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# LIPID PHARMACOLOGY

edited by  
Rodolfo Paoletti



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# LIPID PHARMACOLOGY

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## FOREWORD

The interest of pharmacologists in lipid metabolism was stimulated greatly by a symposium organized by the Institute of Pharmacology and Therapeutics of the University of Milan in 1960. The principal theme of the symposium was the effect of drugs on lipid metabolism. Both lipid biochemistry and lipid pharmacology were entering a period of rapid development, and the symposium organizers endeavored to mirror the problems of lipid research at that time. In earlier years chemical and biochemical work in the lipid field developed slowly, largely because of the relatively poor state of methodology in the field, and very few pharmacologists were interested in lipid problems. Concern about the rising death rate from cardiovascular disorders, particularly atherosclerosis, led to increased emphasis on improved methodology in lipid chemistry and to increased interest in lipid problems in general on the part of chemists, biochemists, and pharmacologists. The realization that lipid metabolism is perhaps even more sensitive than carbohydrate metabolism to environmental changes and to drug influences has been a relatively recent development.

Drugs of many kinds cause lipid metabolic changes. Biosynthetic processes may be affected, and transport and release mechanisms can be studied very effectively by the use of drugs. In fact, the use of drugs to delineate mechanisms of biosynthesis and to study the hormonal control of metabolism is currently one of the most effective ways of studying human biochemistry. Although the study of drug effects is traditionally the province of the pharmacologist, the use of drugs in this way is important to both biochemists and pharmacologists.

The great service done to lipid pharmacology through the interest and endeavors of Professor Emilio Trabucchi and his colleagues at the University of Milan is continued in this volume. Dr. Paoletti has brought together a series of summaries and reviews which reflect the current state of lipid pharmacology. This volume should stimulate pharmacologists everywhere to recognize that many unsolved problems in the lipid field are amenable to study by combined biochemical and pharmacological techniques, and that the effects of drugs on lipid metabolism are de-

serving of more intensive study than has been true in the past. We are indebted to Dr. Paoletti for his effort in organizing the present volume and for his constant interest in stimulating work in this field.

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September, 1963



## CONTENTS

CONTRIBUTORS .....	v
FOREWORD .....	vii

### Chapter 1

#### METHODS FOR THE STUDY OF LIPID CHANGES IN

##### BIOLOGICAL EXPERIMENTS • *Marjorie G. Horning*

I. Isolation of Lipids from Tissue .....	2
II. Separation of Lipid Classes .....	3
III. Chemical Methods for Class Determinations .....	20
IV. Gas Chromatography Procedures .....	23
V. Special Instrumental Methods .....	52
VI. Radioactivity Measurements .....	55
References .....	58

### Chapter 2

#### EXPERIMENTAL ATHEROSCLEROSIS • *David Kritchevsky*

I. Introduction .....	63
II. Definition of Atherosclerosis .....	66
III. Grading of Atherosclerosis .....	66
IV. General Considerations .....	67
V. Experimental Atherosclerosis in Different Animal Species .....	71
VI. Epilogue .....	112
References .....	113

### Chapter 3

#### DRUGS AFFECTING LIPID SYNTHESIS • *William L. Holmes*

I. Introduction .....	132
II. Theoretical Considerations .....	133
III. $\alpha$ -Phenylbutyric Acid and Related Compounds .....	137

IV. Derivatives of Dimethyl- or Diethylaminoethanol .....	145
V. Benzmalecene .....	157
VI. Steroids .....	158
VII. Nicotinic Acid .....	164
VIII. Vanadium .....	168
IX. Analogs of Mevalonic Acid .....	170
X. Terpenes .....	173
XI. Inhibitor of Cholesterol Synthesis (I.C.S.) .....	176
XII. 1- <i>p</i> -Chlorophenylpentylsuccinate .....	177
XIII. Psychotropic Drugs .....	178
References .....	180

## Chapter 4

### DRUGS, HORMONES, AND OTHER FACTORS INFLUENCING STEROID AND STEROL METABOLISM • *M. W. Whitehouse*

I. Introduction .....	186
II. Steroid Pharmacology .....	187
III. Methods of Investigation .....	191
IV. Physiological Control of Steroid and Sterol Metabolism ...	196
V. Some Factors Influencing Steroid and Sterol Transformations in Animal Tissues .....	205
VI. Drugs Inhibiting Steroid Biogenesis in the Adrenal Cortex	215
VII. Drugs and Hormones Influencing Cholesterol Catabolism	220
VIII. Concluding Remarks .....	229
References .....	230

## Chapter 5

### • ESSENTIAL FATTY ACIDS • *H. M. Sinclair*

I. Definition and History .....	237
II. Chemistry .....	240
III. Occurrence .....	249
IV. Metabolism .....	251
V. Function .....	256
VI. Dietary Requirement .....	267
References .....	268

## Chapter 6

## NICOTINIC ACID AND DERIVATIVES

• *O. Neal Miller and James G. Hamilton*

I. Historical Introduction .....	276
II. The Effect of Nicotinic Acid Administration on Serum Lipids of Experimental Animals .....	279
III. The Effect of Nicotinic Acid Administration on Serum Lipids in Man .....	281
IV. The Effect of the Administration of Large Oral Doses of Nicotinic Acid on Metabolism Other Than Lipid Metabolism .....	283
V. The Effect of Compounds Related to Nicotinic Acid on Lipid Metabolism .....	285
VI. The Mechanism of Action of Nicotinic Acid in Lowering Blood Lipids .....	289
References .....	295

## Chapter 7

THE THYROID AND LIPID METABOLISM • *N. B. Myant*

I. Introduction .....	299
II. The Influence of the Thyroid on Cholesterol Metabolism .....	300
III. The Influence of the Thyroid on the Metabolism of Lipids Other Than Cholesterol .....	310
References .....	320

## Chapter 8

## GONADAL HORMONES AND LIPID METABOLISM

• *Norman B. Marshall*

I. Introduction .....	325
II. Sex Differences in Incidences of Atherosclerosis and Related Mortality .....	326
III. Sex Differences in the Concentration and Distribution of Circulating Lipids .....	327
IV. Serum Lipids in the Hypogonadal State .....	330
V. Influence of Exogenous Gonadal Hormones on Circulating Lipids and Mortality .....	331

VI. Sex and Gonadal Hormone Influences on Circulating Lipids and Atherosclerosis in Animals .....	339
VII. Influence of Gonadal Hormones on Lipid Synthesis .....	348
VIII. Summary .....	351
References .....	352

## Chapter 9

### CATECHOLAMINES • *Richard J. Havel*

I. Introduction .....	357
II. Effects of Catecholamines in the Intact Animal on the Concentration of Lipids in Blood Plasma and Tissues .....	358
III. The Action of Catecholamines in Adipose Tissue .....	362
IV. The Role of Catecholamines and the Sympathetic Nervous System in the Physiological Regulation of Fat Metabolism .....	365
V. Summary .....	376
References .....	376

## Chapter 10

### HEPARIN, HEPARINOIDS, AND THE CLEARING FACTOR

• *Georg E. Cronheim*

I. Introduction .....	381
II. The Clearing Factor .....	382
III. Endogenous and Tissue Clearing Factor .....	398
IV. Clearing Factor Inhibitors .....	401
V. Role of Clearing Factor .....	403
VI. Toxicology of Heparinoids .....	407
VII. Absorption of Heparin and Heparinoids from Gastrointestinal Tract .....	408
References .....	410

## Chapter 11

### DRUGS ACTING ON LIPID CATABOLISM AND EXCRETION • *P. Preziosi*

I. Introduction .....	416
II. Essential Physiological Data Regarding the Catabolism and the Excretion of Cholesterol .....	416

III. Pharmacological Actions on the Catabolism and Excretion of Cholesterol .....	424
IV. Essential Physiological Data Regarding the Catabolism and the Excretion of Lipids Other Than Cholesterol .....	446
V. Pharmacological Actions on the Catabolism and on the Excretion of Lipids Other Than Cholesterol .....	449
References .....	450

## Chapter 12

### THE METABOLISM AND DISPOSITION OF HYPOCHOLESTEREMIC DRUGS

• *Robert L. Smith*

I. Introduction .....	460
II. Carboxylic Acids .....	464
III. Derivatives of $\beta$ -Diethylaminoethanol .....	473
IV. The Iodothyronines .....	475
V. Metabolism of Specific Iodothyronines .....	481
References .....	485
AUTHOR INDEX .....	489
SUBJECT INDEX .....	521

# Chapter 1

## METHODS FOR THE STUDY OF LIPID CHANGES IN BIOLOGICAL EXPERIMENTS

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I. Isolation of Lipids from Tissue .....	2
II. Separation of Lipid Classes .....	3
A. Column Chromatography .....	3
B. Thin-Layer Chromatography .....	10
C. Countercurrent Distribution .....	18
D. Paper Chromatography .....	18
III. Chemical Methods for Class Determinations .....	20
A. Cholesterol and Cholesterol Esters .....	20
B. Squalene .....	21
C. Triglycerides .....	21
D. Phospholipids .....	22
E. Free Fatty Acids .....	22
F. Mono-, Di-, and Triglyceride Structural Types ...	22
G. Structural Determination of Polyunsaturated Fatty Acids .....	23
H. Alkali Isomerization .....	23
IV. Gas Chromatography Procedures .....	23
A. Basic Methods .....	25
B. Separation Methods .....	27
C. Problems of Quantification .....	33
D. Identification Methods .....	35
E. Applications in Biological Studies .....	41
V. Special Instrumental Methods .....	52
A. Nuclear Magnetic Resonance .....	52
B. Mass Spectrometry .....	53
C. Infrared Absorption Spectroscopy .....	54

VI. Radioactivity Measurements .....	55
A. Thin-Layer Chromatography .....	55
B. Paper Chromatography .....	55
C. Column Chromatography .....	55
D. Gas-Liquid Chromatography .....	56
References .....	58

## I. Isolation of Lipids from Tissue

A "total lipid" fraction can be isolated from tissues by any one of several solvent extraction procedures. The two most widely employed are those involving the use of ethanol-ether (3:1)<sup>1</sup> and chloroform-methanol (2:1).<sup>2</sup> Lipids which may be found in these fractions include hydrocarbons, cholesterol and other sterols, cholesterol and other sterol esters, mono-, di-, and triglycerides, wax esters, free fatty acids, ceramides, most phospholipids, and fat-soluble vitamins and pigments (Entenman, 1961). For the quantitative extraction of special classes of compounds such as bile acids, urinary or fecal steroids, and highly polar phospholipids, special extraction procedures must be employed.<sup>3</sup>

Chloroform-methanol extraction is generally preferred at present. The operation is usually carried out at room temperature; heating is not necessary (and may be undesirable) in many applications. Liquid samples such as plasma or enzymatic incubation mixtures are extracted with 20-30 volumes of 2:1 (v/v) chloroform-methanol. Samples of tissue such as liver or brain are extracted after homogenization. Since only small quantities of "total lipid" (usually 2-10 mg) are currently required for many investigations, the homogenization and extraction of 5-10 gm of tissue will usually provide adequate amounts of material. This can be carried out with about 20 ml of chloroform-methanol in

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<sup>1</sup> The use of 3:1 (v/v) ethanol-ether for lipid extraction was introduced by Bloor (1914), and for many years this was the accepted method. It is still used for tissues which contain largely neutral lipid; adipose tissue, for example, may be extracted in this way. It is less often used today with tissues containing relatively large amounts of phospholipids and it is unsatisfactory with nervous tissue.

<sup>2</sup> Chloroform-methanol (2:1, v/v) is currently the most widely used solvent mixture for tissue extractions. The use of chloroform-methanol mixtures was initiated by Folch *et al.* (1957), and these conditions are well suited for work with tissues containing relatively large amounts of phospholipids as well as neutral lipids. Nonlipid impurities are removed by the partitioning treatment.

<sup>3</sup> Special procedures must be employed for highly polar compounds, for lipids or steroids occurring in conjugated water-soluble forms, and for tissues which present special problems. For example, bile acid extraction (Bergström and Sjövall, 1951; Norman, 1953; Sjövall, 1953) and the extraction of sulfatides (Lees *et al.*, 1959) are both difficult, and ordinary procedures are not suitable.

a micro Waring Blendor. [The tissue must be finely dispersed so that the cellular lipids may be extracted (see footnote 3).] Homogenization at full speed for two 1-minute periods is usually sufficient. It is often desirable to regulate the speed of the Blendor with a variable transformer during the initial dispersion of the tissue. Smaller amounts of tissue (less than 1 gm) are best homogenized in a glass tube of the Potter-Elvehjem type or in a Servall Micro Omni-mixer with a small volume of solvent. The homogenate is diluted with chloroform-methanol (100–150 volumes for each gram of tissue) and the mixture is allowed to stand at room temperature for at least 30 minutes. After filtration to remove the precipitated proteins, the chloroform-methanol extract is equilibrated with 0.2 volume of water and allowed to stand overnight at 4°C to permit phase separation. If equilibration is carried out with 0.1% sodium chloride solution instead of water, gangliosides are found in the methanol-water layer and cerebroside is found in the chloroform-methanol layer with other lipids. If barium chloride or calcium chloride is substituted for sodium chloride, gangliosides are transferred to the chloroform layer (Folch *et al.*, 1957). Proteolipids (Folch *et al.*, 1951), sulfatides (Lees *et al.*, 1959), phosphatidopeptides (LeBaron and Rothleder, 1960), and triphosphoinositides (Dittmer and Dawson, 1961) require special extraction procedures.

Special care must be taken with lipid extracts because many unsaturated fatty acids (linoleic, linolenic, arachidonic, and C<sub>20</sub> and C<sub>22</sub> fatty acids with five and six double bonds) are rapidly oxidized and polymerized by air at room temperature. Lipid samples should be kept in ice during handling in the laboratory, solvent evaporation should be carried out in an inert atmosphere (nitrogen) at a temperature of 50°C or lower, and samples should be stored in solvents such as benzene, hexane, isooctane, or chloroform at –14 to –20°C in an inert atmosphere. Tubes with glass or Teflon caps, flushed with nitrogen, are employed for storing samples while work is in progress.

## II. Separation of Lipid Classes

### A. Column Chromatography

Silicic acid, Florisil (magnesium silicate), and alumina have been used in column chromatographic separations of both neutral lipids and phospholipids. The fractionation of phospholipids presents a more difficult problem than the separation of neutral lipids, and it is usually desirable to separate these two groups before proceeding with additional class separations by subsequent chromatographic procedures.

The separation of neutral lipids from phosphorus-containing lipids



can be carried out with a small silicic acid column (Borgström, 1952a). A convenient method is to use size-graded (200–325 mesh), acid-washed and dried (100° for 1 hour) silicic acid. A 3–5 in. column is prepared from a slurry of 5 gm of silicic acid in chloroform-methanol (4:1, v/v). The column is washed with 50 ml of solvent mixture and then with chloroform until the column is transparent. The “total lipid” extract is placed on the column with about 5 ml of chloroform. The neutral lipid fraction is eluted with 100 ml of chloroform and the polar lipids (crude phospholipids) are then eluted with 100–150 ml of methanol. The capacity of a column of this kind is about 500 mg of “total lipid.”

An aliquot of each fraction is taken to dryness and weighed to determine recovery from the column. The aliquot of neutral lipids may be used for the colorimetric determination of sterols and triglycerides; the phospholipid aliquot may be used for the colorimetric determination of phosphorus. Samples which have been taken to dryness are not used for analysis of fatty acids by gas chromatography.

The neutral lipids are concentrated and stored under nitrogen in redistilled hexane; the phospholipids are concentrated and stored under nitrogen in benzene or chloroform.

## 1. SILICIC ACID CHROMATOGRAPHY

*a. Neutral Lipids.* The separation of neutral lipids with a silicic acid column has been carried out with mixtures of ether-petroleum ether (Hirsch and Ahrens, 1958; Fillerup and Mead, 1953; Luddy *et al.*, 1958) and hexane-benzene (Borgström, 1952b; M. G. Horning *et al.*, 1960). The latter solvent system has the advantage that relatively large changes in benzene concentration can be used, making the neutral lipid separations particularly easy to accomplish. The degree of resolution and reproducibility obtained with a silicic acid column is dependent on the regulation of the moisture content and on the use of carefully washed and graded silicic acid. A standardized preparation can be obtained with the following procedure (M. G. Horning *et al.*, 1960).

Size grading (Mallinckrodt's silicic acid No. 2847 is generally used) is carried out by dry sieving; all material passing through a 200 mesh screen but held by a 325 mesh screen (about one-half to two-thirds of the sample) is set aside for phospholipid separations. The 100–200 mesh silicic acid is stirred with several changes of 3 N hydrochloric acid (in glass apparatus) until the supernatant is almost colorless, and the material is then washed with successive changes of deionized water until the pH of the supernatant is 4.5–5.5. After washing with acetone by decantation the silicic acid is dried under an infrared lamp to remove acetone and water.