MASS SPECTRAL ANALYSES AND METABOLISM OF ANALOGS OF VITAMIN A

By

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OF ANALOGS OF VITAMIN A

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INTRODUCTION

Numerous reviews and monographs with reference to vitamin A have been published including two excellent summaries by Dr. James Allen Olson (1) (2). In November of 1968 a sympsoium on the metabolic function of vitamin A was held and has since been published (3). For an adequate source of information on vitamin A through 1970, these reviews; a symposium in 1960 (4), Thomas Moore's book Vitamin A (5), the second edition of The Vitamins (6), the recent summaries by Wolf and L. DeLuca (7), Roels (8), and the review by Wasserman and Corradino in the 1971 Annual Review of Biochemistry (9) are recommended.

Nightblindness made man aware of his need for an unknown nutritional substance which was later shown to be vitamin A. With the discovery that substances other than retinol (vitamin A alcohol) may function in specific biological systems, the term vitamin A, as used today, is not limited specifically to retinol, but includes all analogs of retinol that may be similar in either structure or function.

In early investigations concerning vitamin A metabolism the emphasis was upon describing visible signs of vitamin A deficiency. Other than in the visual process, in which retinal (vitamin A aldehyde) interacts with visual pigments, there has been little success in correlating any of the physical signs of vitamin A deficiency with a specific biochemical lesion. It has been proposed that fat-soluble vitamins function at a

hormonal level of control of protein synthesis rather than as a coenzyme (7). A decrease in the number of goblet cells in the small intestine was observed by Wolf et al. (10) as well as a decreased synthesis of a fucose-containing glycolipid in vitamin A deficient rats (11) (12). Injections of retinol restored these conditions to normal after 18 hours. A mannolipid has been found that is composed of a retinol metabolite that incorporates mannose into glycoprotein (13). This and any new information regarding the functions of vitamin A at the molecular level may explain the visible deficiency signs and elucidate the mechanism whereby retinol affects growth and maintains the general health and well being of higher animals.

Arens and van Dorp in 1946 (14) (15) (16) first synthesized retinoic acid (vitamin A acid). Subsequently its physiological activity has been demonstrated in the rat (17) (14) (16), chick (18) and pig (19) (20). An important aspect of the physiological activity of retinoic acid was after injection or administration of retinoic acid, no retinol could be detected in the liver. Rats supplemented with retinoic acid grew normally but became blind, further demonstrating that the acid was not reduced to the aldehyde or the alcohol (17).

Retinoic acid has also failed to support pregnancy in female rats or spermatogenesis in male rats (21) (22). In marked contrast, chicks receiving methyl retinoate produced normal spermatogen and eggs but would not support the normal development of the embryo (23). Nevertheless, retinoic acid can replace retinol in the in vitro biosynthesis of corticosterone from cholesterol (24), effect the release of a protease from rat liver lysosomes (25), restore sulfate transferase activity (26) and reduce cerebrospinal fluid pressure (19).

An uncomplicated vitamin A deficiency was produced in pigs by Nelson et al. (20) who later demonstrated that retinoic acid was biologically equivalent to retinyl palmitate in supporting growth and maintaining a normal cerebrospinal fluid pressure (19). Blood plasma retinol was unrelated to any level of retinoic acid fed but liver retinol concentration increased with retinol or retinoic acid intake. A sparing effect was observed on liver retinol by retinoic acid and it was demonstrated that liver retinol was not preferentially metabolized when retinoic acid was present as was thought to be the case at that time. A requirement in some instances but not others suggested that retinol or retinoic acid might be converted into a form that is active in specific processes.

Retinoic acid could not be detected in the blood or liver by early investigators following oral administration or injection (14) (15) (16) (17). A quantitative method was developed by Nelson et al. (27) for the determination of both retinol and retinoic acid on a single sample of blood plasma. When [6,7-^{14}C] retinoic acid was fed to pigs, retinoic acid concentration in blood plasma reached a maximum in 1.5 to 3 hours then decreased until none could be detected after 12 hours. However, as retinoic acid concentration decreased, the radioactivity present in the ethanol-plasma residue increased. Thus, retinoic acid was being converted to more polar compounds not extractable with petroleum ether. Both retinol and retinal have been shown to yield retinoic acid as a normal metabolite (28) (29) (30) lending support to the concept that the acid might be closer to an active form. Additional evidence for this concept is that retinoic acid produces hypervitaminosis A at concentrations lower than retinol.

It has been shown in our laboratory that retinoic acid is bound to bovine serum albumin (31). Scatchard plots of the data obtained from binding of retinoic acid to bovine serum albumin in vitro show that bovine serum albumin possesses more than one class of binding sites for retinoic acid. The sites were divided into a higher and lower energy class with at least one of the retinoate binding sites on bovine serum albumin binding palmitate.

It would appear to be a simple task to isolate and identify metabolites of vitamin A, especially with the availability of ^{14}C-labeled compound. This has turned out to be far from the case. The search for these compounds has resulted in partial purification of numerous metabolites of retinoic acid (18) (32) (33) (34) (35) (36). Some of these unidentified compounds reportedly have some biological activity. Wolf, Kahn, and Johnson (37), in 1957, observed the appearance of water soluble metabolites in the urine of rats which were injected with [14-^{14}C] retinol. Olson (38) recently summarized their discoveries concerned with the isolation and identification of water soluble metabolites in the bile. They have shown that formation and biliary excretion of retinoyl-β-glucuronide and retinyl-β-glucosiduronate from retinoic acid acid and retinol, respectively, occurs normally in rats, chicks, guinea pigs and rabbits. The origin of other metabolites isolated from bile have been shown to be formed from retinoyl-β-glucuronide during its isolation (35). Lehman has recently separated radioactive compounds derived from [15-^{14}C]retinoic acid into various fractions based on their solubilities in solvents of varying polarities. One of these is retinoyl-β-glucuronide (31).

Other radioactive metabolites of retinoic acid have been reported. Some of these have growth promoting properties but no identification has been made so it is not possible to determine the source of biological activity. One biologically active metabolite from liver (33) was later shown to be 13-cis-retinoic acid which was probably formed during the isolation procedure since 60% of added all-trans-retinoic acid was converted to the 13-cis-isomer (39). Another metabolite of retinol and retinoic acid was reported (32) which did not contain the terminal carbon but was capable of supporting growth in rats.

Metabolic pathways for retinol and retinoic acid have been suggested. These have been based on the rate of appearance of carbon-14 in CO_2, urine and feces when the label was located in the ring ([6,7-^{14}C] retinoic acid), carboxyl ([15-^{14}C]retinoic acid) or side chain ([14-^{14}C] retinoic acid). Roberts and DeLuca (40) proposed that the intact molecule is excreted as retinoyl-β-glucuronide in bile and urine, that the decarboxylated product is excreted in the bile and that the urinary metabolites result from oxidation of the side chain. The data of Sundaresan and Therriault (41) indicates that the ring and the side chain might be excreted in the urine at the same rate and that the lower quantity of radioactivity from [15-^{14}C]retinoic acid in the urine is due to rapid decarboxylation. They also suggest that the side chain could be cleaved prior to decarboxylation. No retinoyl-β-glucuronide was found in the urine (42).

The decarboxylation of retinoic acid has been studied in rat kidney and liver slices (43) and microsomes (34). Effects of various cofactors and inhibitors on the decarboxylation, when incubated with intact microsomes, were reported, but there was no additional purification of the enzymes.

Preparations of rat and chicken liver microsomes (44), an acetone-butanol-ether (ABE) powder prepared from liver microsomes and crystalline horseradish peroxidase (HRP) (45) have been used to decarboxylate [15-^{14}C]retinoic acid. The ABE powder resulted in 40% decarboxylation, as evidenced by the $^{14}CO_2$ collected, in the presence of either 1 mM Fe^{+2}, NADPH or ascorbate. Heat deactivated the NADPH and ascorbate catalyzed reactions. When horseradish peroxidase (2.6 μM) was used up to 48% of the carboxyl labeled ^{14}C-retinoic acid could be collected as $^{14}CO_2$.

Structural alteration of retinol does affect its biological activity (46) (47). The biological activity of 3-dehydroretinol (vitamin A_2, one additional conjugated double bond in the ring) was 30-40% (48) and saturation of one or more of the double bonds in the side chain resulted in complete loss of activity. Alteration of the terminal carbon has less effect upon biological activity. Morton (47) emphasized the apparent indispensability of the four double bonds in the unsaturated side chain.

The preparation, properties and biological activities of several analogs of retinol and retinoic acid have been reported (49) (50) (51) (52) (53) (54). Retro-retinol was biologically active after isomerization to retinol and methyl 5,6-monoepoxyretinoate was converted to the free epoxy acid by rat liver homogenate. Both the 5,6-monoepoxyretinoate and methyl 5,8-monoepoxyretinoate supported growth in rats as did anhydroretinol (55).

The investigation of Vecchi et al. (57) on retinol and isomers of retinol was the first report of mass spectrometric studies of vitamin A compounds. The first comprehensive mass spectral analysis of retinol

and related compounds was performed in our laboratory by Lin et al. (56). Enzell has written a review of mass spectra of vitamin A published in 1972 (64). The volumes of work published on carotenoid derivatives cannot be disregarded. There is an excellent review by Weedon (58) of carotenoid advances as well as other articles by Weedon (59), Enzell (60), and Elliot et al. (65). Epoxy and furanoid oxides of carotinoids were studied by Baldas et al. (61) (62) and Dan and Lederer (63).

Even though a considerable amount of research has been done on the metabolism of retinoic acid, its metabolic fate is not completely understood. The identification of metabolic products would greatly enhance the validity of proposed metabolic pathways and modes of action. The availability of sophisticated instrumentation such as mass spectrometry for identification of microgram quantities of metabolic products should accelerate the progress in this field. The mass spectral analyses of vitamin A analogs in this thesis were reported in order to apply these data to aid in the isolation, characterization and identification of the metabolites of retinoic acid.

CHAPTER II

MASS SPECTRA OF ANALOGS OF VITAMIN A

Mass spectrometry has become an important analytical tool for examination and identification of biological compounds. Attempts to identify metabolites of vitamin A by mass spectral analysis have indicated a need for additional information on the fragmentation patterns of structural analogs of vitamin A. Vecchi et al. (57) and Lin et al. (56) have published mass spectra of some vitamin A compounds. This chapter reports the mass spectral analysis of 5,6-monoepoxyretinoic acid, 5,8-monoepoxyretinoic acid, methyl-5,6-monoepoxyretinoate, methyl-5,8-monopoxyretinoic acid, trimethylsilyl-5,6-monoepoxyretinoate, trimethylsilyl-5,8-monoepoxyretinoate, 5,6-monoepoxyretinyl acetate, 5,8-monoepoxyretinyl acetate, 5,8-monopoxyretinal, β-C_{19}-retinal, β-C_{19}-retinyl acetate, and C_{19}-anhydroretinol.

Experimental

Materials

The compounds investigated were obtained from the following sources. The 5,6 monoepoxy and 5,8 monoepoxy-vitamin A compounds were prepared according to John et al. (67). The β-C_{19}-retinal and all-trans-retinoic acid were provided by Dr. R. H. Bunnell of Hoffmann-LaRoche, Inc., Nutley, New Jersey. The C_{19}-analogs were prepared from β-C_{19}-retinal. The purity of these compounds was checked by thin-layer chromatography on silicic acid, column chromatography on alumina, and gas-liquid

chromatography just prior to analysis and each compound eluted as one peak.

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., 12221 Parklawn Drive, Rockville, Maryland) (68). The direct inlet temperature was varied from ambient temperature to 50°C while the ion source was 310°C. Spectra were taken at 70 ev using an accelerating voltage of 3.5 kv. The source pressure was 5×10^{-6} to 1×10^{-7} mm Hg. All mass spectra were computer-plotted from tabular intensity data. A CalComp 565 plotted data from disc storage of an IBM Systems 360-Model 65.

High resolution mass spectra were obtained on a CEC 21-110-B mass spectrometer (consolidated Electrodynamics Corp., Pasadena, California). Spectra were taken at 70 ev with a trap current of 75 μA. The direct inlet and source temperatures were 120°C and 150°C respectively. Perfluorokerosene was used for peak-matching which was done electronically on the all-trans-retinoic acid while the methyl-5,6-monoepoxyretinoate was matched from a photographic plate.

Results and Discussion

The mass spectra of 5,6-monoepoxyretinoic acid, 5,8-monoepoxyretinoic acid, methyl-5,6-monoepoxyretinoate, methyl-5,8-monoepoxyretinoate, trimethylsilyl-5,6-monoepoxyretinoate, trimethylsilyl-5,8-monoepoxyretinoate, 5,6-monoepoxyretinyl acetate, 5,8-monoepoxyretinyl acetate, 5,8-monoepoxyretinal, β-C_{19}-retinal, β-C_{19}-retinyl acetate and C_{19}-anhydroretinol are shown in Figures 1 through 12 respectively. Structures of

Figure 1. Mass Spectrum of 5,6-Monoepoxyretinoic Acid.

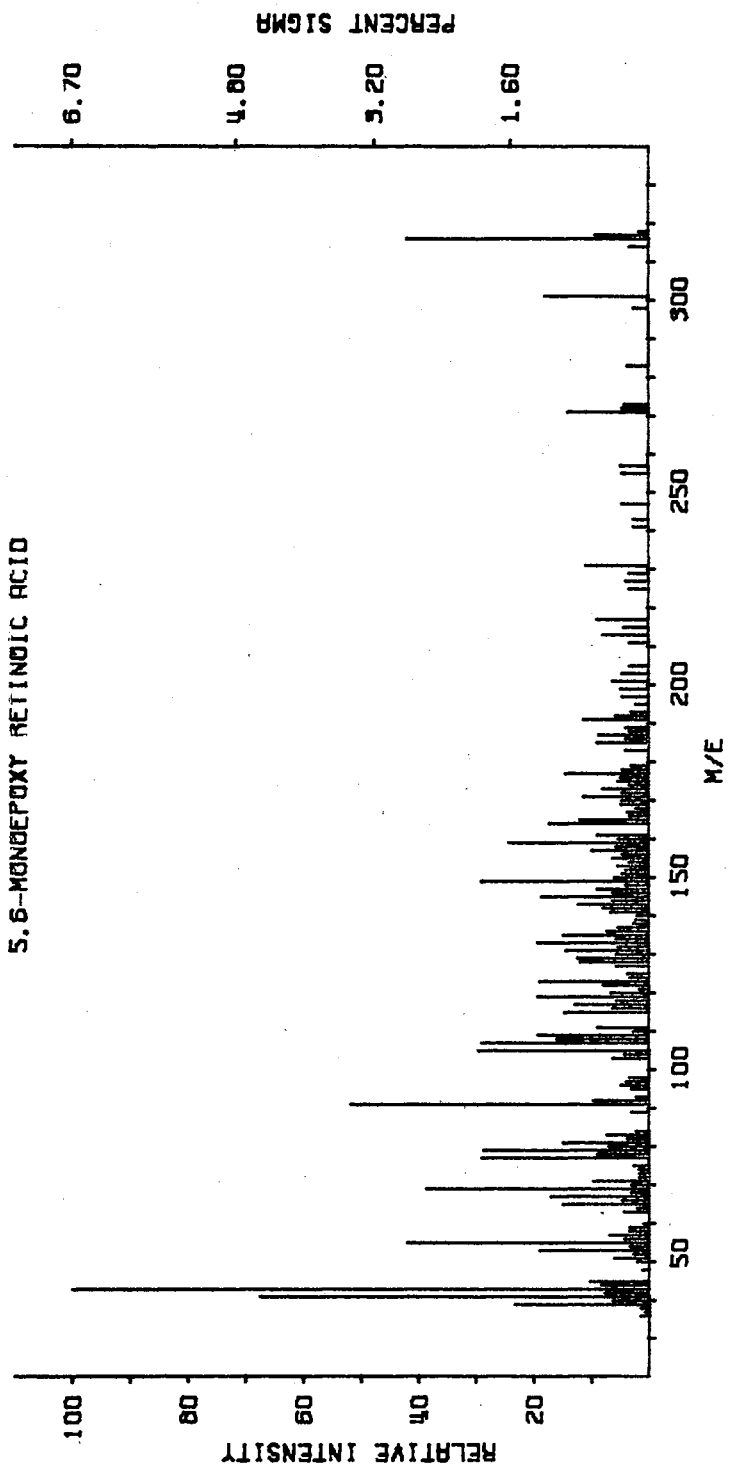

5,6-MONOEPOXY RETINOIC ACID
PERCENT SIGMA
6.70
4.80
3.20
1.60
RELATIVE INTENSITY
100
80
60
40
20
M/E
50
100
150
200
250
300

Figure 2. Mass Spectrum of 5,8-Monoepoxyretinoic Acid.

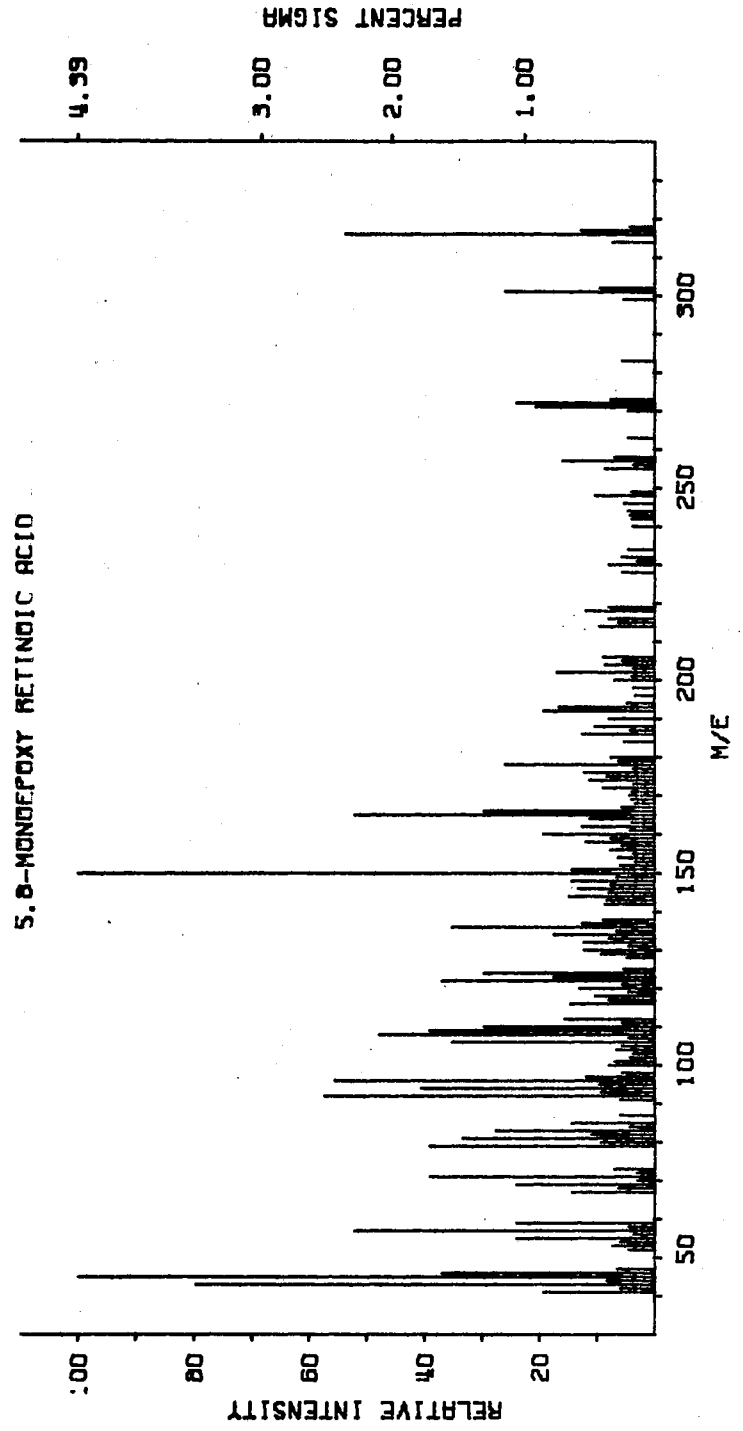

PERCENT SIGMA
4.39
3.00
2.00
1.00
5,8-MONOEPOXY RETINOIC ACID
M/E
50
100
150
200
250
300
RELATIVE INTENSITY
20
40
60
80
100

Figure 3. Mass Spectrum of Methyl-5,6-Monoepoxyretinoate.

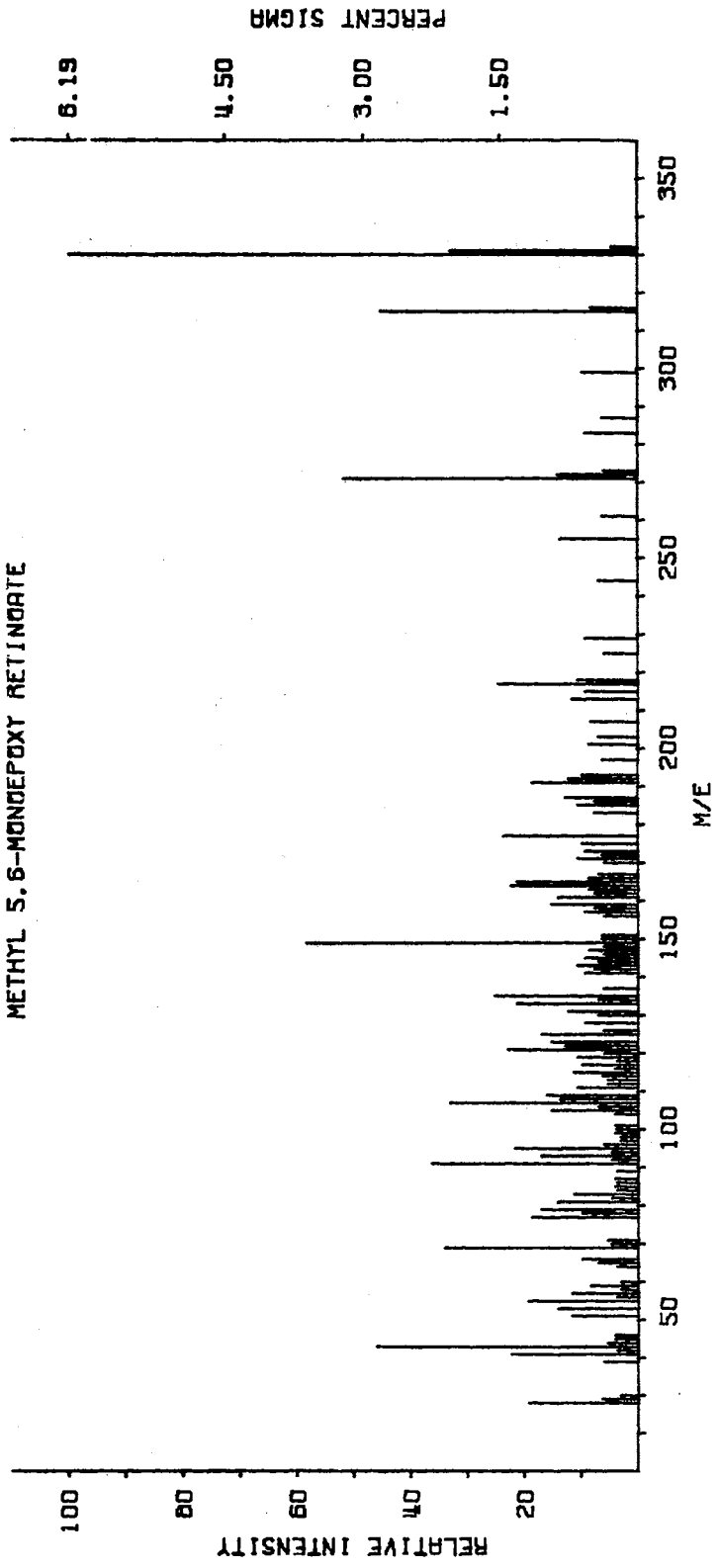

METHYL 5,6-MONOEPOXY RETINOATE
PERCENT SIGMA
6.19
4.50
3.00
1.50
RELATIVE INTENSITY
100
80
60
40
20
M/E
50
100
150
200
250
300
350

Figure 4. Mass Spectrum of Methyl-5,8-Monoepoxyretinoate.

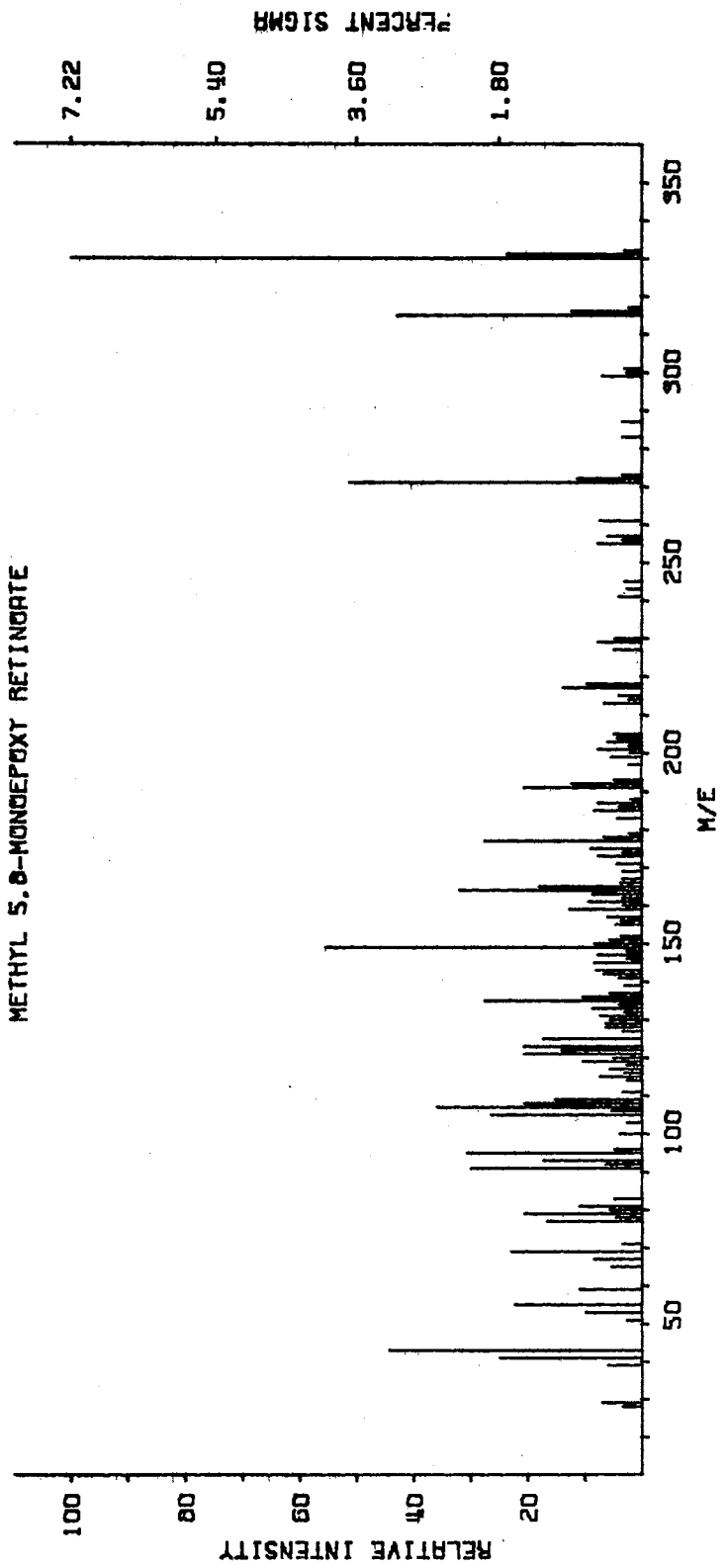

METHYL 5,8-MONOEPOXY RETINOATE
PERCENT SIGMA
7.22
5.40
3.60
1.80
RELATIVE INTENSITY
100
80
60
40
20
M/E
50
100
150
200
250
300
350

Figure 5. Mass Spectrum of Trimethylsilyl-5,6-Monoepoxyretinoate.

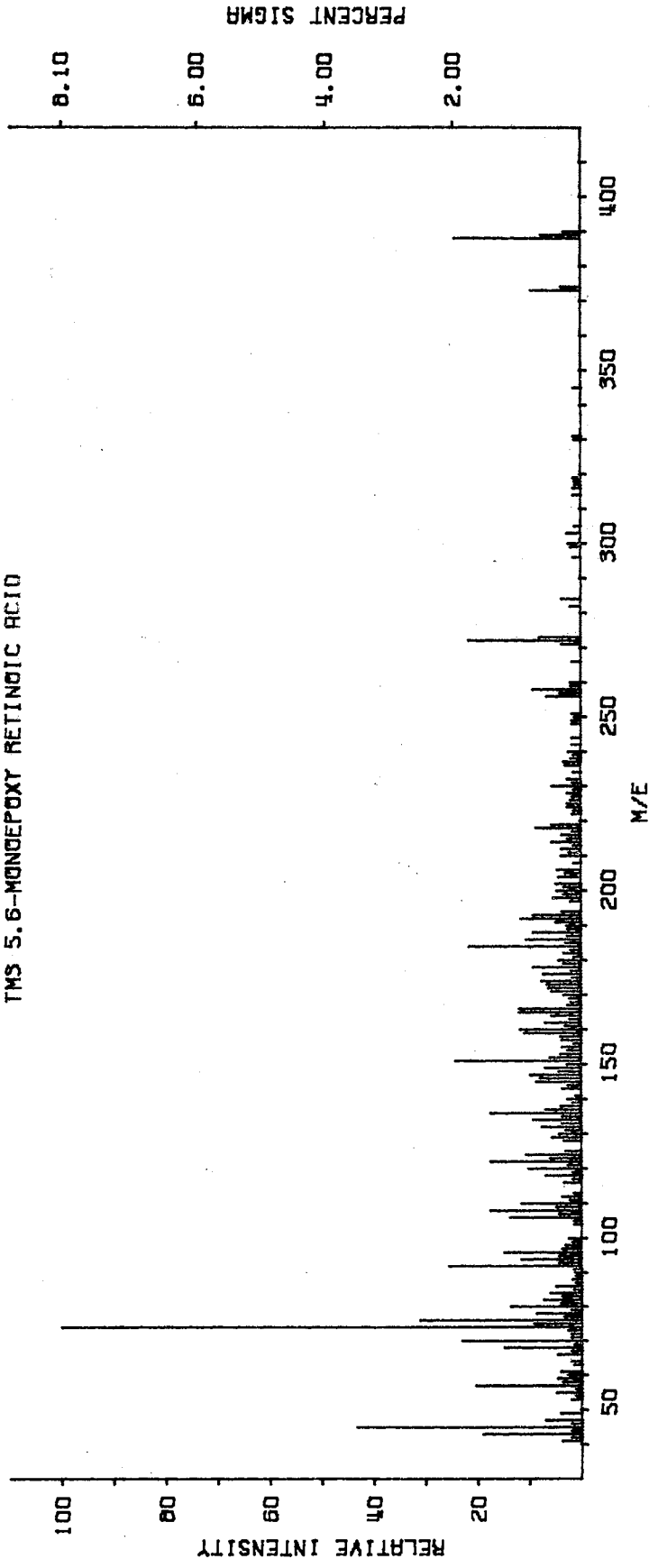

TMS 5.6-MONOEPOXY RETINOIC ACID
PERCENT SIGMA
8.10
6.00
4.00
2.00
RELATIVE INTENSITY
100
80
60
40
20
50
100
150
200
250
300
350
400
M/E

Figure 6. Mass Spectrum of Trimethylsilyl-5,8-Monoepoxyretinoate.

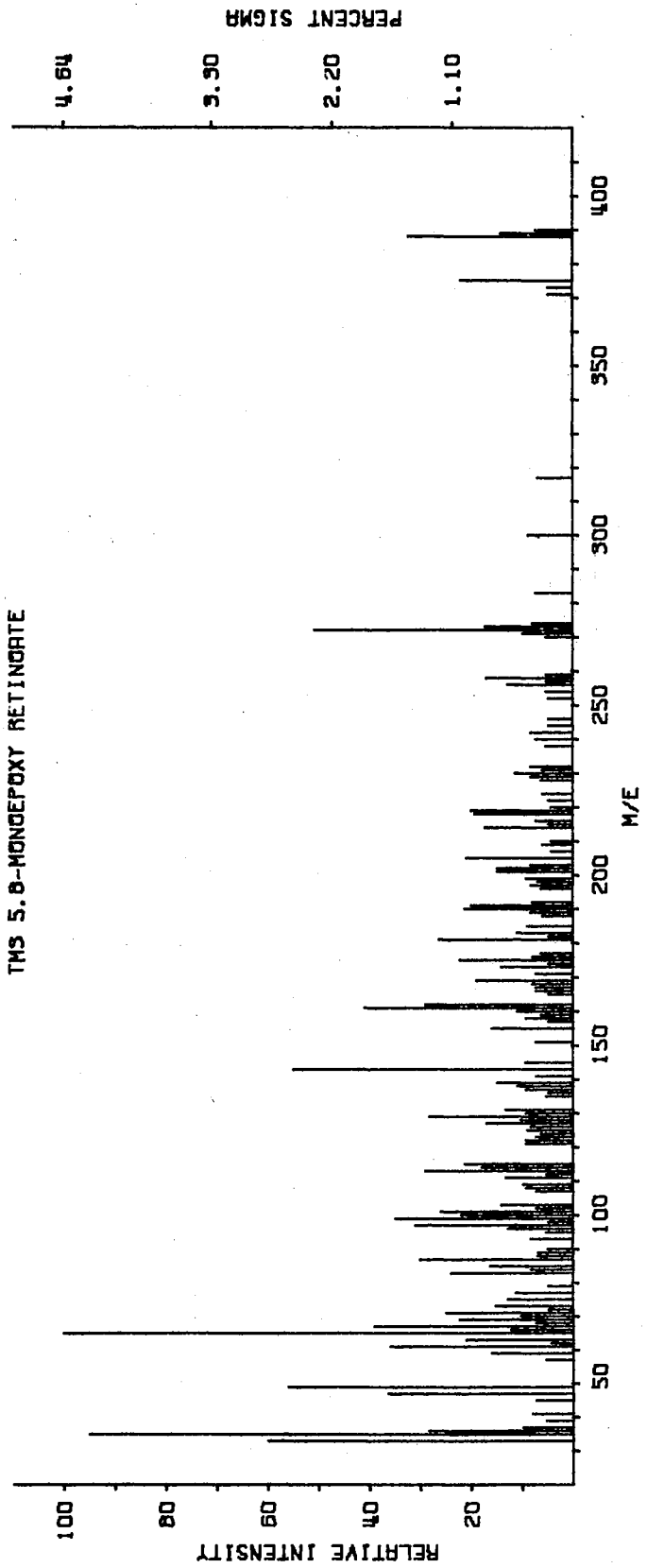

PERCENT SIGMA
4.64
3.30
2.20
1.10
TMS 5,8-MONOEPOXY RETINOATE
RELATIVE INTENSITY
100
80
60
40
20
50
100
150
200
250
300
350
400
M/E

Figure 7. Mass Spectrum of 5,6-Monoepoxyretinyl Acetate.

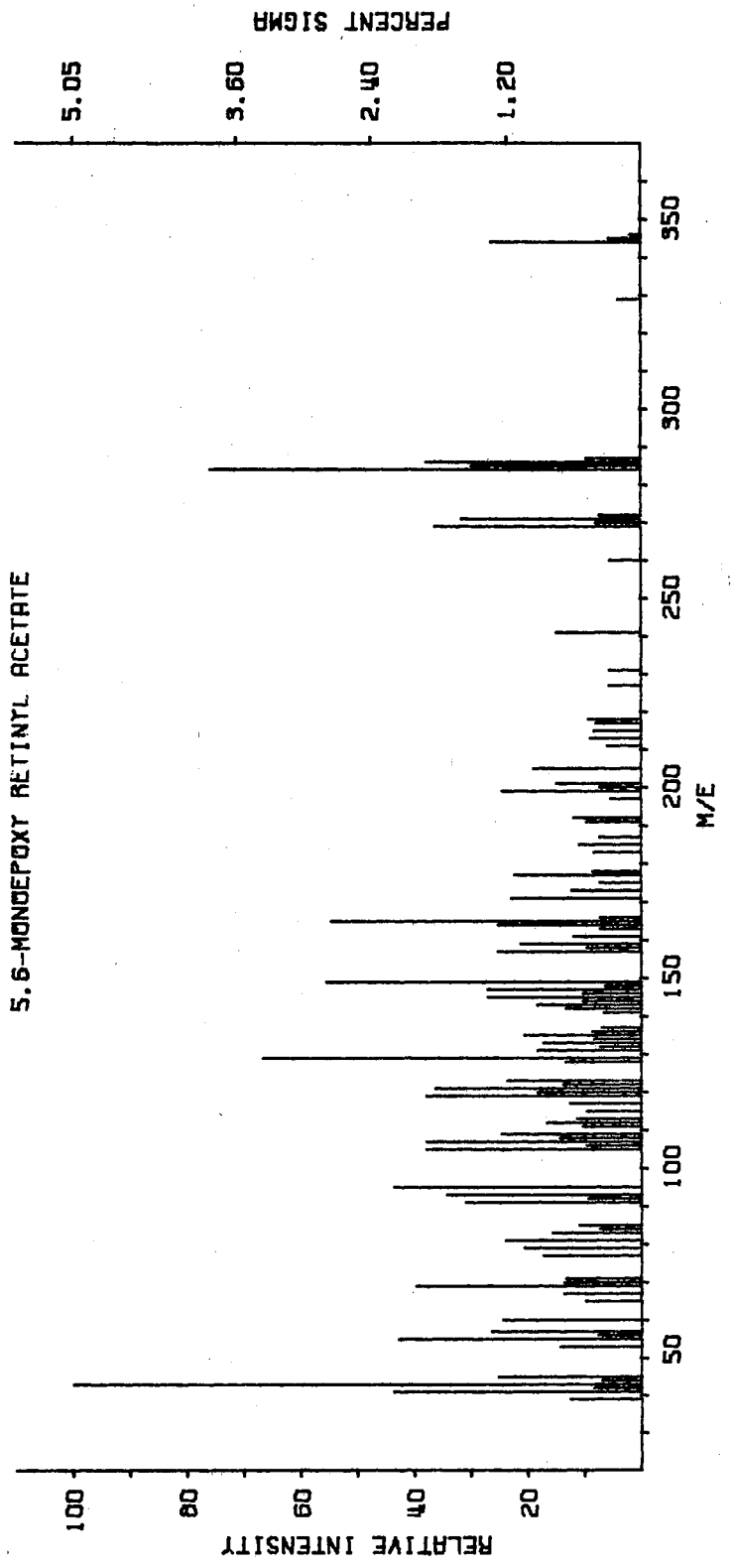

PERCENT SIGMA
5.05
3.60
2.40
1.20
5,6-MONOEPOXY RETINYL ACETATE
M/E
50
100
150
200
250
300
350
RELATIVE INTENSITY
20
40
60
80
100

Figure 8. Mass Spectrum of 5,8-Monoepoxyretinyl Acetate.

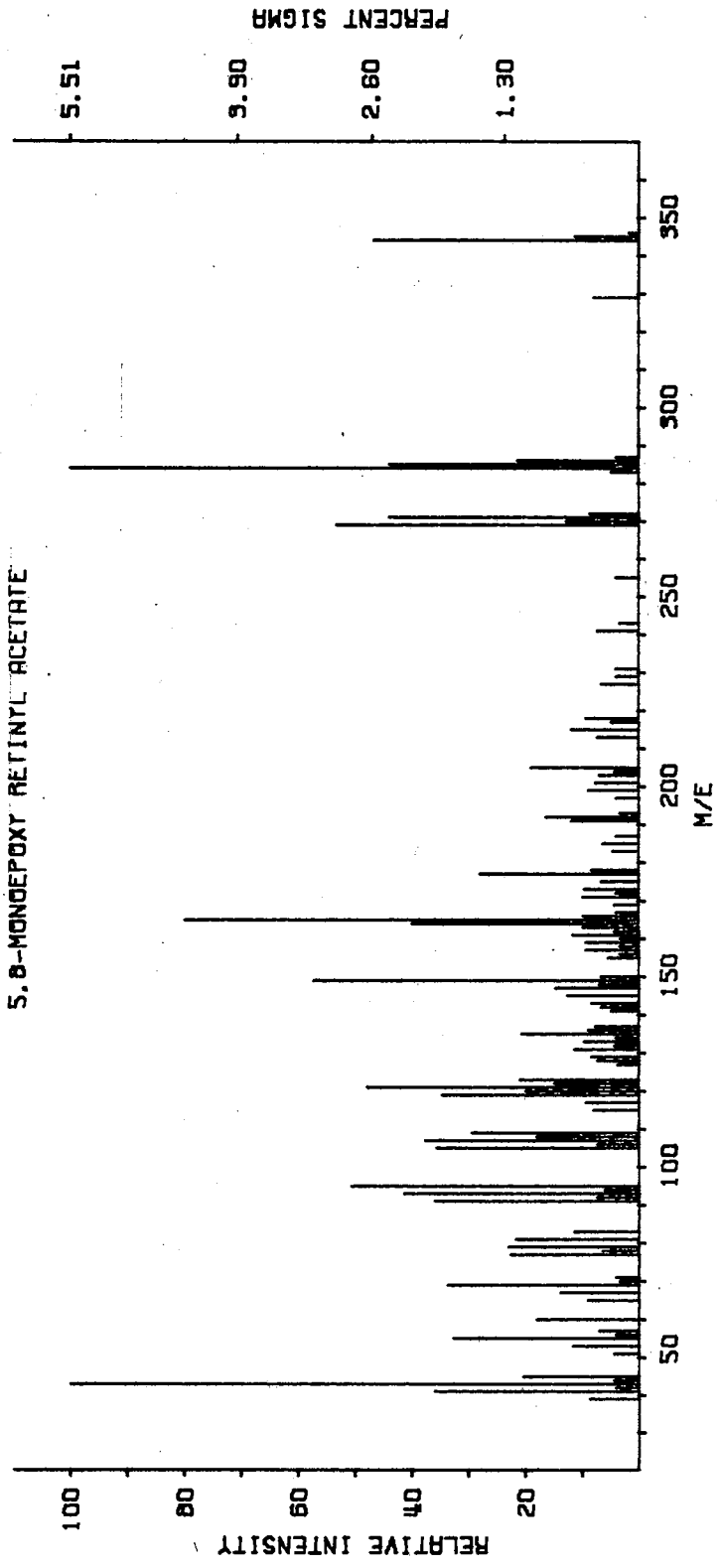

PERCENT SIGMA
5.51
3.90
2.60
1.30
5,8-MONOEPOXY RETINYL ACETATE
M/E
50
100
150
200
250
300
350
RELATIVE INTENSITY
20
40
60
80
100

Figure 9. Mass Spectrum of 5,8-Monoepoxyretinal.

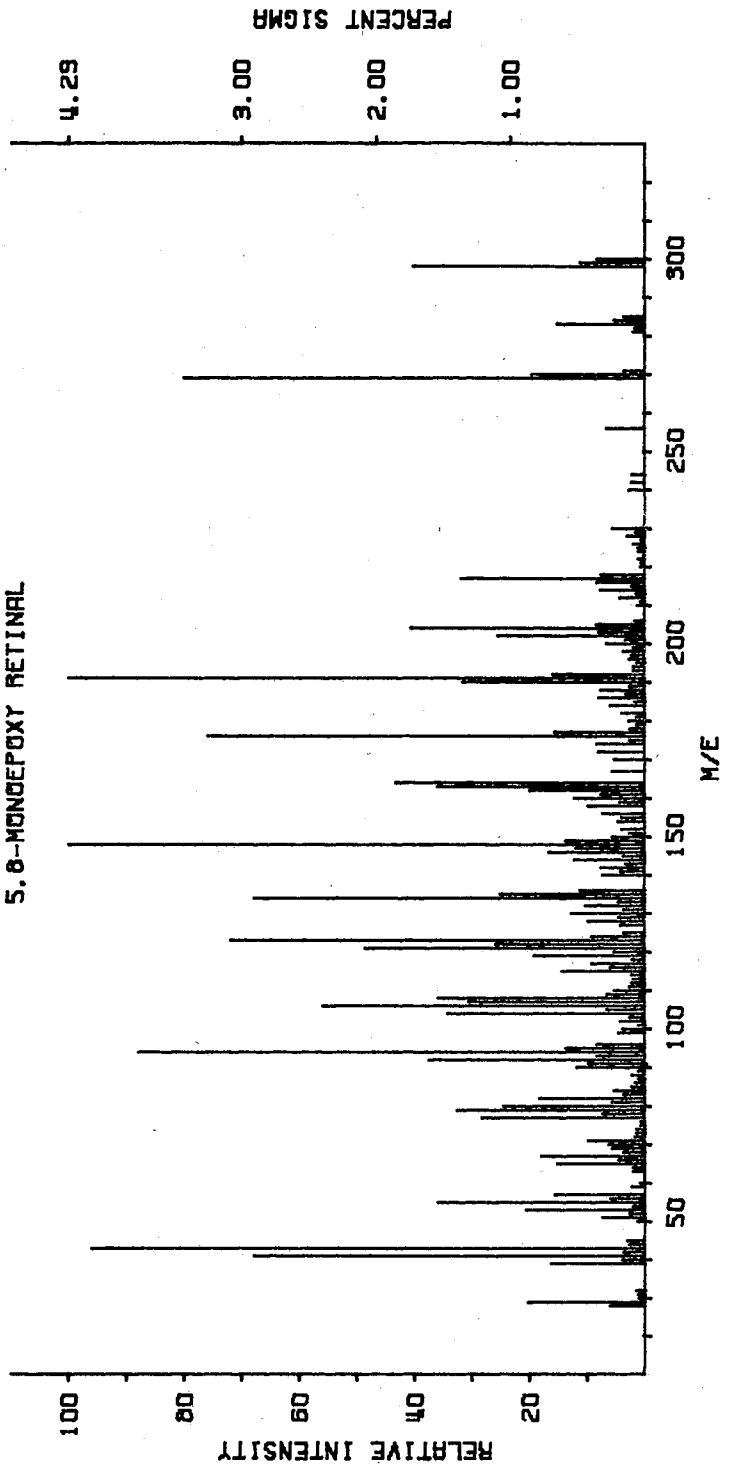

PERCENT SIGMA
4.29
3.00
2.00
1.00
5,8-MONOEPOXY RETINAL
M/E
50
100
150
200
250
300
RELATIVE INTENSITY
20
40
60
80
100

Figure 10. Mass Spectrum of β-C_{19}-Retinal.

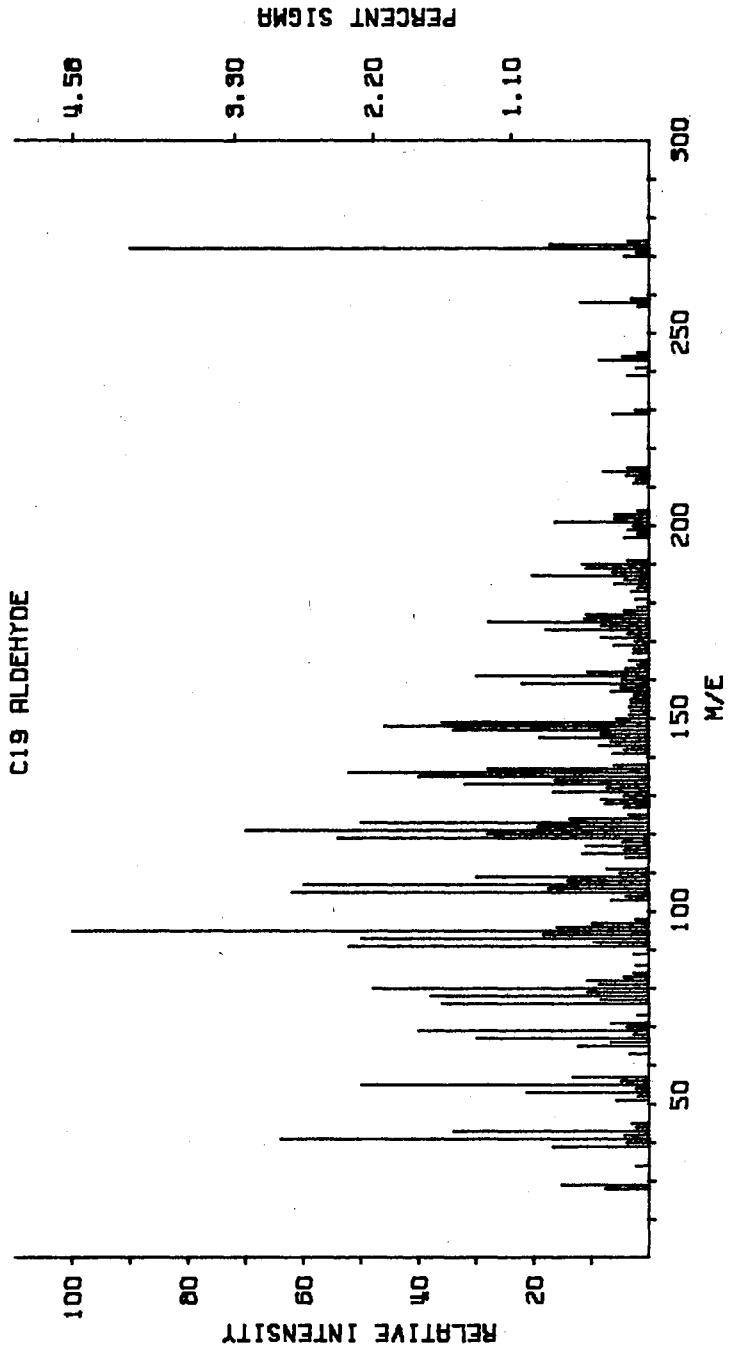

PERCENT SIGMA
4.50
3.30
2.20
1.10
C19 ALDEHYDE
M/E
50
100
150
200
250
300
RELATIVE INTENSITY
20
40
60
80
100

Figure 11. Mass Spectrum of β-C_{19}-Retinyl Acetate.

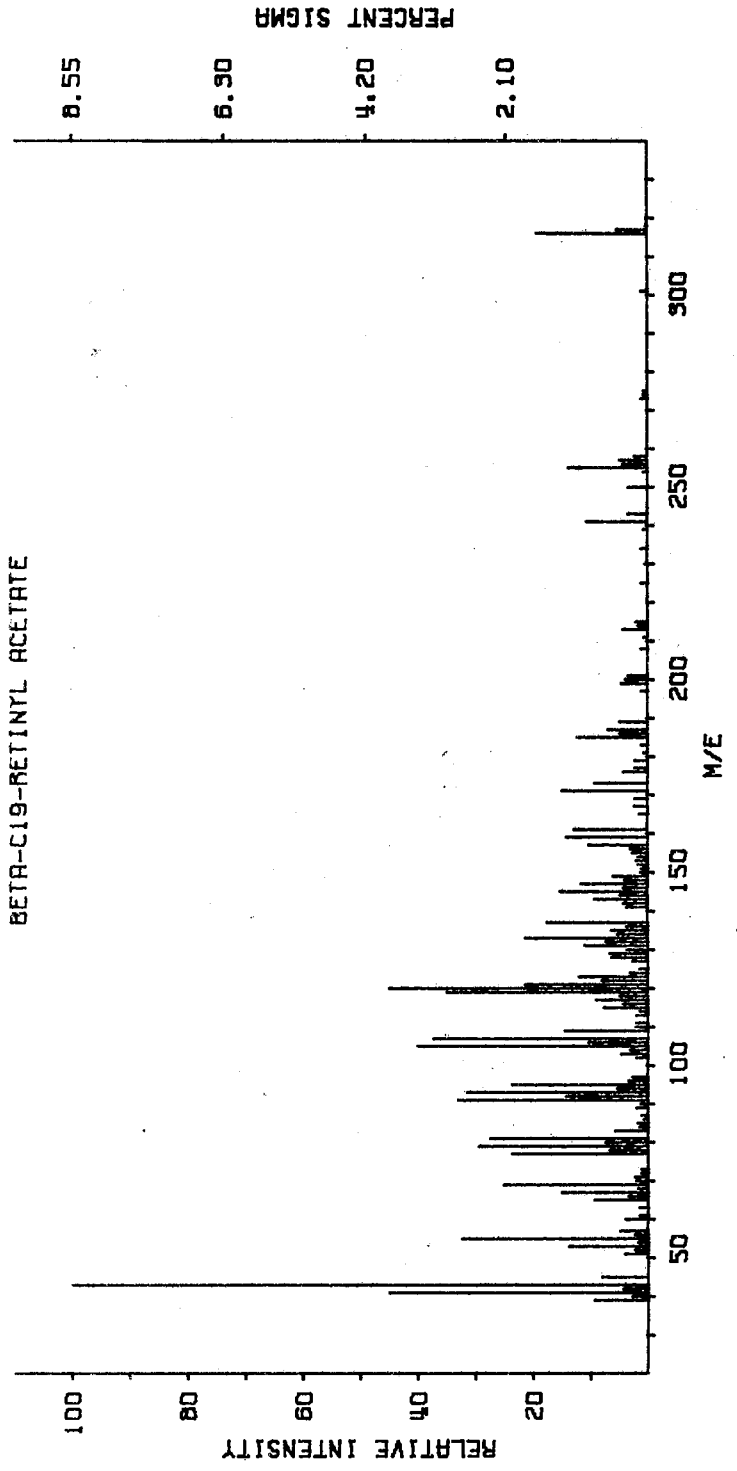
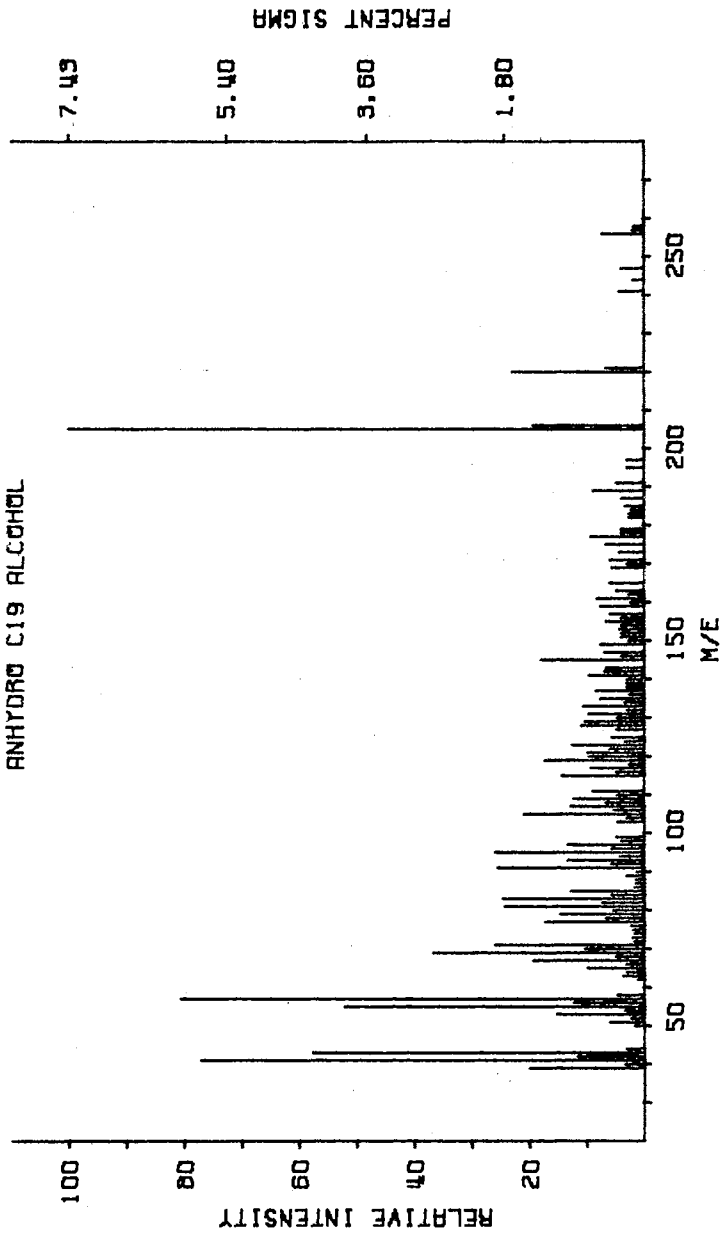

PERCENT SIGMA
8.55
6.30
4.20
2.10
BETA-C19-RETINYL ACETATE
M/E
50
100
150
200
250
300
RELATIVE INTENSITY
20
40
60
80
100

Figure 12. Mass Spectrum of C_{19}-Anhydroretinol.


PERCENT SIGMA
7.49
5.40
3.60
1.80
ANHYDRO C19 ALCOHOL
RELATIVE INTENSITY
100
80
60
40
20
50
100
150
200
250
M/E

these compounds are shown in Table I with the ten most intense peaks tabulated in Table II.

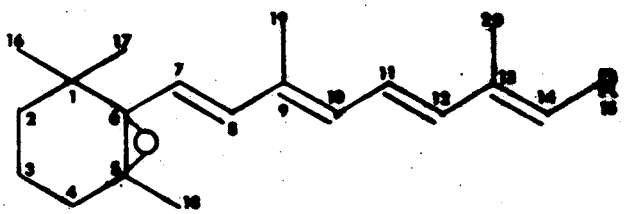
Examination of the monoepoxy-analogs of vitamin A compounds by mass spectrometry has shown that the interpretive areas of the spectra are respresentative of certain portions of the molecule. The importance of these data will become evident as metabolic products of vitamin A are isolated and structural elucidation is attempted. The C_{19}-analogs reinforce the assumptions made with regard to the contribution of the side chain to the lower mass of the spectrum of vitamin A compounds.

Cyclic end groups of conjugated side chains are very stable and lead to cleavage of bonds within the side chain, an effect reinforced by introduction of oxygen substituents in the ring. Aromatic or furanoid end groups delocalize the positive charge and lead to stable fragments with the smaller fragments being more abundant. Otherwise, only the peak due to the larger fragment is in high abundance. Cleavage of C_7-C_8, C_9-C_{10}, and C_{10}-C_{11} with accompanied hydrogen transfers is expected. The relative intensities of these ions will vary greatly, however, partly as a result of the character of the end group present (64).

Intense molecular ions are present in the furan and oxirane vitamin A compounds as was the case with other vitamin A molecules (56). The loss of methyl (M^+-15) from the molecular ion was apparent in all compounds analyzed. From the structural analogs examined in the present study loss of methyl was from the side chain since either a 5,6 or 5,8 monoepoxy analog of the vitamin A compounds would not be conducive to ring methyl loss. Thomas et al. (78) showed the ring methyl was lost from hexadeuterated (on the gem-dimethyl groups) β-ionone not one of the gem-dimethyl carbons.

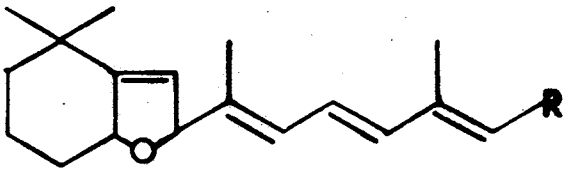
TABLE I

STRUCTURAL ANALOGS OF VITAMIN A



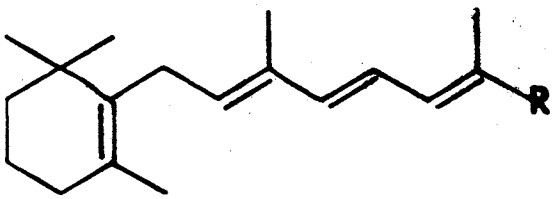
R = COOH

5,6-monoepoxyretinoic acid



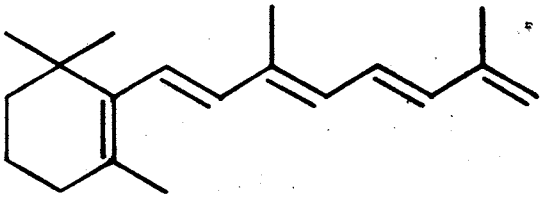
R = COOH

5,8-monoepoxyretinoic acid



R = CHO

β-C_{19}-retinal



C_{19}-anhydroretinol

TABLE II

MOST INTENSE PEAKS IN DECREASING ORDER

OF SOME VITAMIN A ANALOGS

Compound	m/e
5,6-monoepoxyretinoic acid	43, 41, 91, 316, 55, 69, 105, 107, 77, 149
5,8-monoepoxyretinoic acid	45, 150, 43, 92, 96, 316, 165, 57, 108, 94
methyl-5,6-monoepoxyretinoate	330, 149, 255, 43, 315, 91, 69, 107, 135, 217
methyl-5,8-monoepoxyretinoate	330, 149, 271, 43, 315, 107, 164, 95, 91, 177, 135
TMS-5,6-monoepoxyretinoate	73, 45, 76, 92, 388, 151, 272, 184, 57, 43
TMS-5,8-monoepoxyretinoate	65, 49, 43, 272, 161, 47, 36, 99, 388, 97
5,6-monoepoxyretinyl acetate	43, 284, 129, 149, 165, 95, 41, 55, 69, 286, 119, 105, 107
5,8-monoepoxyretinyl acetate	43, 284, 165, 149, 269, 95, 121, 344, 285, 93
5,8-monoepoxyretinal	148, 191, 43, 94, 269, 176, 41, 134, 106, 121
β-C_{19}-retinal	95, 372, 121, 41, 105, 107, 54, 136, 92, 55, 93
β-C_{19}-retinyl acetate	43, 41, 120, 105, 109, 119, 91, 55, 93, 79
C_{19}-anhydrol retinol	205, 57, 41, 43, 55, 69, 95, 71, 91, 83

Cleavage of the bond alpha to the functional group was apparent in all compounds as well as reoccurring mass values indicative of the hydrocarbon side chain m/e 91, 93, 105, 107, 119, 123 and the oxygen substituted ring structure m/e 217, 177, 165, 149. The C_{19}-compounds show an increased intensity of the low mass values from the side chain of these compounds which are more prone to cleave alpha to the ring and a definite decrease in the intensity of the M^+-15. Loss of the functional group was as expected with these compounds.

The mass values m/e 91, 93, 105, 107, 119, and 123 of all-trans-retinoic acid were examined by high resolution mass spectroscopy and the molecular compositions and results are shown in Table III.

Methyl-5,6-monoepoxyretinoate mass values m/e 91, 93, 105, 107, 119, 123, 149, 164, 165, 177, 217, 271 and 330 were analyzed by high resolution mass spectroscopy and the results are presented in Table IV.

Comparison of high resolution data reveals the identical molecular composition of mass values examined between m/e and m/e 123. Higher mass values of the monoepoxy-compound showed the stability of the oxygen as to elimination so the identical peaks most probably are from the side chain. These oxygen containing peaks are as expected if these compounds behave as their corresponding carotenoids (61).

In the present study mass spectra of vitamin A structural analogs have revealed important regions of their spectra. These regions will be of value in determining structures of isolated but as yet unidentified vitamin A metabolites. The low resolution data of nine monoepoxy- and three C_{19}-vitamin A analogs were presented. In addition, high resolution mass spectra were obtained for six all-trans-retinoic acid and thriteen methyl-5,6-monoepoxyretinoate mass values. The oxygen substituent on

TABLE III

MASS VALUES OF RETINOIC ACID

m/e	Fragment Molecular Composition	Mass Expected	Mass Obtained	$\Delta m/e \times 10^3$
91	C_7H_7	91.054773	91.054872	0.099
93	C_7H_9	93.070422	93.081648	1.2
105	C_8H_9	105.070422	105.070534	0.12
107	C_8H_{11}	107.086071	107.085305	0.76
119	C_9H_{11}	119.086071	119.086252	0.2
123	C_9H_{15}	123.117370	123.117065	0.32

TABLE IV

MASS VALUES OF METHYL-5,6-MONOEPOXYRETINOATE

m/e	Fragment Molecular Composition	Mass Expected	Mass Obtained	$\Delta m/e \times 10^3$
91	C_7H_7	91.0547	91.0527	2.0
93	C_7H_9	93.0704	93.0703	0.1
105	C_8H_9	105.0704	105.0699	0.5
107	C_8H_{11}	107.0860	107.0874	1.4
119	C_9H_{11}	119.0860	119.0888	2.8
123	C_9H_{15}	123.1173	123.1164	1.1
149	$C_{10}H_{13}O_1$	149.0966	149.0980	1.4
164	$C_{11}H_{16}O_1$	164.1201	164.1225	2.4
165	$C_{11}H_{17}O_1$	165.1279	165.1288	0.9
177	$C_{12}H_{17}O_1$	177.1279	177.1293	1.4
217	$C_{15}H_{21}O_1$	217.1592	217.1614	2.2
271	$C_{19}H_{27}O_1$	271.2062	271.2095	3.3
330	$C_{21}H_{30}O_3$	330.2195	330.2180	1.5

the ring directs sequential fragmentation of the side chain while interupted conjugation of the side chain promotes cleavage alpha to the ring structure.

CHAPTER III

THE MASS SPECTRA OF TRIMETHYLSILYL GLUCURONIC ACIDS

In developing analytical separation techniques for metabolites of retinoic acid it was necessary to make volatile derivatives of the more polar compounds such as retinoyl-β-glucuronide by gas-liquid chromatography (GLC). Imanari et al. (79) separated glucuronic acid derivatives as the methyl ester and trimethylsilyl ethers. Glucuronic acid conjugates were chromatographed as the methyl ester and trimethylsilyl ethers respectively by Knaak (80). The mass spectra (MS) of the trimethylsilyl ester and ethers of free glucuronic acid would aid in identification of ring size and linkage of metabolic conjugates. Urinary acids and their conjugates were chromatographed as the trimethylsilyl ester and ethers with good results by Horning et al. (81) (82). Lippel et al. (35) reported the isolation of retinoyl-β-glucuronide from rat bile and that retinoyl-β-glucurono-γ-lactone was formed during the isolation. Identification was by chromatography on anion-exchange resin and silicic acid columns and characterization on thin layer plates of silica gel G. Mass spectral analyses of glucuronic acid will aid in the separation and positive identification of metabolites and artifacts of retinoic acid formed in vivo and in vitro.

Experimental Materials

Reagents were purchased from the following sources: bis-(trimethylsilyl)acetamide (BSA) from Analabs, Inc., Hamden, Connecticut; reagent

grade glucuronic acid and glucuronic acid lactone from Sigma Chemical Company, St. Louis, Missouri, and trimethylsilylimidazole (TSIM) in pyridine as Tri-Sil "Z" was purchased from Pierce Chemical Company, Rockford, Illinois.

Methods

The prototype LKB-9000 gas chromatograph-mass spectrometer was used (68). The total ion current record served as the GLC tracing.

An 8' x ¼" glass column packed with 1% OV-1 on Gas-Chrom Q (Applied Science Laboratories, State College, Pennsylvania) was used. The operating conditions were as follows: injection port, 230°C; column, 180°C; molecular separators, 220°C; ion source, 250°C. The helium flow rate was 35 ml/min. Mass spectra were taken at 70 ev and with varied electron-multiplier voltages and magnet positions to resolve the isotope peaks of the small molecular ions.

Silylation was accomplished by adding BSA (0.2 ml) to a vial containing 0.4 ml of pyridine and 0.05 g of either D-glucuronic acid or γ-D-glucuronolactone. After 15 minutes no changes in gas chromatographic peak heights were noticed and the reaction was assumed to be complete. The reaction mixture was injected directly for GLC-MS analysis. Mass spectra of BSA and pyridine were subtracted for background contribution.

Silylation of 0.05 gm of carbohydrates was accomplished by addition of 0.2 ml of trimethylsilylimidazole (TSIM), and the mass spectra were compared to the reaction products of BSA silylation.

Results and Discussion

Either BSA or TSIM gave identical peaks under gas chromatographic analysis but the sugars silylated with BSA changed from colorless to

orange or dark red in 15 to 30 minutes reaction time. The comparison of the mass spectra of the two yields showed an increase of background peaks and extraneous peaks during scans of the BSA silylated compounds. When TSIM was the silylation reagent spectra were relatively clear with no detectable loss of complete silylation of the carbohydrates.

The fragmentation patterns of the TMS derivatives of the α- and β-anomers of D-glucopyranuronic acid easily distinguishes these compounds from the D-glucofuranuronic acid and the 6,3-glucuronolactone. The mass spectra of these compounds are shown in Figure 13 through 16 and their structures are illustrated in Table 5. The molecular ions of these compounds are of the order of less than 1% of the base peak and the M^+-15 can be used to ascertain molecular weight since a strong loss of methyl groups from TMS derivatives is indicative of this class of compounds (83). The relative intensity of the M^+-15 is also indicative of the stabilities of the pyranose or furanose form of the carbohydrate (70). The less stable furan ring generates a higher percentage of methyl group cleavage than its pyranose form. The m/e 217/204 ratio is also indicative of the ring structure of the carbohydrate.

Earlier work (70) on the fragmentation of carbohydrates can be consulted for theoretical fragmentation patterns since no high resolution data has been obtained for the present compounds. Pierce (71) reviewed several mass spectra of TMS carbohydrates with analyses of the following peaks. The m/e 147 is $(CH_3)_2$ Si = $^+$O-Si$(CH_3)_3$ while TMS cleavage results in m/e 73 with HO=CH-CH=CH-OH also a possible contributor. The m/e 217 is TMS-O-CH=CH-CH=$^+$O-TMS while m/e 204 is [TMS-O-CH=CH-O-TMS]$^+$, m/e 305 is TMS-O-CH=C-[OTMS]-CH=$^+$O-TMS, and m/e 147 is TMS-O$^+$=Si$(CH_3)_2$. Table VI shows the relative intensities of peaks that are indicative of

Figure 13. Mass Spectrum of TMS-β-D-Glucopyranuronic Acid with Scale Multiplication x 100 for m/e 539-557.

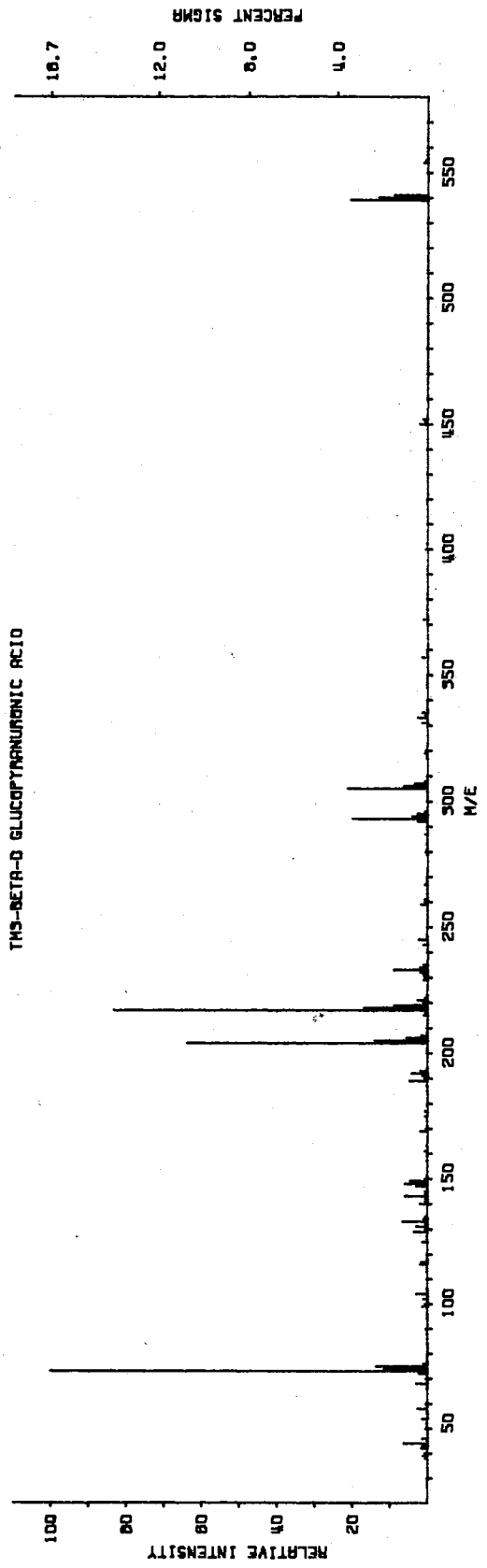

PERCENT SIGMA
16.7
12.0
8.0
4.0
TMS-BETA-D GLUCOPYRANURONIC ACID
M/E
RELATIVE INTENSITY

Figure 14. Mass Spectrum of TMS-α-D-Glucopyranuronic Acid with Scale Multiplication x 10 for m/e 450-463 and x 100 for m/e 538-557.

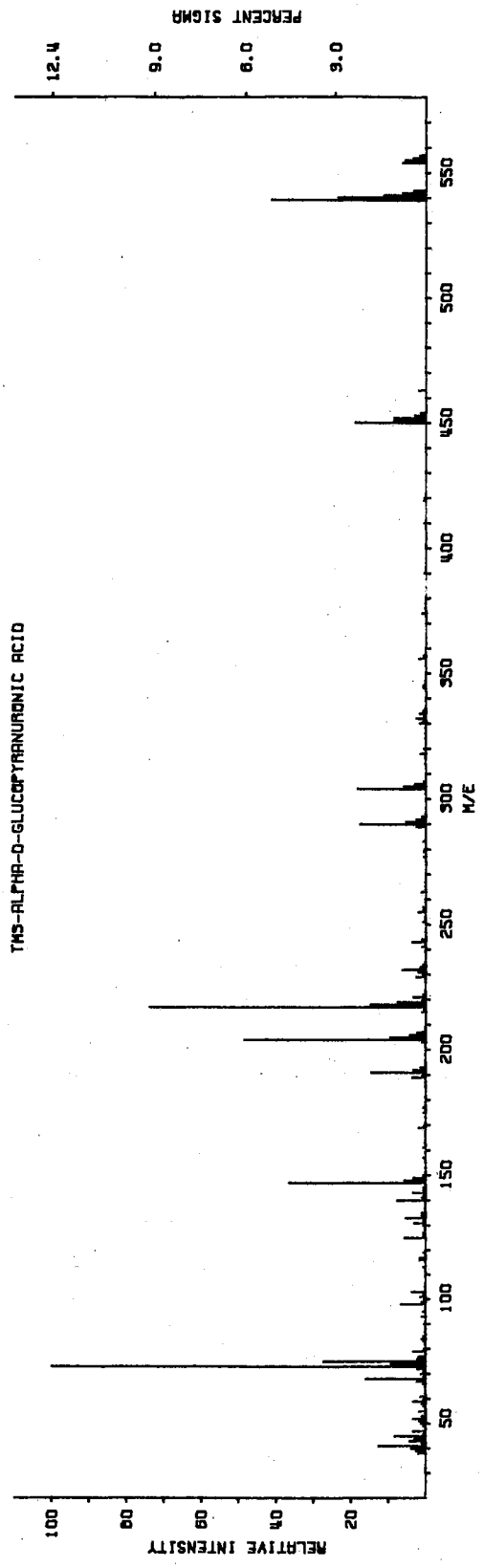

PERCENT SIGMA
12.4
9.0
6.0
3.0
RELATIVE INTENSITY
100
80
60
40
20
M/E
50
100
150
200
250
300
350
400
450
500
550
TMS-ALPHA-D-GLUCOPYRANURONIC ACID

Figure 15. Mass Spectrum of TMS-D-Glucofuranuronic Acid with Scale Multiplication x 10 for m/e 539-542 and x 100 for m/e 554-557.

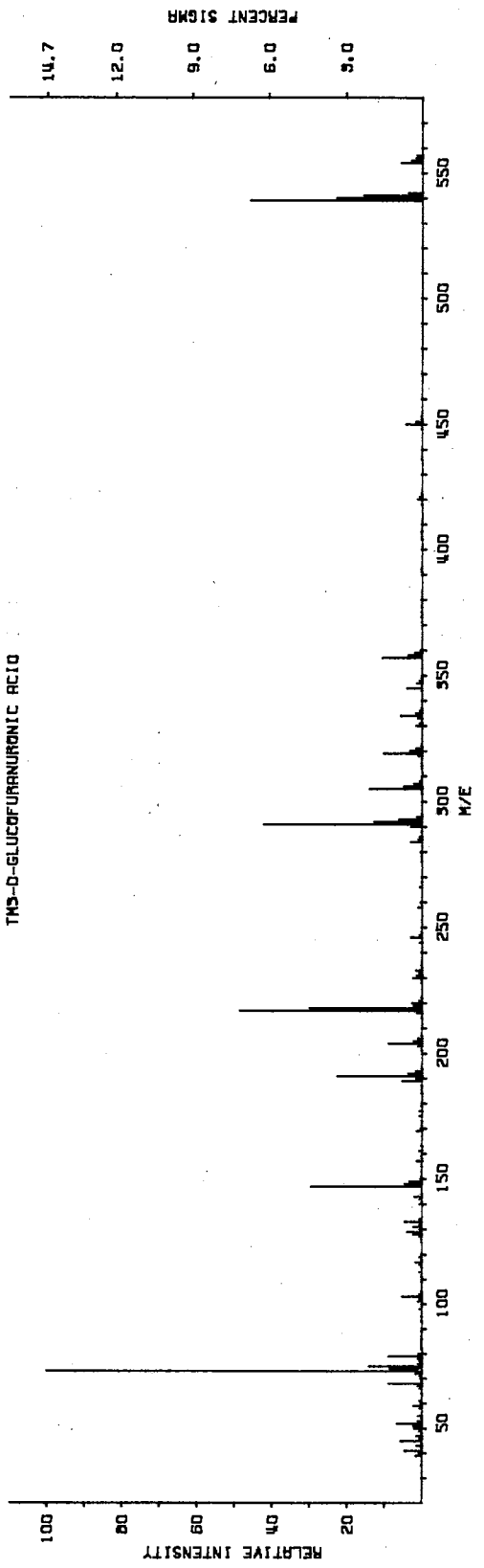

TMS-D-GLUCOFURANURONIC ACID
PERCENT SIGMA
14.7
12.0
9.0
6.0
3.0
RELATIVE INTENSITY
100
80
60
40
20
M/E
50
100
150
200
250
300
350
400
450
500
550

Figure 16. Mass Spectrum of TMS-6,3-Glucuronolactone with Scale Multiplication x 10 for m/e 392-395.

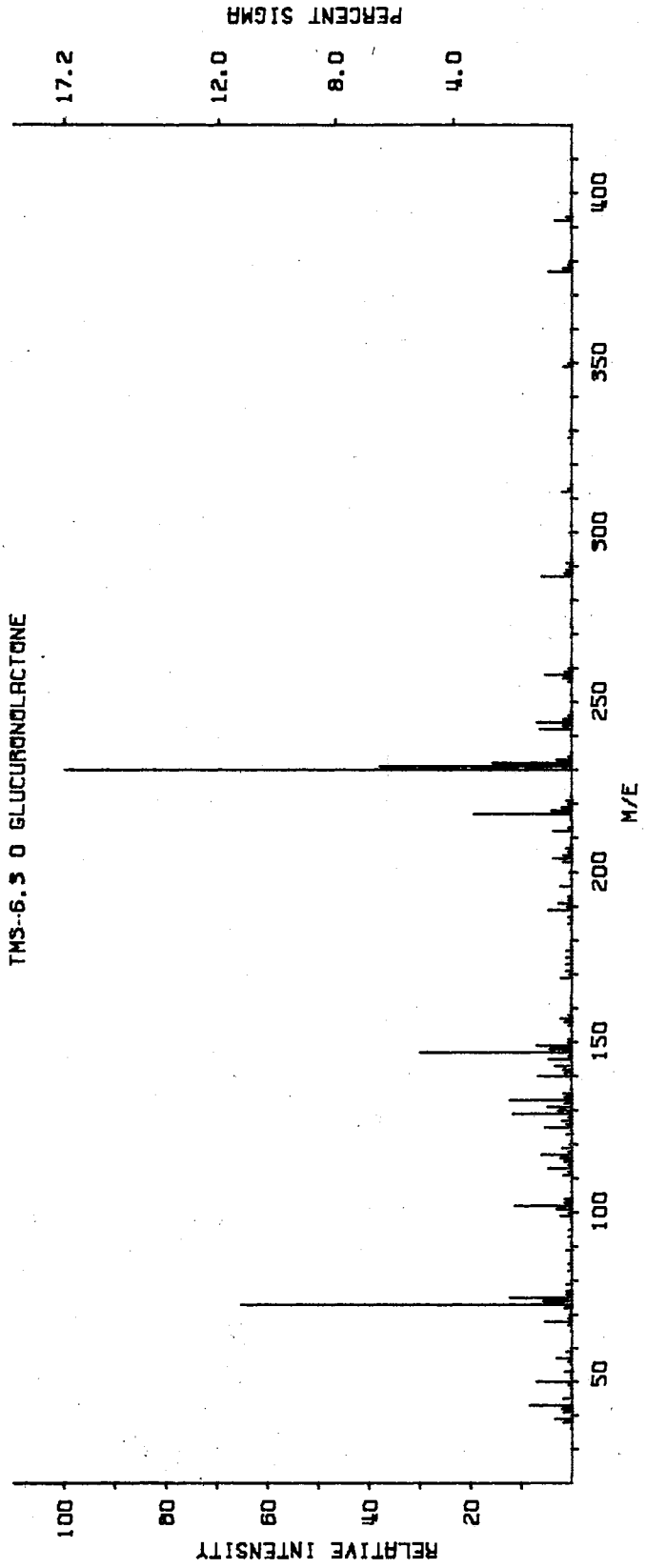
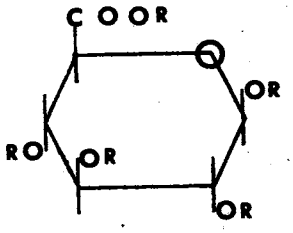

PERCENT SIGMA
17.2
12.0
8.0
4.0
TMS--6.3 O GLUCURONOLACTONE
RELATIVE INTENSITY
100
80
60
40
20
50
100
150
200
250
300
350
400
M/E

TABLE V

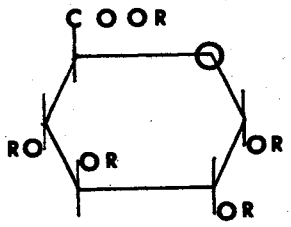
CARBOHYDRATE STRUCTURES AND MOLECULAR WEIGHTS



β-D-Glucopyranuronic Acid

R = H M.W. = 194

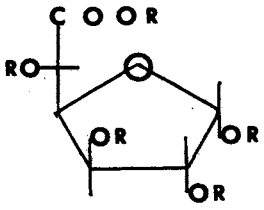
R = TMS M.W. = 554



α-D-Glucopyranuronic Acid

R = H M.W. = 194

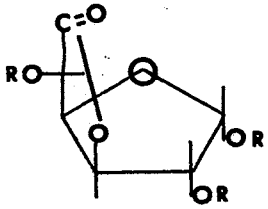
R = TMS M.W. = 554



D-glucofuranuronic Acid

R = H M.W. = 194

R = TMS M.W. = 554



6,3 or γ-D-Glucuronolactone

R = H M.W. = 176

R = TMS M.W. = 392

TABLE VI

CHARACTERISTIC PEAKS AND RELATIVE INTENSITIES

OF SOME TRIMETHYLSILYL GLUCURONIC ACIDS

m/e	M^+	M^+-15	450	305	217	204	147
			Relative Intensity				
Lactone	0.34	4.81	-	-	19.38	3.75	29.91
Furan acid	0.05	4.59	4.24	13.96	48.58	9.01	29.78
α-pyranose	0.06	0.41	1.99	18.49	73.95	48.81	36.77
β-pyranose	0.01	0.21	1.91	21.38	83.33	64.15	2.85

α, β, pyranose, or furanose ring structure. In other studies (72) the lactone formed from the pyranose acid during the inlet injection contaminated the sample. This is prohibited by the TMS derivation of the carboxyl and hydroxyl groups with subsequent separation by gas chromatography. Complete silylation is indicated by the presence of the molecular ions and strong M^{+}-15 ions.

Thus it is possible to ascertain ring size without fear of rearrangement during inlet injection. TMS derivatives of biological samples are becoming increasingly important for gas chromatographic separations and with combination gas chromatography-mass spectrometry of column eluants, it is possible to identify carbohydrate moieties.

CHAPTER IV

METABOLISM OF RETINOIC ACID

Retinol is oxidized to retinoic acid *in vivo*, but the degradative fate of retinoic acid has not been elucidated. Although numerous reports of metabolites of retinoic acid have appeared (18) (32) (33) (34) (35) (36) only two have been identified. One of these, 13-*cis* retinoic acid (33), has been found to be an artifact of the isolation procedure (39) and the other, retinoyl-β-glucuronide, was isolated from rat bile (35) (38) (31). Retinoyl-β-glucuronide is not a degradative metabolite but a conjugate with glucuronic acid and may be a detoxifying mechanism.

Retinoic acid is capable of undergoing rapid decarboxylation *in vitro* by a partially purified enzyme from chicken liver (44) and by horseradish peroxidase (45). The objective of the present investigation was to isolate some of the products resulting from the decarboxylation of retinoic acid by these two enzyme systems *in vitro* and by the rat *in vivo*.

Materials

Radioactive Retinoic Acid

The [15-^{14}C]all-*trans*-retinoic acid and [10,11-^{3}H]all-*trans*-retinoic acid were gifts from Hoffmann-LaRoche, Inc., Nutley, New Jersey. The specific activity of the tritiated and ^{14}C retinoic acid was 352 μCi/mg and 58 μCi/mg respectively. The radioactive purity of the ^{14}C-compound was checked by thin-layer chromatography on silicic acid and subsequent

confirmation by scanning on a gas flow strip counter (Actigraph III, Model 1006, Nuclear Chicago, Des Plaines, Illinois). Only one radioactive spot was observed. The ^{3}H-compound was chromatographed and the thin-layer chromatogram scraped and sequential sections counted in Bray's scintillation solution (66) with a liquid scintillation spectrometer (Packard Tricarb Model 3320 Packard Instrument Company, Inc., 2200 Warrenville Road, Downers Grove, Illinois).

Solvents and Reagents

Reagents were analytical reagent grade unless otherwise noted. Solvents were spectroanalyzed ACS grade. Crystalline horseradish peroxidase (type VI, RZ-3.1) was purchased from Sigma Chemical Company, St. Louis, Missouri. All-*trans*-retinoic acid was a gift from Hoffmann-LaRoche, Inc., Nutley, New Jersey. It was purified by recrystallization several times from 2-propanol until the corrected melting point and ultraviolet spectrum agreed very closely with reported values (84) (85). In addition, retinoic acid was submitted to mass spectral analyses (56).

Methods

Enzyme Incubation Preparations

Microsomes were prepared from liver obtained from fasted chicks or rats which had been fed retinoic acid as their only source of vitamin A. The liver was removed, immediately placed in ice-cold 0.25 M sucrose and partially homogenized by expulsion through a 1 mm stainless steel screen by means of a screw press (Harvard Apparatus, Inc., Cambridge, Mass.). Ten percent homogenates of liver in 0.25 M sucrose were made in a Potter-Elvejhem glass tissue grinder with a motor driven, grooved

teflon pestle. The microsomal fraction was prepared as follows. The 10% homogenate was centrifuged at 7000 x g for 10 minutes. The supernatant solution was decanted and centrifuged for 60 minutes at 105,000 x g. The microsomal pellet was layered over an equal volume of 1.5 M sucrose and centrifuged at 105,000 x g for 75 minutes. The decarboxylase activity was located in the microsomal layer. The incubation mixtures contained 0.2-0.4 ml of resuspended microsomes in phosphate buffer (equivalent to 40-80 mg liver), retinoic acid (10 μM), $FeCl_2$ (1 mM), and sodium phosphate buffer, pH 6.4 (0.1 M) in a total volume of 2 ml.

Crystalline horseradish peroxidase (HRP) was suspended in 0.1 M-sodium phosphate buffer, pH 6.4. Unless stated otherwise, incubation mixtures were composed of horseradish peroxidase (2.6 μM), approximately 10^4 c.p.m. retinoic acid (10 μM), assayed for each set of incubations and ferrous chloride (1 mM), diluted to a final volume of 2 ml with 0.1 M-sodium phosphate buffer, pH 6.4. Retinoic acid and ferrous chloride were added separately at zero time and the mixture was incubated at 37°C for the desired time.

At the end of the incubation period, the reaction mixture (either the microsomal or HRP) was acidified with hydrochloric acid, and $^{14}CO_2$ was collected in a glass vial containing a filter-paper wick and 0.3 ml of ethanolamine-ethylene glycol monomethyl ether (1:2, v/v). Two hours after addition of the acid the trapping solution was transferred to a counting vial, 10 ml of scintillation fluid (2-methoxyethanol-toluene, 1:2, v/v, containing 2,5-diphenyloxazole, 5.5 g/l) was added and the radioactivity was counted on a liquid-scintillation counter. No attempt was made to isolate metabolites from these flasks.

Batch incubations were performed for isolation of the decarboxylation products. The horseradish peroxidase reaction contained 6 mg of retinoic acid (labeled with ^{3}H and ^{14}C) in 20 ml of 95% ethanol, two liters of 0.1 M phosphate buffer pH 6.4, 10 mg of HRP and 200 mg of ferrous chloride. The reaction mixtures were incubated at 37°C for 1 hr in a shaking water bath (Eberbach Corporation, Ann Arbor, Michigan).

Animals

Rats of the Holtzman albino strain (Holtzman Company, P. O. Box 4068, Madison, Wisconsin) were bred in our laboratory animal colony and fed a vitamin A deficient diet (General Biochemicals, Laboratory Park, Chagrin Falls, Ohio) supplemented with 500 µg retinoic acid/kg diet. The retinoic acid was stabilized in gelatin beadlets. A pair of 200 g male rats were injected intraperitoneally with 10 µCi of [15-^{14}C, 10,11-^{3}H] retinoic acid (26 µg) dissolved in ethanol. The rats were sacrificed after 3 hrs by cervical dislocation and the livers, kidneys and spleens were removed. The organs were frozen in acetone-dry ice and lyophilized to dryness. The tissue was minced in duplicates in a Sorvall omni-mixer (Ivan Sorvall, Inc., Newtown, Connecticut) with 25 ml of methanol. The methanol samples were allowed to set for 15 min and then centrifuged at 1100 x g for 20 min. The supernate was assayed for radioactivity, decanted from the pellet, reduced in volume and applied to an alumina column slurried in acetone. The column was eluted with methanol in 10 ml fractions, each counted for radioactivity.

Microsomal incubations contained corresponding amounts of all substrates and buffers with the substitution of a microsomal fraction prepared from chicks 2 weeks of age, obtained from Dr. R. H. Thayer, Animal Science Department, Oklahoma State University, Stillwater,

Oklahoma. All chicks, from the time of hatching, had been fed a vitamin A deficient diet 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.

Methylene Chloride Extraction and $LiAlH_4$ Reduction

Microsomal and horseradish peroxidase (HRP) incubations were extracted with methylene chloride immediately after incubation, dried over anhydrous sodium sulfate and evaporated to dryness on a flash evaporator. The residue was dissolved in 10 ml of anhydrous diethyl ether and cooled to $4^{o}C$ in an ice bath. Lithium aliminum hydride, four times the weight of the incubated retinoic acid, was cooled to $4^{o}C$ in diethyl ether and the extract was mixed with the cold $LiAlH_4$-ether suspension and allowed to stand over ice for 30 minutes. Water was added to expend the excess $LiAlH_4$ and the reduction products were extracted with diethyl ether. The ether solution was dried over anhydrous sodium sulfate and evaporated to dyrness on a flash evaporator. The residue was dissolved in petroleum ether (boiling range 37^{o}-$38^{o}C$) and applied to a 10% water deactivated alumina column and eluted with petroleum ether with increasing concentrations of diethyl ether. The substrate containing ^{14}C and ^{3}H was separated from the decarboxylation product. The decarboxylation product contained no ^{14}C but did contain tritium. The tritium fraction was evaporated to dryness on a flash evaporator and a trimethylsilyl derivative (TMS) was prepared. The TMS product was injected on a 1% OV-1 on Gas Chrom Q column at $180^{o}C$ column temperature, $200^{o}C$ injector, $250^{o}C$ detector, 50 ml/min He_2 flow. A hydrogen flame detector was used to assay the column eluant.

Methanol Extraction

Batch incubation mixtures containing microsomes or horseradish peroxidase were centrifuged at 12,100 x g for 20 min. The supernate was frozen, lyophilized to dryness and extracted 12-18 hr with methanol.

Column Chromatography

An analytical column (1.5 x 8 cm) containing 10 g of 10% water deactivated aluminum oxide (acid washed, reagent grade, Merck and Co., Inc., Rahway, New Jersey) slurried in petroleum ether (37-38° boiling range redistilled and chromatographed over silicic acid) was used to chromatograph methylene chloride extracts of the reaction mixtures both before and after lithium aluminum hydride ($LiAlH_4$) reduction. The column was eluted with a 10% stepwise gradient from 100% petroleum ether to 100% diethyl ether and collected in 10 ml fractions. A 0.2 ml aliquote of each fraction was transferred to a scintillation vial containing 10 ml of Brays (66) scintillation solvent and counted in a liquid scintillation spectrometer with quench correction provided by automatic external standardization.

A 10 g alumina column was slurried in acetone and the methanol extract of the lyophilized incubations was evaporated to dryness on a flash evaporator and dissolved in acetone. The acetone fraction was applied to the column and eluted with acetone until no radioactivity was observed in the eluant. Methanol was then applied to elute the more polar compounds from the column. Retinoic acid and other carboxylic acids were adsorbed to the alumina and could be recovered by deactivating the alumina with water.

A slurry of 10 g silica acid (Bio-Gel HA, minus 325 mesh, Bio-Rad Laboratories, 32nd and Griffin Avenue, Richmond, California) in benzene: chloroform:methanol (4:1:1) was poured in a 1.5 x 8 cm column and the

acetone extract was applied to the column and eluted with benzene: chloroform:methanol (4:1:1).

Thin-Layer Chromatography (TLC)

Samples were applied to pre-coated analytical TLC plates (Q1, Silica Gel, and Q3, Alumina, 5 x 20 cm and 20 x 20 cm; layer thickness, 250 μ ± 4% and 500 μ ± 4% preparative) which were purchased from Quantum Industries, Fairfield, New Jersey. Plates were pre-developed in the solvent system used, before spotting. Silica capillaries or 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) Benzene:chloroform:methanol (4:1:1)

(b) Diethyl ether:hexane (1:10)

(c) Cyclohexane:chloroform:methanol (8:1:1)

(d) Petroleum ether

(e) Hexane

(f) Cyclohexane

Separated components were detected by fluorescence under ultraviolet light at 366 nm, adsorbance of 254 nm ultraviolet light, iodine and antimony trichloride positive spots were located by scraping into 10 ml of Bray's (66) scintillation fluid and counting in a liquid scintillation spectrometer

Gas Liquid Chromatography (GLC)

A modified Barber-Coleman Gas Chromatograph (Barber-Coleman Company, Rockford, Illinois) Model 5000 equipped with hydrogen flame detector (68) was used for gas chromatography. A stream splitter 9:1

was used to collect radioactive samples on a Packard tricarb gas chromatograph fraction collector. Samples were condensed on glass beads and counted in 10 ml of Bray's scintillation solution by a liquid scintillation spectrometer. The column used in this instrument is interchangeable with the mass spectrometer-gas chromatograph. Suitable conditions for separating compounds are developed on this instrument prior to using the mass spectrometer-gas chromatograph.

A ¼ inch x 8 feet helical glass column packed with 1% OV-1 on Gas Chrom Q (Applied Science Laboratories, State College, Pennsylvania) and equipped with a septum liquid injection system.

The metabolites were converted to the more volatile and thermally stable trimethylsilyl ethers (TMS-derivative) by the use of trimethylsilyl imidazole (TSIM) as commercially prepared in TriSil Z (TSIM in pyridine, Pierce Chemical Company, Rockford, Illinois). The column conditions were: column 180°C; injector 200°C; detector 250°C; helium flow 50 ml/min.

Results and Discussion

Metabolites of retinoic acid were isolated following decarboxylation by liver microsomes or horseradish peroxidase *in vitro*, and from rat liver *in vivo*. Products were similar in all three of the systems investigated.

Reaction mixtures containing either microsomes or horseradish peroxidase were extracted with methylene chloride, reduced with $LiAlH_4$ and silylated. The microsomal decarboxylation product was chromatographed on a Barber-Coleman gas chromatograph with a 9:1 stream splitter and the eluant collected on glass beads and radioactivity measured. Of the

three major TMS peaks one was radioactive and highly enriched in tritium. The same procedure was followed using the HRP reaction product and the identical gas chromatographic peak was labeled with tritium.

Methylene chloride extractions of microsomal and HRP reaction mixtures were spotted on alumina TLC plates and developed in benzene: chloroform:methanol (4:1:1). The spots were visualized with ultraviolet light and iodine, scraped and assayed for radioactivity. The results are shown in Table VII. Reaction products with similar migration rates were formed by the horseradish peroxidase system with addition of Fe^{2+} or H_2O_2 and by the microsomal system with Fe^{2+}.

Horseradish peroxidase batch incubations were centrifuged at 12,100 x g for 20 min and the supernatant solution decanted from the pellet. The supernate was assayed for ^{3}H and ^{14}C by liquid scintillation spectroscopy and corrected for quench by external standarization. After incubation, 43% of the ^{14}C was absent from the reaction mixture. Recovery in the aqueous supernate was 90% for ^{3}H and 71% for the remaining ^{14}C. The supernate was frozen, lyophilized to dryness and extracted with methanol. Of the initial radioactivity 70% of the ^{3}H and 51% of the ^{14}C was recovered. The methanol extraction was 80% efficient for ^{14}C and ^{3}H recovery. The methanol volume was reduced by flash evaporation and chromatographed on alumina in acetone. The first peak eluted contained only ^{3}H. Methanol was then used and a second ^{3}H peak was eluted. All ^{14}C, which represented the carboxyl function of retinoic acid, was tightly bound to the alumina. It could be partially recovered by elution with water which deactivated the alumina. Total disintegrations per minute in the acetone fraction was 14.5% of the initial substrate activity with 10% found in the methanol fraction.

TABLE VII

THIN-LAYER CHROMATOGRAPHY OF RADIOACTIVE INCUBATION PRODUCTS[1]

Incubation	Fe + HRP	H_2O_2 + HRP	Fe + Microsome
	rf	rf	rf
Retinoic Acid	0	0	0
Spot 1	.54	.54	.52
Spot 2	.64	.66	.67

[1]TLC on alumina developed in benzene:chloroform:methanol (4:1:1).

Microsomal batch incubations were centrifuged at 12,100 x g for 20 min and the supernate decanted from the pellet. The radioactivity was assayed in the same manner as the HRP incubations. Recovery was 58% complete of the ^{3}H and 37% of the ^{14}C as compared to the initial substrate. After incubation 40% of the ^{14}C was absent from the reaction mixture. The same isolation procedure was followed as for the HRP incubations. The acetone eluted ^{3}H was 3% of the initial substrate while methanol eluted ^{3}H equivalent to 4% of the initial value.

Thin-layer chromatography (TLC) on silicic acid in cyclohexane: chloroform:methanol (8:1:1) of the acetone and methanol fractions resulted in multiple product isolation from both the microsome and HRP incubations. Three hours after intraperitoneal injection of retinoic acid into a rat, tissues were removed and extracted as outlined in the methods section. The liver contained the greatest percentage of isotope (14%) followed by the kidney (3%) while the spleen contained only background radiation. The radioactive fraction separated by alumina chromatography of the liver extract was reduced in volume and portions were applied to silicic acid TLC plates and developed in cyclohexane:chloroform:methanol (8:1:1). The plates were scraped and counted for radioactivity. The results are shown in Table VIII along with standards and comparative data from *in vitro* microsomal and HRP products. Although the metabolite had the same migration rate as 13-*cis*-retinoic acid, the absence of the carboxyl ^{14}C rules out this possibility.

One of the products formed *in vitro* by horseradish peroxidase and microsomal incubations had the same separation characteristics as the ^{3}H-labeled decarboxylation product isolated from rat liver. The other products formed during this reaction hold important information as to

TABLE VIII

A COMPARISON BY THIN-LAYER CHROMATOGRAPHY OF ALUMINA COLUMN ACETONE ELUANT WITH SOME VITAMIN A COMPOUNDS

Compound	Rf
All-*trans*-retinoic acid	0.31
All-*trans*-retinol	0.40
All-*trans*-retinal	0.55
13-*cis*-retinal	0.44
Metabolite (*in vivo*)	0.43
Metabolite (*in vitro* microsomes)	0.44
Metabolite (HRP)	0.43

TLC on silicic acid developed in cyclohexane:chloroform:methanol (8:1:1).

mechanism of decarboxylation and the multiplicity of steps in retinoic acid metabolism. As discussed earlier the incubation products were divided into two fractions by alumina chromatography. One fraction was eluted from alumina with acetone and the second fraction eluted with methanol. Subsequent TLC on silicic acid of these two fractions with cyclohexane:chloroform:methanol (8:1:1) resulted in separation of multiple radioactive components. The products are shown in Table IX.

The whole incubation methylene chloride extract was spotted on silicic acid and developed with cyclohexane:chloroform:methanol (8:1:1). The same products were separated before being chromatographed on alumina as after the column chromatography. Retinoic acid was chromatographed by alumina column chromatography to detect any radioactive substrate impurities or isolation procedure degradation products. The microsomal and in vivo isolation products were heavily contaminated with lipids and steroids as detected by mass spectrometry. The horseradish peroxidase system was free from contaminating lipids or steroids.

The extraction products were submitted to gas chromatographic analysis on 1% OV-1 on Gas Chrom Q, 1% Carbowax 1540 on Chromasorb W, and 14.5% DEGS on ABS. All reagents were purchased from Applied Science Laboratories, Inc., State College, Pennsylvania, except ABS which was purchased from Analabs, Inc., North Haven, Connecticut. The results obtained were unsatisfactory when the eluants were examined by mass spectrometry since there was evidence of thermal degradation and incomplete separation of compounds.

Yagishita et al. (32) reported two radioactive metabolites of retinoic acid which were thought to be a hydroxy-acid and its ester. The metabolite appeared about two hours after injection of radioactive

TABLE IX

TLC OF ALUMINA COLUMN ELUANTS[1]

	HRP		Microsome	
	Acetone	MeOH	Acetone	MeOH
	Rf	Rf	Rf	Rf
Spot 1	.06	.06	.06	.06
Spot 2	.31	.31	.31	.31
Spot 3	.43	.43	.44	.44
Spot 4	.59	.59	.59	.59

[1]TLC on silicic acid developed in cyclohexane:chloroform:methanol (8:1:1).

retinoic acid. Physiological doses of radioactive retinol or retinoic acid yielded three similar labeled anion exchange fractions from rat, guinea pig and chicken (86). Intraperitoneal injection of non-physiological amounts of [15-^{14}C]-, [14-^{14}C]-, and [6,7-^{14}C]retinoic resulted in fecal excretion of greater than 50% of the isotope, probably as the glucuronide (41). Radioactivity in the urine was lower for [15-^{14}C]retinoic acid than for either [14-^{14}C]- or [6,7-^{14}C]retinoic acid which were about the same. Rat liver and kidney slices were used by Roberts and De Luca (43) to decarboxylate retinoic acid. Oxidation of [14-^{14}C] retinoic acid was faster in kidney tissue than liver and inhibition studies showed that [15-^{14}C]retinoic acid did not require Kreb's Cycle enzymes while [14-^{14}C] did. Formation of CO_2 from the product decarboxylation of [15-^{14}C]retinoic acid was postulated. Further work was reported by Roberts and De Luca (34) on the isolation and identification of metabolites of retinoic acid from rat liver and kidney microsomal preparations. One of the products isolated was non-acidic which may be similar to one of the tritium labeled products isolated in this work. Roberts and De Luca (34) state that until products and intermediates of the reaction were positively identified, direct evidence for the mechanism could not be obtained. On the basis of a lowered absorption maximum, a shortened side chain was proposed and polar products from silicic acid chromatography showed other products than the proposed C_{14}-aldehyde (34) were formed. In the present work multiple reaction products have been isolated from double label substrate and partially characterized. With the loss of the terminal carboxyl label only tritium labeled metabolites are found. Geison and Johnson (87) described differential metabolism in the kidney and liver of rats of physiological levels of

intraperitoneally injected of [6,7-^{14}C]retinoic acid. In their study the major route of metabolism was through the kidney with urinary excretion of 60% of the injected dose within 24 hrs. The use of [15-^{14}C] retinoic acid along with the ring labeled compound resulted in recovery of only 32% of the injected dose in 24 hrs. Methanol was used to extract the radioactive metabolites with separation products and carrier retinoic acid on silicic acid. All protein bound radioactivity was easily extracted with methanol. Only 50% of the applied radioactivity was recovered from the column and it was noted that kidney levels of radioactivity remain constant for the time of assay. Retinoyl-SCoA was not formed in rat liver in the studies of Lippel et al. (73). Mitochondrial metabolism was minor compared to glucuronide formation and decarboxylation in the microsomes, but only [15-^{14}C]retinoic acid was used in the experiments and no decarboxylation products were identified. Urinary excretions of retinoic acid metabolites were studied by Sundareson et al. (42) using [15-^{14}C], [14-^{14}C] and [6,7-^{14}C]retinoic acid. Injections of physiological doses were made into retinol deficient rats with the collected urine divided into an ether soluble, acid fraction and water soluble fraction. No retinoyl-β-glucuronide was found in the urine although six urinary metabolites of retinoic acid were reported in the urine. Retinoic acid formation in the kidney has been reported by Büssaler and De Luca (88). Identification was by co-chromatography of methylated products and carrier retinoic acid on silicic acid thin-layer plates. The authors noted that their experimental methods did not permit detection of any new vitamin A metabolites. Geison and Johnson (87) stated that a future problem would be to obtain sufficient quantities of the various metabolites for chemical characterizations and

biological assays. In the present investigation methods and procedures have been developed to prepare and isolate preparative quantities of reaction products of retinoic acid. By the use of the methods and procedure in this study along with mass spectral information of Vecchi *et al.* (57), Lin *et al.* (56), and the information presented in this thesis, preparation, isolation, and identification of metabolic products of vitamin A should be facilitated.

At the present time work is underway to further purify these products so that identification can be accomplished by mass spectrometry and a degradation mechanism as well as metabolic pathway formulated. The products of these incubations are being prepared in physiological amounts so that biological activity can be ascertained. As identification is completed experiments can be undertaken to determine the mode of action of vitamin A in maintenance of growth and fertility.

CHAPTER V

SUMMARY

The mass spectra of 12 analogs of vitamin A were obtained. Compounds studied were 5,6-monoepoxyretinoic acid, 5,8-monoepoxyretinoic acid, methyl-5,6-monoepoxyretinoate, methyl-5,8-monoepoxyretinoate, trimehtylsilyl-5,6-monoepoxyretinate, trimethylsilyl-5,8-monoepoxyretinoate, 5,6-monoepoxyretinyl acetate, 5,8-monoepoxyretinyl acetate, 5,8-monoepoxyretinal, β-C_{19}-retinal, β-C_{19}-retinyl acetate and C_{19}-anhydroretinol. Carbohydrate derivatives were examined by mass spectrometry. The mass spectra of TMS-β-D-glucopyranuronic acid, and TMS-6,3-glucuronolactone were analyzed. The metabolism of retinoic acid *in vivo* and *in vitro* was studied and major products were isolated.

Interpretation of the mass spectra of twelve analogs of vitamin A revealed important details of their fragmentation, resulting from electron impact, that will also be of value in determining structures of other analogs and metabolites of vitamin A. The low resolution data of nine monoepoxy- and three C_{19}-vitamin A analogs were presented. In addition, high resolution mass spectra were obtained for six fragment ions of all-*trans*-retinoic acid and thirteen fragment ions of methyl-5,6-monoepoxyretinoate. The oxygen substituent on the ring directed sequential fragmentation of the side chain while interrupted conjugation of the side chain promoted cleavage alpha to the ring sturcture. The mass spectra of trimethylsilyl derivatives of glucuronic acids were

presented. This information may be used for determination of ring size and linkage of metabolic conjugates. Decarboxylation products of retinoic acid were isolated from incubation systems containing horseradish peroxidase or chicken liver microsomes *in vitro*, and from rat livers *in vivo*. One product was found to have the same separation characteristics from all three incubation systems. Multiple products were isolated and separation techniques were presented for vitamin A metabolites.

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