INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again – beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

Xerox University Microfilms
300 North Zeeb Road
Ann Arbor, Michigan 48106

73-23,932

BUSSJAEGER, Carolee Emley, 1942-
A COMPARATIVE STUDY OF BILE SALTS IN SUCKER FISHES (FAMILY CATOSTOMIDAE).

The University of Oklahoma, Ph.D., 1973
Biochemistry

University Microfilms, A XEROX Company, Ann Arbor, Michigan

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED.

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

A COMPARATIVE STUDY OF BILE SALTS IN SUCKER FISHES (FAMILY CATOSTOMIDAE)

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

CAROLEE EMLEY BUSSJAEGER

Oklahoma City, Oklahoma

1973

A COMPARATIVE STUDY OF BILE SALTS IN SUCKER FISHES (FAMILY CATOSTOMIDAE)

APPROVED BY

Thomas Briggs

Albert M. Chandler

Hubert Frings

Jerry S. Mayes

Leon Unger

DISSERTATION COMMITTEE

ACKNOWLEDGEMENTS

My major professor, Dr. Thomas Briggs, introduced me to the study of bile salts. His help, guidance and criticisms throughout this study and the writing are gratefully acknowledged. Thanks are also due to my committee members: Drs. Albert M. Chandler, Hubert Frings, Jary S. Mayes and Leon Unger for reading and criticizing this manuscript.

Financial support for the first three years of this study was provided by a National Science Foundation pre-doctoral traineeship. Mrs. Marjorie Bradley, Assistant to the Dean of the Graduate College in Norman, was most helpful in administering the research funds from the traineeship. A student research grant from Sigma Xi provided some travel funds for collecting trips.

Without the help of Mr. Sam Jackson, Fisheries Biologist for the City of Tulsa, and many other fisheries biologists throughout the state of Oklahoma as well as friends from other states, a sufficient number and variety of gallbladders would not have been available for this study.

I especially thank my husband, Louis, and son, Christopher, for their patience, support and understanding. Many other friends contributed in various ways to the completion of this study and to all of them a debt of gratitude is owed.

TABLE OF CONTENTS

page

LIST OF TABLES vi

LIST OF ILLUSTRATIONS vii

LIST OF TRIVIAL AND SYSTEMATIC NAMES OF COMPOUNDS ix

Chapter

I. INTRODUCTION 1

II. METHODS AND MATERIALS 12

Collection and identification of animals 12

Isolation, purification and subsequent treatment of bile salts 13

Bile Alcohol Sulfates 13

Bile Acid Conjugates 14

Anhydro-bile alcohols 15

Analytical and Chemical Procedures 15

Thin-layer Chromatography 15

Column Chromatography 16

Gas-liquid Chromatography 16

Melting Points 17

Specific Rotations 17

Infrared Spectra 17

Nuclear Magnetic Resonance 18

Mass Spectra 18

Table of Contents-- continued

Derivatives 18

Chemicals 19

III. RESULTS 20

Genus _Carpiodes_ 22

Genus _Catostomus_ 25

Genus _Chasmistes_ 28

Genus _Cycleptus_ 28

Genus _Hypentelium_ 29

Genus _Ictiobus_ 31

Genus _Minytrema_ 33

Genus _Moxostoma_ 34

IV. DISCUSSION 37

V. SUMMARY 54

REFERENCES 55

APPENDIX I 59

II 76

III 85

LIST OF TABLES

Table Page

1. Bile Acids and Alcohols Present in the Various Classes of Vertebrates (Haslewood, 1967a) 7

2. A List of Common and Scientific Names of Fishes Used in this Study and Major Biliary Component Found in Each. 21

3. Gas Liquid Chromatography Data 23

4. Melting Points of the Major Component Crystallized from Various Species. 24

5. Specific Rotation of the Major Biliary Component of the Various Species Studied. 26

6. Sulfate Conjugation as Determined by $BaSO_4$ Precipitation . . 30

LIST OF ILLUSTRATIONS

Figure		Page
1.	Proposed Stages in the Biosynthesis of Various Bile Acids and Alcohols from Cholesterol	4
2.	A Phylogenetic Representation of the Relationships of Various Families of Ostariophysan Fishes.	9
3.	Structures of the Three Compounds Identified from the Bile of Catostomid Fishes: 5α-cyprinol, 5α-chimaerol and Allocholic Acid	38
4.	Miller's (1958) "Hypothetical Phylogeny of the Family Catostomidae"	46
5.	A Proposed Alteration of Miller's "Hypothetical Phylogeny of the Family Catostomidae"	50
6.	Infrared Spectrum of Allocholic Acid from <u>Carpiodes carpio</u>	60
7.	Infrared Spectrum of 5α-chimaerol from <u>Catostomus macrocheilus</u>.	62
8.	In rared Spectrum of Anhydro-5α-chimaerol from <u>Catostomus plebius</u>	64
9.	Infrared Spectrum of 5α-chimaerol from <u>Chasmistes brevirostris</u>.	66
10.	Infrared Spectrum of 5α-cyprinol from <u>Ictiobus cyprinellus</u>	68
11.	Infrared Spectrum of Anydro-5α-cyprinol from <u>Ictiobus</u> spp.	70
12.	Infrared Spectrum of Alcohol Complex from <u>Moxostoma duquesnei</u>	72
13.	Infrared Spectrum of Anhydro Alcohol Complex from <u>Moxostoma erythrurum</u>.	74
14.	Nuclear Magnetic Resonance Spectrum of 5α-chimaerol from <u>Catostomus macrocheilus</u>	77

15. Nuclear Magnetic Resonance Spectrum of 5α-chimaerol from Chasmistes brevirostris. 79

16. Nuclear Magnetic Resonance Spectrum of 5α-cyprinol from Ictiobus spp. 81

17. Nuclear Magnetic Resonance Spectrum of 5α-chimaerol-5α-cyprinol complex from Moxostoma erythrurum 83

18. Mass Spectrum of 5α-chimaerol from Catostomus macrocheilus 86

19. Mass Spectrum of 5α-cyprinol from Ictiobus spp. 88

20. Mass Spectrum of Redhorse Alcohol Complex. 90

LIST OF TRIVIAL AND SYSTEMATIC NAMES OF COMPOUNDS

Trivial Name	Systematic Name
allocholic acid	5α-cholane,3α,7α,12α-triol, 24-oic acid
5α-bufol	5α-cholestane, 3α,7α,12α,25ξ,26(or 27)-pentol
5β-bufol	5β-cholestane, 3α,7α,12α,25ξ,26(or 27)-pentol
5α-chimaerol	5α-cholestane,3α,7α,12α,24,26-pentol
5β-chimaerol	5β-cholestane,3α,7α,12α,24,26-pentol
cholic acid	5β-cholane,3α,7α,12α-triol,24-oic acid
5α-cyprinol	5α-cholestane,3α,7α,12α,26,27-pentol
latimerol	5α-cholestane,3β,7α,12α,26,27-pentol
myxinol	5β-cholestane,3β,7α,16α,26(or 27)-pentol
5α-ranol	5α-27-nor-cholestane,3α,7α,12α,24ξ,26-pentol
5β-ranol	5β-27-nor-cholestane,3α,7α,12α,24ξ. 26-pentol
scymnol	5β-cholestane,3α,7α,12α,24ξ,26,27-hexol
trihydroxy coprostanic acid	5β-cholestane,3α,7α,12α-triol, 26(or 27)-oic acid

A COMPARATIVE STUDY OF BILE SALTS IN

SUCKER FISHES (FAMILY CATOSTOMIDAE)

CHAPTER I

INTRODUCTION

Bile salts are substances, derived from sterols in vertebrates, which have been perfected during evolution to assist in the emulsification, hydrolysis and absorption of fats. Haslewood (1962) lists three distinct chemical types fulfilling these conditions which have been found in nature. These are: 1) alcohols found primarily in the primitive fishes (Cyclostomata and Chondrichthyes), cyprinid fishes and amphibians; 2) C_{27} acids found in some amphibians and reptiles; 3) C_{24} acids (the predominant type) found in snakes, birds and mammals. In nature, all bile acids and alcohols are believed to occur in bile as the conjugated forms; bile alcohols are conjugated with sulfate, bile acids may be conjugated either with glycine or taurine.

The biogenesis of bile acids from cholesterol in higher animals, and their functions in regulating sterol metabolism and in gastrointestinal physiology have been elucidated by the development of elegant methodological approaches during the last two decades. Investigations into the nature of the compounds present in bile actually date back to the first decade of the nineteenth century and possibly earlier. The

history of this early work has been reviewed by Kritchevsky and Nair (1971). Matschiner (1971) designates three phases in the history of scientific investigations of bile salts. The first phase began in the middle of the nineteenth century when bile acids were first obtained in crystalline form. This phase terminated around the end of the nineteenth century and was characterized by uncertainty and confusion concerning the constituents of bile. The second phase included the development of methods of isolation and characterization of bile salts. This provided a strong background of structural knowledge concerning these compounds. In addition, these studies recognized the relationship between the bile acids and cholesterol, and later, the steroid hormones. Fieser and Fieser (1959) reviewed the contribution of Wieland and his collaborators to the structure of bile acids. Their work, which began about 1912, is classic in the field of steroid biochemistry.

The third, and current, scientific phase of bile salt investigation began about 1950. The application of reversed-phase partition chromatography to the separation of free (Bergström and Sjövall, 1951; and Sjövall, 1953) and conjugated (Bergström and Norman, 1953; and Norman, 1953) bile acids was probably the greatest single advance in the separation and purification of bile acids. Thin-layer and gas chromatography were introduced separately for the separation of bile acids. Together they provide a valuable means for the identification of bile acids and alcohols (Grundy, _et al_., 1965 and Ali, _et al_., 1966). Mass spectrometry, which was applied to the characterization of bile acids as early as 1958 (Bergström, _et al_.) may be coupled with gas-liquid chromatography. Used in conjunction with thin-layer chromatography, these

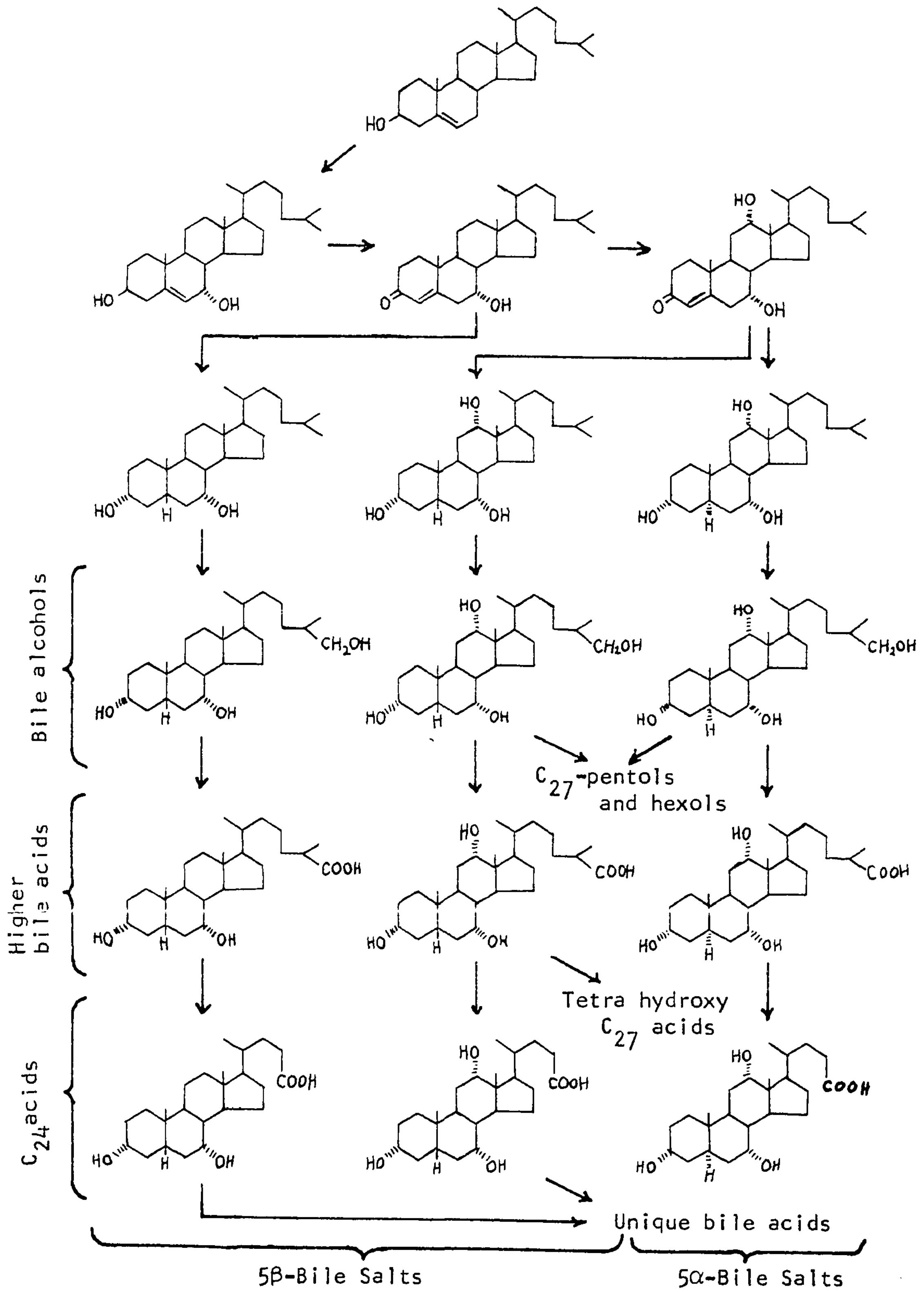
methods provide a means of thorough analysis of any source of naturally occurring bile salts (Eneroth, et al., 1966). As a result of these improved techniques, nearly half of the known bile acids and alcohols have been reported since 1960 (Matschiner, 1971).

The bile salts, as principal end products of cholesterol metabolism, occupy a focal position in our understanding of the role of steroids in biological systems. The molecular pleiomorphism exhibited by the bile acids and bile alcohols in the animal kingdom is a classic example of their role in biochemical evolution. Haslewood (1964) suggests that bile salts may be used as a criterion for comparisons of evolutionary relationships. The breakdown of cholesterol (C_{27}) to C_{24} bile acids occurs stepwise (Fig. 1).

1. The cholesterol ring structure is hydrogenated and extra hydroxyl groups are added to it.
2. The end of the side-chain is oxidized to give alcohols and then acids.
3. Carbon atoms are removed from the side chain, resulting finally in C_{24} acids.

On the basis of these biosynthetic pathways, substances that lie in the pathway between cholesterol and a C_{24} bile acid are less highly evolved chemically than the C_{24} acids. Therefore these substances, if they are found in the bile, and the organism that produces them are regarded as being less highly evolved biologically as well. However, some substances may also represent a branch line that biochemically cannot evolve to C_{24} acids. There is apparently an evolutionary change with respect to the type of conjugate formed since the bile alcohols are conjugated with

Figure 1. Proposed stages in the biosynthesis of various bile acids and alcohols from cholesterol. The pathways from cholesterol to the 5β bile acids have been studied and shown to occur in rat liver (Haslewood, 1967). The 5α counterparts of these compounds are thought to follow a parallel biosynthetic sequence. However, this has not yet been demonstrated.


Bile alcohols
Higher bile acids
C24 acids
C27-pentols and hexols
Tetra hydroxy C27 acids
Unique bile acids
5β-Bile Salts
5α-Bile Salts

sulfate while the bile acids are conjugated with either glycine or taurine. Finally, the 5α configuration is considered to be more primitive than the 5β configuration (Haslewood, 1967a).

In bile salts, the progression from substances containing the entire C_{27} skeleton of cholesterol to C_{24} acids entails several changes which seem to be of evolutionary significance: 1) the change from alcohols to acids, 2) the change from C_{27} compounds to C_{24} compounds, and 3) the change from sulfate as the conjugate with alcohols to glycine or taurine as the conjugate with acids (Haslewood, 1967a). In this progression each chemical stage can be regarded as more advanced in an evolutionary sense, than those previous to it. However, more than one stage may be represented at the same time in a single species. Also, there are bile salts apparently confined within vertebrate groups and peculiar to the group, but which do not seem to be more or less advanced in the sense discussed above.

Table I summarizes the major groups of vertebrates and the major type of bile salt found in each group. This shows the close correlation between chemical types and systematic classification which can be readily interpreted in terms of evolution. It is apparent also, that the evolution of bile salts leading from C_{27} alcohols through C_{27} acids to C_{24} acids must have occurred independently at least twice (i.e. cholic acid is seen to occur in the bony fishes as well as in amphibians, reptiles, birds and mammals). Such evolution may have gone on separately in each of the vertebrate classes in which the apparent evolutionary progression can be demonstrated. Bile salt evolution in some groups (e.g. Crocodilia) seems to have ceased and in others (e.g. Ranidae) it appears to be still in progress (Haslewood, 1964).

Table I. Bile Acids and Alcohols Present in the Various Classes of Veretbrates (Haslewood, 1967a).

Vertebrate Class	Bile Alcohols or Acids Found in Class	Type of Conjugation	Some Examples
Cyclostomata	C_{27} Alcohol	Sulfate	Myxinol
Chondrichthyes	C_{27} Alcohol	Sulfate	5β-Chimaerol Scymnol
Osteichthyes	C_{27} Alcohol	Sulfate	5α-Chimaerol Latimerol 5α- & 5β-Cyprinol
	C_{24} Acid	Taurine	Cholic acid Allocholic acid
Amphibia	C_{27} Alcohol	Sulfate	5α- & 5β-Bufol 5α- & 5β-Ranol
	C_{27} Acid	Taurine	3α,7α,12α-trihydroxy-5β-cholestanoic acid
	C_{24} Acid	Taurine	Cholic acid
Reptilia	C_{27} Acid	Taurine	3α,7α,12α-trihydroxy-5β-cholestanoic acid
	C_{24} Acid	Taurine	Cholic acid
Aves	C_{24} Acid	Taurine	Cholic acid
Mammalia	C_{24} Acid	Taurine	Cholic acid

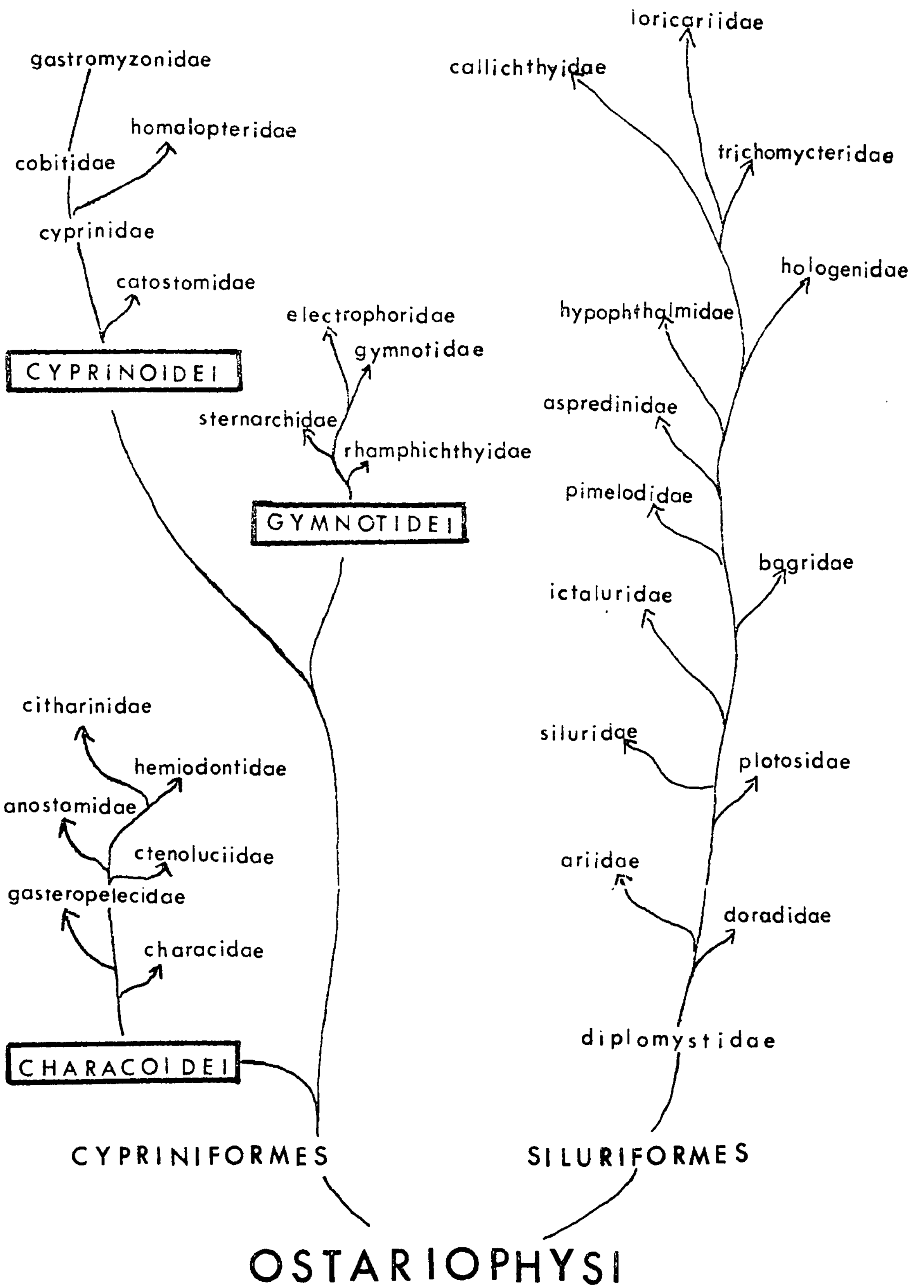
This study is centered on teleostean fishes, specifically the family Catostomidae. Figure 2 is a phylogenetic representation of the superorder Ostariophysi, the large group of teleostean fishes to which the family Catostomidae belongs. A comparison of the two orders of ostariophysan fishes provides an interesting contrast since members of the Siluriformes (catfishes) have taurocholate as their major bile salt, as do most teleostean fishes. Members of the Cypriniformes (suckers and minnows) have C_{27} alcohol sulfates as their major biliary components, with the exception of the genus *Carpiodes*.

The discovery of allocholic acid in the River carpsucker, *Carpiodes carpio* (Briggs and Bussjaeger, 1972), suggested an unusual diversity of bile salts in the family Catostomidae. The principal components of bile are usually invariant within any given vertebrate family. Hence, the discovery of a C_{24} bile acid in a family previously known only to have C_{27} bile alcohols suggested that bile salt evolution is currently in progress in catostomid fishes.

The family Catostomidae is comprised of 11 genera containing 57 species in Central and North America. In addition, one genus (*Myxocyprinus*) which has three species occurs in northeastern Siberia and Yangtse Kiang. The catostomid fishes are well represented in Oklahoma with eight of the 11 extant genera (14 of 57 species) occurring in the state. Furthermore, one genus which does not occur in the state (*Lagochila*) is thought to be extinct (Moore, 1968).

Hubbs (1930) states, "In few groups of North American freshwater fishes have ichthyologists exhibited as little agreement as to specific limits or as to nomenclature, as in the suckers or Catostomidae.

Figure 2. A phylogenetic representation of the relationships of various families of Ostariophysan fishes. Adapted from Greenwood, *et al.*, 1966.


gastromyzonidae
homalopteridae
cobitidae
cyprinidae
catostomidae
CYPRINOIDEI
electrophoridae
gymnotidae
sternarchidae
rhamphichthyidae
GYMNOTIDEI
citharinidae
hemiodontidae
anostomidae
ctenoluciidae
gasteropelecidae
characidae
CHARACOIDEI
CYPRINIFORMES
loricariidae
callichthyidae
trichomycteridae
hologenidae
hypophthalmidae
aspredinidae
pimelodidae
bagridae
ictaluridae
siluridae
plotosidae
ariidae
doradidae
diplomystidae
SILURIFORMES
OSTARIOPHYSI

Specific names have been juggled back and forth between distinct species and even between different genera, and the concept of the species variously broadened or restricted." To judge from the number of taxonomic changes in the Catostomidae in the most recent edition of the list of Common and Scientific Names of Fishes (Bailey, *et al.*, 1970) Hubbs' statement is still true.

The purpose of this study was to isolate and identify the major biliary component in each available genus of the family Catostomidae. From this, we hoped to see the extent of the diversity in catostomid bile salts and perhaps later be able to determine what selective pressures are involved in the evolution of bile salts. Haslewood (1964) indicates that systematic use of bile salts as a taxonomic character is too 'coarse' or too 'insensitive' in its variation to be useful at a specific or even generic level. However, bile salt variation might be a character which can be used to clarify relationships of the various genera within the family.

CHAPTER II

METHODS AND MATERIALS

Collection and Identification of Animals

Animals from which the bile samples were taken for this study were obtained in a variety of ways. Those collected by the author were taken with the use of seines and by electro-fishing. Many specimens were taken with the co-operation of various fisheries biologists in Oklahoma and Arkansas during their population samplings with rotenone and gill nets. Bile samples from Buffalo fishes, genus *Ictiobus*, were collected by a commercial fisherman and sampies from *Chasmistes brevirostris* were taken from fresh fishes discarded by fishermen around Antero Reservoir, near Fairplay, Colorado. Specimens of the Blue sucker, *Cycleptus elongatus*, were captured in the draft tubes of Denison Dam, Lake Texoma.

In all cases, gallbladders were taken either from freshly caught specimens or from specimens which had been frozen soon after capture. The gallbladders were immediately put in an excess of 95% ethanol to avoid bacterial contamination. If there was any suspicion that the animal had begun to decay so that bacterial contamination may have altered the structure of the bile salts, the specimen was not used.

Specimens were identified with the aid of *The Freshwater Fishes* (Eddy, 1957), "Fishes" (Moore, 1968) and "Know Your Oklahoma Fishes"

(Oklahoma Department of Wildlife Conservation, 1965). The common and scientific names of the fishes referred to in this study are those approved by the American Fisheries Society (Bailey, et al., 1970).

Isolation, Purification and Subsequent Treatment of Bile Salts

General procedures for isolating and purifying bile samples are those outlined by Haslewood (1967a). Gallbladders which had been collected and stored in an excess of 95% ethanol were cut into tiny pieces. After the debris was removed by filtration, the solvent was evaporated leaving a solid residue which was washed with light petroleum (b.p. 30-60°) to remove fatty materials. This crude bile sample was then analyzed by thin-layer chromatography.

Bile Alcohol Sulfates

Those bile salts composed of bile alcohol sulfates were subjected to solvolysis by a procedure designed in our laboratory. Crude defatted bile salts (200 mg) were dissolved in concentrated formic acid (5 ml) with warming (50-70°), and left several hours or overnight. (Partial formylation yields a product which is soluble in dioxane). The solution was evaporated under N_2 and finally in a vacuum dessicator to remove all formic acid. The formylated bile salt mixture was dissolved in 1,4-dioxane (10 ml) and a minute amount of sulfuric acid in dioxane (0.2 ml of a solution containing 0.1 ml concentrated H_2SO_4 in 10 ml dioxane) added as catalyst. Solvolysis usually occurred immediately as indicated by the formation of a fluffy precipitate.

On completion of solvolysis, the dioxane was evaporated under N_2. Formyl groups were then removed by saponification with ethanolic

KOH, leaving the free bile alcohol. Acidification, extraction with ethyl acetate and drying with Na_2SO_4, left the product in a clear, pale yellow solution. Evaporation of the ethyl acetate followed by warming with acetone yielded the crystalline product in some cases. In other instances, the product had to be purified by reversed-phase column chromatography before it could be crystallized. In all cases, repeated crystallization to a constant melting point concluded the purification procedure.

The water solution from which the bile alcohol was extracted was treated with $BaCl_2$. The resultant precipitation of $BaSO_4$ confirmed the conjugation with sulfate. In some cases the $BaSO_4$ was filtered, dried and weighed to determine the molar relationship between the amount of sulfate vs. the amount of bile alcohol in the conjugate. During the purification process, the course of the reactions for formylation, solvolysis and saponification were monitored by thin-layer chromatography.

Bile Acid Conjugates

In the case of the river carpsucker, *Carpiodes carpio*, (whose major biliary component was a bile acid conjugated with taurine) the crude bile salts (1.18 g) were dissolved in 2*N* NaOH (8 ml) and heated in a stainless steel bomb at 125° for 5 hr. The hydrolysate was diluted with water, acidified with 12*N* HCl (2 ml) and extracted with ethyl acetate. The washed extract was filtered through Na_2SO_4 and taken to small volume, whereupon a precipitate formed and was collected (320 mg). The free bile acids were analyzed by thin-layer chromatography. Purification of the bile acid was achieved through a series of crystallizations with the final crystallization from acetone.

Anhydro-bile Alcohols

Anhydro-forms of the bile alcohols were isolated from Buffalo fishes (Ictiobus spp.), the Rio Grande sucker (Catostomus plebius), and the Redhorses (Moxostoma spp.) by using a modification of the procedure of Bridgwater, et al. (1963). Crude bile salts (1 g) were dissolved in H_2O (2.5 ml) in a stainless steel bomb. To this mixture, 2N NaOH (7.5 ml) was added so that the final concentration was 1 g crude bile salts in 10 ml 1.5N NaOH. The sealed bomb was heated for 16 hr at 140°. The contents were rinsed out with water and diluted to 200 ml. The hydrolysate was extracted with butanol to remove the anhydro-alcohol.

Analytical and Chemical Procedures

Thin-layer Chromatography

As indicated previously, thin-layer chromatography (TLC) was used extensively throughout this study. Analyses were performed using silica gel H (Brinkmann Instruments, Westbury, N.Y.) in 250-μm layers on glass plates preground to that depth (Kontes Glass Co., Vineland, N.J.). Solvent systems are described below. Samples were detected with an anisaldehyde spray reagent (Kritchevsky, et al., 1963) which made it possible to distinguish some samples on the basis of color.

Thin-layer chromatography was used in the initial qualitative analysis of the crude bile. Color and R_f of spots derived from samples whose bile salt components were unknown were compared with those from samples whose bile salt components were known. The solvent system used for crude conjugated bile salts was modified from Sasaki (1966): ethyl acetate-butanol-acetic acid-water (40:30:10:10). Free bile acids and

alcohols were analyzed with ethyl acetate-methanol-acetic acid (85:10:5). Permanent records were made by photographing TLC plates with Polaroid color film using a Polaroid MP Land Camera.

Column Chromatography

In some cases the free bile alcohol did not crystallize readily after extraction. It was then purified by reversed-phase column chromatography. The support was Hyflo Supercel (Bergström and Sjövall, 1951) with chloroform-octanol (1:1 v/v) as the stationary phase. Five-milliliter fractions of the moving phase, methanol-water (52:48 v/v), were collected in tared tubes, evaporated to dryness, and the residues detected by weight. Fractions which contained a significant amount of material were analyzed by TLC to determine the presence of bile alcohols.

Gas-liquid Chromatography

Gas-liquid chromatography (GLC) was performed with a Perkin-Elmer Model 880 gas chromatograph having a 180-cm coiled glass column, 2mm ID, at 260° and a N_2 flow of 35 ml/min. In some cases GLC was performed with a Varian Model 1740 gas chromatograph having a 150-cm coiled glass column, 2 mm ID, at 275° and a N_2 flow of 50 ml/min. Columns were packed with 3% OV 17 on Gas Chrom Q (Applied Science Laboratories, State College, Pa.). Samples were converted to trimethylsilyl (TMS) ethers for GLC as follows (Horning <u>et al</u>., 1967): a sample (50 μg) was dissolved in bistrimethylsilyl acetamide (25 μl); trimethyl chlorosilane (5 μl) was added as catalyst. The sample was diluted with methylene chloride (25 μl) prior to analysis. All samples contained methyl cholate TMS ethers as an internal standard. One-microliter portions (about 1 μg

of original sample) were injected into the instrument. Relative proportions of the components present in GLC samples were determined by comparison of peak areas (height x base at half height).

Melting Points

Melting points were determined with a Nalge Company (Rochester, N.Y.) hot-stage melting-point apparatus and are uncorrected. The melting points reported are those taken after the final crystallization when the crystals had been dried _in vacuo_ for 1 hr at 100° over P_2O_5.

Specific Rotations

Specific rotations were determined at 25° with a Franz Schmidt and Hoensch polarimeter having a 2-dm light path. Samples of the highest purity obtainable having a constant melting point, were dried _in vacuo_ at 100° over P_2O_5 for at least one hour. Ethanol solutions were used for rotation determinations.

At least seven readings were made with each eye. The high and low readings for each eye were omitted and the other ten readings averaged to obtain the observed rotation. Specific rotations were then calculated according to the formula: $[\alpha]_D = \frac{\alpha}{c \times l}$ where $[\alpha]_D$ is the specific rotation of the sample using the sodium D line as the light source; α, the observed rotation; c, the concentration of the material in g/ml; and l, the length of the light path in dm.

Infrared Spectra

Infrared spectra (IR) were recorded with a Perkin-Elmer model 21 spectrophotometer and a Beckman IR 10 spectrophotometer, using KBr

pellets. Prior to analysis, samples were dried *in vacuo* over P_2O_5 at 100° for at least one hour.

Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectra were recorded by Mr. Bob Hayes (Department of Chemistry, University of Oklahoma, Norman, Oklahoma) with a Varian A-60 spectrometer. Samples were dissolved in deuterated pyridine with trimethyl silane as the internal standard. Spectra were recorded with a sweep time of 250 sec, sweep width 500 hz and spin rate 40 rps.

Mass Spectra

Mass spectra were recorded by Mr. Keith Kinneberg (Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma) using the procedures of Waller (1968) on the prototype LKB-9000 gas chromatograph-mass spectrometer (LKB instruments, Inc., Rockville, Md.). The ion source temperature was 310°, probe temperature 140°. Spectra were recorded by direct probe at 70 ev using an accelerating voltage of 3.5 kv and a brief current of 60 μA. The source pressure was 5×10^{-6} to 1×10^{-7} mm Hg. Perfluorokerosene was used for peak-matching. The spectra were plotted from tabular intensity data.

Derivatives

The ethyl ester of allocholic acid from the River carpsucker (*Carpiodes carpio*) was formed by dissolving the acid in ethanol which contained 2% H_2SO_4 and allowing it to stand overnight. It was crystallized from acetone. The methyl ester was formed by treating the acid dissolved in a minute amount of methanol with diazomethane in ether.

The tetra-acetates of 5α-cyprinol from Buffalo fishes (*Ictiobus* spp.) and of the 5α-chimaerol-5α-cyprinol complex from the Golden redhorse (*Moxostoma erythrurum*) were prepared by dissolving the sample in pyridine, adding acetic anhydride and leaving overnight at room temperature. The solution was diluted with water, acidified and extracted with ether from which the product was obtained after evaporation. The tetra-acetate was recrystallized several times with the final crystallization from a mixture of ethyl ether and light petroleum.

Chemicals

The deuterated pyridine used in the NMR studies was 99% pure, purchased from Aldrich Biochemical Co. (Atlanta, Ga.). Potassium bromide used in making pellets for IR was of spectral quality, purchased from Matheson, Coleman and Bell (Cincinnati, Ohio). All other chemicals were reagent grade. Solvents were redistilled before use.

CHAPTER III

RESULTS

Thirteen species of sucker fishes were collected for this study. This collection included representatives from eight of the 11 extant genera of the family Catostomidae occurring in North America. Specimens used in this study, their scientific and common names, along with their major biliary component are shown in Table 2. The two genera not represented in this study (*Erimyzon* and *Xyrauchen*) are both limited in numbers and distribution. The Creek chubsucker, *Erimyzon oblongus* (Mitchill), occurs in eastern Oklahoma. However, in recent years very few specimens have been collected by the Oklahoma Biological Survey (Stevens, pers. comm.). The Humpback sucker, *Xyrauchen texanus* (Abbott), occurs in the Colorado and Gila River systems in Utah and Arizona. In 1972, an attempt was made by the author to collect "Humpies." On that occasion, several small populations of "Humpies" were sighted in the Colorado River 0-20 miles below Hoover Dam in Arizona. However, in all cases the animals were in deep pools (20-30 feet) where the current was swift and it was impossible to capture any specimens with the equipment available at the time. One other genus, *Lagochila*, is thought to be extinct (Moore, 1968).

There was little or no variation in the bile salts found in species which were members of the same genus. Hence the results are

Table 2. A List of Scientific and Common Names of Fishes used in this Study and the Major Biliary Component in Each.

Scientific Name	Common Name	Major Biliary Component (s)
Carpiodes carpio (Rafinesque)	River carpsucker	allocholic acid
Catostomus commersoni (Lacépède)	White sucker	5α - chimaerol
Catostomus macrocheilus Girard	Largescale sucker	5α - chimaerol
Catostomus plebius Baird & Girard	Rio Grande sucker	5α - chimaerol
Chasmistes brevirostris Cope	Shortnose sucker	5α - chimaerol
Cycleptus elongatus (Lesueur)	Blue sucker	5α - chimaerol (65%) 5α - cyprinol (35%)
Hypentelium nigricans (Lesueur)	Northern hogsucker	5α - chimaerol (74%) 5α - cyprinol (22%)
Ictiobus bubalus (Rafinesque)	Smallmouth buffalo	5α - cyprinol
Ictiobus cyprinellus (Valenciennes)	Bigmouth buffalo	5α - cyprinol
Minytrema melanops (Rafinesque)	Spotted sucker	5α - chimaerol (49%) 5α - cyprinol (51%)
Moxostoma carinatum (Cope)	River redhorse	5α - chimaerol (80%) 5α - cyprinol (20%)
Moxostoma duquesnei (Lesueur)	Black redhorse	5α - chimaerol (87%) 5α - cyprinol (13%)
Moxostoma erythrurum (Rafinesque)	Golden redhorse	5α - chimaerol (82%) 5α - cyprinol (18%)

reported for each genus studied. Infrared, NMR and mass spectra are recorded in Appendices I, II, and III, respectively.

Genus *Carpiodes*

The River carpsucker, *Carpiodes carpio*, is the only species collected from this genus. This is the only catostomid genus whose major biliary component was a C_{24} acid conjugated with taurine.

Thin-layer chromatography of the crude bile salts showed two spots; the major component had an R_f slightly less than that of taurocholate and the minor component had an R_f similar to that of 5α-cyprinol sulfate. Thin-layer chromatography of the free bile acid showed a single substance which was more polar than cholic acid. Gas-liquid chromatography of the TMS methyl ester of the acid showed a retention time of 0.885 relative to TMS methyl cholate (Table 3). This corresponds closely with the 0.875 relative retention time for TMS methyl allocholate reported by Anderson and Haslewood (1970). Gas-liquid chromatography of the neutral material from alkaline hydrolysis of River carpsucker bile showed a single peak with a relative retention time of 1.76. This corresponds to the relative retention for anhydro-5α-cyprinol (1.75) reported by Anderson and Haslewood (1970).

The free acid, crystallized from acetone, showed a melting point of 246-248° (Table 4). This is close to the melting point (250-251°) reported by Mitra and Elliott (1968) for their synthetic allocholic acid. Anderson and Haslewood (1962) report a melting point of 239-241° for allocholic acid, but they indicated that their crystals contained acetone. The melting point for the ethyl ester was 226-227°, which agrees with the value for ethyl allocholate reported by Haslewood

Table 3. Gas-liquid chromatography data. For each sample, the type of compound, the number of peaks, retention time relative to TMS methyl cholate and per cent of the sample are shown.

Species	Compound	Peak No.	Rel.Ret.Time	% Sample
Carpiodes carpio	methyl ester	1	0.885[a]	100
	neutral fraction	1	1.76[a]	100
Catostomus commersoni	alcohol	1	1.45[b]	100
Catostomus macrocheilus	alcohol	1	1.47[b]	100
Catostomus plebius	alcohol	1	1.46[b]	100
	anhydro-alcohol	1	1.21[a]	100
Chasmistes brevirostris	alcohol	1	1.47[b]	100
Cycleptus elongatus	alcohol	1	1.40[a]	65
		2	1.77[a]	35
Hypentelium nigricans	alcohol	1	1.40[a]	79
		2	1.75[a]	22
		3	1.27[a]	4
Ictiobus bubalus	alcohol	1	1.86[b]	100
Ictiobus cyprinellus	alcohol	1	1.86[b]	100
Minytrema melanops	alcohol	1	1.40[a]	49
		2	1.76[a]	51
Moxostoma carinatum	alcohol	1	1.40[a]	80
	alcohol	2	1.76[a]	20
Moxostoma duquesnei	alcohol	1	1.46[b]	87
		2	1.87[b]	13
Moxostoma erythrurum	alcohol	1	1.46[b]	82
		2	1.86[b]	18
	anhydro-alcohol	1	1.19[a]	74
		2	1.67[a]	26

a = samples run on Varian at 275°; N_2 flow 50 ml/min.

b = samples run on Perkin-Elmer at 260°; N_2 flow 35 ml/min.

Table 4. Melting points of the major component crystallized from various species. In cases where some other form of the major component was produced, a melting point is listed for the derivative.

Species	Melting Point (°C)	
	Major Component	Derivatives
Carpiodes carpio	246-248	225-226 (ethyl ester)
Catostomus commersoni	234-235	
Catostomus macrocheilus	233-234	
Catostomus plebius	235-236	220-221 (anhydro-alcohol)
Chasmistes brevirostris	234-236	
Ictiobus bubalus	243-244	
Ictiobus cyprinellus	243-244	109-111 & 135-138 °C (tetra-acetate)
Minytrema melanops	239-241	
Moxostoma duquesnei	239-240	
Moxostoma erythrurum	238-239	133-137 (tetra-acetate)

(1961) and by Mitra and Elliott (1968) (225-226° in both cases). Ethyl allocholate has $[\alpha]_D$ + 24.8°, $\pm 2^{\circ}$ (Table 5, c = 2.0 in ethanol). Haslewood (1961) reports + 23°, $\pm$ 2°. The infrared spectrum (Appendix I) for the free acid is identical to that published for allocholic acid (Anderson and Haslewood, 1962).

The acid was probably conjugated with taurine since the R_f of the crude bile salt on TLC was closer to the R_f of taurocholate than to that of glycocholate. However, no specific test was done to prove the presence of taurine in the conjugate.

Genus *Catostomus*

The genus *Catostomus* was represented by three species: *C. commersoni*, *C. macrocheilus*, and *C. plebius*. The data for all three were similar. Thin-layer chromatography showed two spots: the major component had an R_f slightly greater than that of 5α-cyprinol sulfate with the solvent system EtOAc:BuOH:HOAc:H_2O:MeOH (40:30:10:10:10). This R_f difference was reproducible, but not great enough to separate the compounds. In addition, this major substance differed from 5α-cyprinol sulfate in that its TLC color was more blue. The minor component was slightly more polar than the major one. However, it appeared to be less than 5% of the total and was not further investigated. Analysis by TLC of the free alcohol showed only a single spot which was not separable at all from 5α-cyprinol, although it did still show the color difference. The anhydro compound obtained by alkaline hydrolysis of bile from *C. plebius* showed the same R_f on TLC as anhydro-5α-cyprinol. The anhydro-form of the compound also showed the blue color difference.

Table 5. Specific rotation of the major biliary component of the various species studied. For *Carpiodes carpio*, the specific rotation was determined for the derivative, ethyl allocholate. In all other species, the specific rotation was determined for the free alcohol. α is the observed rotation using the sodium D line as a light source; c is the concentration of the compound in absolute ethanol, expressed in g/100 ml; $[\alpha]_D$ is the calculated or specific rotation.

Species	α_D	c	$[\alpha]_D \pm 2^\circ$
Carpiodes carpio	1.0	2.02	24.8
Catostomus macrocheilus	1.03	1.59	32.4
Chasmistes brevirostris	1.36	2.10	32.3
Ictiobus bubalus	1.24	2.15	28.8
Ictiobus cyprinellus	1.12	2.02	27.7
Minytrema melanops	1.13	2.10	26.9
Moxostoma duquesnei	1.18	2.06	28.7
Moxostoma erythrurum	1.38	2.20	31.4

Gas-liquid chromatography of the free alcohol showed a single peak with a retention time of 1.45-1.47 (Table 3) relative to TMS methyl cholate. This corresponded closely to the relative retention time of 1.42 reported by Anderson and Haslewood (1970) for 5α-chimaerol. The anhydro-alcohol from C. plebius appeared as a single peak on GLC with a relative retention time of 1.21. Anderson and Haslewood (1970) report a relative retention time of 1.20 for anhydro-5α-chimaerol.

Melting points for the alcohol from these three species were: 234-235°, 233-234° and 235-236°, respectively. These are essentially the same as the melting point (234-235°) reported for 5α-chimaerol by Anderson and Haslewood (1970). The anhydro-alcohol had a melting point of 220-222° which also corresponded to Anderson and Haslewood's (1970) reported melting point of 221-222°.

The alcohol from C. macrocheilus (Table 5) had $[\alpha]_D$ +32.4°. This is in the ± 2° range of +33.5° reported by Anderson and Haslewood (1970). The infrared spectrum was similar to that of 5α-cyprinol; Anderson and Haslewood (1970) also found the spectra of 5α-cyprinol and 5α-chimaerol to be similar. The IR spectra of the alcohol from all three species were identical (Appendix I). Nuclear magnetic resonance spectra from these samples show chemical shifts which are the same as those reported for 5α-chimaerol (Tökes, 1970) and are all identical (Appendix II). The mass spectrum of the alcohol from C. macrocheilus (Appendix III) shows the same molecular ion and m/e peaks as Tökes (1970) reported for 5α-chimaerol.

The alcohol was shown to be conjugated with sulfate. However, the $BaSO_4$ was not quantitatively determined. The mobility of

the conjugated bile salt on TLC indicated one sulfate group per alcohol moiety.

Genus Chasmistes

Chasmistes brevirostris is the only representative studied from this genus. Thin-layer chromatography of the crude bile showed a single spot with the same R_f as that of 5α-cyprinol sulfate, but with the blue color difference that was seen with the bile salt from members of the genus Catostomus. Thin-layer chromatography of the free alcohol also showed a single spot with the same R_f as that of 5α-cyprinol, but with the blue color. Gas-liquid chromatography showed a single peak with the relative retention time of 1.47 (Table 3), characteristic of 5α-chimaerol (Anderson and Haslewood, 1970). The crude bile was not subjected to alkaline hydrolysis to obtain the anhydro-form of the alcohol.

The melting point (235-236°) and specific rotation (+32.3^{o}) of the free alcohol corresponded to the values reported by Anderson and Haslewood (1970) for 5α-chimaerol. The NMR spectrum from this alcohol (Appendix II) is also similar to that reported by Tökes (1970).

The alcohol was shown to be conjugated with sulfate. However, the $BaSO_4$ was not quantitatively determined. The mobility of the conjugated bile on TLC indicated one sulfate group per alcohol moiety.

Genus Cycleptus

Cycleptus is a monotypic genus containing only the Blue sucker, C. elongatus. Thin-layer chromatography of the crude bile salts showed three distinct spots: the major spot had the same R_f as that of 5α-

cyprinol sulfate with an indeterminate color--sometimes appearing more red like that of 5α-cyprinol sulfate, sometimes more blue like that of 5α-chimaerol sulfate. One minor component migrated slightly faster than, but was separate from, the major component, the other moved more slowly than, but was just separate from, the major component. Neither of the two minor spots had an R_f characteristic of any known bile salt. Neither of these minor substances was analyzed further.

Gas-liquid chromatography of the free alcohol from the Blue sucker (which showed a single spot, R_f like that of 5α-cyprinol on TLC) showed two distinct peaks (Table 3). The first (and larger) peak had a relative retention time of 1.40 and comprised 65% of the total fraction. The second peak had a relative retention time of 1.77 and comprised 35% of the fraction. These retention times suggest 5α-chimaerol and 5α-cyprinol, respectively.

Although the alcohol from the Blue sucker was purified by reversed-phase column chromatography, it was not possible to obtain crystals. Therefore there are no data for melting point, IR spectra or specific rotation. The bile alcohol from the Blue sucker was shown to be conjugated with sulfate (Table 6), having one sulfate per alcohol moiety in the conjugate.

Genus Hypentelium

The Northern hogsucker, H. nigricans, is the only representative studied from this genus. Thin-layer chromatography of the crude bile showed a single spot with an R_f similar to that of 5α-cyprinol sulfate, but having an indeterminate color like that of the main component from the Blue sucker. Thin-layer chromatography of the free

Table 6. Sulfate conjugation as determined by $BaSO_4$ precipitation. Comparison of molar amounts of free alcohol with sulfate released indicates the number of moles of sulfate conjugated with each alcohol moiety.

Species	mg alcohol	mmole alcohol	mg $BaSO_4$	mmole $BaSO_4$
Cycleptus elongatus	535	1.18	280	1.19
Hypentelium nigricans	158	0.334	79	0.338
Minytrema melanops	493	1.09	209	0.94
Moxostoma duquesnei	603	1.33	322	1.38
Moxostoma erythrurum	610	1.35	320	1.36

alcohol also showed a single spot with an R_f like that of 5α-cyprinol and an indeterminate color.

Gas-liquid chromatography of the free alcohol showed three peaks (Table 3). One tiny peak (4% of the sample), had a relative retention time of 1.27, which was not comparable to that of any known bile alcohol. The major component (74% of the sample) had a relative retention time of 1.40 and was identifiable with 5α-chimaerol (Anderson and Haslewood, 1970). The third peak (22% of the sample), had a relative retention time of 1.76 which was comparable to that of 5α-cyprinol (Anderson and Haslewood, 1970).

Although the free alcohol from the Northern hogsucker was purified by reversed-phase column chromatography, it was not possible to obtain crystals. Hence there are no data for melting point, IR spectra or specific rotation. The bile alcohol(s) from the Northern hogsucker was shown to be conjugated with sulfate (Table 6), having one sulfate per alcohol moiety in the conjugate.

Genus *Ictiobus*

The genus *Ictiobus* is represented by samples known to come from the Smallmouth buffalo, *I. bubalus*, and the Bigmouth buffalo, *I. cyprinellus*. In addition, a large sample of bile collected by a commercial fisherman from "Buffalo fishes" which undoubtedly contained bile from the Black buffalo, *I. niger*, as well as from *I. bubalus* and *I. cyprinellus*, was analyzed. No differences were found in any of these samples.

Thin-layer chromatography of the crude bile from the Buffalo fishes resulted in a major spot with R_f and color identical to those of

5α-cyprinol sulfate. A very minor component had R_f and color suggesting tauroallocholate, although its identity was not confirmed. Thin-layer chromatography of the free alcohol showed a single spot with the same R_f and color as 5α-cyprinol.

Gas-liquid chromatography of the free alcohol from both I. bubalus and I. cyprinellus produced a single peak with a relative retention time of 1.86 in both cases (Table 3). This agrees with the relative retention time of 5α-cyprinol reported by Anderson and Haslewood (1970).

Melting points for the crystalline alcohol from Buffalo fishes were 243-244°. This is close to the 242° melting point reported for 5α-cyprinol by Anderson et al. (1964). The tetra-acetate derivative of the alcohol underwent the double melting point (Table 4) reported for 5α-cyprinol tetra-acetate. Anderson et al. (1964) reported 110.5° and 137.5-139° as the melting point for their 5α-cyprinol tetra-acetate. The tetra-acetate from the Buffalo fishes melted at 109-111° and 135-138°. The specific rotation of the free alcohol (Table 5) was 28.8° for the sample from I. bubalus and 27.7° for the sample from I. cyprinellus. Both values are slightly lower, but within the ± 2° range of the $[\alpha]_D$ + 29° reported by Anderson et al. (1964) for 5α-cyprinol.

The IR spectra from both the alcohol and the anhydro-alcohol from the Buffalo fishes (Appendix I) are identical to those published for 5α-cyprinol and anhydro-5α-cyprinol (Anderson, et al., 1964). In addition, the NMR spectrum (Appendix II) shows the same chemical shifts as those reported for 5α-cyprinol (Cross, 1964).

No $BaCl_2$ precipitation test was done to determine the type of conjugation. However, conjugation with one mole of sulfate was

inferred from the similarity in R_f between the crude bile from Buffalo fishes and known 5α-cyprinol sulfate from the carp.

Genus *Minytrema*

Minytrema is a monotypic genus containing only the Spotted sucker, *M. melanops*. Thin-layer chromatography of the crude bile sample produced a single spot with the R_f of 5α-cyprinol sulfate and with the indeterminate color seen with the samples from the Blue sucker and the Hog sucker. Thin-layer chromatography of the free alcohol also resulted in a single spot with the R_f of 5α-cyprinol and the indeterminate red-blue color.

Gas-liquid chromatography of the free alcohol showed two distinct peaks of about equal intensity (Table 3). The first peak had a relative retention time of 1.40 and contained 49% of the sample; the second peak had a relative retention time of 1.76 and contained 51% of the sample. These peaks correspond to 5α-chimaerol and 5α-cyprinol, respectively (Anderson and Haslewood, 1970).

The melting point of the crystals obtained from this sample was constant and sharp, 239-241°. This melting point is somewhat low for 5α-cyprinol, which was reported as 242° (Anderson, *et al.*, 1964) and found to be 243-244° in the present study (sample from *Ictiobus* spp.). However, it is too high for 5α-chimaerol which was reported as 234-235° (Anderson and Haslewood, 1970), and also found to be 234-235° in the present study (samples from *Catostomus* spp.). The IR spectrum is similar to that of 5α-cyprinol and 5α-chimaerol; but these two are similar to each other as indicated by Anderson and Haslewood (1970).

The specific rotation for the free alcohol was + 26.9°. This is lower than the + 29° reported for 5α-cyprinol by Anderson, et al. (1964). The bile alcohol(s) from the Spotted sucker was shown to be conjugated with sulfate (Table 6); having one sulfate per alcohol moiety in the conjugated bile salt.

Genus Moxostoma

This genus is represented by three species: the River redhorse, *M. carinatum*; the Black redhorse, *M. duquesnei*; and the Golden redhorse, *M. erythrurum*. No significant differences were noted in the data for the bile salts of these three species. Thin-layer chromatograms of the crude bile showed a single spot with the R_f of 5α-cyprinol sulfate, whose color was indeterminate--being neither the reddish color from 5α-cyprinol, nor blue as with 5α-chimaerol. Thin-layer chromatography of the free alcohol produced a single spot with the R_f of 5α-cyprinol, but with the same indeterminate color.

The melting point for the crystals obtained from *M. duquesnei* and *M. erythrurum* was 239-240° and 238-239°, respectively (Table 4). This suggested the possibility of a new compound, since the melting point was intermediate between that reported for 5α-chimaerol (234-235°, Anderson and Haslewood, 1970) and that reported for 5α-cyprinol (242°, Anderson, et al., 1964). The tetra-acetate was prepared and its melting point was 133-137° (Table 4), a value which is compatible with the upper melting point of the double melting point of 5α-cyprinol tetra-acetate (110.5° and 137.5-139°, Anderson, et al., 1964). However, repeated re-crystallization of the tetra-acetate from the redhorse alcohol failed to give the lower melting point so characteristic of 5α-cyprinol tetra-

acetate. The tetra-acetate from 5α-chimaerol has a single melting point, 141-143° (Anderson and Haslewood, 1970).

A number of tests were performed to determine the identity of the single spot on TLC which did not match other data. The crystals from the redhorse alcohol showed a melting point depression with both pure 5α-cyprinol and 5α-chimaerol. The presence of a 3β-OH group was tested with digitonin. However, no insoluble digitonide resulted. The anhydro-alcohol was formed by alkaline hydrolysis and purified by reversed-phase column chromatography. One fraction from this column crystallized on avaporation of the solvent, and had a melting point of 210-214°. Since all fractions showing the same R_f by TLC were thought to contain the same compound, they were combined and repeated attempts made to crystallize the substance. However, it was not possible to regain the crystals from the Redhorse anhydro-alcohol. The IR spectra of the compounds (Appendix II) were also inconclusive since they all appeared similar to 5α-cyprinol (or 5α-chimaerol, as the case may be).

Finally, the compounds were analyzed by GLC. In all three species, the alcohol showed two peaks (Table 3) with relative retention times that suggested 5α-chimaerol and 5α-cyprinol (Anderson and Haslewood, 1970). The 5α-chimaerol comprised 80-87% of the samples and the 5α-cyprinol comprised 13-20% of the samples. The anhydro-alcohol from the Golden redhorse also showed two peaks with the relative retention times that suggested anhydro-5α-chimaerol and anhydro-5α-cyprinol (Anderson and Haslewood, 1970). The anhydro-5α-chimaerol comprised 74% of that sample.

Specific rotations of the free alcohols from M. duquesnei and M. erythrurum were +28.7° and +31.4°, respectively (Table 5). Since the specific rotations reported for 5α-chimaerol (+33.5°, Anderson and Haslewood, 1970) and 5α-cyprinol (+29°, Anderson, et al., 1964) are fairly close and the specific rotations for the Redhorses were both nearly the same as the rotations reported for 5α-chimaerol and 5α-cyprinol, this data was considered inconclusive.

The bile alcohol from the Redhorses was shown to be conjugated with sulfate (Table 6); having one sulfate per alcohol moiety in the conjugate. Nuclear-magnetic resonance spectra (Appendix II) and mass spectra (Appendix III) showed chemical shifts and m/e peaks intermediate between those resulting from 5α-chimaerol and 5α-cyprinol. Hence, this data is not definitive except that it is consistent with the hypothesis that the crystals from which the spectra were taken contained both 5α-chimaerol and 5α-cyprinol.

To confirm the hypothesis that the crystalline material obtained from the isolation and purification of the bile alcohols from the Redhorses and the Spotted sucker contained a complex formed from 5α-cyprinol and 5α-chimaerol, 50 mg each (5α-cyprinol, m.p. 243-244° from Ictiobus sp., and 5α-chimaerol, m.p. 234-235° from Catostomus commersoni) were dissolved together in acetone, taken to a small volume and crystallized twice. The crystals had a melting point of 237-239° and on gas chromatography produced two distinct peaks, with relative retention times of 1.46 and 1.86, respectively. Each represented approximately half the total sample. Liqours from this crystallization produced the same two peaks on GLC, in the same relative proportions.

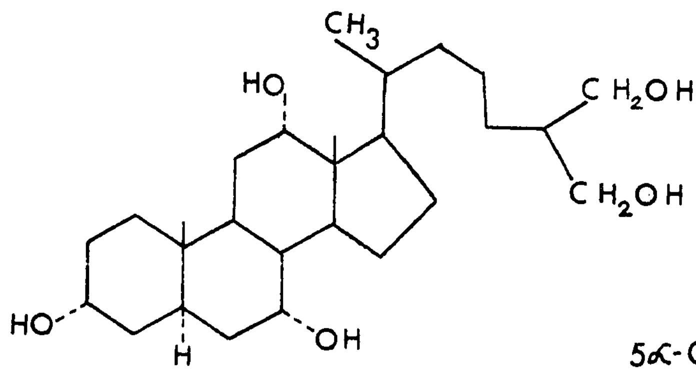
CHAPTER IV

DISCUSSION

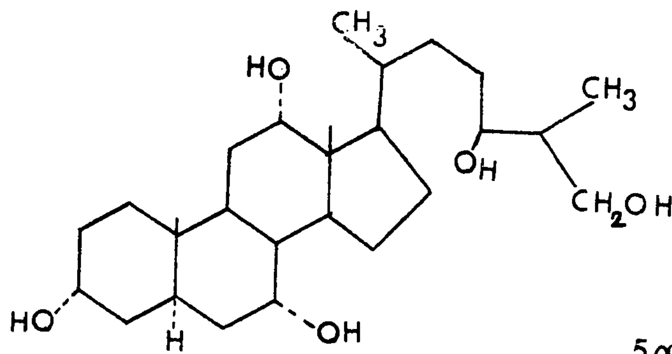
Three different compounds were found to be present as major biliary components in catostomid fishes. The structures of these compounds, 5α-chimaerol, 5α-cyprinol and allocholic acid, are shown in Figure 3. The data indicate that on the basis of bile salt composition the various genera of the family Catostomidae may be placed in four groups. 1) Members of the genus Carpiodes (carpsuckers) alone possess allocholic acid, a C_{24} acid, conjugated with taurine as their major biliary component. However, they may also contain some 5α-cyprinol as a secondary component. 2) Members of the genus Ictiobus (Buffalo fishes) alone possess 5α-cyprinol as their major biliary component. Their bile may contain traces of allocholic acid, though this was not confirmed. 3) Members of both Catostomus and Chasmistes contain 5α-chimaerol as their major biliary component. 4) Members of the genera Moxostoma, Hypentelium, Minytrema and Cycleptus all contain both 5α-chimaerol and 5α-cyprinol, in varying proportions, as major biliary components (Table 2).

Criteria for identification, such as physical properties and chromatographic behavior, of the allocholic acid with its taurine conjugation and the 5α-chimaerol and 5α-cyprinol as sulfate conjugates, all corresponded closely with published values. The crystalline complex

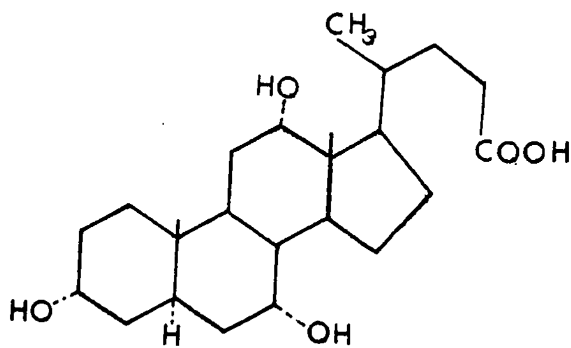
Figure 3. Structures of the three compounds identified from the bile of catostomid fishes: 5α-cyprinol, 5α-chimaerol and allocholic acid.



5α-CYPRINOL



5α-CHIMAEROL



ALLOCHOLIC ACID

formed by 5α-cyprinol and 5α-chimaerol, however, was not nearly so easy to identify. The fact that the material formed crystals with a sharp melting point which was different from all melting points published to date for bile alcohols suggested that the material was a new, undescribed bile alcohol.

Several avenues were explored in the elucidation of this complex. The first specific rotation determined for the substance from the Golden redhorse was $+36^{\circ}$. This was higher than the $+33^{\circ}$ rotation reported by Anderson and Haslewood (1970) for 5α-chimaerol. Furthermore, they indicated that 5α-chimaerol from <u>Catostomus commersoni</u> showed the greatest degree of rotation that would be predicted for any of the possible isomers of 5α-chimaerol. However, there was a change in the zero point on the polarimeter (which is of unknown, but venerable, vintage) during the time when the Golden redhorse rotation was recorded, so this datum was questionable.

Another possibility considered was that the Golden redhorse alcohol was a 3β isomer of 5α-chimaerol since such isomers do exist (e.g., latimerol is the 3β isomer of 5α-cyprinol and has a specific rotation 4° greater than 5α-cyprinol, Haslewood, 1967a). Preparation of the digitonide disproved this possibility because 3β digitonides are insoluble and this reaction did not form an insoluble compound.

Mixed melting points showed a melting point depression with both pure 5α-cyprinol and pure 5α-chimaerol. The melting point of the tetra-acetate derivative ($133-137^{\circ}$) did not show the double melting point (110.5° and $137.5-139^{\circ}$) characteristic of 5α-cyprinol tetra-acetate and is too low for the melting point of 5α-chimaerol tetra-acetate ($141-143^{\circ}$).

Both NMR and IR spectra were inconclusive. As Anderson and Haslewood (1970) note, the IR spectra of 5α-cyprinol and 5α-chimaerol (which are positional isomers, Fig. 3) are very similar. The IR spectrum from the Redhorses resembles these two, but that did not indicate whether the alcohol from the redhorses was 5α-cyprinol, 5α-chimaerol, or another closely related positional isomer. The NMR spectra of 5α-cyprinol and 5α-chimaerol show different chemical shifts (Tökes, 1970, and Cross, 1964). The NMR spectrum from the Redhorse alcohol (Appendix II) was intermediate between these two. The mass spectrum of the alcohol from the Redhorses showed the molecular ion to be 452, the same as for 5α-cyprinol and 5α-chimaerol. However, the spectrum showed the m/e peaks characteristic of the 5α-chimaerol mass spectrum as well as the m/e peaks characteristic of the 5α-cyprinol mass spectrum.

Access to a gas chromatograph finally solved the puzzle of the Redhorse bile alcohol. The two distinct peaks produced by GLC had the same relative retention times that Anderson and Haslewood (1970) reported for 5α-chimaerol and 5α-cyprinol. Furthermore, if known 5α-chimaerol and/or 5α-cyprinol was added to the injection sample of Redhorse alcohol, there was no evidence that either of these compounds separated, even partially, from the respective components of the Redhorse sample. The particular peak was merely heightened with no broadening or other evidence of partial separation.

The bile samples from the Blue sucker and the Hog sucker were analyzed after the problem of the composition of the Redhorse bile had been solved. Hence, these samples were not subjected to the variety of tests that were performed during the analysis of the Redhorse bile.

The case of the Spotted sucker reinforced to this author the need for thoroughness in analyses such as these. The bile of the Spotted sucker was analyzed prior to that of the Redhorses and was initially thought to contain 5α-cyprinol with a slightly low melting point (239-241° as opposed to 243-244° for the 5α-cyprinol we had purified in our laboratory or 242° reported by Anderson, et al., 1964). The single spot seen on TLC indicated 5α-cyprinol. The indeterminate nature of the color of the spot from the 5α-cyprinol-5α-chimaerol complex is more evident in retrospect and with subsequent tests and comparisons than it was initially. Gas-liquid chromatographic analysis of the crystals from the Spotted sucker bile alcohol consistently shows the presence of both 5α-chimaerol and 5α-cyprinol. The greater percentage of 5α-cyprinol (Table 2) in the Spotted sucker bile would account for the fact that these crystals have a slightly higher melting point than those from the Redhorses.

To substantiate the theory that the crystalline material obtained from the Redhorses and the Spotted sucker was a complex containing both 5α-cyprinol and 5α-chimaerol, 50 mg of pure material of each of these compounds was dissolved in and recrystallized twice from acetone. The crystals resulting from this mixture had a melting point of 236-238°. On GLC these crystals produced the two peaks that had been seen from the crystals from Redhorses and Spotted suckers.

Although the co-crystallization of two closely related compounds to form a crystalline complex with a constant melting point is rare, it is not unknown. In a similar circumstance, Ohta (1939), in his analysis of the bile from the Gigi fish, *Pelteobagrus nudiceps*, reported

what he thought was a new compound. The acid which Ohta isolated from the Gigi fish (tetrahydroxy-norsterocholanic acid, $C_{27}H_{46}O_6$, is the name and formula he assigned to the compound) crystallized from acetone and from ethanol-water. The crystalline needles showed a constant melting point of 212-214°. In elucidating the structure of his acid, Ohta found that when he oxidized the acid carefully he obtained two triketo acids, an α-triketo acid with a melting point of 198° and a β-triketo acid with a melting point of 234°. These he thought were oxidation products from his acid, according to his interpretation of the structure as $3\alpha,6\alpha,12\alpha,x$,tetrahydroxy cholestanoic acid. However, Anderson and Haslewood (1962) showed that what Ohta had interpreted to be a single substance was actually a combination of cholic and allocholic acids. It is not surprising that Ohta's acid was thought to be a single substance, for Anderson and Haslewood (1962) further indicate that its methyl and ethyl esters as well as the free acids consist of apparently homogeneous crystals, which have been chromatographically inseparable. It seems that methyl (and ethyl) cholate form mixed crystals with their corresponding allo esters. The composition of such mixtures may be variable, but Anderson and Haslewood (1962) suggest that what have been described as ester of Ohta's acid are approximately 2:1 (w/w) methyl (or ethyl) allocholate:methyl (or ethyl) cholate. Such a composition agrees also with results of measurements of optical rotation. These results are suprisingly consistent with the data from this study. Ratios of 5α-chimaerol:5α-cyprinol were 1:1 (Spotted sucker), 2:1 (Blue sucker) and 4:1 (Redhorses) in this study.

The discovery of tauro-allocholate as the major bile salt in the River carpsucker represents some significant "firsts" in the study

of bile salts in general, and for fish bile salts in particular (Briggs and Bussjaeger, 1972). This is the first report of an "allo" (5α) bile acid as the main component of bile from fishes of the superorder Ostariophysi. Haslewood (1967) reports cholic acid (5β) as the major component from several fishes of the order Siluriformes, and Ohta's acid from the "Gigi" fish has been shown to contain allocholic acid (Anderson and Haslewood, 1962) but it has not been verified as being the principal component of the bile. In addition, this is the first report of a bile acid being the chief component of bile from fishes in the order Cypriniformes. Yamasaki, et al. (1972) have since reported the presence of allocholic acid in carp bile. However, the percentage of allocholic acid present was in that case exceedingly small. Previous reports show a bile alcohol, specifically 5α-cyprinol or 5α-chimaerol, to be the principal component of bile in cypriniform fishes (Anderson and Haslewood, 1970). Finally, the relative abundance of the River carpsucker in Oklahoma makes this a substantial reservoir of naturally occurring allocholic acid. The lizard Anolis lineatopus does have tauroallocholate as almost its only bile salt (Haslewood, 1967a), but its small size and relatively small numbers would limit it as a natural source of allocholic acid.

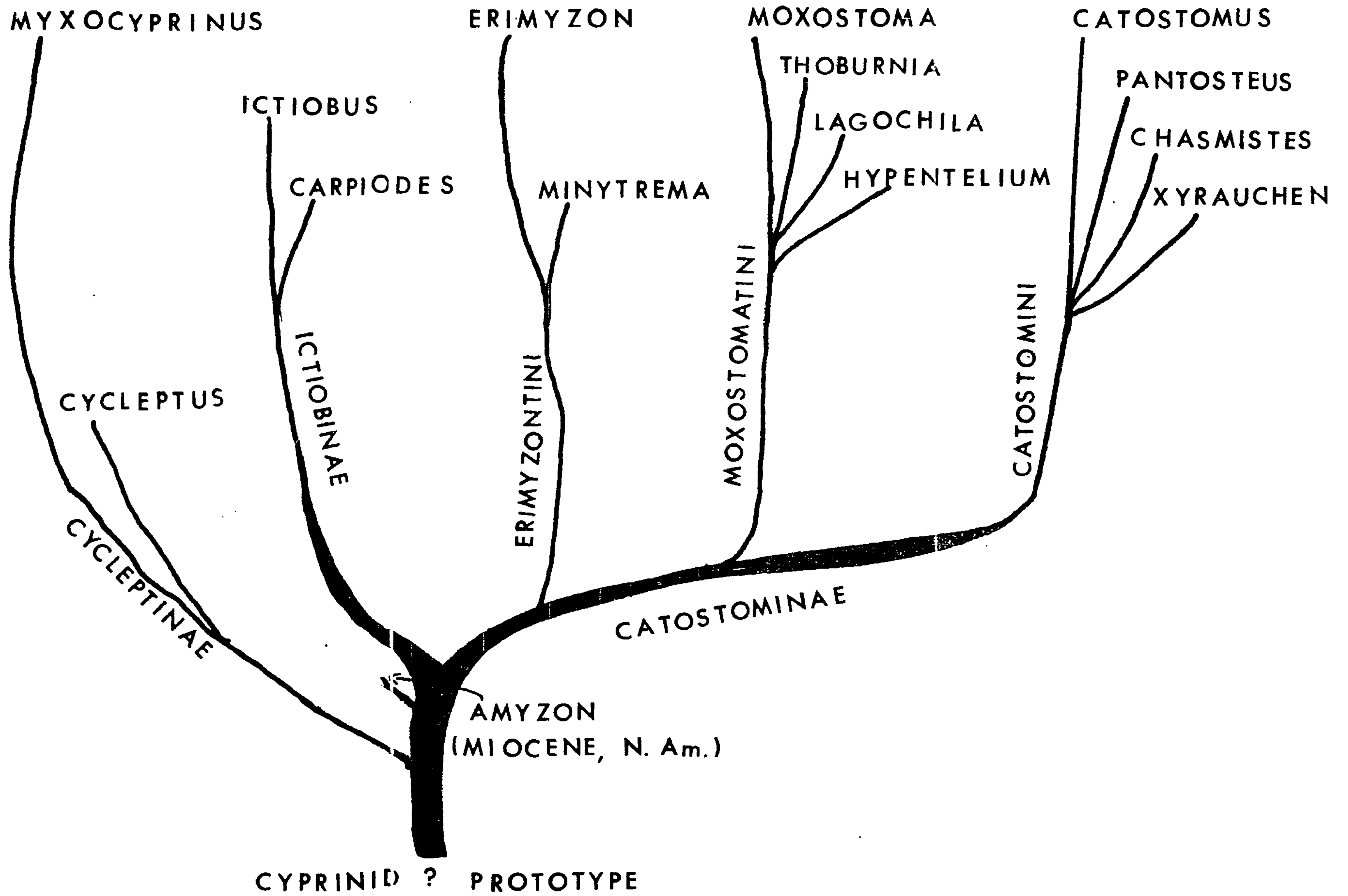
The availability of large amounts of allocholic acid makes possible a variety of experimental work, such as the synthesis (Briggs, 1970) of metabolic precursors of 5α-bile salts, and the work being done in other laboratories (Mitra and Elliott, 1968) which requires allocholic acid. Also, this may provide the resources necessary for determining the relationship between the 5α and 5β configuration and the structure and function of enzymes in bile acid formation.

A number of studies dealing with the biochemical taxonomy of catostomids have been conducted in recent years. Tsuyuki et al. (1967) studied variations in the electrophoretic patterns of muscle myogens and plasma proteins in several species of catostomids. Variations in the serum esterases of Catostomus clarkii were shown by Koehn and Rasmussen (1967) and polymorphisms in the hemoglobin of a number of species of catostomids have been described (Tsuyuki et al., 1967 and Koehn, 1969). In addition, Huntsman (1970) studied blood sera and muscle extracts from catostomid fishes. Powers and Edmundson (1972, 1972a) have isolated isohemoglobins from C. clarkii and determined the amino acid sequence of the major α chain.

Catostomids provide a suitable group for studies in biochemical taxonomy. Since much of their taxonomy has been well worked out on morphological, anatomical and meristic characters, a good background exists by which to evaluate the findings of biochemical studies. There are many interesting taxonomic problems in the Catostomidae to which the application of biochemical studies may be of real value.

Miller (1958) presents the most recent phylogeny of the family Catostomidae (Fig. 4). This phylogeny is based largely on Nelson's (1948, 1949) division of the family into three subfamilies primarily on the basis of morphology of the four highly modified anterior vertebrae (the Weberian apparatus) that connect the gas bladder with the inner ear. Miller indicates, however, that since the sub-family Cycleptinae has a primitive genus in each continent, it might justifiably be segregated as two subfamilies with Cycleptus as the North American and Myxocyprinus as the Asian representative.

Figure 4. Miller's (1958) "Hypothetical phylogeny of the family Catostomidae."


MYXOCYPRINUS
ERIMYZON
MOXOSTOMA
CATOSTOMUS
THOBURNIA
PANTOSTEUS
ICTIOBUS
LAGOCHILA
CHASMISTES
HYPENTELIUM
CARPIODES
MINYTREMA
XYRAUCHEN
MOXOSTOMATINI
CATOSTOMINI
ICTIOBINAE
ERIMYZONTINI
CYCLEPTUS
CYCLEPTINAE
CATOSTOMINAE
AMYZON
(MIOCENE, N. Am.)
CYPRINID ? PROTOTYPE

The four groups of catostomids based on bile salt composition generally correlate quite well with Miller's hypothetical phylogeny. However, some minor changes may be in order, based on this and other studies. First, since the tribes Erimyzontini and Moxostomatini both show the 5α-cyprinol-5α-chimaerol complex as does the genus Cycleptus, it would seem that these groups should show a closer phylogenetic relationship than may be inferred from Miller's representation (Fig. 4). This change could be accomplished simply by a three-dimensional reorientation of the Cycleptus "branch" of the phylogenetic tree.

Secondly, the tribes Erimyzontini and Moxostomatini are quite close to each other according to bile salt composition. These data as well as Huntsman's (1970) data on blood sera and muscle myogens support the conclusion that these two tribes are closer to the Catostomini than to the branch point of the subfamily Ictiobinae. In addition, Huntsman (1970) indicates that his data do not resolve whether Hypentelium is most closely related to the Moxostomatini or the Catostomini. Bile salt data indicates that Hypentelium is more closely related to the Moxostomatini. Eastman (pers. comm.) also affirms that his data on the pharyngeal teeth of catostomids support this conclusion. From these data, it is tempting to suggest that the tribes Erimyzontini and Moxostomatini be combined and perhaps put into their own subfamily separate from the Catostominae. However, more supporting evidence would need to be gathered before such a change would be seriously proposed.

Finally, the major bile salt component of Carpiodes is more "advanced" chemically that that from Ictiobus inasmuch as a C_{24} acid

is not only chemically more changed from the cholesterol molecule than is a C_{27} alcohol, but also because an acid is more highly oxidized than an alcohol. An argument for switching their positions in the phylogenetic tree could also be supported by the fact that the carpsuckers are a highly specialized genus, particularly with respect to their food habits. No alterations are in order for the Catostomini, since bile salt data indicate this group to be quite discrete.

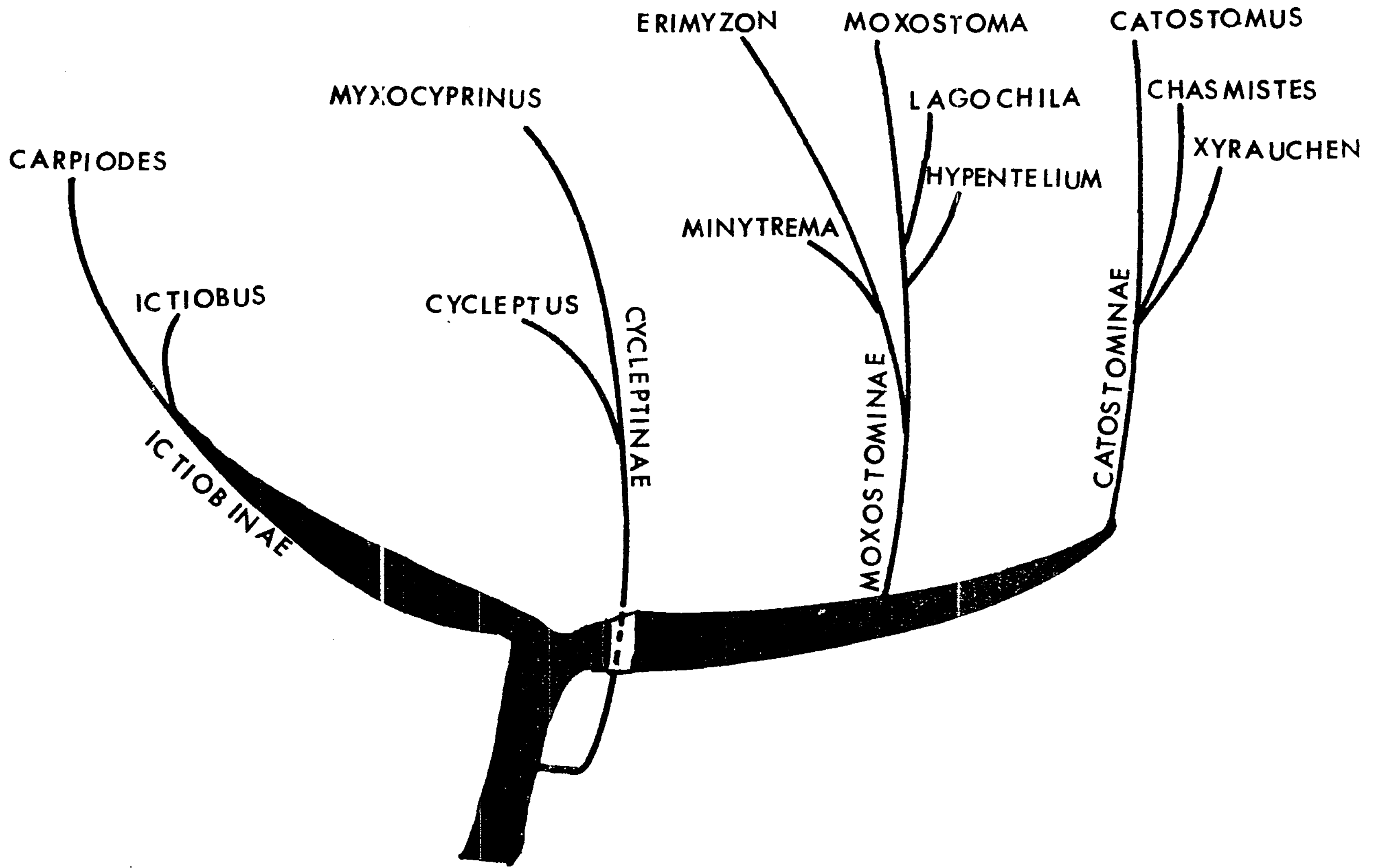
A proposed altered hypothetical phylogeny of the family Catostomidae, incorporating these changes, is presented in Figure 5. It should also be pointed out that since Miller's (1958) publication, _Pantosteus_ and _Catostomus_ have been combined in a single genus, _Catostomus_; and _Thoburnia_ and _Moxostoma_ have been combined in a single genus, _Moxostoma_ (Bailey, _et al._, 1970).

A more nearly comprehensive study would require samples from _Xyrauchen_, _Erimyzon_ and (if they are still extant) _Lagochila_. However, the conclusions presented here do correspond closely with already-postulated phylogenetic relationships. Furthermore, these data confirm and extend the use of bile salts as a phylogenetic character, at least above the generic level.

As with most research, this study leaves questions unanswered and points out many avenues for further investigation. For example, a close comparison of members of the genus _Ictiobus_ with members of the genus _Carpiodes_, particularly in the area of habitat and food habits might indicate a correlation between these factors and reasons for difference in bile salt composition.

Carpiodes, since they contain tauro-allocholate as their major biliary component, provide an ideal system for comparing the biosynthetic

Figure 5. A proposed alteration of Miller's "Hypothetical phylogeny of the family Catostomidae."


CARPIODES
ICTIOBUS
ICTIOBINAE
MYXOCYPRINUS
CYCLEPTUS
CYCLEPTINAE
ERIMYZON
MINYTREMA
MOXOSTOMA
LAGOCHILA
HYPENTELIUM
MOXOSTOMINAE
CATOSTOMUS
CHASMISTES
XYRAUCHEN
CATOSTOMINAE

sequences of 5α-bile salts with the already-determined formation of their 5β-counterparts in mammals. *Carpiodes* would also be a good genus to use in experimental work for determining the role of 26,27 (cyprinol) versus 24,27 (chimaerol) hydroxylation patterns as possible intermediates in C_{24}-acid formation. The fact that those catostomid species which have allocholic acid as their major biliary component (*Carpiodes*) or as a secondary biliary component (*Ictiobus*) also possess 5α-cyprinol, but do not have 5α-chimaerol may be interpreted two ways. It is possible that 5α-cyprinol is an intermediate in allocholic acid biosynthesis and the relative efficiencies of the enzymes involved in this conversion determine whether allocholic acid is present in major or minor amounts. If this is the case, one must infer that the enzymes necessary for the 24-hydroxylation of 5α-chimaerol either have not arisen in this group or have been lost.

A second, chemically more sound, argument is that 5α-chimaerol is an intermediate in allocholic acid biosynthesis and is not present because it is converted to allocholic acid. Chemically this seems to be the stronger argument since with the presence of the hydroxyl group at C_{24} in 5α-chimaerol, one would predict cleavage of the side chain at this point upon oxidation. The 26,27 hydroxylation pattern in 5α-cyprinol would not lead directly to side-chain cleavage, a circumstance which would explain the presence of 5α-cyprinol in these animals. These theories could easily be tested by injecting labeled 5α-cyprinol and 5α-chimaerol into *Carpiodes* and determining if either of them produced any labeled allocholic acid.

The genetic control of the enzymes regulating bile salt production could be pursued through the formation of artificial hybrids.

In his summary of the naturally occurring hybrids of North American fishes, Hubbs (1955) reported approximately 70 natural hybrids in the faimly Cyprinidae. This author (unpublished data) has artificially produced viable hybrids between two members of different subfamilies in the family Cyprinidae (*Pimephales vigilax* X *Notropis boops*). The bile salts from artificial hybrids from the family Catostomidae such as *Carpiodes* (a bile acid-former) X *Catostomus* (a producer of bile alcohols) should provide interesting data on the genetic control of bile salt production.

Finally, a comprehensive comparison of the entire catostomid family in terms of habitat, food habits, climate and genetics (karyotypes and hybridization) might indicate the relative influence of these factors as selective pressures in bile salt evolution.

CHAPTER V

SUMMARY

1. The major biliary component of 13 species (8 genera) of the family Catostomidae was isolated and identified.

2. A new, more simple, procedure for solvolysis of the bile alcohol sulfate conjugates is described.

3. Allocholic acid found in the river carpsucker is the first "allo" (5α) bile acid reported to be the main component of bile from fishes of the superorder Ostariophysi and the first bile acid reported to be the main component of bile from fishes in the order Cypriniformes.

4. A crystalline complex formed from varying ratios of 5α-chimaerol and 5α-cyprinol was discovered in several genera.

5. Biliary components in catostomid fishes fall into four categories which correspond fairly closely to previously proposed phylogenies for the family Catostomidae based primarily on anatomy and morphology.

6. Some minor changes in Miller's phylogeny of the family Catostomidae are proposed.

REFERENCES

Ali, S., A. Kuksis and J. Beveridge. 1966. Excretion of bile acids by three men on a fat free diet. Can. J. Biochem. 44: 957-969.

Anderson, I.G., T. Briggs and G.A.D. Haslewood. 1964. Comparative study of 'bile salts' 18. The chemistry of cyprinol. Biochem. J. 90: 303-308.

Anderson, I.G. and G.A.D. Haslewood. 1962. Comparative studies of 'bile salts' 15. The natural occurrence and preparation of allocholic acid. Biochem. J. 85: 236-242.

Anderson, I.G. 1970. Comparative studies of bile salts: 5α-chimaerol, a new bile salt from the White sucker, Catostomus commersoni Lacépède. Biochem. J. 116: 581-585.

Bailey, R.M., J. Fitch, E. Herald, E. Lachner, C. Lindsey, C. Robins, and W. Scott. 1970. A list of common and scientific names of fishes from the United States and Canada. Amer. Fish Soc. Spec. Publ. 6: 150 pp.

Bergström, S. and J. Sjövall. 1951. Separation of bile acids with reversed phase partition chromatography. Acta Chem. Scand. 5: 1267-1270.

Bergström, S. and A. Norman. 1953. Metabolic products of cholesterol in bile and feces of rat. Proc. Soc. Exp. Biol. Med. 83: 71-74.

Bergström, S., R. Ryhage and E. Stenhagen. 1958. Mass spectrometric studies on bile acids and other steroid derivatives. Acta Chem. Scand. 12: 1349-1358.

Briggs, T. 1970. Partial Synthesis of 25D- and 25L-Cholestanoic Acids from some common Bile Acids. J. Org. Chem. 35: 1431.

Briggs, T. and C. Bussjaeger. 1972. Allocholic acid, the major component in bile from the river carpsucker, Carpiodes carpio (Rafinesque) (Catostomidae). Comp. Biochem. Physiol. 42B: 493-496.

Bridgwater, R.J., G.A.D. Haslewood and J.R. Watt. 1963. Comparative studies of 'bile salts' 17. A bile alcohol from Chimaera monstrosa. Biochem. J. 87: 28-31.

Cross, A.D. 1964. A nuclear-magnetic-resonance spectral study of cyprinol. Biochem. J. 90: 308-309.

Eastman, J. 1973. Dept. Anat. Sci. OUHSC, Oklahoma City, Personal Communication.

Eddy, S. 1957. The freshwater fishes. Wm. C. Brown Co., Dubuque, Iowa 253 pp.

Eneroth, P., B. Gordon, R. Ryhage and J. Sjövall. 1966. Identification of mono and dihydroxy bile acids in human feces by gas-liquid chromatography and mass spectrometry. J. Lipid Res. 7: 511-523.

Fieser, L.F. and M. Fieser. 1959. Steroids. Reinhold, New York. 945 pp.

Greenwood, P., D. Rosen, S. Weitzman and G. Myers. 1966. Phyletic studies of teleostean fishes with a provisional classification of living forms. Bull. Am. Mus. Nat. Hist. 131: 4, New York. 455 pp.

Grundy, S., E. Ahrens, Jr. and T. Miettinen. 1965. Quantitative isolation and gas-liquid chromatographic analysis of total fecal bile acids. J. Lipid Res. 6: 397-410.

Haslewood, G.A.D. 1961. Comparative studies of 'bile salts' 13. Bile acids of the leopard seal, Hydrurga leptonyx and of two snakes of the genus Bitis. Biochem. J. 78: 352-359.

Haslewood, G.A.D. 1962. Bile salts: structure, distribution, and possible biological significance as a species character, pp. 205-229. In M. Florkin and H.S. Mason (eds.). Comparative Biochemistry, Vol. 3, pt. 3. Academic Press, New York. 959 pp.

Haslewood, G.A.D. 1964. The biological significance of chemical differences in bile salts. Biol. Rev. 39: 537-574.

Haslewood, G.A.D. 1967. Bile salt evolution. J. Lipid Res. 8: 535-550.

Haslewood, G.A.D. 1967a. Bile salts. Methuen & Co. Ltd., London. 116 pp.

Horning, E.C., M. Horning, N. Ikekawa, E.M. Chanbaz, P. Jaakomaki and C. Brooks. 1967. Studies of analytical separations of human steroids and steroid glucuronides. J. Gas Chromat. 5: 283-289.

Hubbs, C.L. 1930. Materials for a revision of the catostomid fishes of eastern North America. Univ. Mich. Mus. Zool. Misc. Publ. 20. 47 pp.

Hubbs, C.L. 1955. Naturally occurring hybrids in cyprinid fishes. Systematic Zool. 4: 1-20.

Huntsman, G.R. 1970. Disc gel electrophoresis of blood sera and muscle extracts from some catostomid fishes. Copeia, No. 3 pp. 457-467.

Koehn, R.K. 1969. Hemoglobins of fishes of the genus Catostomus in western North America. Copeia, No. 1 21-30.

Koehn, R.K. and D.I. Rasmussen. 1967. Polymorphic and monomorphic serum esterase heterogeneity in catostomid fish populations. Biochem. Genet. 1: 131-144.

Kritchevsky, D., S. Martak and G. Rothblat. 1963. Detection of bile acids in thin-layer chromatography. Anal. Biochem. 5: 388-392.

Kritchevsky, D. and P.P. Nair. 1971. Chemistry of the bile acids, pp. 1-3. In D. Kritchevsky and P.P. Nair (eds.). The bile acids: chemistry, physiology and metabolism, Vol. 1 Plenum Press, New York. 372 pp.

Matschiner, J.T. 1971. Naturally occurring bile acids and alcohols and their origins, pp. 11-46. In D. Kritchevsky and P. Nair (eds.). The bile acids: chemistry, physiology and metabolism, Vol. 1. Plenum Press, New York. 372 pp.

Miller, R.R. 1958. Origin and affinities of the freshwater fauna of western North America. In Zoogeography. Carl L. Hubbs (ed.) Pub. No. 51 of Am. Assoc. for Advanc. Sci. Washington, D.C. 509 pp.

Mitra, M.N. and W.H. Elliott. 1968. Bile Acids XIII. A new direct synthesis of allocholic acid and its 3β isomer. J. Org. Chem. 33: 175-181.

Moore, G.A. 1968. Fishes. pp. 21-166. In Blair, W.F., A.P. Blair, P. Brodkorb, F. Cagle and G.A. Moore Vertebrates of the United States. 2nd ed. McGraw-Hill, New York. 616 pp.

Nelson, E.M. 1948. The comparative morphology of the Weberian apparatus of the catostomidae and its significance in systematics. J. Morph. 83: 225-252.

Nelson, E.M. 1949. The opercular series of the catostomidae. J. Morph. 85: 559-567.

Norman, A. 1953. Separation of conjugated bile acids by partition chromatogarphy. Bile acids and steroids 6. Acta Chem. Scand. 7: 1413-1419.

Ohta, K. 1939. Tetraoxy-norsterocholansäure aus der "Gigi"-Fisch Galle (Pelteobagrus nudiceps) Z. Physiol. Chem. 259: 53-61.

Oklahoma Department of Wildlife Conservation. 1965. Know your Oklahoma Fishes.

Powers, D. A. and A. B. Edmundson. 1972. Multiple hemoglobins of catostomid fish I. Isolation and characterization of the Isohemoglobins from Catostomus clarkii. J. Biol. Chem. 247: 6686-6693.

Powers, D. A. and A. B. Edmundson. 1972a. Multiple hemoglobins of catostomid fish II. The amino acid sequence of the major α chain from Catostomus clarkii hemoglobins. J. Biol. Chem. 247: 6694-6707.

Sasaki, T. 1966. Stero-bile acids and bile alcohols LXXXII. Comparative studies on the bile salts of fishes by thin-layer chromatography. J. Biochem. (Tokyo) 60: 56-62.

Sjövall, J. 1953. On the separation of bile acids by partition chromatography. Bile acids and steroids 4. Acta Physiol. Scand. 29: 232-240.

Stevens, M. 1972. Oklahoma Biological Survey, Norman, Oklahoma. Pers. Comm.

Tökés, L. 1970. Nuclear-magnetic-resonance and mass-spectral examination of the principal bile alcohol from Catostomus commersoni and its anhydro derivative. Biochem J. 116: 585-587.

Tsuyuki, H., R. H. Kerr, J. F. Uthe and L. W. Clarke. 1967. Comparative electropherograms of the family Catostomidae. J. Fish Res. Bd. Canada. 24: 299-304.

Waller, G. R. 1968. A description of the LKB-9000 prototype mass spectrometer-gas chromatgraph combination. Proc. Okla. Acad. Sci. 47: 271-283.

Yamasaki, K., S. Ikawa, Y. Ayaki, and Y. Yamamoto. 1972. Natural occurrence of allocholic acid in ox and carp bile. J. Biochem. (Tokyo). 72: 769-772.

APPENDIX I

Figure 6. Infrared spectrum of allocholic acid from *Carpiodes carpio*.

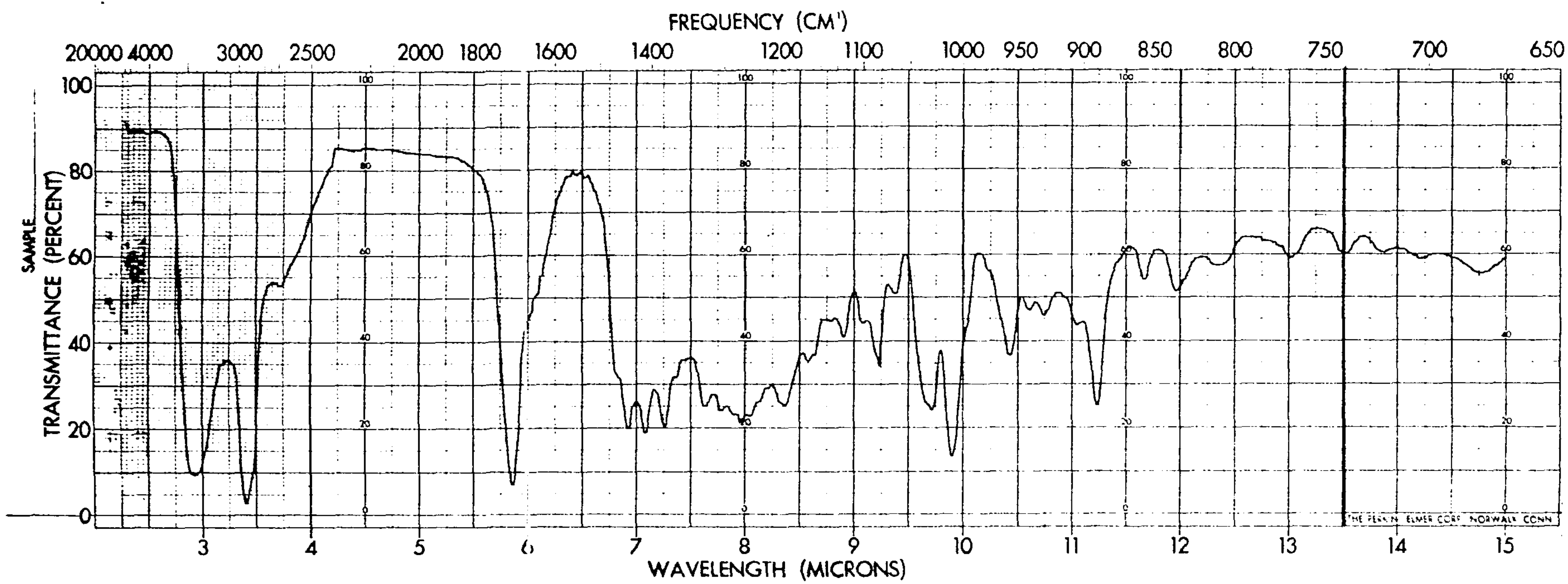

FREQUENCY (CM')
20000 4000
3000
2500
2000
1800
1600
1400
1200
1100
1000
950
900
850
800
750
700
650
100
80
60
40
20
0
SAMPLE
TRANSMITTANCE (PERCENT)
3
4
5
6
7
8
9
10
11
12
13
14
15
WAVELENGTH (MICRONS)

Figure 7. Infrared spectrum of 5α-chimaerol from *Catostomus macrocheilus*.

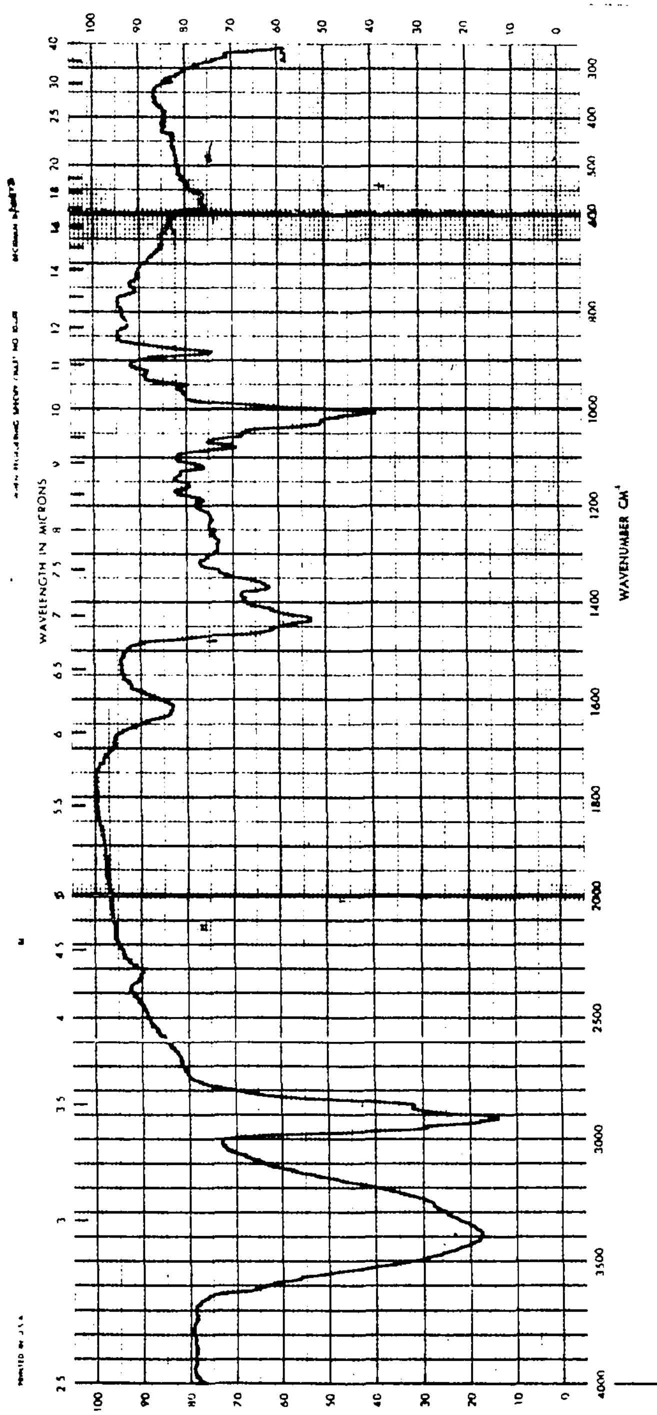

WAVELENGTH IN MICRONS
WAVENUMBER CM-1

Figure 8. Infrared spectrum of anhydro-5α-chimaerol from *Catostomus plebius*

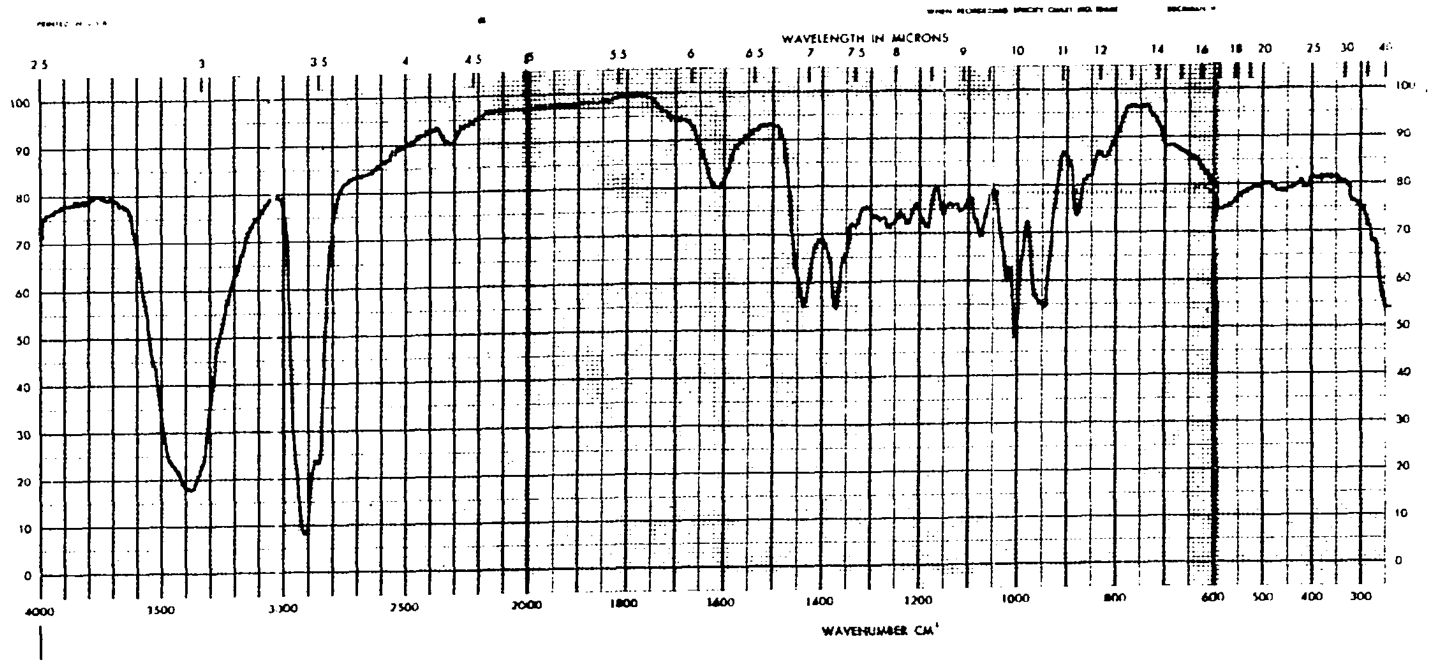
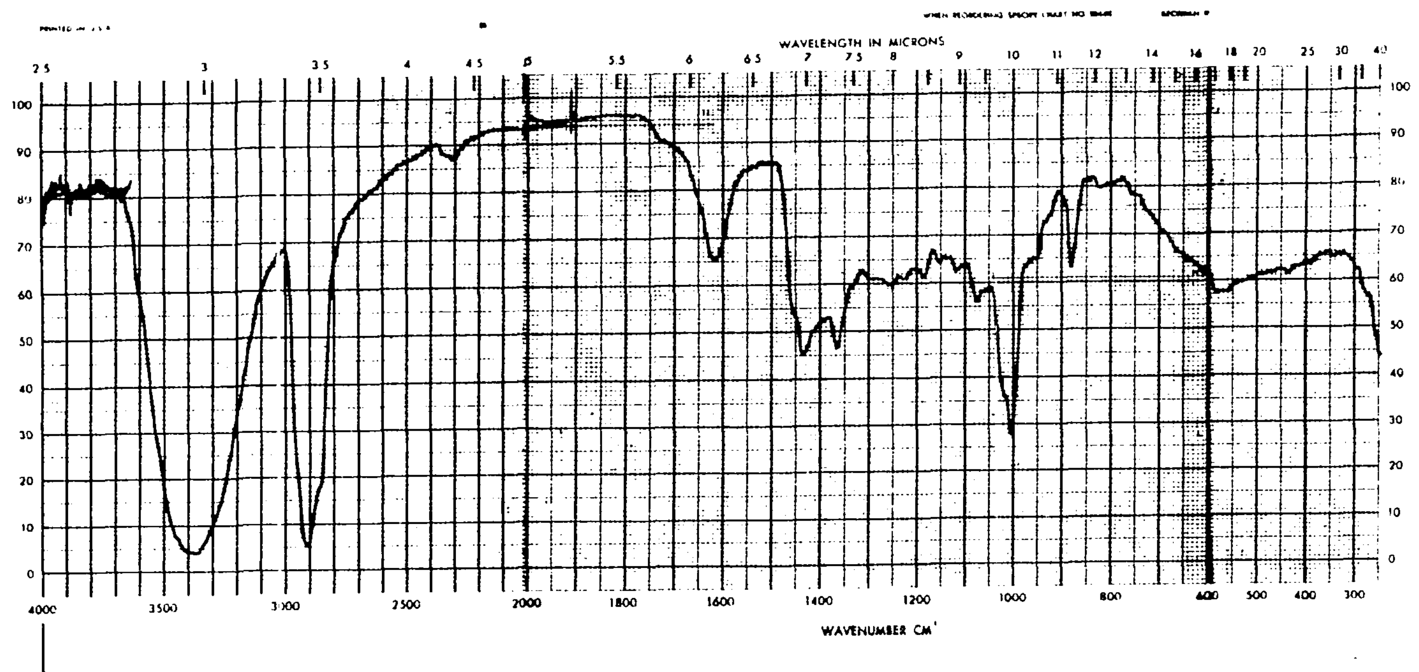

WAVELENGTH IN MICRONS
2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8 9 10 11 12 14 16 18 20 25 30 40
100 90 80 70 60 50 40 30 20 10 0
4000 3500 3000 2500 2000 1800 1600 1400 1200 1000 800 600 500 400 300
WAVENUMBER CM⁻¹

Figure 9. Infrared spectrum of 5α-chimaerol from *Chasmistes brevirostris*



WAVELENGTH IN MICRONS
2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8 9 10 11 12 14 16 18 20 25 30 40
100 90 80 70 60 50 40 30 20 10 0
4000 3500 3000 2500 2000 1800 1600 1400 1200 1000 800 600 500 400 300
WAVENUMBER CM⁻¹

Figure 10. Infrared spectrum of 5α-cyprinol from *Ictiobus cyprinellus*

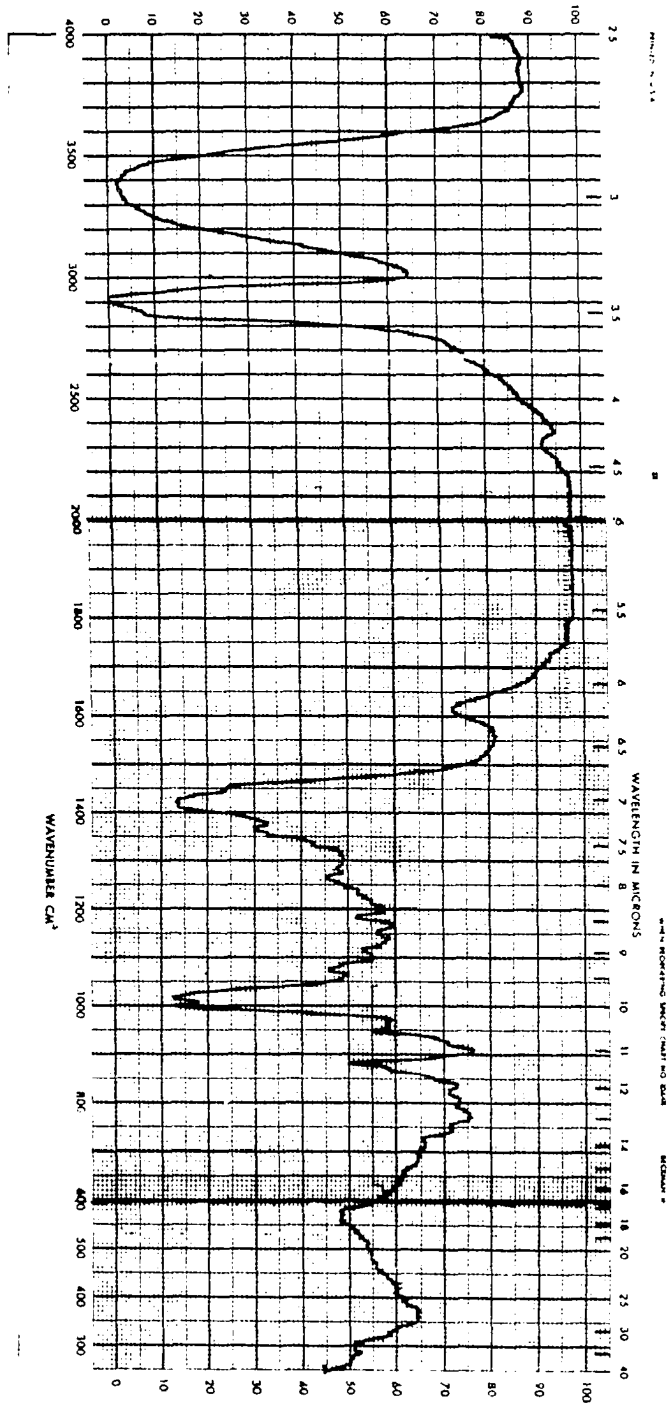

WAVELENGTH IN MICRONS
WAVENUMBER CM-1

Figure 11. Infrared spectrum of anydro-5α-cyprinol from *Ictiobus* spp.

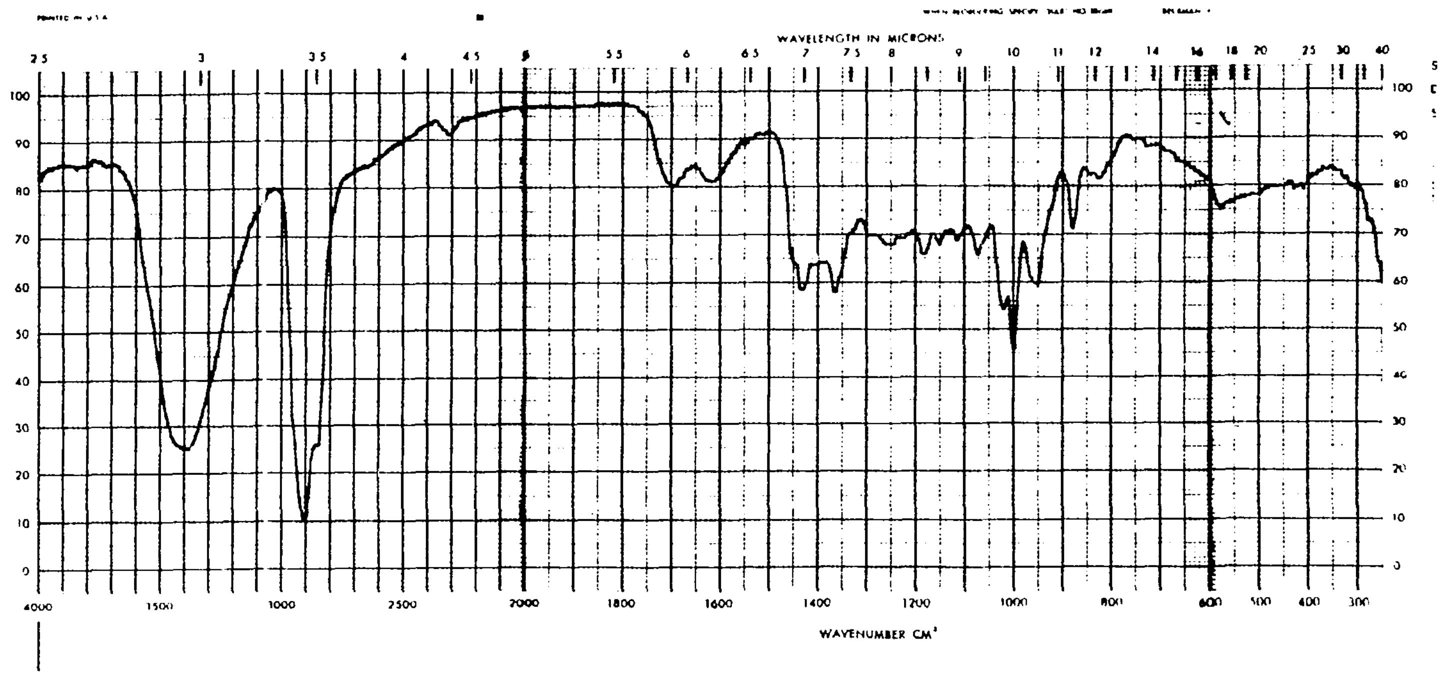

WAVELENGTH IN MICRONS
2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8 9 10 11 12 14 16 18 20 25 30 40
100 90 80 70 60 50 40 30 20 10 0
4000 3500 3000 2500 2000 1800 1600 1400 1200 1000 800 600 500 400 300
WAVENUMBER CM⁻¹

Figure 12. Infrared spectrum of alcohol complex from *Moxostoma duquesnei*

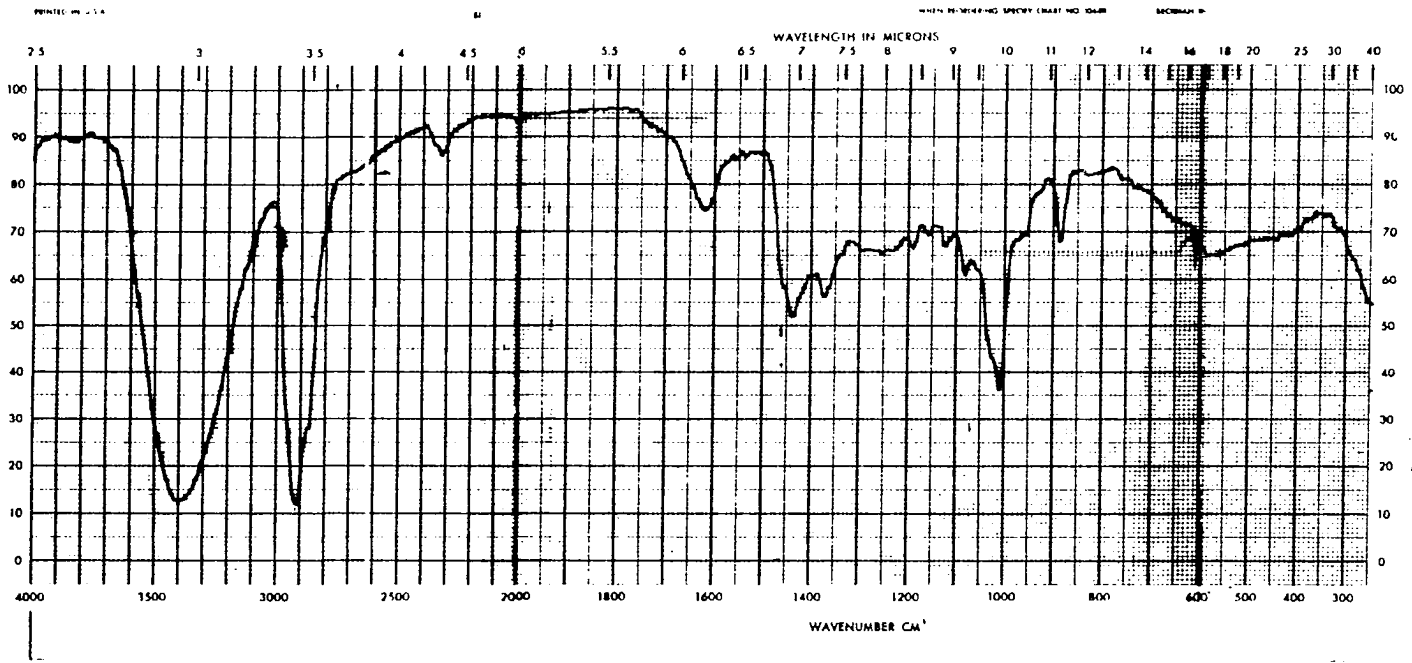
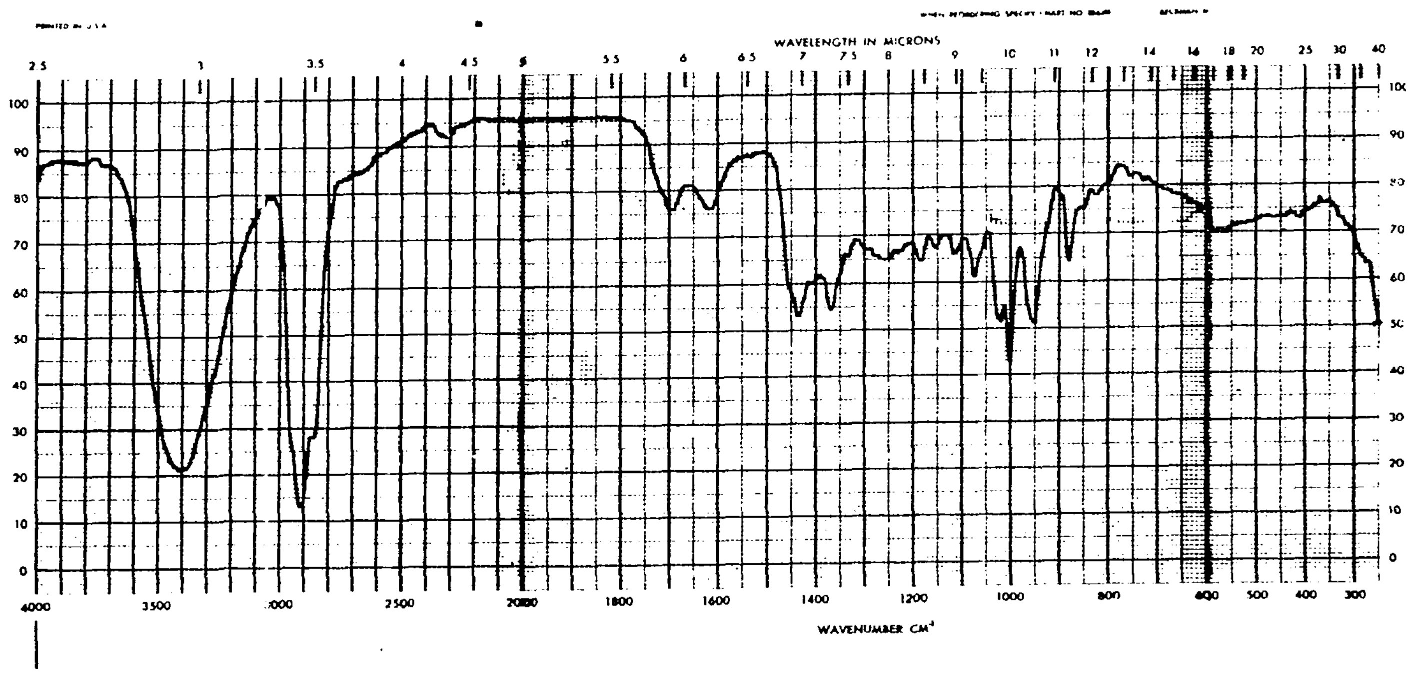

WAVELENGTH IN MICRONS
2.5
3
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
9
10
11
12
14
16
18
20
25
30
40
100
90
80
70
60
50
40
30
20
10
0
4000
3500
3000
2500
2000
1800
1600
1400
1200
1000
800
600
500
400
300
WAVENUMBER CM⁻¹

Figure 13. Infrared spectrum of anhydro alcohol complex from *Moxostoma erythrurum*


WAVELENGTH IN MICRONS
2.5 3 3.5 4 4.5 5 5.5 6 6.5 7 7.5 8 9 10 11 12 14 16 18 20 25 30 40
100 90 80 70 60 50 40 30 20 10 0
4000 3500 3000 2500 2000 1800 1600 1400 1200 1000 800 600 500 400 300
WAVENUMBER CM⁻¹

APPENDIX II

Figure 14. Nuclear magnetic resonance spectrum of 5α-chimaerol from Catostomus macrocheilus.

CHART S-60T
MADE IN U.S.A.

500 400 300 200 100 0 Hz

8.0 7.0 6.0 5.0 PPM (δ) 4.0 3.0 2.0 1.0 0

MANUAL		AUTO	SAMPLE: Chimaerol	REMARKS: Sample from *Catostomus macrocheilus*
SWEEP OFFSET (Hz):	SWEEP TIME (SEC): 250	(250)		
SPECTRUM AMPLITUDE:	SWEEP WIDTH (Hz): 500	(500)		
INTEGRAL AMPLITUDE:	FILTER: 2	(2)		
SPINNING RATE (RPS):	RF POWER LEVEL: 0.05	(.05)	SOLVENT: $CDCl_3$/TMS	

DATE: 11-30-72 OPERATOR: RSH 60 MHz NMR SPECTRUM NO.

Figure 15. Nuclear magnetic resonance spectrum of 5α-chimaerol from *Chasmistes brevirostris*.

CHART S-60T
MADE IN U.S.A.

500 400 300 200 100 0 Hz

8.0 7.0 6.0 5.0 PPM (δ) 4.0 3.0 2.0 1.0 0

MANUAL AUTO ☐ SAMPLE: Chasmistes REMARKS:

SWEEP OFFSET (Hz): —
SPECTRUM AMPLITUDE: 80
INTEGRAL AMPLITUDE: 1.2
SPINNING RATE (RPS):
SWEEP TIME (SEC): 50 250 (250)
SWEEP WIDTH (Hz): 25 50 100 250 500 (500)
FILTER: 1 2 3 4 5 6 7 8 (2)
RF POWER LEVEL: 0.05 (.05)
SOLVENT: C_6D_6/TMS

varian
analytical instrument division

DATE: 11-30-72
OPERATOR: RH

60 MHz NMR
SPECTRUM NO.

Figure 16. Nuclear magnetic resonance spectrum of 5α-cyprinol from *Ictiobus* spp.

CHART S-60T
MADE IN U.S.A.

500 400 300 200 100 0 Hz

acetone

11

15

8.0 7.0 6.0 5.0 PPM (δ) 4.0 3.0 2.0 1.0 0

MANUAL AUTO

SAMPLE: Cyprinol

REMARKS:

SWEEP OFFSET (Hz): ....

SPECTRUM AMPLITUDE: ....

INTEGRAL AMPLITUDE: ....

SPINNING RATE (RPS): ....

SWEEP TIME (SEC): 5 | 250 (250)

SWEEP WIDTH (Hz): 25 | 50 | 100 | 250 | 500 (500)

FILTER: 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 (2)

RF POWER LEVEL: ....0.05.... (.05)

SOLVENT: CD_3OD/TMS

DATE: 11-30-72

OPERATOR: RBH

60 MHz NMR

SPECTRUM NO. ....

Figure 17. Nuclear magnetic resonance spectrum of 5α-chimaerol-5α-cyprinol complex from *Moxostoma erythrurum*.

CHART S-60T
MADE IN U.S.A.

500 400 300 200 100 0 Hz

120Hz offset

710

14

12

8.0 7.0 6.0 5.0 PPM (δ) 4.0 3.0 2.0 1.0 0

MANUAL

SWEEP OFFSET (Hz): —	SWEEP TIME (SEC): 50 250	AUTO ☐ (250)	SAMPLE: GR
SPECTRUM AMPLITUDE: 63	SWEEP WIDTH (Hz): 25 50 100 250 500	(500)	REMARKS:
INTEGRAL AMPLITUDE: 1	FILTER: 1 2 3 4 5 6 7 8	(2)	
SPINNING RATE (RPS): ~40	RF POWER LEVEL: 0.05	(.05)	SOLVENT: C₆D₅/TMS

varian
analytical instrument division

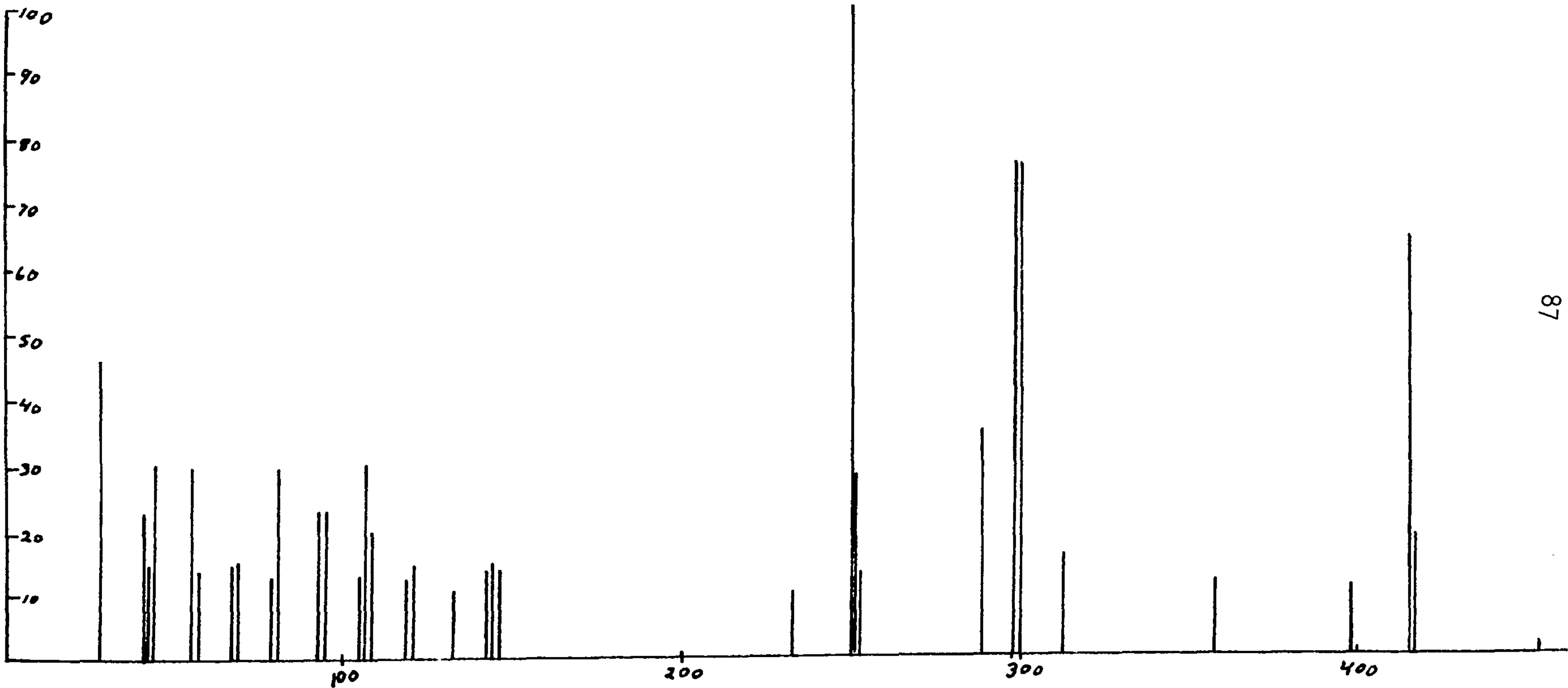
DATE: 11-30-72

OPERATOR: RH

60 MHz NMR
SPECTRUM NO.

APPENDIX III

Figure 18. Mass spectrum of 5α-chimaerol from Catostomus macrocheilus.



100
90
80
70
60
50
40
30
20
10
100
200
300
400

Figure 19. Mass spectrum of 5α-cyprinol from *Ictiobus* spp.

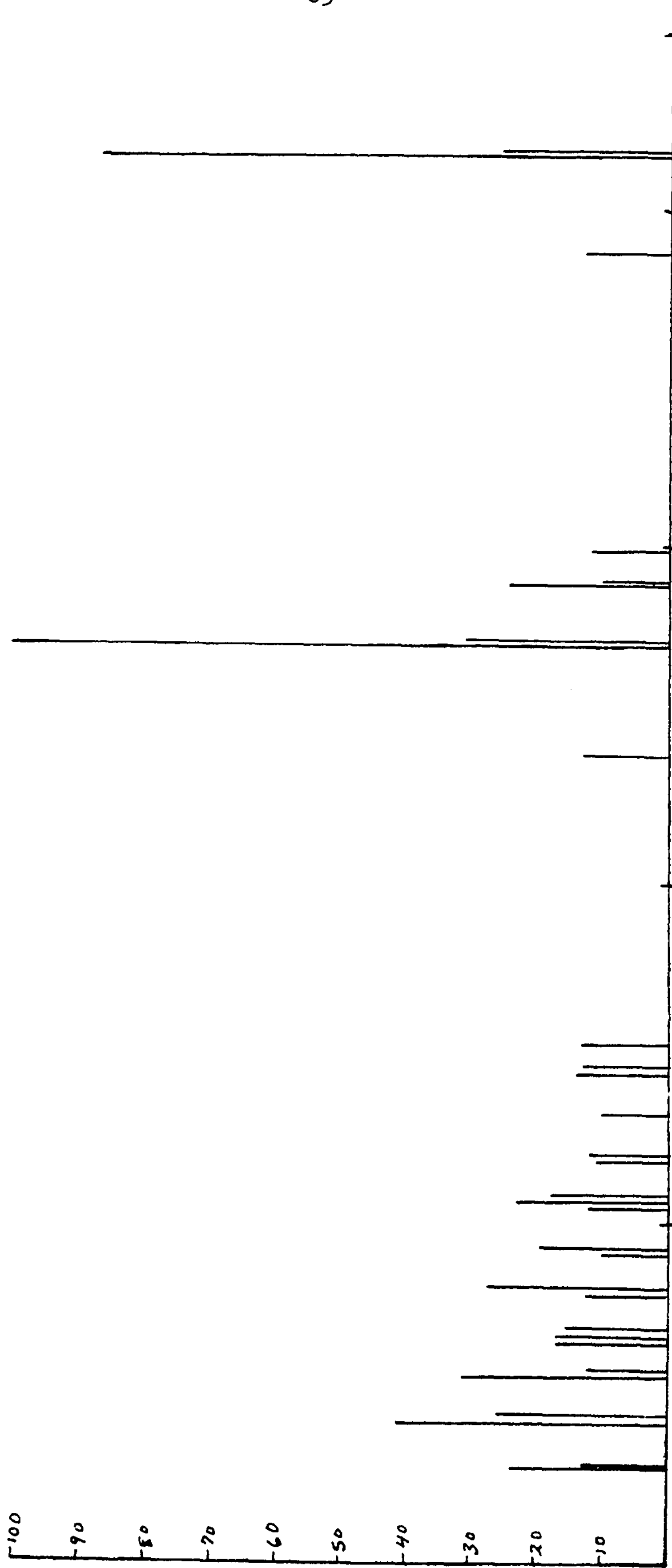
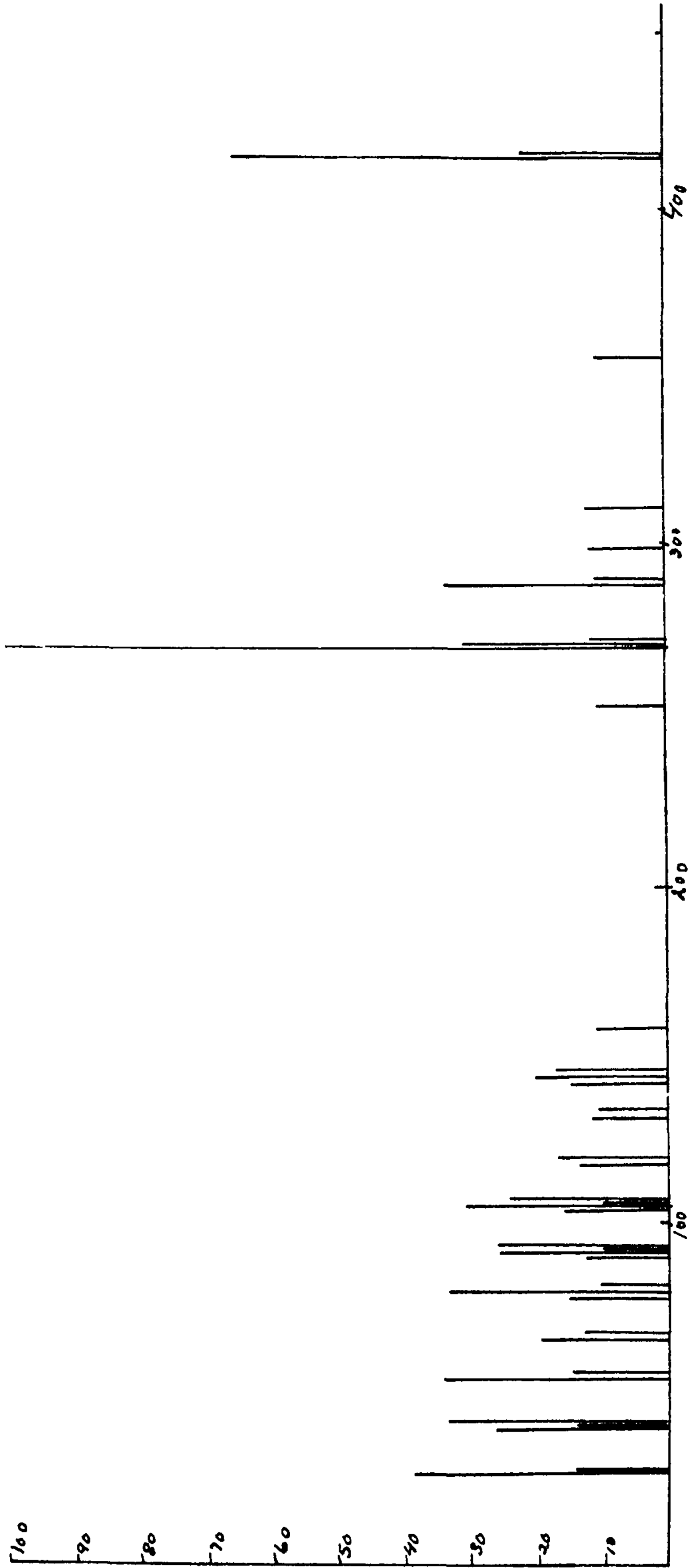

100
90
80
70
60
50
40
30
20
10

Figure 20. Mass spectrum of Redhorse alcohol complex.


100
90
80
70
60
50
40
30
20
10
100
200
300
400