A Biochemical Analysis of Secretions from the Uropygial Gland of Selected Passeriform Birds

by

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Paul C Peterson — March 10, 1977

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ABSTRACT

A Biochemical Analysis of Secretions
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Passeriform Birds

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The fatty acids of the uropygial gland waxes of 99 individuals of 4 species of birds including the common grackle, Quiscalus quiscula (Icteridae); starling, Sturnus vulgaris (Sturnidae); red-winged black bird, Agelaius phoeniceus (Icteridae) and brown-headed cowbird, Molothrus ater (Icteridae) were analyzed using a new and simplified technique for the extraction of fatty acids and subsequent analysis of their methyl ester derivatives. The principle components were found to be methyl branched and unbranched fatty acids ranging from C_{11} to C_{21}. Resulting chromatograms depict four basic patterns; intraspecific variation, including sexual differences was minimal or absent within each species. One instance of a possible avian subspecific difference in fatty acid components was noted. The potential use of fatty acids and chemotaxonomy is discussed.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Paul C. Peterson for his encouragement, direction and guidance throughout the course of this study. Special recognition is extended to Mr. John Klosterman, Director and Mr. Frederick Posey, Criminalist, of the Northeastern Ohio Forensics Laboratory for their expert technical assistance, use of major and minor items of equipment, and consumable supplies. The efforts of Mr. Norman Vujavic and Mr. William Kurey who aided in the collection of numerous avian species is also greatly appreciated.

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INTRODUCTION

The uropygial gland or preen gland of birds is a sebaceous holocrine gland positioned immediately beneath the skin in a mass of fatty tissue dorsal to the levator muscle of the tail and the free coccygeal vertebrae. This gland, the only sebaceous gland in birds, is present in most avian orders with a few notable exceptions among the Ratites and a few species of the avian orders Galliformes, Gruiformes, Caprimulgiformes, and Apodiformes.

The gland is a bilobed structure with each lobe terminating in an expanded central cavity. The lobes are closely associated, separated only by a very thin interlobar septum. Secretions from these cavities surface by one or more openings at the apex of the papilla. The papilla is generally devoid of feathers except at the oriface where it is circled by tufts of filoplume feathers. Often these feathers are usually inundated with solidified yellowish secretions from the uropygial gland. The shape of the gland is remarkably uniform among avian taxa; however, intergroup variations do occur in the number of nipple-like openings to the outside (Grasse, 1950) and the arrangement of the specialized tufts of feathers which surround the papilla.

There have been few studies on the cytology and histology of the uropygial gland. However, it is known that numerous secretory tubules radiate from the periphery of the primary cavity. The glandular secretions are synthesized in the specialized cells within the

basal ends of the tubules. The cytoplasm of these cells gradually fill with lipid droplets and show increased cellular degeneration as they near the cavity of each lobe of the gland. With final cellular breakdown the lipid secretion is liberated into the lumen of the cavity.

Numerous functions have been attributed to uropygial gland secretions. Among the most frequently mentioned are bill maintenance, vitamin D supplementation, water repellency of feathers and plumage maintenance (Elder, 1954; Bollinger, 1960). Most recent studies indicate that the prime function of this gland is to provide a dressing for maintenance of feather structure from one molt to the next. Without this secretion the feathers lose much of their normal function as a flight mechanism and as a heat-insulating medium. In waterfowl the secretion may also supplement the water repellent qualities of feathers.

Recent studies of the uropygial gland have been directed to the area of chemical analysis of the glandular secretion (Odham, 1967a; Jacob and Zeman, 1970; Jacob and Poltz, 1975). Major components of the glandular secretion have been identified as waxes, free fatty acids, triglycerides, phospholipids, cholesterol and glycerin (Weitzel, 1951). In subsequent studies, uropygial gland waxes of various avian species have been analyzed and have been determined to vary in the kind and quantity of wax present. In the order Anseriformes the uropygial waxes of three species were analyzed: the water duck Anser anser, the Peiking duck Anas platyrhynchos, and the mute swan Cygnus olor (Odham, 1963, 1965, 1966, 1967a). These studies revealed a difference

in the fatty acid composition of the waxes of the three species. The water duck waxes contained only tetramethyl-substituted aliphatic acids; the swan waxes were mainly trimethyl-substituted acids and the Peiking duck waxes were monomethyl-branched fatty acids and straight chained acids. The alcohol moieties were mainly n-octadecanol-1 and monomethyl-branched alcohols.

Jacob and Zeman (1970) in their analysis of the uropygial gland wax of the tufted duck Aythya fuligula (Anseriformes) found the major acidic fraction of the wax to be 2,4,6-trimethyl-nonanoic (39%), 2,4,6-trimethyl-octanoic (8.7%), and 2,6-dimethyl-nonanoic acid (5.9%). The main alcohol components of this bird were determined as n-hexadecanol and n-octadecanol with minor components of 2-, 6-, 10-, 12- and 14-methyl-branched primary alcohols.

Whereas the uropygial gland waxes of the order Anseriformes were esters of monohydric alcohol, the uropygial gland waxes of the order Galliformes are diester waxes consisting of 2,3-n-alkanediols esterified with straight-chained fatty acids. The major diols from chicken Gallus gallus wax esters have been identified as threo and erythro isomers of chain lengths C_{22}, C_{23} and C_{24} (Haahti and Fales, 1967); whereas, in the turkey Meleagris gallopavo only erythro isomers of the C_{20}-C_{23} diols were present (Hansen, Tang and Edkins, 1969). Analysis of the uropygial gland waxes of the mallee fowl Leiopoa ocellata and the stubble quail Coturnix pectoralis reveals the mallee fowl wax to contain mainly C_{14} and C_{16} acids and erythro 2,3-octadecanediol and the stubble quail wax to contain C_{11} - C_{20} acids and C_{14} - C_{24} diols (Edkins and Hansen, 1971).

Until recently very little analytical research of the uropygial waxes of Passeriformes had been performed. The two studies reported by Jacob et al. (1970, 1975) revealed that the major fatty acids components of the uropygial waxes of twelve european species were primarily 2-methyl; 2,x-dimethyl- and 2,x,y,-trimethyl-branched acids ranging in chain length from C_{10} - C_{20}. The secretion also revealed smaller amounts of unbranched fatty acids. The primary alcoholic fraction in the passerine species was identified as n- and 2-methyl-alcohols.

The purpose of this investigation was to further study and compare the uropygial waxes of selected Passeriform birds. Additionally, in an effort to elucidate any intraspecific differences in bird groups it was also the main intent of this study to develop a simple, repetitive technique for the extraction, separation and analysis of lipid fatty acids from single sample collections.

MATERIALS AND METHODS

Collection

Uropygial gland secretions were obtained immediately from freshly collected birds by gently squeezing the gland in an effort to minimize the destruction of undifferentiated peripheral cells which might inundate the secretion with other cellular components, particularly the phospholipids. Secretions were collected on filter paper and immediately transferred to a chloroform-methanol mixture (2:1 v/v), labelled and stored at room temperature for future chemical analysis

Isolation of Wax

An initial lipid separation was necessary to separate the lipid and nonlipid fractions present in the sample. At the outset of the study silicic acid column chromatography was attempted. A modified technique of Hirsch and Ahrens (1958) was used to separate lipid classes on Bio-Sil HA silicic acid by stepwise elution with solvents of differing polarity. The technique was abandoned because of the large sample loss incurred. An alternate technique was employed using a modification of the methods of Knudsen (1976). The lipid separation was carried out on silicic acid coated chromatographic plates. To prepare the secretion for this procedure the sample was oven heated at 100°C and the excess chloroform-methanol mixture evaporated. Ten microliters of the concentrated sample were spotted on silica gel (1000 micron) thin layer chromatographic (TLC) plates

and developed in petroleum ether-diethyl ether-acetic acid (90:10:1 v/v). The resulting lipid bands were developed by spraying the plate with a nondestructive detection reagent, 0.0012% Rhodamine 6G, and were observed as yellow bands under ultraviolet irradiation. The wax component with Rf value of 0.85 was scraped off the plate. This band corresponded to long chained triglyceride standards which were developed simultaneously with the sample. The scrapings were transferred to 1 ml ampules for further analysis.

This initial separation employing TLC essentially accomplished two steps simultaneously. First, the nonlipid and lipid fractions were separated and secondly, the lipid fraction was separated into its respective classes. This method proved most satisfactory for small sample quantities in the microliter range.

Hydrolysis of the Wax

There are three basic techniques for the hydrolysis and subsequent methylation of fatty acids from their waxes. These include alkaline hydrolysis, enzymic hydrolysis and transesterification.

Alkaline hydrolysis, or saponification, involves refluxing in alcohol-alkali hydroxide solution. The products of this reaction are the fatty acids in the form of soaps (R^1COOK) and their respective alcohols (R^2OH). After the reflux period the alcohol solvent is distilled off and water added to the soap solution. Unsaponifiable materials such as sterols, ethers and hydrocarbons are then extracted with petroleum or diethyl ether. The remaining soap solution is treated with an excess of mineral acid to liberate the fatty acids. These

fatty acids can be recovered by extraction with petroleum or diethyl ether. Methyl ester derivatives of fatty acids are more suitable for gas liquid chromatography (GLC) than fatty acids because of their low polarity. Their retention time in the column is shortened and their resolution and separation is more pronounced than the fatty acids. Therefore the fatty acids obtained in this study were methylated to form methyl ester derivatives. Thus, subsequent to hydrolysis of the wax, esterification of the resulting fatty acids to their respective methyl esters may be accomplished by treatment of the fatty acids with an excess of anhydrous methanol in the presence of an acidic catalyst.

Enzymic hydrolysis employs ester-splitting enzymes and lipases which can catalyze the hydrolysis of various types of lipids. The success of enzymic hydrolysis is dependent upon carefully controlled laboratory conditions necessary to maintain specificity during the hydrolysis. No attempt was made to utilize this procedure.

Transesterification or interesterification is a one step procedure in which methyl esters of fatty acids are prepared directly from waxes in the presence of methanol and an acid or alkaline catalyst. Of the three procedures described for the separation and isolation of waxy fatty acids and their subsequent methylation, transesterification was demonstrated as the most satisfactory methodology for this study. Transesterification, as opposed to the other techniques, allowed for analysis of small amounts of lipid secretions from individual birds. Very little sample loss occurred since the number of fraction extractions and washings were minimal.

After the wax fraction was isolated by TLC, the wax band was

scraped from the plate into a 1 ml ampule and 0.2 ml of methylation reagent (anhydrous methanol-sulfuric acid 15:1 v/v) was added. The ampule was sealed and placed in an oven for 3 hours at 100° C. The resulting fatty acid methyl ester-fatty alcohol mixture was extracted with n-hexane. The excess n-hexane was evaporated in a water bath to a volume of 6 microliters. The sample (2 microliters) was subjected to analysis by gas liquid chromatography (Fischer 4800).

Gas Liquid Chromatography Analysis

It was necessary to use temperature programming to achieve satisfactory resolution of methyl ester derivatives. The following parameters were found to produce the best possible separation:

Initial Hold: 2 min.

Program: 17 C/min.

Initial Temperature: 80° C

Final Temperature: 200° C

Final Hold: 10 min.

Two methods of data collection were used. In the first method, peaks were recorded using a Beckman double pen recorder without an integrator or automatic timing device. Retention times were determined by means of calibrated chart paper and the area under the peaks quantified by mathematical triangulation.

In the second procedure, the GLC was interfaced with an automatic data capture device (Hewlett-Packard, Model 3800A). The computer provided a printout of the retention times, area under peaks and peak percentages automatically. Both internal and external

standards were at different times used to calculate retention times and peak percentages more accurately.

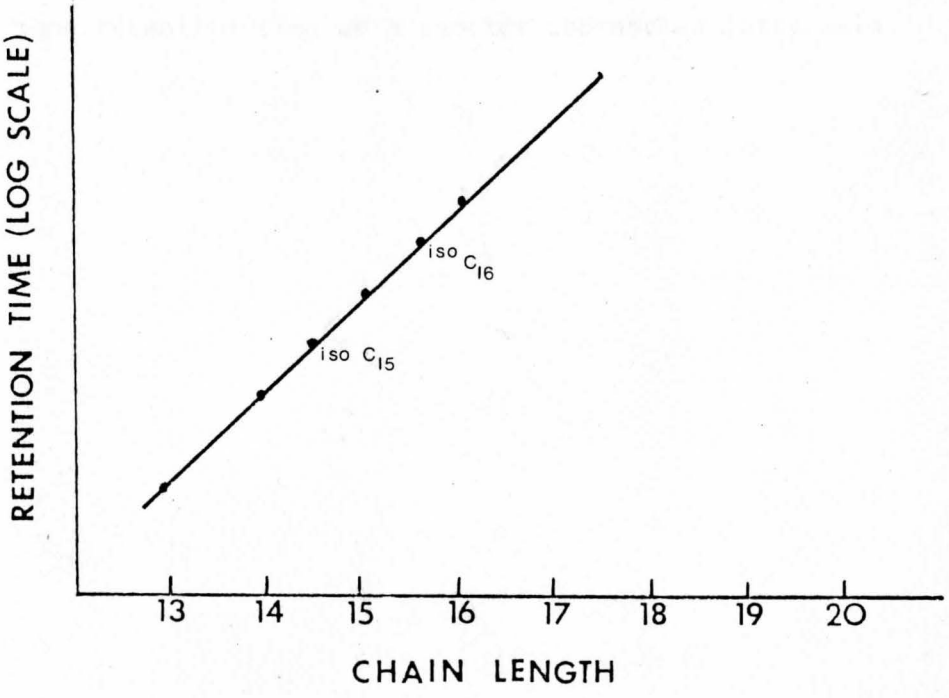
Purified methyl ester extracts of each gland were injected into the GLC and the resulting peaks determined by either computer or pen recorder. Prior to each run a mixture of straight chained standards (C_6 - C_{22}) was injected into the GLC to facilitate identification and comparison of the bird samples. In addition to the direct comparison of retention times, peak identification was aided by the use of graphs depicting a linear correlation between the logarithm of the retention time of the standard fatty acid derivatives and that of a homologous series of methyl esters of straight chained fatty acids. Retention times of the uropygial esters were located on these slopes in an effort to identify their chain lengths.

Comparison of retention times by these methods made it possible to tentatively identify the unbranched fatty acid methyl esters. Methyl esters eluted between the straight chained esters were tentatively identified as branched based upon the fact that methyl branched fatty acid derivatives elute prior to their straight chained esters. Figure 1 shows the position of standard iso-methyl esters run with their respective n-methyl esters. Multiple methyl branches decrease the retention time even further than the single branched derivatives. Therefore, branched and unbranched fatty acid methyl esters elute from the GLC column in the following order: trimethyl branched, dimethyl branched, monomethyl branched and unbranched fatty acid methyl esters.

According to the studies of Apandi and Edwards (1964), Odham (1967a), Jacob and Poltz (1975) and others, unsaturated fatty acids

Figure 1. Logarithm of retention time of standard straight and iso-branched fatty acid methyl esters vs. chain length.

Fig. 1


RETENTION TIME (LOG SCALE)
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CHAIN LENGTH

comprise an insignificant amount of the total lipid fraction. Based on these findings no attempt was made to determine any unsaturated components.

It must be emphasized that other variables including type and location of the branched groups can significantly affect the elution of the component. A possibility therefore exists that some of the acids tentatively identified as straight chained fatty acids could be multiple branched higher chained fatty acids that happen to possess the same retention time as a shorter unbranched fatty acid.

RESULTS

The gas-liquid chromatograms of the uropygial gland waxes of thirty-four grackles (Quiscula quiscala) were analyzed and compared to determine the components and the extent of intraspecific variability of the fatty acid component of the wax moiety. Significant attempts were made to detect any sexual differences among individuals within the species and to determine whether there were seasonal changes in the composition of the gland lipids.

With two exceptions, the resulting chromatograms of all bird glands analyzed demonstrate a technique with remarkable reproducibility. There was a marked homogeneity in pattern of each sample including both types of components and relative amounts. From the total number of chromatograms, four were elected representative of the basic "grackle pattern" and are illustrated in figures 2 - 5.

The typical pattern consists of 12 - 15 prominent peaks 8 - 10 of which were considered to be primary and the remainder, secondary peaks. The primary peaks were arbitrarily determined as being those reaching a height of 8 cm or higher on the chromatogram. Each peak is identified by a number. Peaks inconsistent in the bird specimens were designated as a,b,c, etc. on the chromatogram and corresponding retention slope. Much of the total variation occurring in the "pattern" was due to the presence or absence of the secondary peaks.

Figure 2. Gas-liquid chromatogram of the methyl esters of the fatty acids of grackle A.

Fig. 2

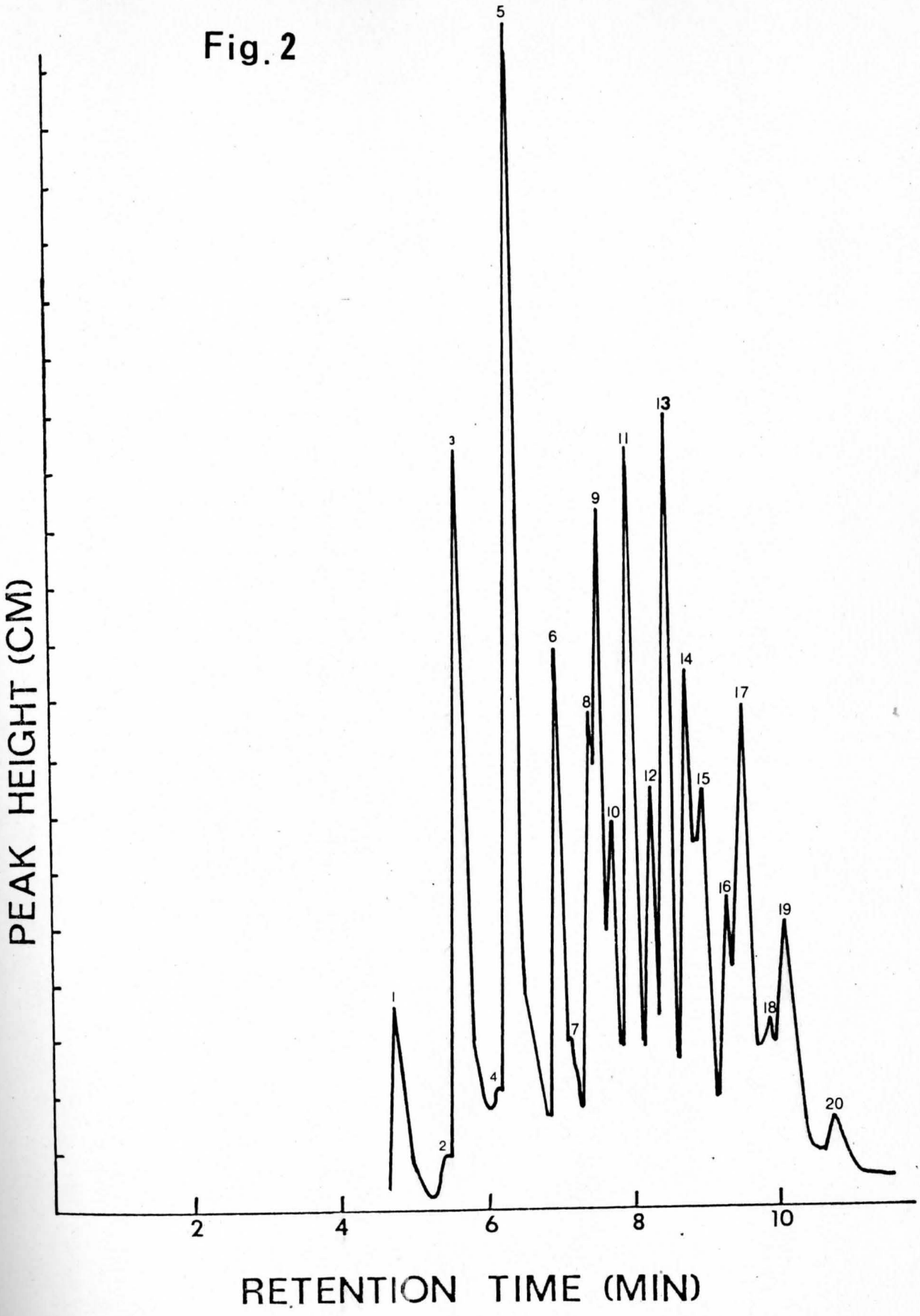
PEAK HEIGHT (CM)
RETENTION TIME (MIN)
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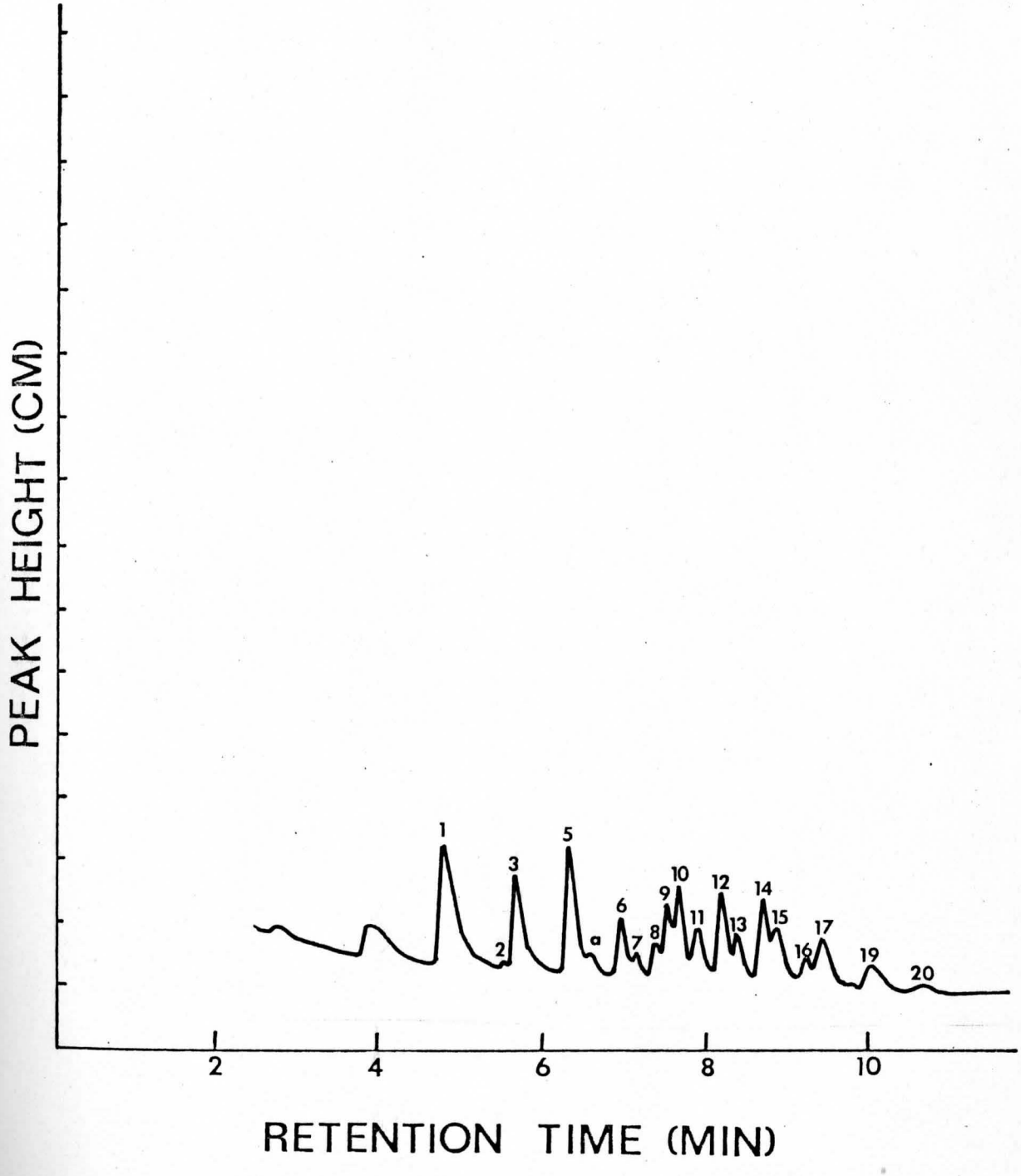
Figure 3. Gas-liquid chromatogram of the methyl esters of the fatty acids of grackle B.

Fig. 3


PEAK HEIGHT (CM)
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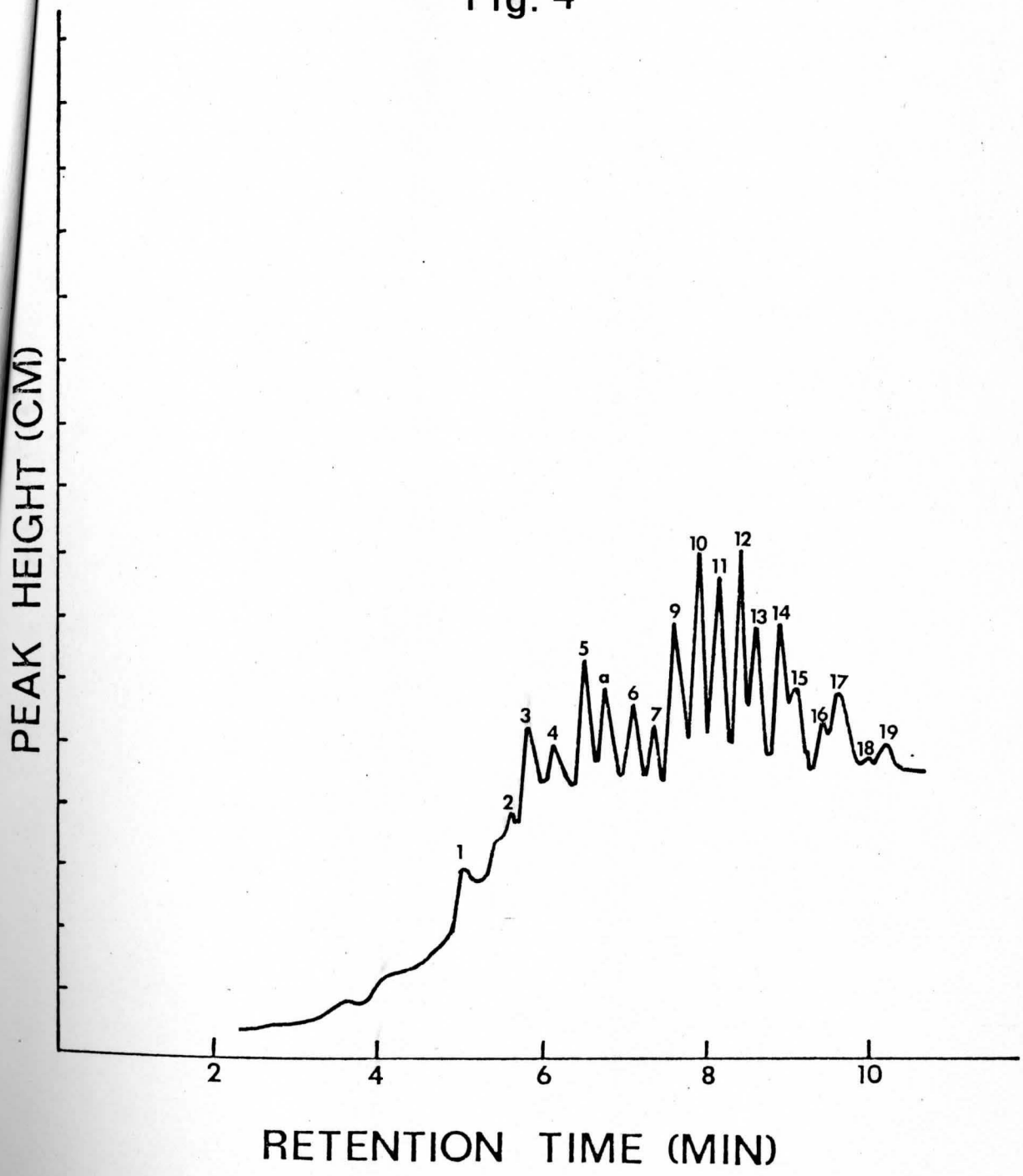
RETENTION TIME (MIN)

Figure 4. Gas-liquid chromatogram of the methyl esters of the fatty acids of grackle C.

Fig. 4


PEAK HEIGHT (CM)
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RETENTION TIME (MIN)

Figure 5. Gas-liquid chromatogram of the methyl esters of the fatty acids of grackle D.

Fig. 5

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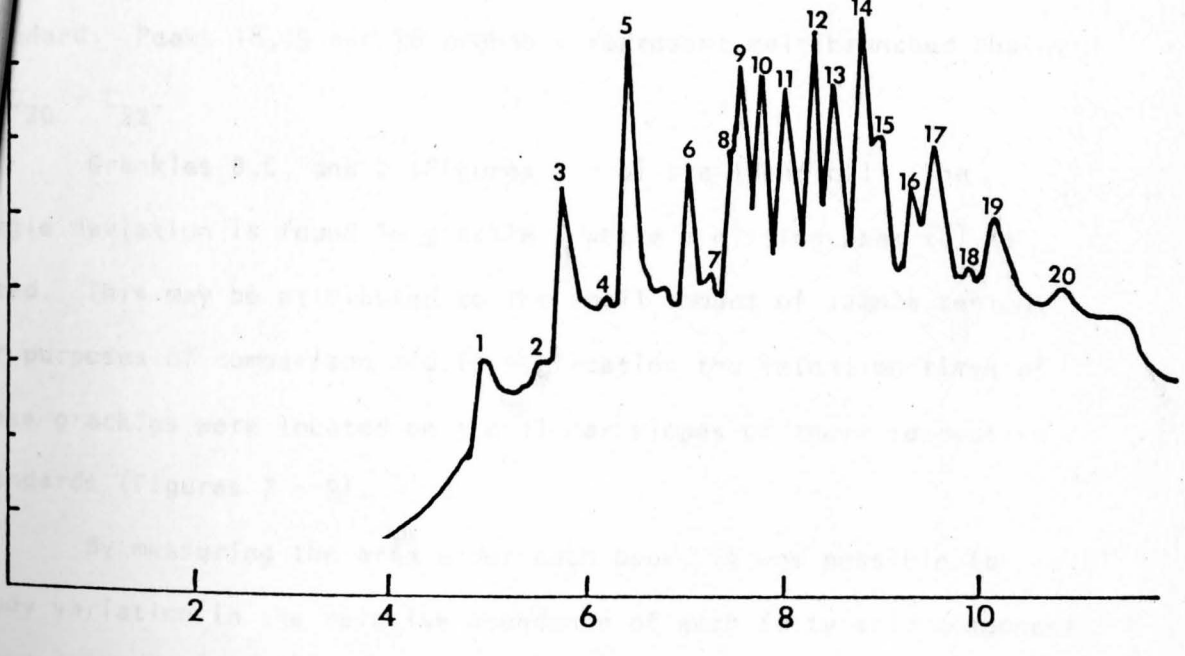


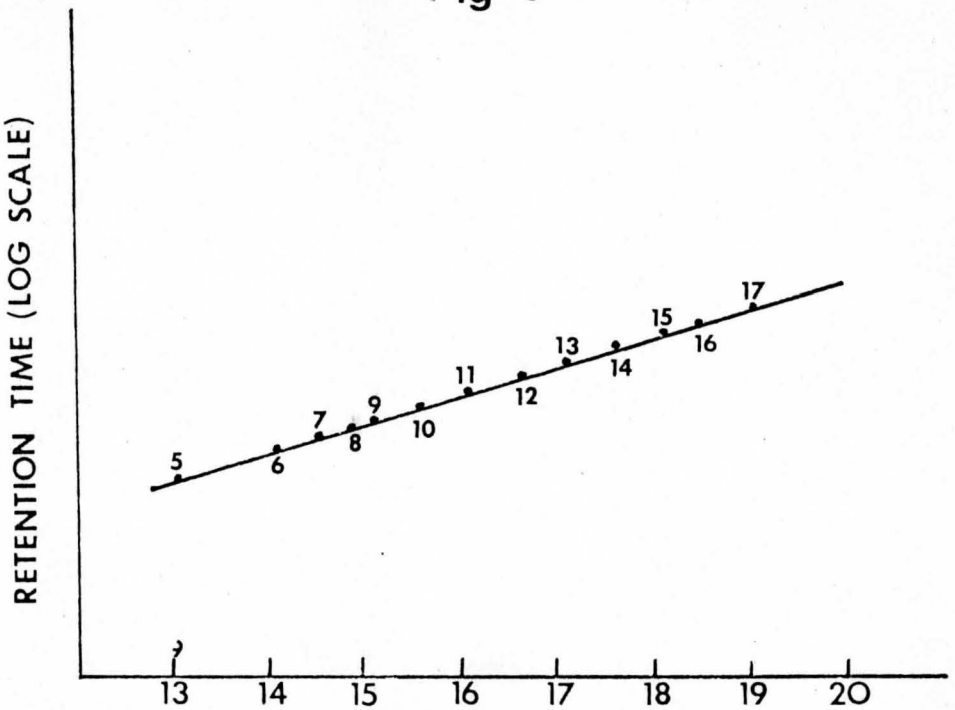
The chromatograms of grackle A (Figure 2) revealed 10 primary peaks tentatively identified as unbranched methyl esters of carbon chains varying in length from C_{11} to C_{19}. The single exception identified as peak 14 and eluting between C_{17} and C_{18} was tentatively identified as a branched ester of C_{18+n}. The retention times of these fractions were located on the linear slope of the methyl ester standards and their peak numbers were identified on the line (Figure 6). Peaks 5,6,9,11,13,15 and 17 eluted at regular intervals and corresponded exactly with the unbranched series of fatty acids C_{13} - C_{19}. Peaks 7,10,12,14, and 16 which are located approximately midway between the unbranched fractions were tentatively identified as iso-or mono- branched fractions of C_{15} - C_{19} respectively. Peak 8 may represent a multibranched higher fatty acid. Tentative identification of peaks 1 and 3 as unbranched C_{11} and C_{12} was made by direct comparison of the fraction's retention time with that of the standard. Peaks 18,19 and 20 probably represent multibranched chains of C_{20} - C_{22}.

Grackles B,C, and D (Figures 3 - 5) are identical. The single deviation is found in grackle C where a missing peak (8) is noted. This may be attributed to the small amount of sample tested. For purposes of comparison and identification the retention times of these grackles were located on the linear slopes of their respective standards (Figures 7 - 9).

By measuring the area under each peak, it was possible to study variation in the relative abundance of each fatty acid component (Table 1). Grackles A,B,C, and D reveal an equal distribution of

Figures 6 - 7. Comparison of retention times of sample methyl esters to standard methyl ester slope (logarithm of retention time of fatty acid methyl esters vs. chain length). Figure 6, grackle A. Figure 7, grackle C.

Fig. 6


RETENTION TIME (LOG SCALE)
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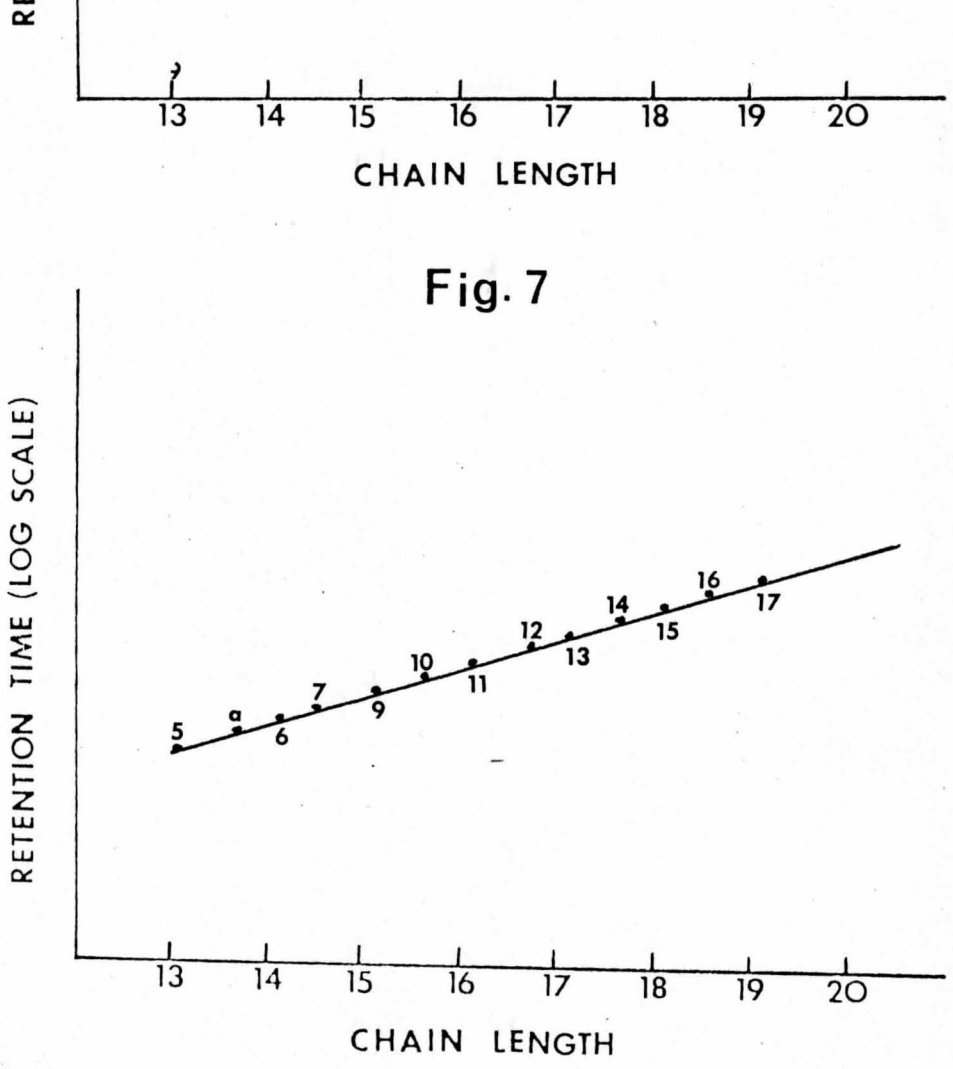
CHAIN LENGTH

Fig. 7


RETENTION TIME (LOG SCALE)
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CHAIN LENGTH

Figures 8 - 9. Comparison of retention times of sample methyl esters to standard methyl ester slope (logarithm of retention time of fatty acid methyl esters vs. chain length). Figure 8, grackle D. Figure 9, grackle B.

Fig. 8

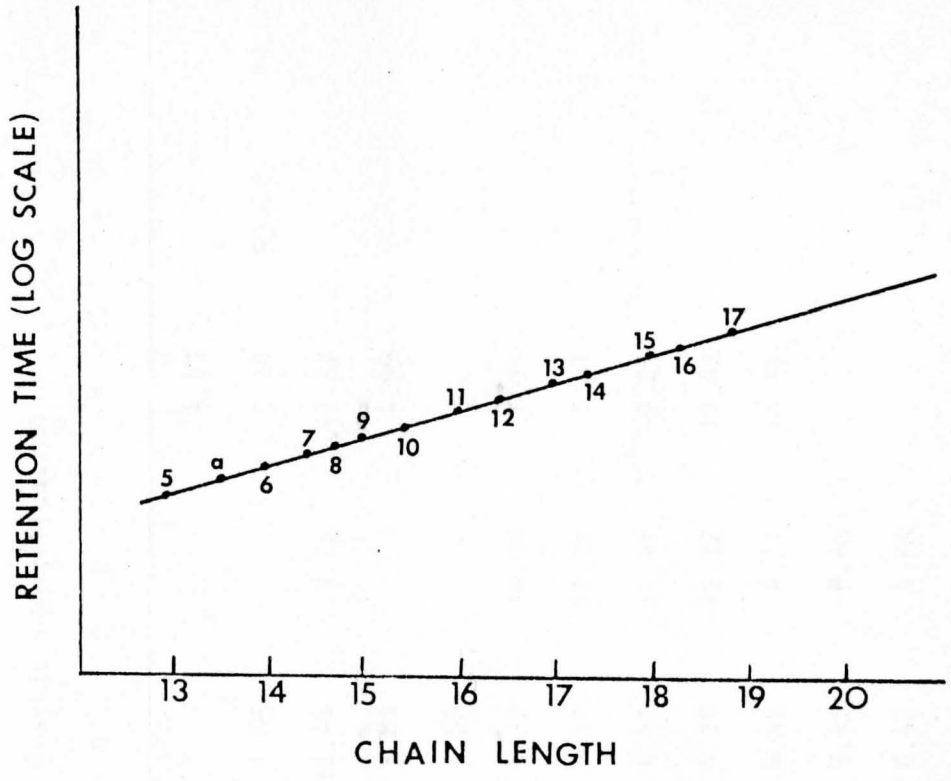

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Fig. 9

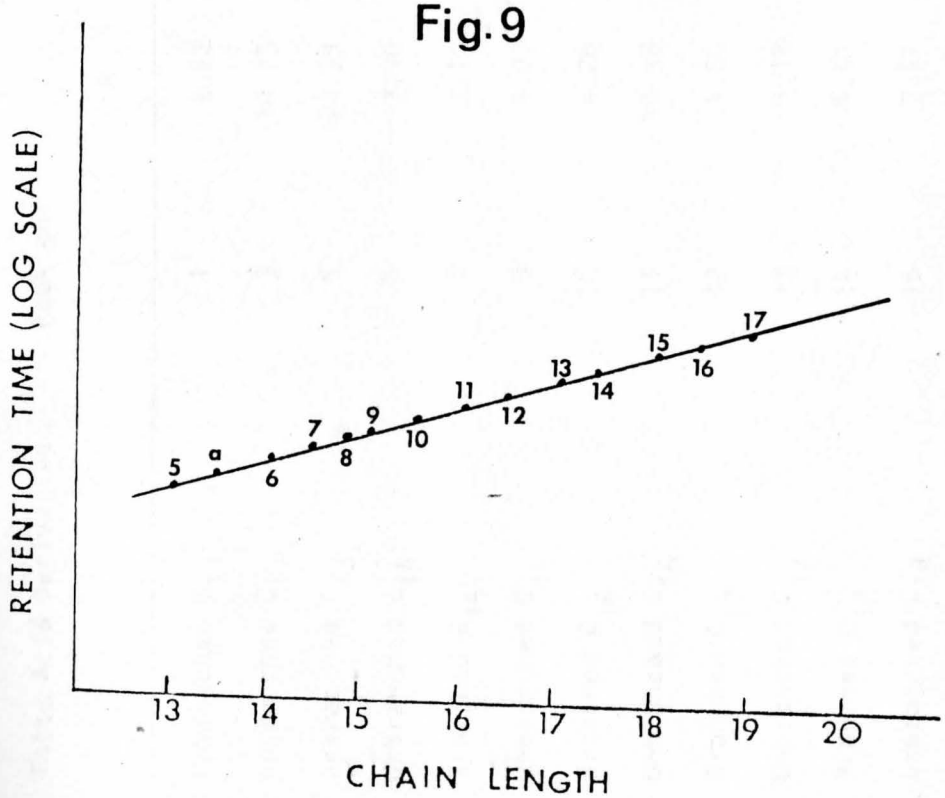


TABLE 1

PERCENTAGE UNDER PRIMARY PEAKS OF GRACKLE CHROMATOGRAMS

Fatty Acid Derivative	Peak no.	Grackle Identification					
		A	B	C	D	E	F
Unbranched C_{11}	1	4.22			4.38		
Unbranched C_{12}	3	10.73	9.65		5.24	99.00	99.00
Unbranched C_{13}	5	24.39	10.46	3.26	12.27		
Unbranched C_{14}	6	7.90	3.84		4.99		
Branched C_{15}	8	2.12	1.86				
Unbranched C_{15}	9	6.07	4.22	10.00	1.75		
Branched C_{16}	10	4.26	7.19	12.72	7.01		
Unbranched C_{16}	11	10.98	4.41	11.41	9.20		
Branched C_{17}	12	4.66	4.78	12.72	12.62		
Unbranched C_{17}	13	9.19	4.48	8.49	10.52		
Branched C_{18}	14	4.53	5.42	8.80			
Unbranched C_{18}	15	3.51	6.42	4.89			
Unbranched C_{19}	17	3.86	5.88				

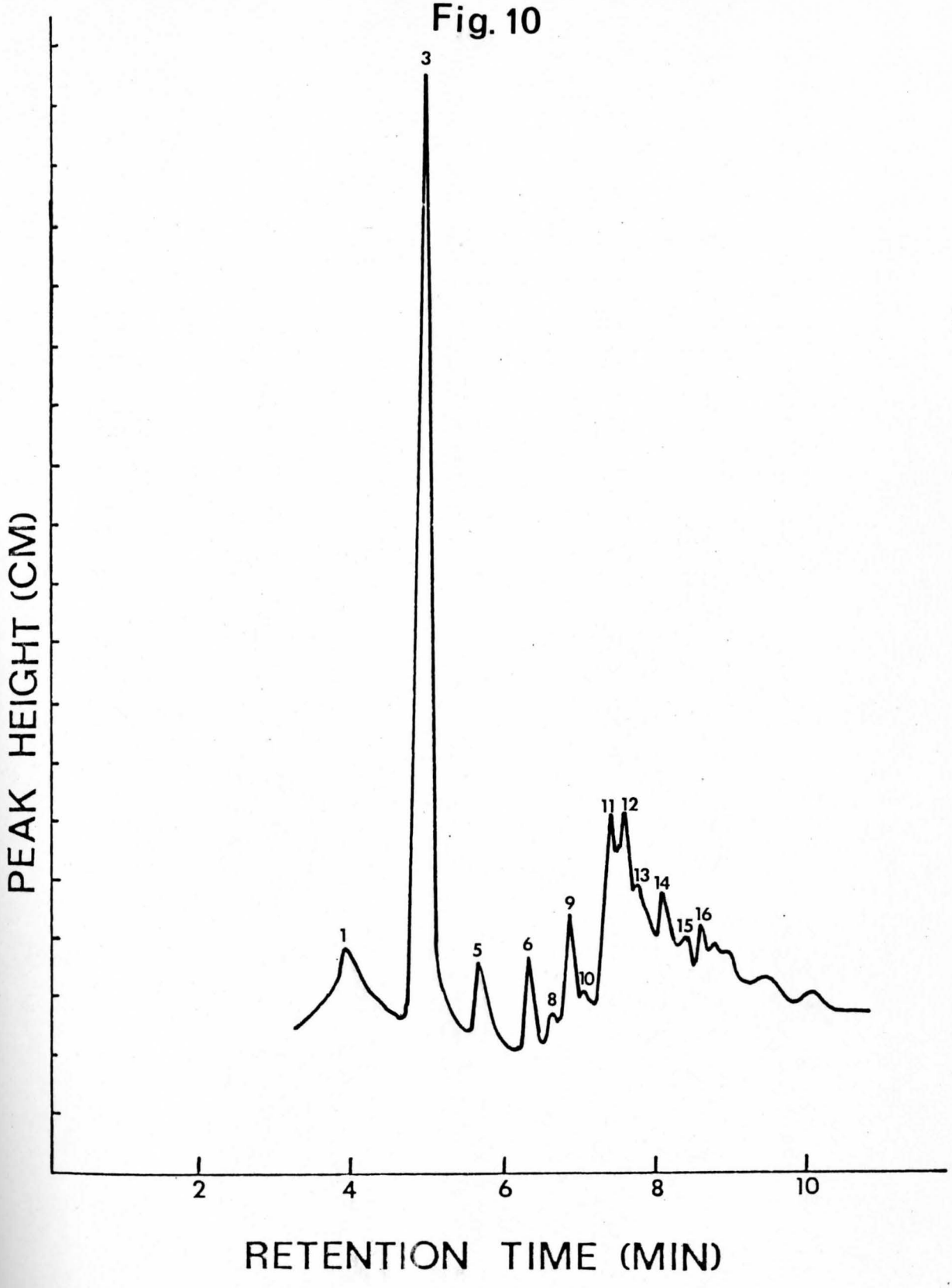
relative amounts of both unbranched and branched chains. The chromatograms of the uropygial secretions of grackles E and F (Figures 10 and 11) do not exhibit the same "grackle pattern" as the four previous specimens; they possess a single primary peak tentatively identified as unbranched C_{12} which accounts for over 99% of the methyl esters analyzed. Many of the components present in grackles A,B,C and D were also noted but because of the extremely small amount of lipid fraction eluted after peak 2 in grackle E and F it is possible that many of the esters were lost (Figures 12 - 13).

Grackle E and F present a different chromatographic pattern than that of A,B,C and D and may indeed be a subspecies of the common grackle studied. Further examination of the specimens in question did reveal notable morphological differences. Plummage of grackles A,B,C and D possessed a purple coloring; whereas, specimens E and F appeared more brown in color.

The remarkable conformity in chromatographic patterns which characterized the grackles pattern is paralleled in the "pyrimidal pattern" of starlings. Of the thirty-one starlings examined the basic pattern consisted of 5 major or primary peaks and numerous secondary peaks. The two consistent major peaks which form the apex of the pyramid have numerous shoulders and probably represent branched methyl esters of fatty acids. Four typical starling patterns are illustrated in figures 14 - 17.

The five principle peaks of the chromatograms of starling G (Figure 14) are tentatively identified as methyl branched C_{12} (peak 5), unbranched C_{12} (peak 6), methyl branched C_{13} (peaks 7 and 8), and

Fig. 10


PEAK HEIGHT (CM)
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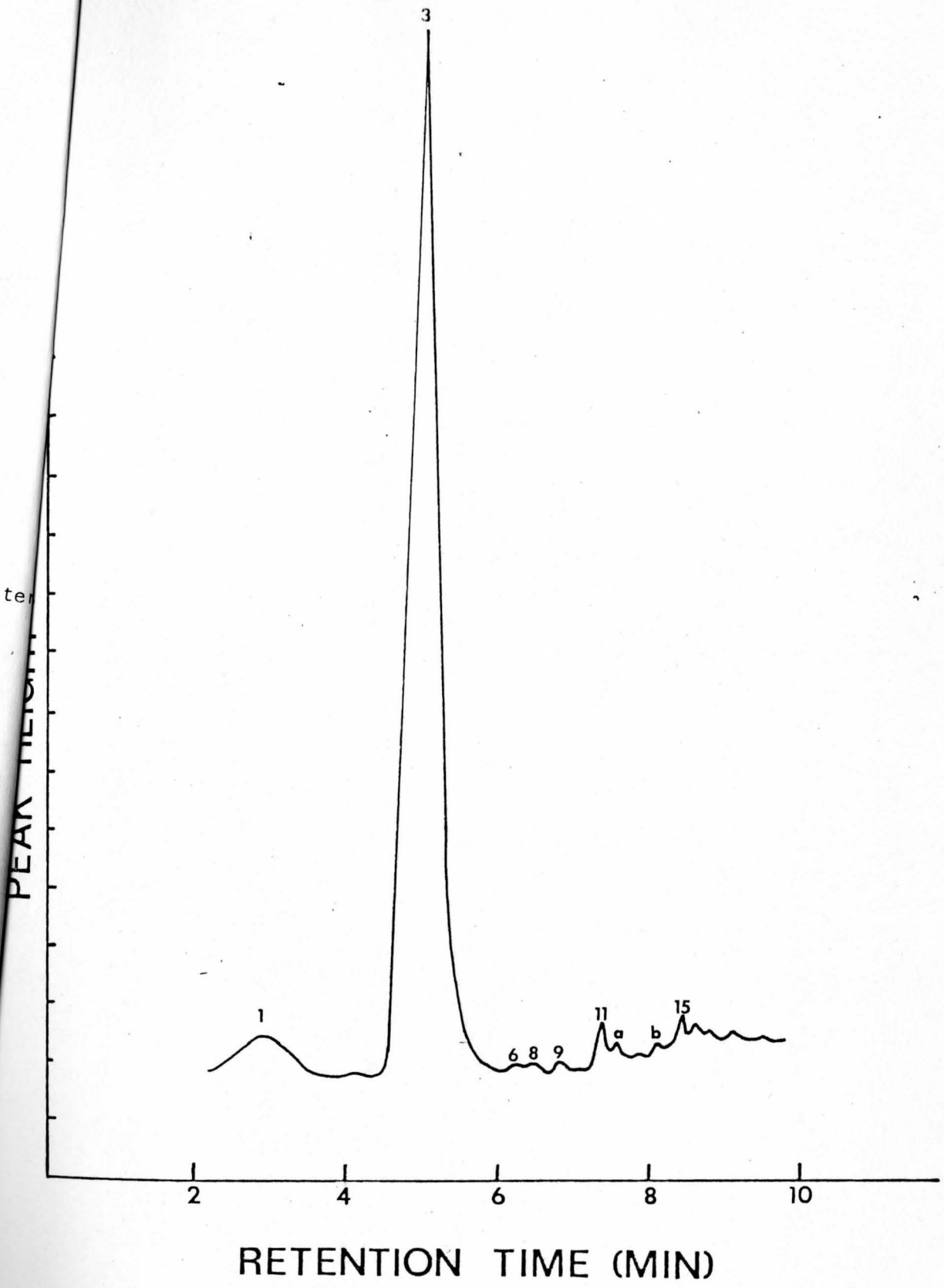
RETENTION TIME (MIN)

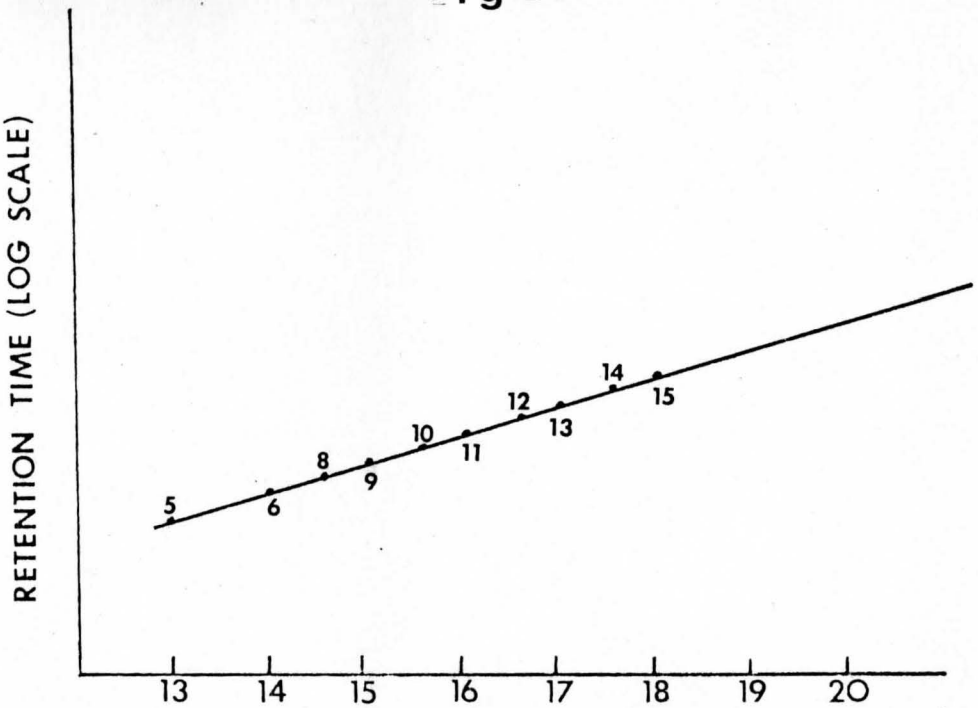
Figure 11. Gas-liquid chromatogram of the methyl esters of the fatty acids of grackle F.

Fig. 11



Figures 12 - 13. Comparison of retention times of sample methyl esters to standard methyl ester slope (logarithm of retention time of fatty acid methyl esters vs. chain length). Figure 12, grackle E. Figure 13, grackle F.

Fig.12


RETENTION TIME (LOG SCALE)
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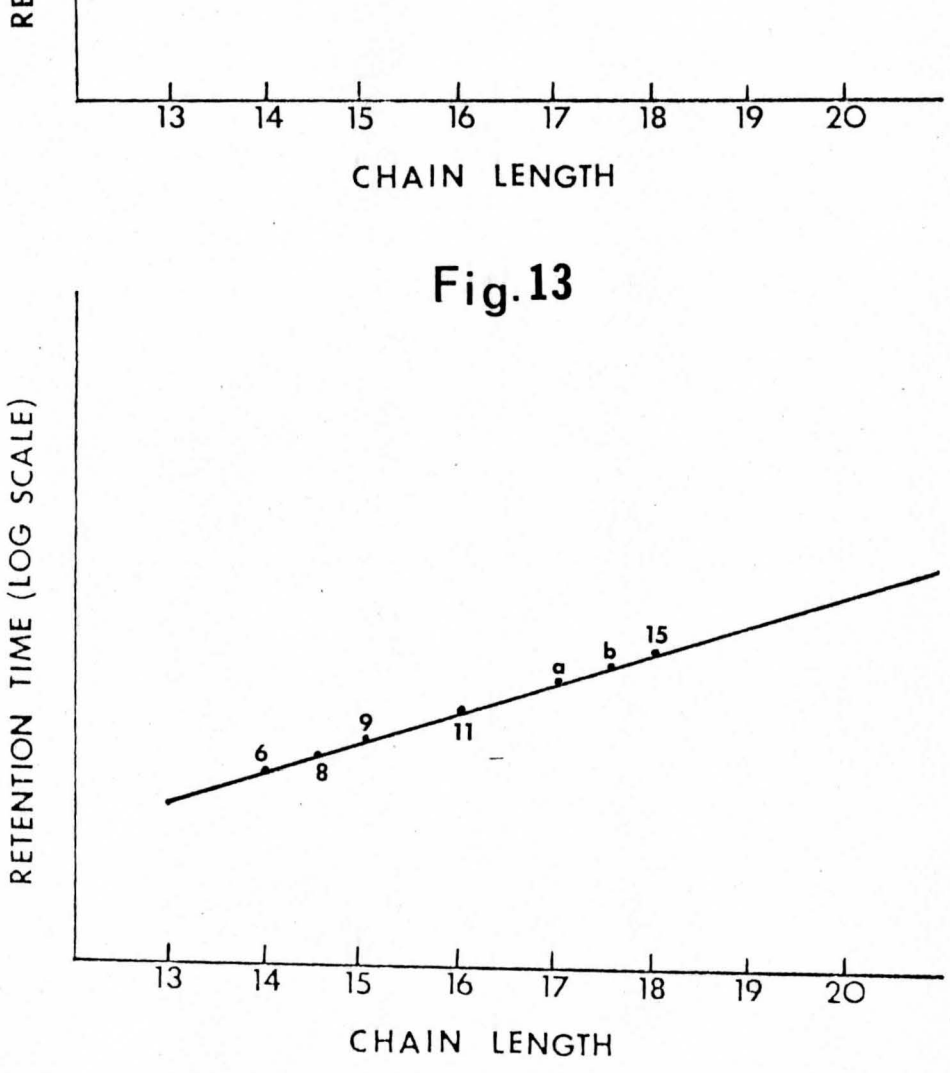
CHAIN LENGTH

Fig.13


RETENTION TIME (LOG SCALE)
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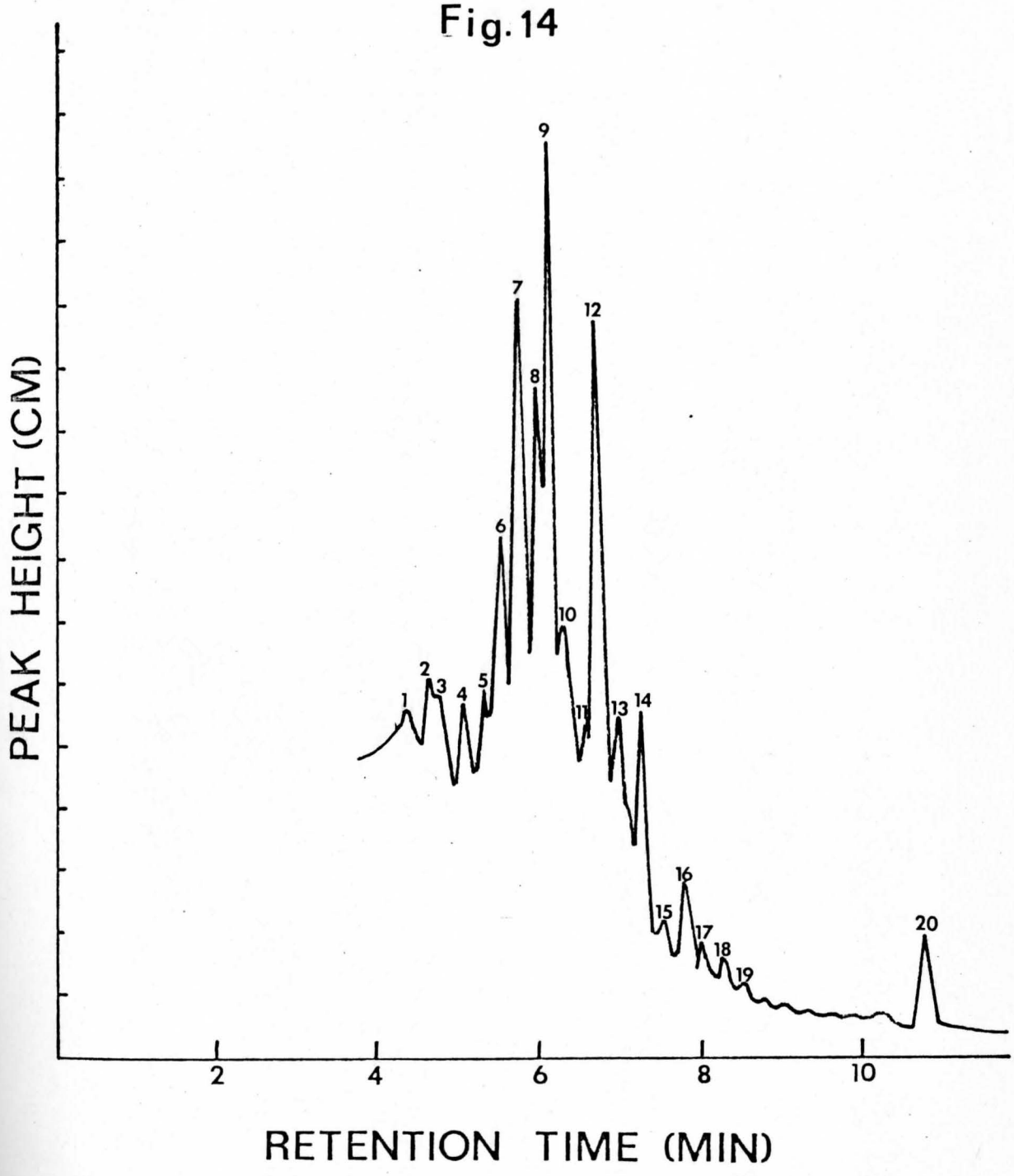
CHAIN LENGTH

Figure 14. Gas-liquid chromatogram of the methyl esters of the fatty acids of starling G.

Fig. 14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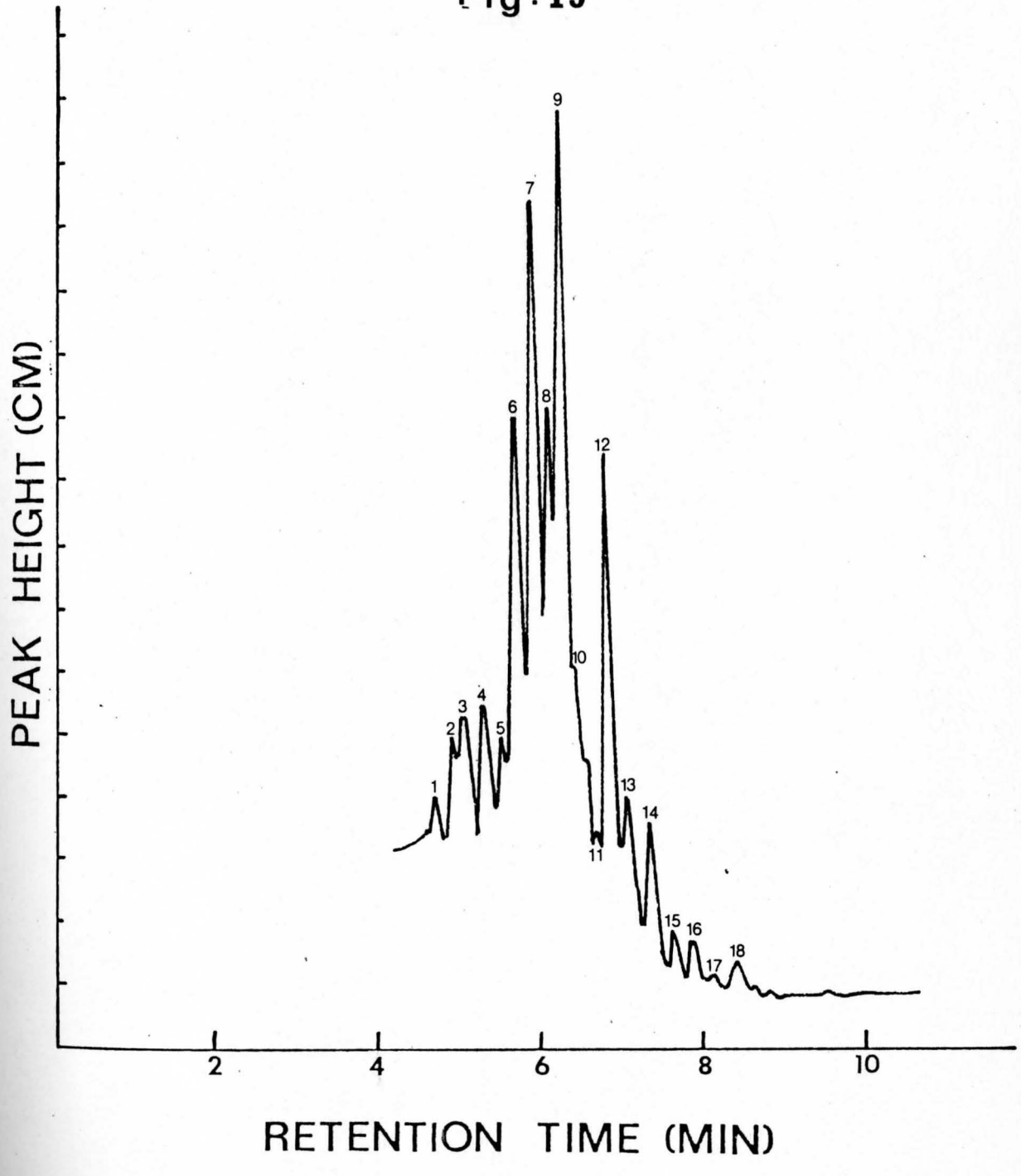
RETENTION TIME (MIN)

Figure 15. Gas-liquid chromatogram of the methyl esters of the fatty acids of starling H.

Fig. 15


PEAK HEIGHT (CM)
1
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3
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16
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18
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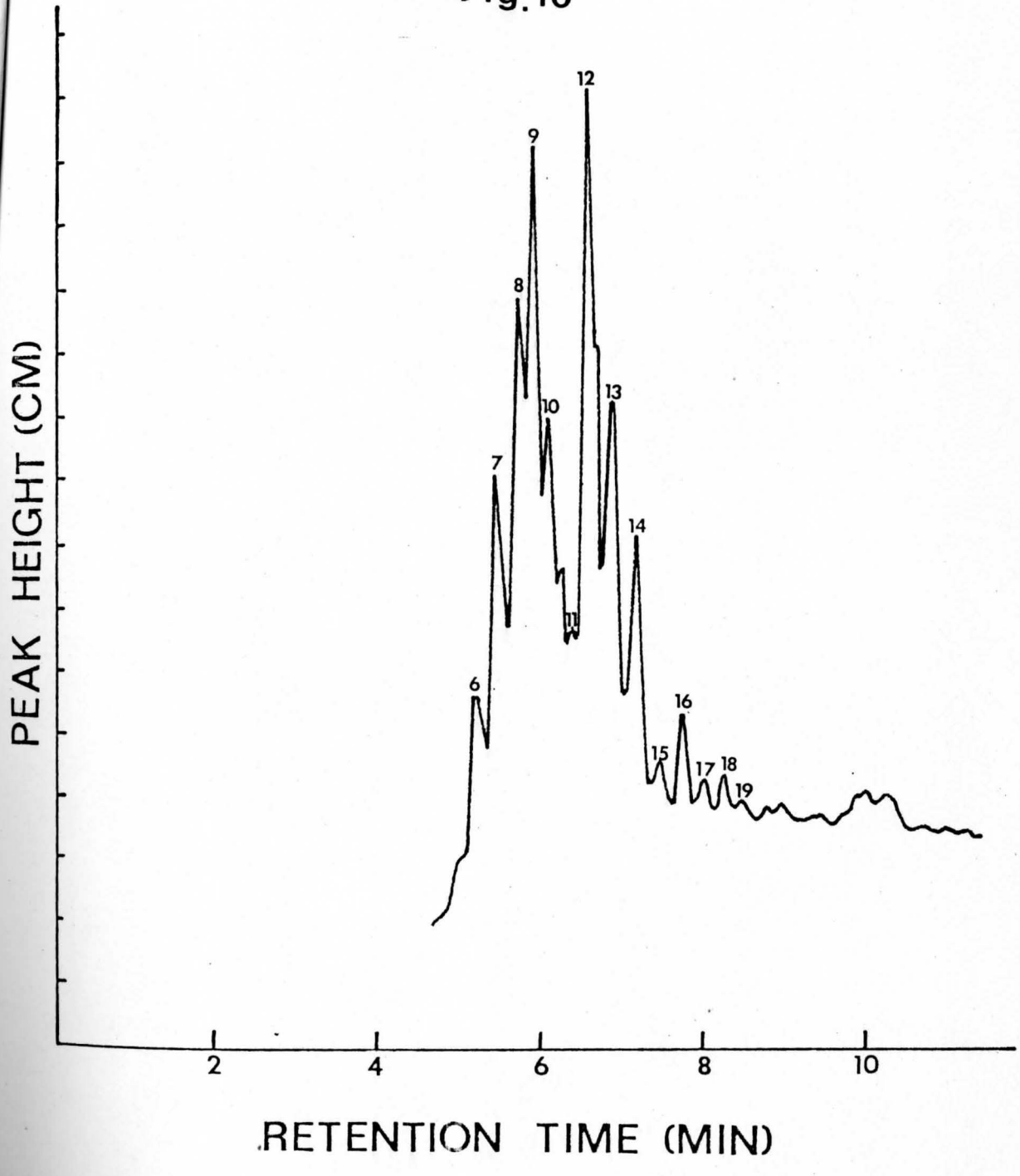
8
10
RETENTION TIME (MIN)

Figure 16. Gas-liquid chromatogram of the methyl esters of the fatty acids of starling 1.

Fig.16


PEAK HEIGHT (CM)
6
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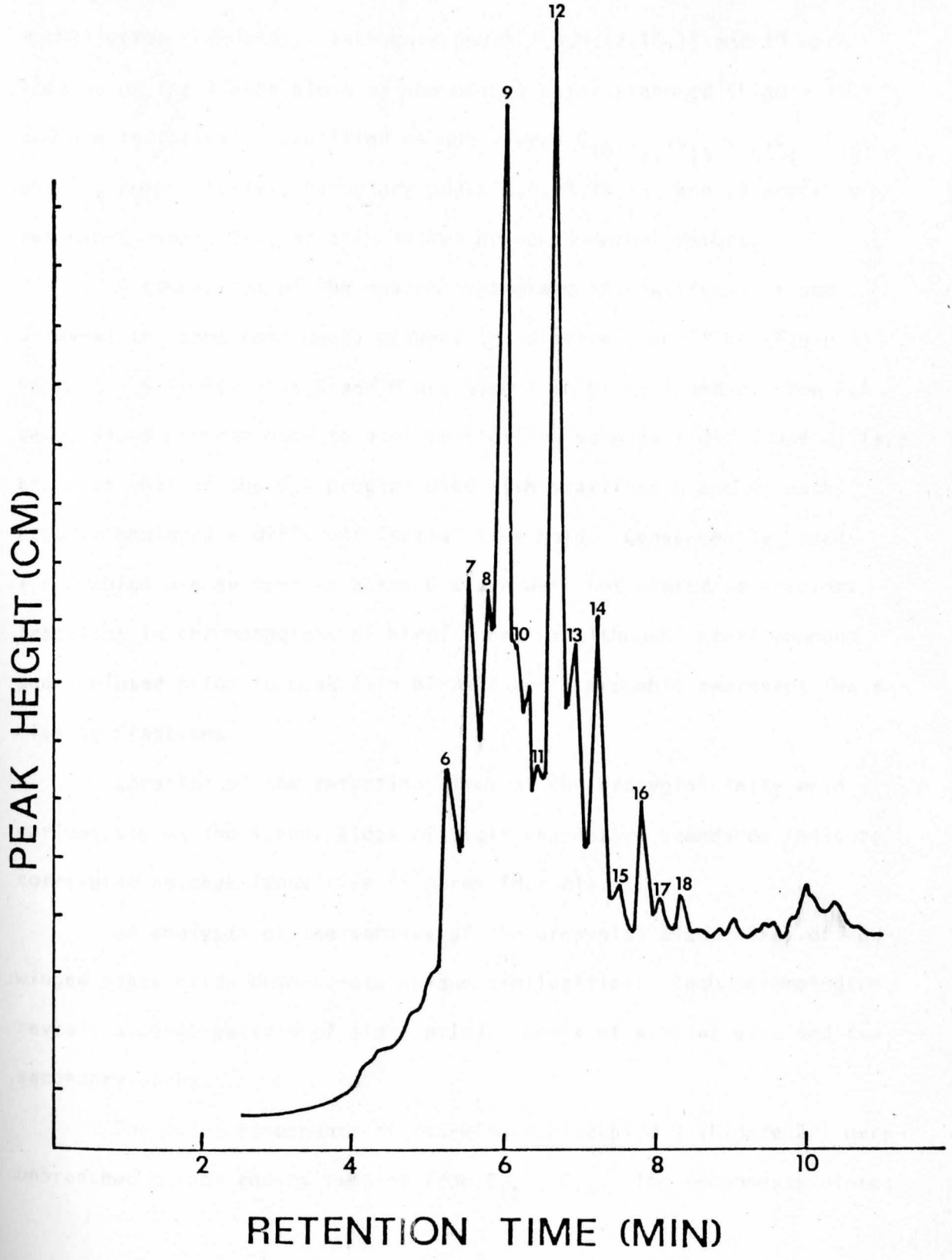
RETENTION TIME (MIN)

Figure 17. Gas-liquid chromatogram of the methyl esters of the fatty acids of starling J.

Fig. 17


PEAK HEIGHT (CM)
RETENTION TIME (MIN)
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17
18

methyl branched C_{14} (peak 11). Numerous secondary peaks were present, many of which eluted with the same retention times as the unbranched methyl ester standards. Secondary peaks 1,3,9,12,15,16 and 18 were located on the linear slope of the methyl ester standard (Figure 18) and are tentatively identified as unbranched C_{10},C_{11},C_{13},C_{14},C_{15},C_{16}, and C_{18} respectively. Secondary peaks 2,4,10,12,15, and 17 probably represent mono-, di-, or tri- methyl branched methyl esters.

A comparison of the gas chromatograms of starlings H,I and J reveal the same components present in relative quantities (Table 2). Peaks 1 - 5 in starling G and H are absent in birds I and J. The GLC temperature program used to analyse starling samples I and J was different than that of the GLC program used with starlings G and H: each program employed a different initial time hold. Consequently, peaks 1 - 5 which are evident in birds G and H were not eluted as distinct fractions in chromatograms of birds I and J: although, small rounded peaks eluted prior to peak 1 in birds I and J probably represent these missing fractions.

Location of the retention times of the uropygial fatty acid derivatives on the linear slope of their respective standards indicate corresponding peak identities (Figures 18 - 21).

An analysis of the samples of the uropygial gland waxes of red-winged black birds demonstrate unique similarities. Each chromatogram reveals a basic pattern of eight primary peaks of similar size and few secondary peaks.

The major components of red-winged blackbird K (Figure 22) were unbranched carbon chains ranging from C_{12} - C_{19}. The components eluted

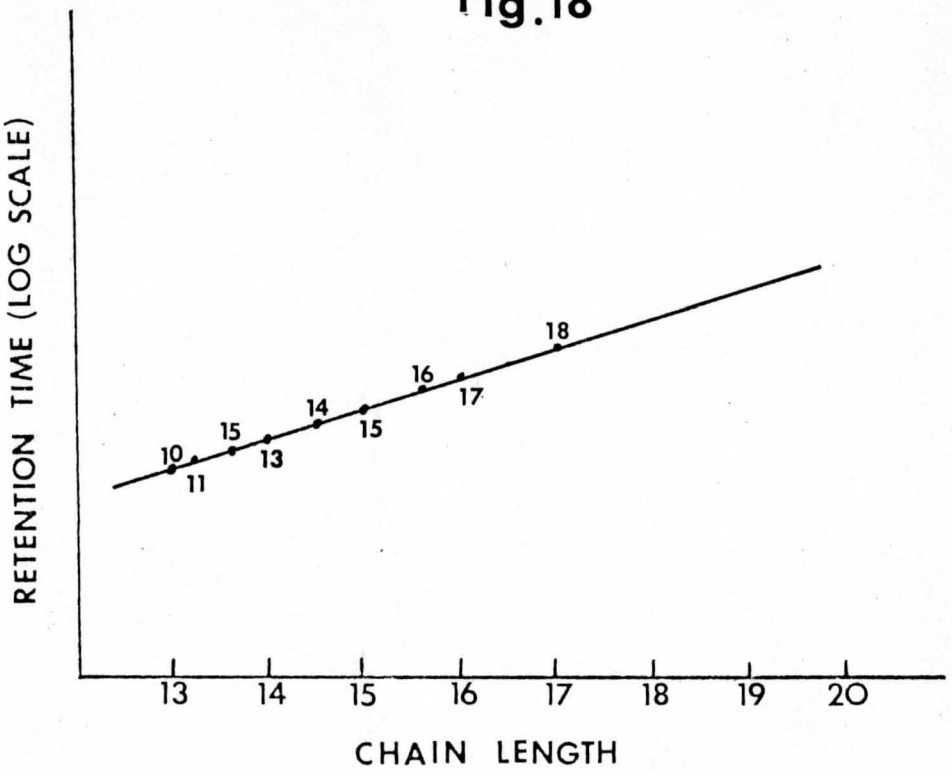
TABLE 2

PERCENTAGE UNDER PRIMARY PEAKS OF STARLING CHROMATOGRAMS

Fatty Acid Derivative	Peak no.	Starling Identification			
		G	H	I	J
Branched C_{12}	6	7.89	11.81	7.60	3.25
Unbranched C_{12}	7	12.98	16.45	14.51	10.74
Branched C_{13}	8	8.66	8.91	19.69	8.34
Branched C_{13}	9	17.38	27.18	11.19	25.09
Branched C_{14}	12	16.36	12.74	12.09	21.92

Figures 18 - 19. Comparison of retention times of sample methyl esters to standard methyl ester slope (logarithm of retention time of fatty acid methyl esters vs. chain length). Figure 18, starling G. Figure 19, starling H.

Fig.18


RETENTION TIME (LOG SCALE)
10
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17
18
13
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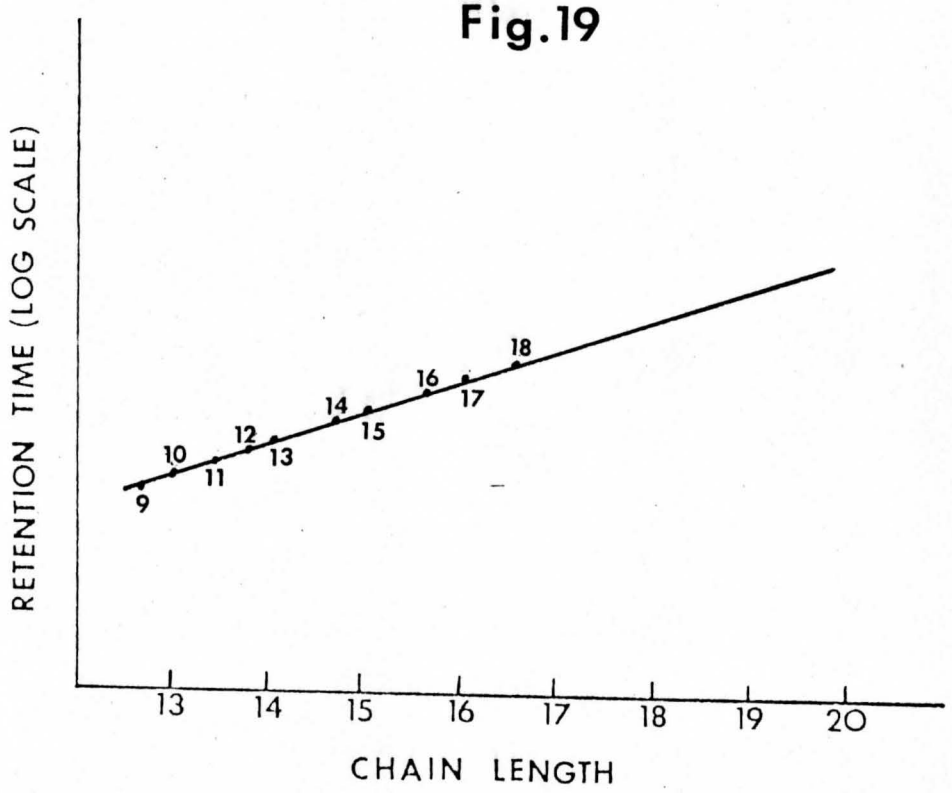
17
18
19
20
CHAIN LENGTH

Fig.19


RETENTION TIME (LOG SCALE)
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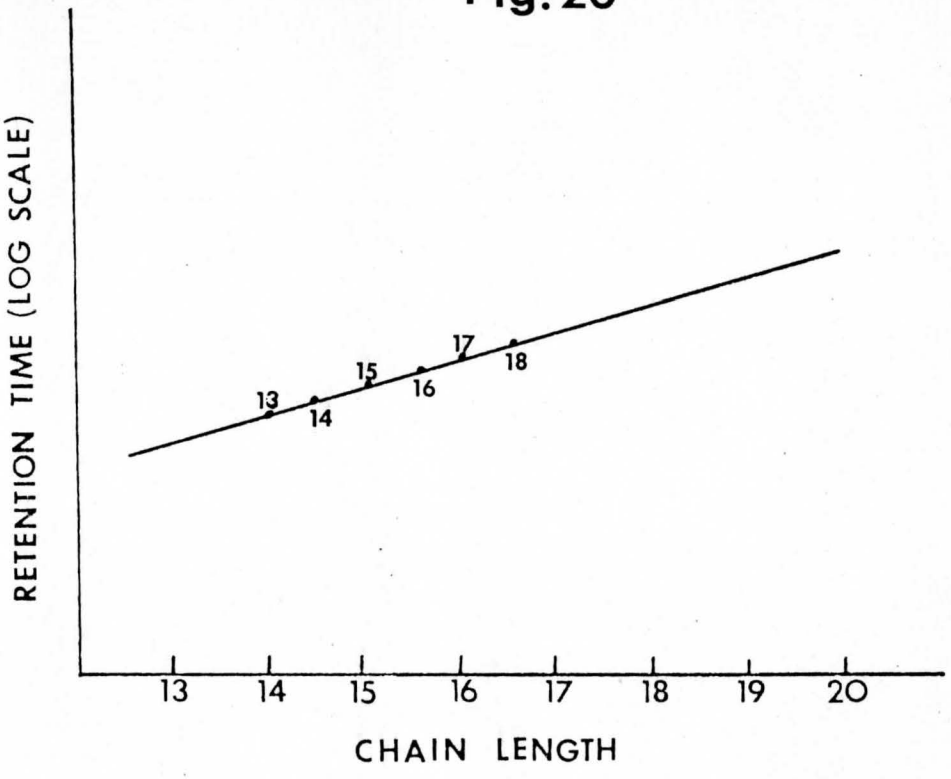
19
20
CHAIN LENGTH

Figure 20 - 21. Comparison of retention times of sample methyl esters to standard methyl ester slope (logarithm of retention time of fatty acid methyl esters vs. chain length). Figure 20, starling I. Figure 21, starling J.

Fig. 20


RETENTION TIME (LOG SCALE)
13
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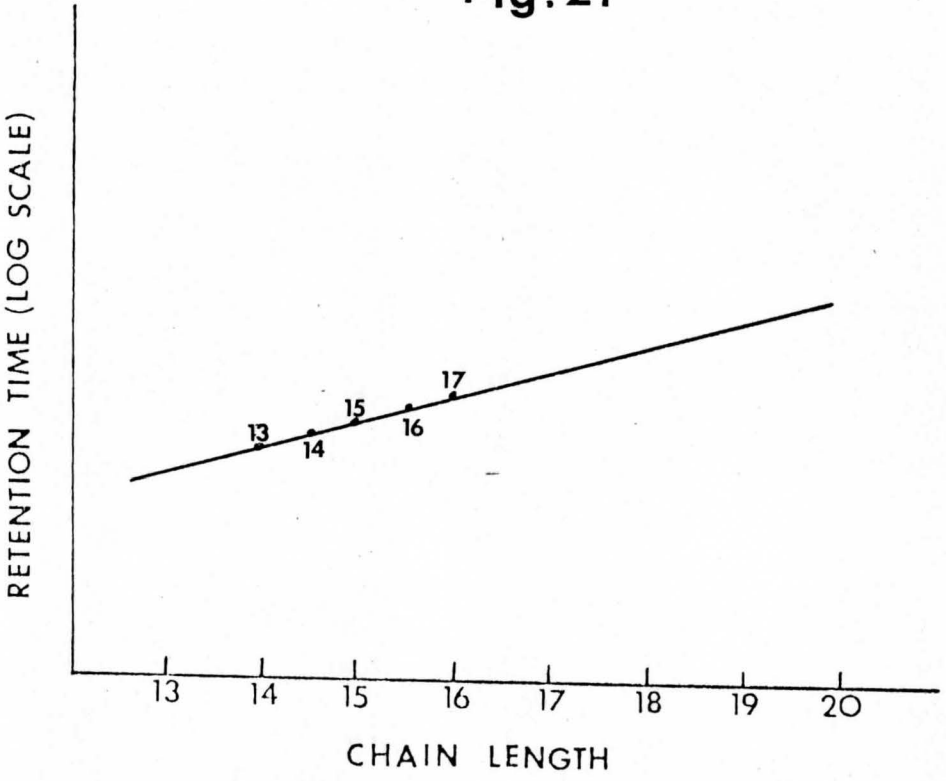
CHAIN LENGTH

Fig. 21


RETENTION TIME (LOG SCALE)
13
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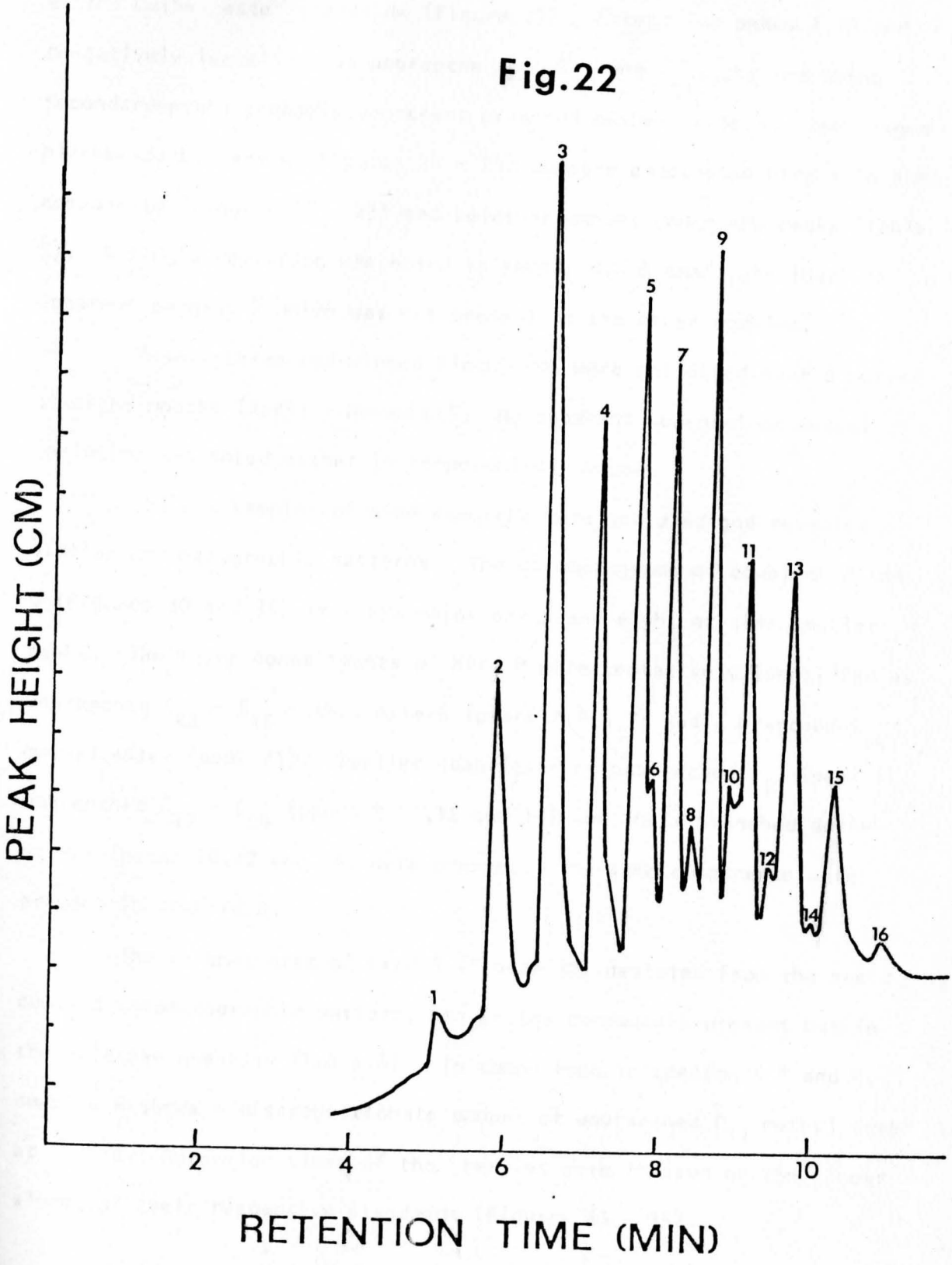
CHAIN LENGTH

Figure 22. Gas-liquid chromatogram of the methyl esters of the fatty acids of red-winged blackbird K.

Fig.22


PEAK HEIGHT (CM)
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RETENTION TIME (MIN)

at regular intervals and corresponded exactly with the retention times of the methyl ester standards (Figure 23). Except for peaks 1,15 and 16, tentatively identified as unbranched C_{11},C_{20} and C_{21}, the remaining secondary peaks probably represent branched methyl esters. Red-winged blackbirds L,M and N (figures 24 - 26) compare exactly to bird K in both components (Figures 27 - 29) and relative amount under the peaks (Table 3). A single deviation was noted in sample N. A small shoulder was apparent on peak 7 which was not present in the other samples.

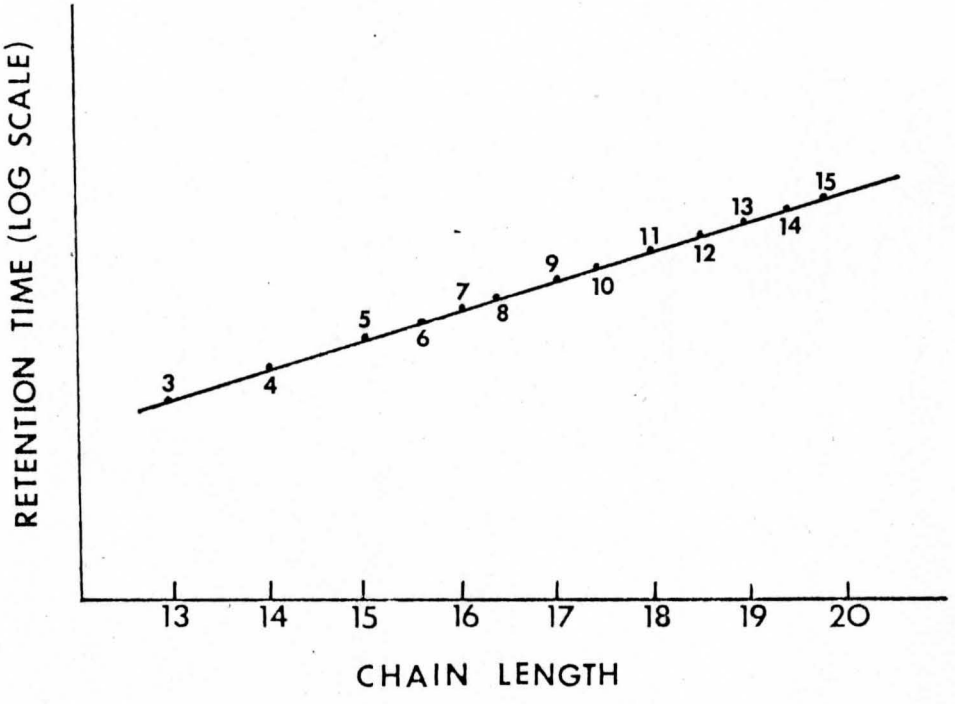
Twenty-three red-winged blackbirds were collected over a period of eight months (April - November). No apparent seasonal or sexual variation was noted either in component or amount.

The wax samples of nine cowbirds were analyzed and revealed similar chromatographic patterns. The chromatograms of cowbirds P and Q (Figures 30 and 31) show six major peaks and eight or nine smaller peaks. The major constituents of bird P were tentatively identified as unbranched C_{12} - C_{16} methyl esters (peaks 2,4-6,7) and a branched C_{19} methyl ester (peak 21). Smaller quantities of unbranched C_{11} (peak 1), unbranched C_{17} - C_{20} (peaks 9,11,12 and 15) and their branched derivatives (peaks 10,12 and 14) were present. The same components were present in cowbird Q.

The chromatogram of bird R (figure 32) deviates from the basic cowbird chromatographic pattern, not in the components present but in the relative quantity (Table 4). In comparison to specimens P and Q, cowbird R shows a disproportionate amount of unbranched C_{12} methyl ester at peak 2. Retention times of the samples were located on the linear slopes of their respective standards (Figures 33 - 35).

Figure 23. Comparison of retention times of sample methyl esters of standard methyl ester slope (logarithm of retention time of fatty acid methyl esters vs. chain length). Red-winged blackbird K.

Fig. 23


RETENTION TIME (LOG SCALE)
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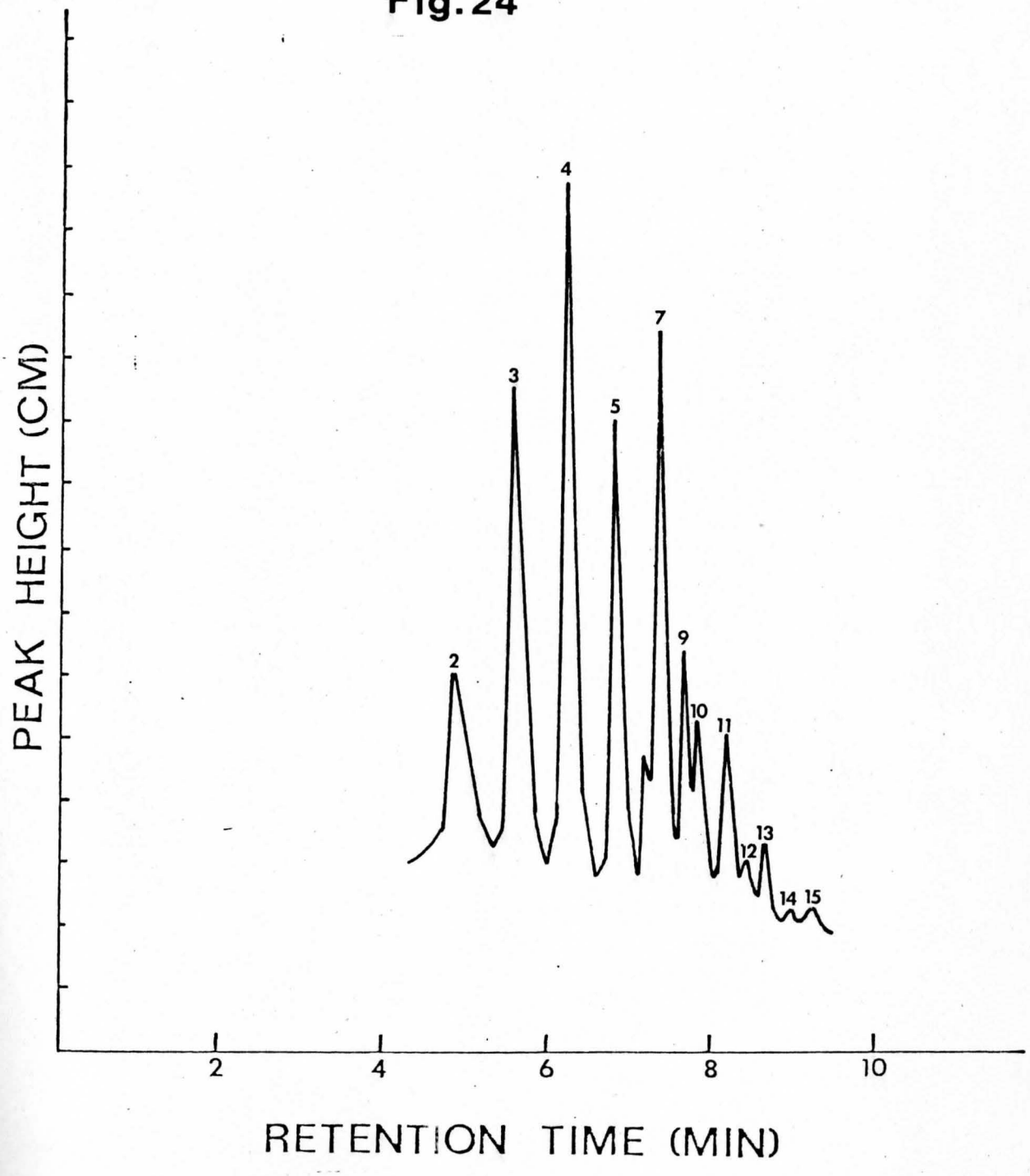
CHAIN LENGTH

Figure 24. Gas-liquid chromatogram of the methyl esters of the fatty acids of red-winged blackbird L.

Fig. 24


PEAK HEIGHT (CM)
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RETENTION TIME (MIN)

Figure 25. Gas-liquid chromatogram of the methyl esters of the fatty acids of red-winged blackbird M.

Fig. 25

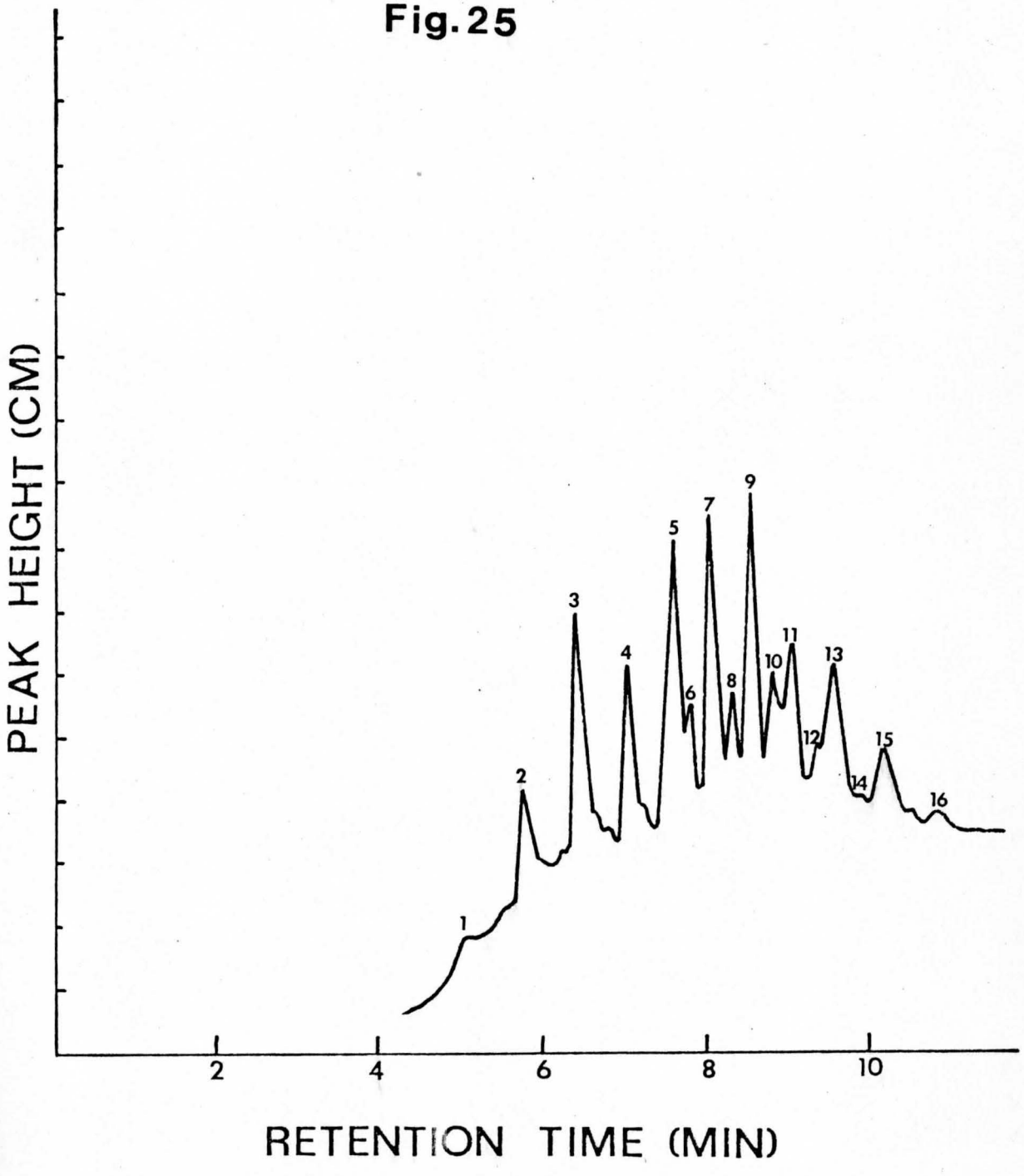
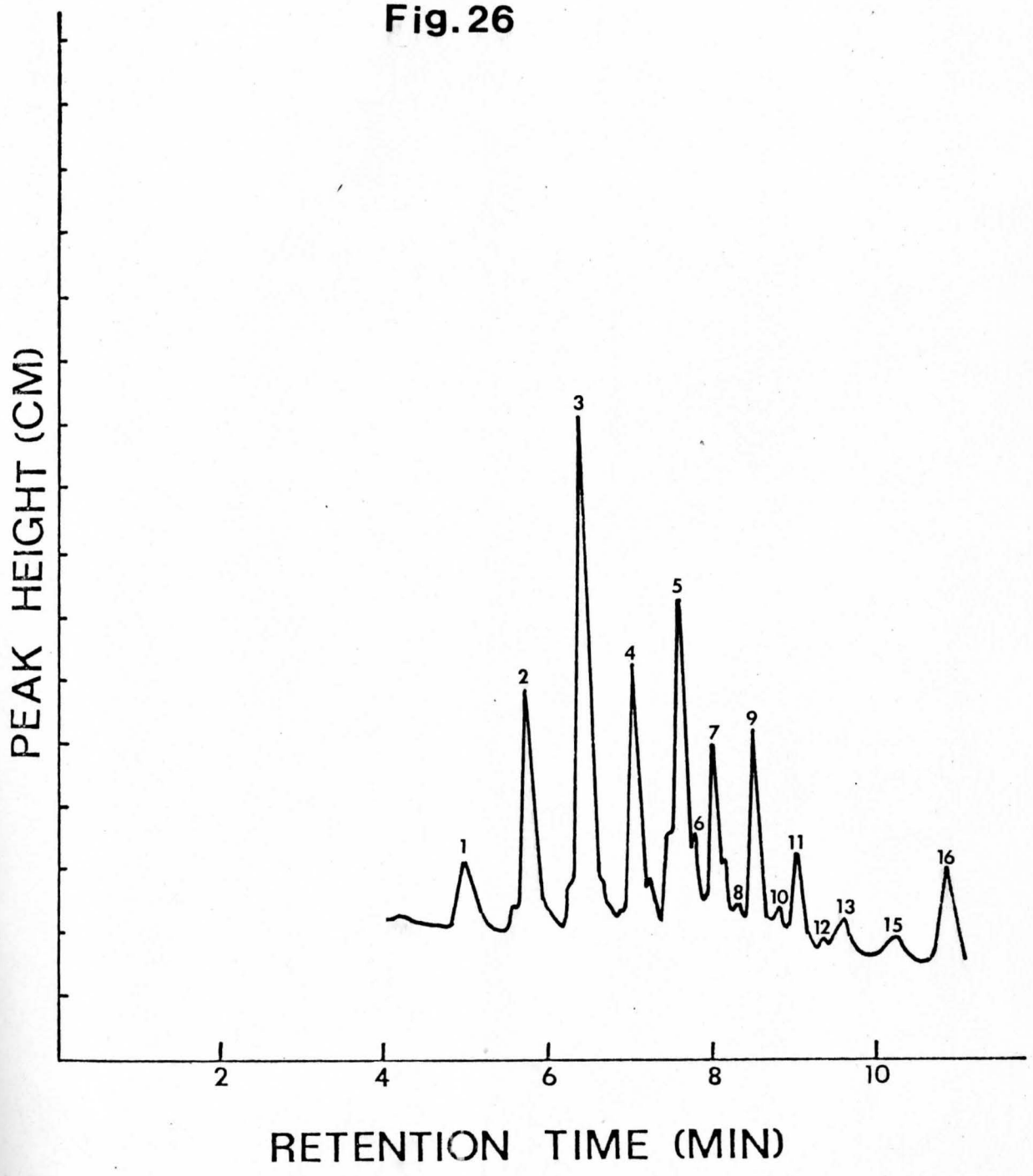


Figure 26. Gas-liquid chromatogram of the methyl esters of the fatty acids of red-winged blackbird N.

Fig. 26


PEAK HEIGHT (CM)
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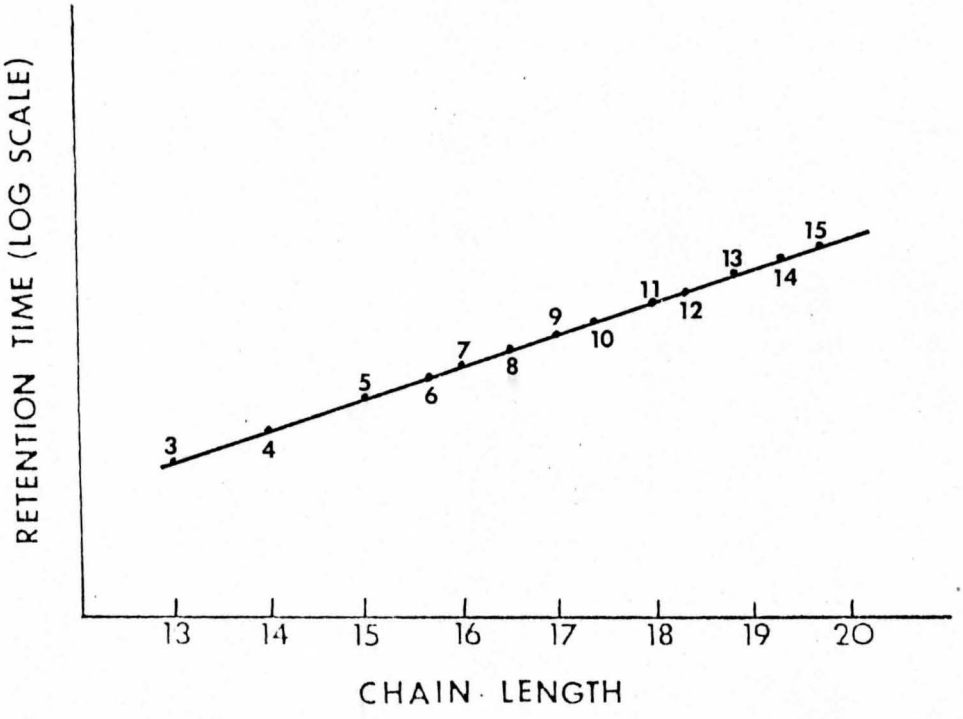
RETENTION TIME (MIN)

Figure 27. Comparison of retention times of sample methyl esters to standard methyl ester slope (logarithm of retention time of fatty acid methyl esters vs. chain length). Red-winged blackbird M.

Fig. 27


RETENTION TIME (LOG SCALE)
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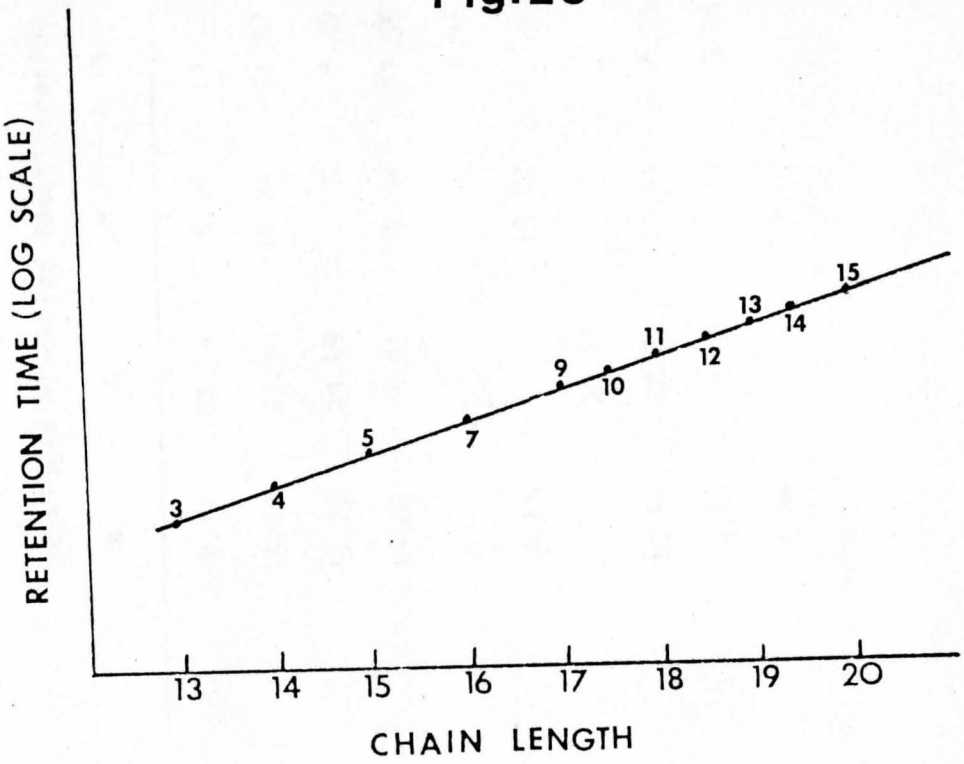
CHAIN LENGTH

Figures 28 - 29. Comparison of retention times of sample methyl esters to standard methyl ester slope (logarithm of retention time of fatty acid methyl esters vs. chain length). Figure 28, red-winged blackbird L. Figure 29, red-winged blackbird N.

Fig.28


RETENTION TIME (LOG SCALE)
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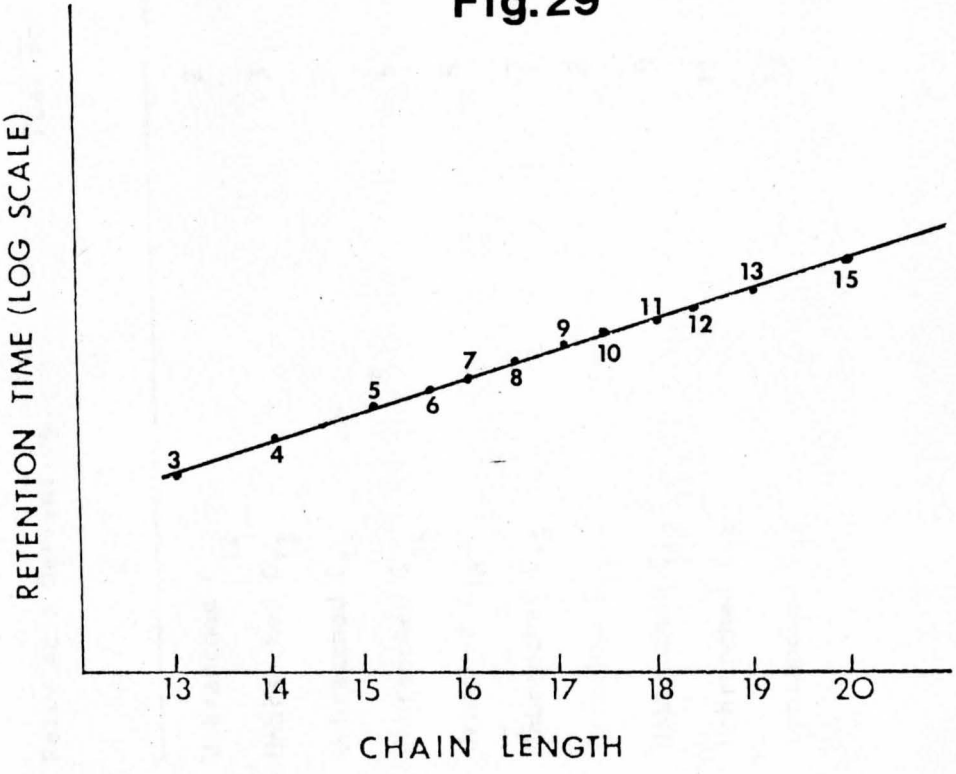
CHAIN LENGTH

Fig.29


RETENTION TIME (LOG SCALE)
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CHAIN LENGTH

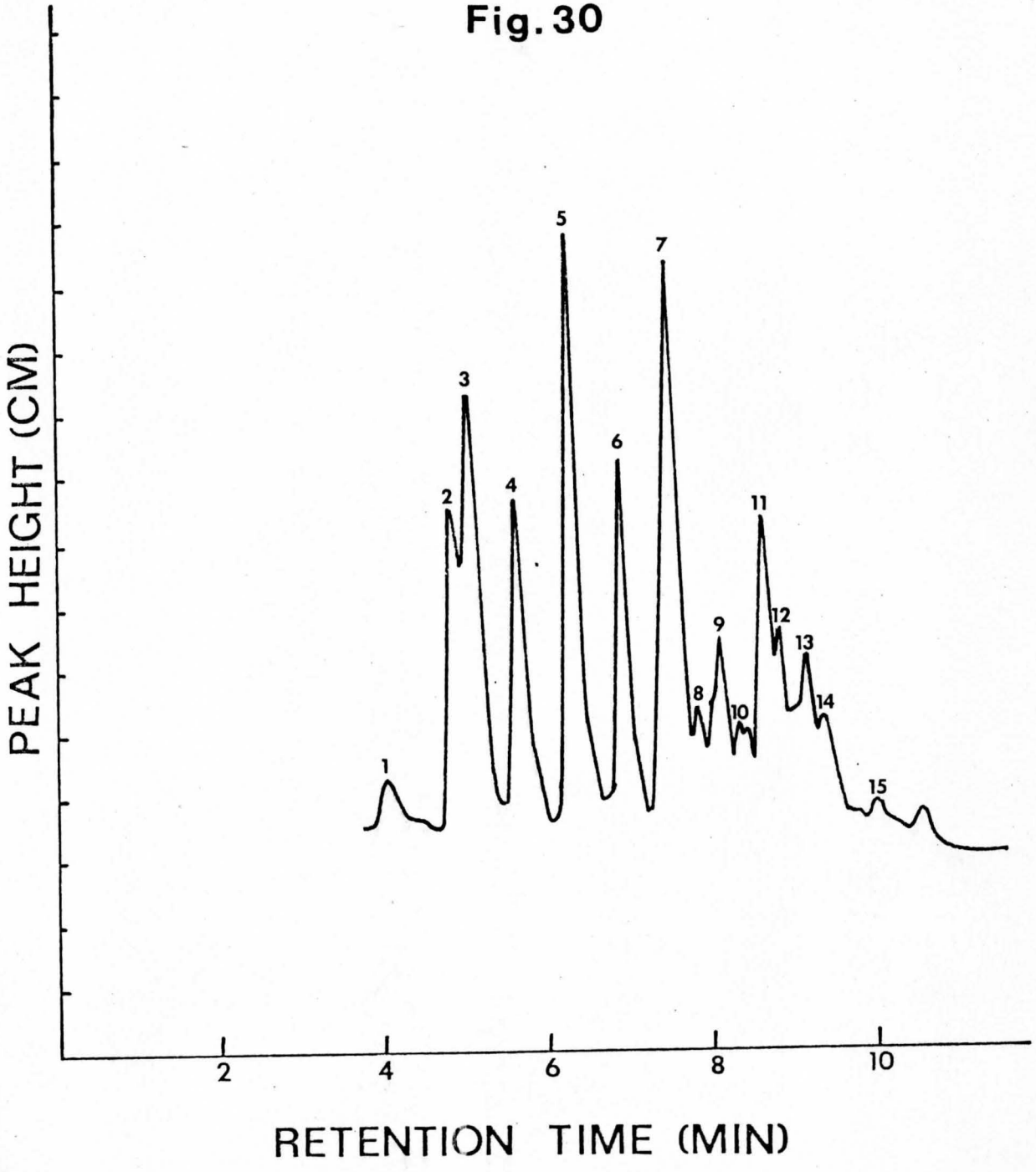
TABLE 3

PERCENTAGE UNDER PRIMARY PEAKS OF RED-WINGED BLACKBIRD

Fatty Acid Derivative	Peak no.	Red-winged Blackbird Identification			
		K	L	M	N
Unbranched C_{12}	2	9.12	10.54	5.56	10.52
Unbranched C_{13}	3	23.23	22.39	19.78	21.87
Unbranched C_{14}	4	13.27	29.49	7.05	9.29
Unbranched C_{15}	5	18.04	16.86	13.79	13.55
Branched C_{16}	6				8.91
Unbranched C_{16}	7	8.65		13.47	
Branched C_{17}	8		20.71		
Unbranched C_{17}	9	10.81	12.82	13.46	8.19
Unbranched C_{18}	11	4.43	2.50	4.28	3.76
Unbranched C_{19}	13	5.89		8.97	

Figure 30. Gas-liquid chromatogram of the methyl esters of the fatty acids of cowbird P.

Fig. 30


PEAK HEIGHT (CM)
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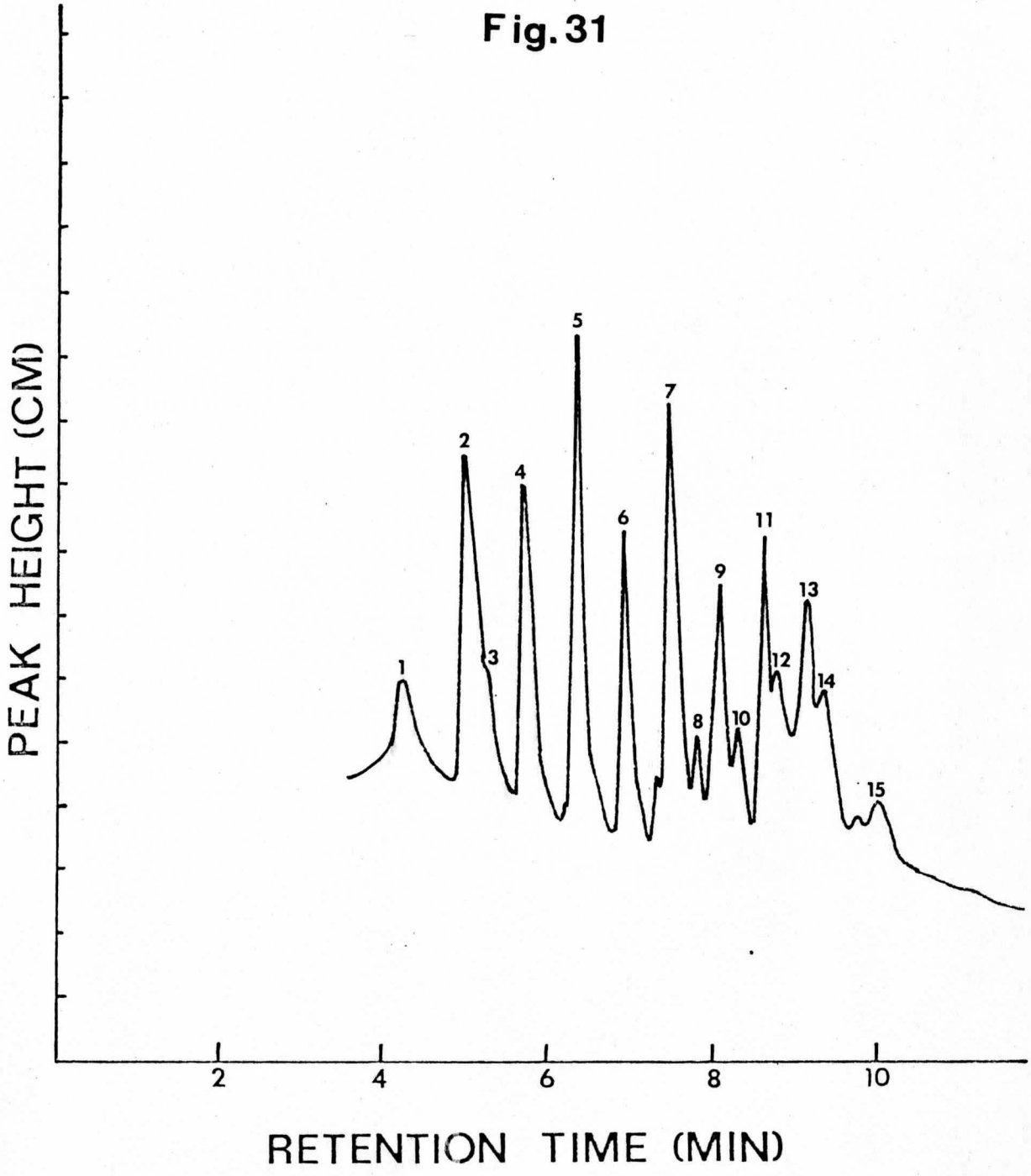
RETENTION TIME (MIN)

Figure 31. Gas-liquid chromatogram of the methyl esters of the fatty acids of cowbird Q.

Fig. 31


PEAK HEIGHT (CM)
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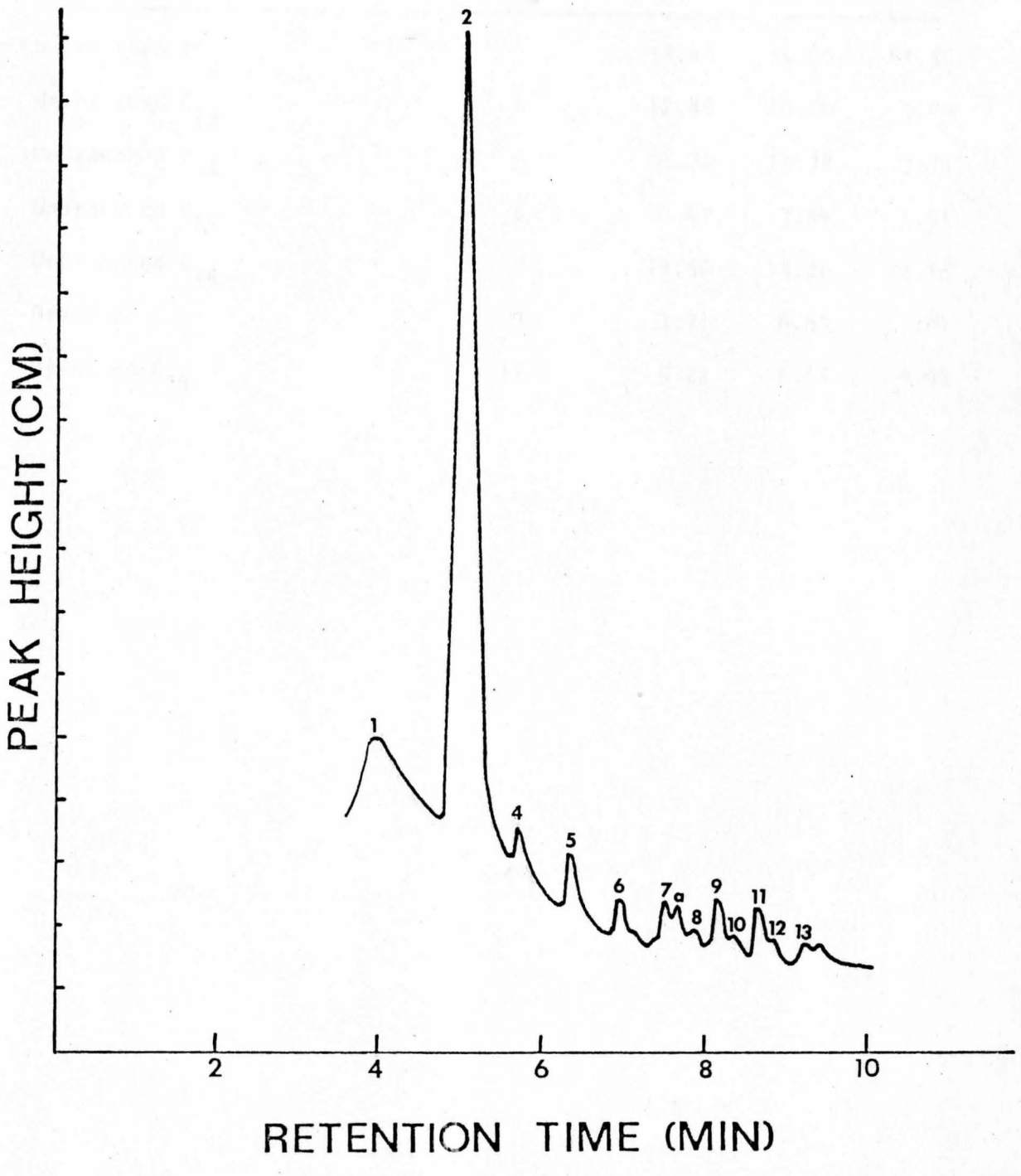
RETENTION TIME (MIN)

Figure 32. Gas-liquid chromatogram of the methyl esters of the fatty acids of cowbird R.

Fig. 32


PEAK HEIGHT (CM)
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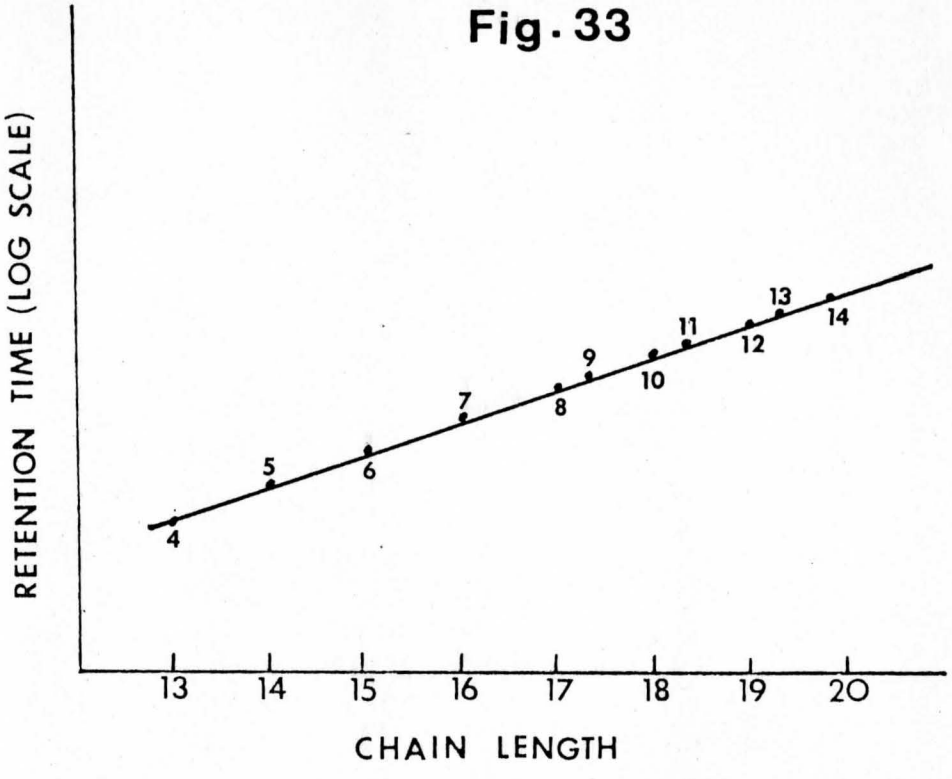
RETENTION TIME (MIN)

Figures 33 - 34. Comparison of retention times of sample methyl esters to standard methyl ester slope (logarithm of retention time of fatty acid methyl esters vs. chain length). Figure 33, cowbird P. Figure 34, cowbird Q.

Fig. 33


RETENTION TIME (LOG SCALE)
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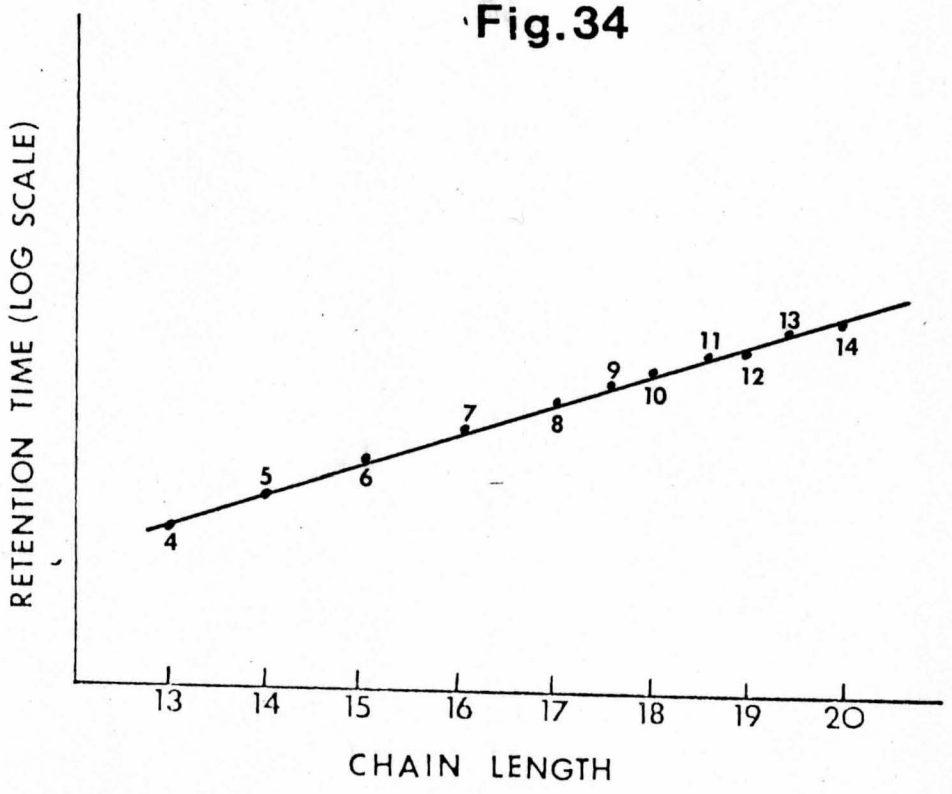
CHAIN LENGTH

Fig. 34


RETENTION TIME (LOG SCALE)
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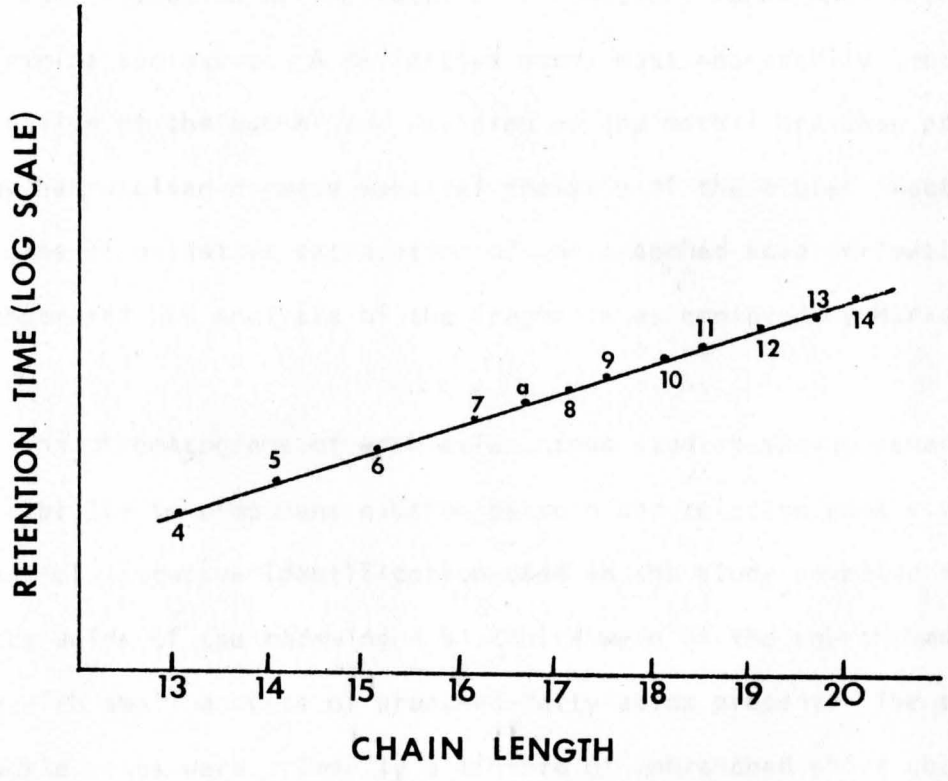
CHAIN LENGTH

Figure 35. Comparison of retention times of sample methyl esters to standard methyl ester slope (logarithm of retention time of fatty acid methyl esters vs. chain length). Cowbird R.

Fig. 35


RETENTION TIME (LOG SCALE)
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CHAIN LENGTH

DISCUSSION

In order to test individual uropygial gland samples, it was necessary to develop a technique which could facilitate the extraction, separation and analysis of extremely small quantities of wax. Thin layer separation followed by transesterification and gas-liquid chromatography analysis proved most effective for this purpose.

Identification of the fatty acid fractions based upon retention times remains tentative. A definitive study must necessarily include a determination of the number and position of the methyl branches present. This may be resolved by mass spectral analysis of the eluted fractions or by means of oxidative degradation of the branched acid derivatives with subsequent GLC analysis of the fragments as employed by Murray (1959).

The chromatograms of each avian group studied showed remarkable reproducibility in component elution pattern and relative peak sizes. The means of tentative identification used in the study revealed that the fatty acids of the red-winged blackbird were of the unbranched variety with small amounts of branched fatty acids present. The cowbird and grackle waxes were primarily a mixture of unbranched short chained fatty acids (C_{11} - C_{14}) combined with both branched and unbranched longer chained fatty acids (C_{15} - C_{20}). The starlings, unlike the other groups studied, revealed mainly branched fatty acids with the exception of an unbranched fraction identified as C_{12}. Although the chromatograms of the three species of Icteridae were more closely related to

each other than to the starling (Sturnidae), definite conclusions about these relationships must be tentative pending collections from numerous other avian hosts.

During the past decade there has been an intensified interest in the use of biochemical characteristics for improving systematic conclusions. This interest is not new but has been accelerated with the development of rapid and relatively simple techniques for chemical component isolation such as paper, thin-layer and gas-liquid chromatography, mass spectrophotometry and electrophoresis. As a result these data have provided new insights into systematics particularly with respect to phylogenetic inference.

Numerous authors (Odham, 1966: Jacob and Poltz, 1975 and Jacob and Grimmer, 1975) have suggested the usefulness of uropygial fatty acids as a valid chemotaxonomic character in elucidating interspecific vatiations. However, such data must necessarily be speculative due primarily to the fact that many previous analytical studies have used a composite rather than a single collection. Thus, distinct inter and intraspecific patterns may be obscured and unreliable.

It must also be noted when using these chemotaxonomic characters that each chemical, series of chemicals or chemical reactions have their own specific rate of evolutionary change (mosaic evolution). Such systems may be homologous to morphological characters which are known to evolve at different evolutionary rates. It is extremely important that such data be analyzed delineating the different evolutionary phenomena at the molecular and organismic level before definite conclusions be determined.

In this study data obtained from the uropygial gland waxes tend to define basic patterns among species and furthermore a probable pattern within a species population. If such data can be repeated for other species and subspecies within the Passeriformes, the potential exists for this technique as a useful tool in making certain taxonomic decisions. For example, chemotaxonomic data coupled with morphological, behavioral or ecological data could be successfully applied toward discrimination between two subspecies or between sibling species. It is reasonable to assume that geographical races may differ markedly in the wax moiety as the result of differences in habitat parameters. This data however must be coupled with morphological, behavioral, ecological and genetic studies in an effort to make the best possible taxonomic or systematic conclusions.

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