18$ peak of the metabolite indicates that the metabolite could be secondary or tertiary alcohol (104, 105, 106, 107). The similarity of the hydrocarbon portion below m/e 213 of the mass spectra of the metabolite, retinoic acid, and related compounds, the retention of a peak at m/e 213 ($C_{16}H_{21}$), m/e 255 ($C_{19}H_{27}$) in all these spectra and also the loss of a methyl group from the β-ionone ring provides strong support to the concept that the β-ionone ring and the isoprenoid side chain carbon atoms, except at C-15, still remains in the metabolite. There seems to be good evidence that the peaks below m/e 213 are the same as those of retinoic acid and related compound. Therefore, assuming the $C_{16}H_{21}$ (m/e 213) part of the molecule is unchanged, and based upon the fragmentation patterns of the metabolite and the composition of fragment ions in the high resolution mass spectrum, the possible structure of the metabolite is proposed as follows:



The molecular ion loses water to give a stable hydrocarbon fragment (M^+ - 18) which probably has five conjugated double bonds. At least two structures which would have the same elemental composition could be written for this m/e 368 ion,; one of which would have the fifth double bond in a non-conjugated position. The M^+ - 18 ion with the double bond may fragment at branch points c or e to give m/e 213 or five conjugated m/e 255 which are present in all vitamin A and related compounds studied in Chapter II. In another mode of fragmentation the M^+ - 18 ion may lose C_3H_6, or C_3H_5 at branch point g and by a proton shift give m/e 326 and m/e 327 ions respectively. The ion m/e 327 may further lose a C_2H_2 group from the end of the side chain to give a stable ion m/e 301 which may possess 6 conjugated double bonds. The molecular ion gives m/e 371 (M^+ - 15) from the loss of one methyl group and m/e 353 (M^+ - 15 - 18) can be formed from either M^+ - 15 or M^+ - 18 by losing H_2O and CH_3 respectively. The explanation of the formation of other oxygen-containing fragment ions are more complicated. However, the oxygen-containing fragment ions containing more than 19 carbon atoms may come mainly from the fragments which still contain the β-ionone ring. The fragmentation at branch point e and a proton shift could give a C_{19} series of oxygen-containing fragments. The oxygen-containing fragments containing less than 19 carbon atoms may come from the side chain with oxygen retained, after loss of the β-ionone ring. For instance, fragmentation at branch point b, gives a C_{16} oxygen-containing fragment which may lose another C_8 and C_6 from branch points e and f to give a C_8 and C_{10} series of oxygen-containing fragments respectively. The fragmentation at branch point d may result in the formation of a C_9 series of oxygen-containing fragments which may

lose another C_3 from branch point g to give C_6 series of oxygen-containing fragments. The fragmentation at branch point c may give the C_{11} series of oxygen-containing fragments which may lose another C_6 unit from the bfanch point f to give the C_5 series of oxygen-containing fragments.

This is only a "proposed" structure and its justification mostly rests on the fragment ions appearing in the mass spectrum of the metabolite and the compositions of fragments shown in the high resolution mass spectrum of the metabolite. The proposed structure explains most of the fragment ions but not all, therefore, the actual structure and the stereochemistry of the metabolite remains unknown.

The formation of this metabolite might be similar to the mechanism of peroxidation (108) with oxygen attacking the double bond of vitamin A acid at C - 13 to form a hydroxyl group at C - 14. Subsequent decarboxylation with loss of CO_2 from C - 15 and synthesis of a new C - C bond at C - 14 with a C_8H_{17} group attached could result. The source of C_8H_{17} might come from cleavage of the β-ionone ring or from other degenerate isoprenoid compounds such as geranyl pyrophosphate. However, the actual source of C_8H_{17} and the actual mechanism of the formation of the metabolite must await further study.

The relationship of the presently described metabolite to those reported by others is not clear, since the metabolites previously reported were not completely characterized and their compositions and molecular weights were not determined. In 1964, Wolf, et al. (30) claimed that an acidic metabolite was isolated from rat small intestine. Its UV absorption maximum was 252 nm and its IR spectra showed a -OH, C=O and COO^- group. Zile and DeLuca (40) also reported that one

metabolite which does not possess the UV spectra of retinoic acid was isolated. A metabolite of retinoic acid was completely identified by Olson et al. (35) as retinoyl β-glucuronide which is not a degradative product but is a product of the conjugation of retinoic acid and β-glucuronic acid. Although both the retinoic acid decarboxylation enzyme and the β-glucuronide synthesizing enzyme are microsomal enzymes, the product isolated from the decarboxylation reaction is not retinoyl β-glucuronide. Recently, Roberts and DeLuca (48) reported that the decarboxylation product of retinoic acid by microsomes of rat liver and kidney appeared to lack only C - 15 of the original retinoic acid molecule. It was not retained by DEAE-cellulose, was more polar than retinoic acid upon silicic acid chromatography, had a lower UV absorption maximum (295 nm) than retinoic acid, and seemed to have an aldehyde group at C - 14. The molecular weight and the composition of this metabolite were not reported. The physiological significance of the decarboxylation product remains to be assessed.

SUMMARY

The mass spectra of all-*trans* retinol (vitamin A), anhydroretinol, retinyl acetate, retinal, retinoic acid, methyl retinoate and 9-*cis* retinal were investigated with the view of using this technique as a rapid identification method and as an aid in identifying metabolites of retinol and retinoic acid. The decarboxylation of retinoic acid by chicks was studied *in vivo* and *in vitro*. In order to simplify the isolation of the reaction products, an enzyme system which carries out the decarboxylation of retinoic acid was partially purified and the properties of the enzyme were studied. Products resulting from the decarboxylation of retinoic acid were isolated and purified.

The mass spectra of retinol and related compounds shows that each compound gave its molecular ion, m/e = M^+, and certain characteristic peaks representing the loss of its functional group, thereby providing a quick and unambiguous method of identification. A characteristic mode of fragmentation for these compounds was the cleavage of the bond between the ring and the side-chain resulting in the formation of the ion, m/e = 123 which corresponds to the β-ionone ring and the formation of positively charged ions representing the side-chain and the hydrocarbon portion of the side-chain after the functional group was lost. Certain useful interpretive peaks were observed.

The experiments *in vivo* with chicks demonstrated that radioactive CO_2 was respired to the extent of 4.5% of the oral dose when retinoic acid-15-^{14}C was administered, and to the extent of 0.4% of the oral

dose when retinoic acid-6,7-^{14}C was administered. The enzyme system which carries out the decarboxylation of retinoic acid is a microsomal enzyme and was purified approximately 292 fold from liver homogenate and 13 fold from liver microsomes. It has an optimum pH of 6.4, optimum temperature of 37°C, requires oxygen and requires ferrous ion as a cofactor. Other biological reducing agents such as L-cysteine, ascorbic acid, and NADPH also enhanced the enzyme activity but were not equivalent to Fe^{++}. Metal ions (Mg^{++}, K^{+}, Ca^{++}, Zn^{++}, Ba^{++}, and Fe^{+++}) and bile salts (sodium glycocholate and sodium taurocholate) had little effect on enzyme activity. Cyanide, DPPD, and phenazine-metho-sulfate inhibited the enzyme.

A metabolite resulting from the decarboxylation of retinoic acid by a partially purified enzyme was isolated and purified by column, thin-layer, and gas chromatography. The mass spectra of this metabolite and its TMS-derivative and the high resolution mass spectrum of the metabolite were obtained. This metabolite has a molecular weight of 386 with the composition of $C_{27}H_{46}O$. It has a UV absorption maximum at 285 nm and its IR spectra shows OH-group, CH_3 and CH_2 group. The mass spectra revealed that the metabolite is an alcohol, and retains the β-ionone ring and the isoprenoid side-chain carbon atoms except C-15. A possible structure was proposed.

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