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Glucocorticoids and Glycolytic Inhibition by Lipoproteins

Theodore S. Musiala
Loyola University Chicago

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GLUCOCORTICOIDS AND GLYCOLYTIC INHIBITION BY LIPOPROTEINS

by

Theodore S. Musiala

A Dissertation Submitted to the Faculty of the Graduate School
Loyola University of Chicago
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

February, 1973
ACKNOWLEDGMENT

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The author is also indebted to the staff of the Library of the Loyola Medical Center for their aid in the documentation of this work and to colleagues in the Department of Biochemistry and Biophysics for their assistance.
BIOGRAPHY

Theodore S. Musiala was born in Chicago, Illinois on February 14, 1943.

He graduated from Saint Patrick High School, Chicago, in June, 1961. He was awarded a four-year academic scholarship to Loyola University of Chicago, and graduated with the degree of Bachelor of Science in Chemistry in June, 1965. In September, 1965, the author began his graduate studies at Loyola University, Stritch School of Medicine, Department of Biochemistry and Biophysics, under the direction of Dr. H. J. McDonald.

During the years 1965-1969, the author served as a teaching assistant in the Department of Biochemistry and Biophysics and was supported by a National Science Foundation Traineeship, Number GE-7828. In 1967, the author received the degree of Master of Science in Biochemistry. His Master's Thesis is entitled "Serum Lipoproteins and Orotic Acid." During this period he co-authored abstracts
entitled "Thermodynamic Confirmation of the Reversibility of Lipid Uptake by Rat Serum High Density Lipoproteins" and "The Effect of Uridine-5'-triphosphate on the Uptake of Rat Liver Lipids by Serum High Density Lipoproteins." From September, 1970, to September, 1971, the author served as Assistant Clinical Chemist in the Department of Pathology, University Hospitals, University of Iowa, under the direction of Dr. J. I. Routh.

The author is also co-author of publications entitled "The Effects of Phospholipids on Lipoprotein Biosynthesis in Control and Orotic Acid-Fed Rats," Federation Proceedings 26, 848 (1967) and "Amniotic Fluid Lecithin/Sphingomyelin Ratio as a Measure of Fetal Pulmonary Maturity," Iowa State Medical Society Journal, 279-284 (June, 1972).
PREFACE

The material in chapters I–IX is intended to be an introduction to the experiments of the author and the reader who is familiar with the glucocorticoid effects treated in these chapters may elect to omit them. However, each of these chapters is intended to be a complete summary of an aspect of glucocorticoid action related to the experimental problem. Since most of these aspects of glucocorticoid action are still under investigation, no final evaluation can be made. Rather, these chapters may suggest interrelations between glucocorticoids, growth hormone and insulin, one of which has been chosen for experimental study.

It may be helpful to the reader to put these chapters into context. Chapter I explores the relationship between hereditary diabetes and cortisol metabolism. Having found support for such a relationship, Chapter II examines the relationship of other hormones to the diabetogenic effect of cortisol. Chapter III describes those insulin inhibitors
which have been reported but not yet characterized. These chapters clarify the concept of insulin inhibition.

Chapter IV discusses the relationship between glucocorticoids and mineralcorticoids with respect to the use of the latter in maintenance therapy in the study of glucocorticoid effects in adrenalectomized rats. Chapter V relates current concepts of the action of glucocorticoids in glycolytic inhibition. Chapter VI describes the role of growth hormone and glucocorticoids in lipolysis and shows that lipolysis is not necessary for the anti-insulin effect of cortisol. In Chapter VII the mechanism of the glucocorticoid effect is discussed in light of known control mechanisms.

Chapter VIII describes lipoprotein structure relative to the problem of the lipoprotein insulin inhibitor. The transport function of the lipoprotein is emphasized. Chapter IX compares the effect of glucocorticoids and other steroids on serum lipoproteins. Similarities between the effects of glucocorticoids and estrogens are noted, especially with respect to their interaction with growth hormone.

Lipoprotein lipase is discussed because it also affects serum lipids. In addition, the effects of steroid hormones on its activity are reviewed. The mechanism

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which decreases the activity of this enzyme in diabetes is discussed in conjunction with the mechanism of action of insulin. Finally, an analysis of the efficacy of steroids in the treatment of mammary cancer leads to the concept that glycolytic inhibition is an important factor in the effects of glucocorticoid analogs.
GLUCOCORTICOIDS AND GLYCOLYTIC INHIBITION OF LIPOPROTEINS (Abstract) by Theodore S. Musiala, Loyola University of Chicago.

The effect of the synthetic glucocorticoid $6\alpha$-fluoro-$11\beta,17\alpha,21$-trihydroxy-1,4-pregnadiene-3,20-dione (fluprednisolone, m.p 206-208°C) on the production of physiologic inhibition of glucose oxidation in male alloxan-diabetic adrenalectomized Sprague-Dawley rats was studied. Fluprednisolone was chosen for study to minimize mineralcorticoid effects on glucose oxidation. Electrolyte balance was maintained in the adrenalectomized rats by the oral administration of sodium chloride and the sodium salt of $\beta$-glycyrrhetinic acid. The rats were maintained on a diet containing approximately 55% casein, 21% fructose, 4.4% succinate, 5% safflower oil, plus vitamins, minerals and fiber.

Only those anti-insulin factors transported by $\beta$-lipoproteins at least 6 hours after final injection of 10 mg/kg of steroid were evaluated. In order to define the relative specificity of the effect, the fluprednisolone metabolite $6\alpha$-fluoro-$11\beta,17\alpha,20\beta,21$-tetrahydroxy-1,4-pregnadiene-3-one (m.p 188-192°C) was synthetized and administered to control donor rats. Anesthetized steroid-treated donor rats were exsanguinated via the abdominal aorta and the $\beta$-lipoprotein fraction was isolated by dextran sulfate and transferred to
isotonic tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.40 at 37°C, through use of a gel exclusion column containing diethylaminoethyl anion exchange groups. In alternate experiments one week apart, each recipient rat received intraperitoneally the β-lipoprotein fraction from fluprednisolone-treated donors and weight-matched 6α-fluoro-11β,17α,20β,21-tetrahydroxy-1,4-pregnadiene-3-one treated donors. The recipient rats anesthetized with 2,2-dichloro-1,1-difluoroethyl methyl ether also received a priming dose of glucose-6-¹⁴C followed by a continuous infusion of excess insulin and labeled glucose so that glucose transport was not rate-limiting for glucose oxidation. The specific activity of expired carbon dioxide was measured at 45-minute intervals during a 225-minute period by liquid scintillation spectrometry.

The rate of glucose oxidation was apparently less following the administration of β-lipoproteins from fluprednisolone-treated rats than following the administration of β-lipoproteins from rats treated with 6α-fluoro-11β,17α,20β,21-tetrahydroxy-1,4-pregnadiene-3-one. Provided that the latter steroid treatment did not enhance glucose oxidation, the apparent effect is indicative of high specificity for C-21 steroids. It is possible that structurally related compounds with hyperglycemic effects act by binding to the same growth hormone-dependent receptor protein hypothesized to bind glucocorticoids and mediate their anti-insulin effects.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION TO DIABETES MELLITUS</td>
<td>1</td>
</tr>
<tr>
<td>Etiology of Beta Cell Exhaustion</td>
<td></td>
</tr>
<tr>
<td>Relationship to Obesity, Cancer, and Vascular Disease</td>
<td></td>
</tr>
<tr>
<td>The Heredity and Detection of the Diabetic Genotype</td>
<td></td>
</tr>
<tr>
<td>Glucocorticoid-Glucose and Fucose Tolerance Tests</td>
<td></td>
</tr>
<tr>
<td>Problems in the Study of Cortisol Metabolism in Diabetes</td>
<td></td>
</tr>
<tr>
<td>II. DIABETOGENIC FACTORS</td>
<td>34</td>
</tr>
<tr>
<td>Human Placental Lactogen</td>
<td></td>
</tr>
<tr>
<td>Growth Hormone</td>
<td></td>
</tr>
<tr>
<td>Alloxan-Diabetes</td>
<td></td>
</tr>
<tr>
<td>Interaction of Growth Hormone and Cortisol</td>
<td></td>
</tr>
<tr>
<td>Effects of Steroids and Related Compounds on the Pancreas</td>
<td></td>
</tr>
<tr>
<td>III. INSULIN INHIBITORS</td>
<td>61</td>
</tr>
<tr>
<td>Human Insulin Antagonists</td>
<td></td>
</tr>
<tr>
<td>Synalbumin in Vivo and in Vitro Inhibition by Beta Lipoproteins</td>
<td></td>
</tr>
<tr>
<td>The Specificity of Glucocorticoid Effects</td>
<td></td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV.</td>
<td>MINERALCORTICOIDS AND ELECTROLYTE BALANCE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Problem of Mineralcorticoid Therapy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACTH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adrenal Insufficiency and Diabetes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mellitus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Electrolyte Therapy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycyrrhetic Acid</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Effects of Steroids on Blood Pressure</td>
<td>76</td>
</tr>
<tr>
<td>V.</td>
<td>GLUCOCORTICOIDS AND HEXOKINASE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glycolysis in Muscle Cells and Erythrocytes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Effects of Sulphydryl Compounds and Reagents</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Properties of Hexokinase Isoenzymes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Effect of Insulin on Isoenzymes</td>
<td>100</td>
</tr>
<tr>
<td>VI.</td>
<td>GLUCOCORTICOIDS AND CYCLIC ADENYLATE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Effects on Triglyceride Synthesis and Lipolysis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Effect of Growth Hormone on Fatty Acid Oxidation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Influence of Dietary Carbohydrate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Toxicity of Growth Hormone</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Role of Phosphodiesterase and Theophylline</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N⁶-2'-Dibutryl Cyclic Adenylate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hepatic Glucocorticoid Receptor Proteins</td>
<td></td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI.</td>
<td></td>
</tr>
<tr>
<td>GLUCOCORTICOIDS AND CYCLIC ADENYLATE (continued)</td>
<td></td>
</tr>
<tr>
<td>Effect of Triiodothyronine in Hypophysectomized Rats</td>
<td></td>
</tr>
<tr>
<td>Effect of Diabetes on Citrate and Pyruvate Levels</td>
<td></td>
</tr>
<tr>
<td>Control of Lipogenesis</td>
<td></td>
</tr>
<tr>
<td>Effect of Ethanol on Gluconeogenesis</td>
<td></td>
</tr>
<tr>
<td>Effects of Clavine Alkaloids</td>
<td></td>
</tr>
<tr>
<td>Anti-Lipolytic Effects of Oral Hypoglycemic Agents</td>
<td>115</td>
</tr>
<tr>
<td>VII.</td>
<td></td>
</tr>
<tr>
<td>METABOLIC EFFECTS OF GLUCOCORTICOIDS</td>
<td></td>
</tr>
<tr>
<td>Effects of Hormones on Glycolysis in Rat Diaphragm</td>
<td></td>
</tr>
<tr>
<td>Effect of Bicarbonate</td>
<td></td>
</tr>
<tr>
<td>Effect on Peripheral Glucose Utilization</td>
<td></td>
</tr>
<tr>
<td>Changes in Hepatic Glycolytic Intermediates</td>
<td></td>
</tr>
<tr>
<td>The Catabolic Effect of Cortisol</td>
<td></td>
</tr>
<tr>
<td>Effects of Steroids on Glycogen</td>
<td></td>
</tr>
<tr>
<td>Control of Gluconeogenesis and Glycogenesis</td>
<td></td>
</tr>
<tr>
<td>Regulation of Fructolysis</td>
<td></td>
</tr>
<tr>
<td>Effects on Sugar Transport</td>
<td></td>
</tr>
<tr>
<td>Lipogenesis and Ketosis</td>
<td></td>
</tr>
<tr>
<td>Effects of Hormones on Adipose Tissue</td>
<td></td>
</tr>
<tr>
<td>Role of Glutamic Dehydrogenase in Gluconeogenesis</td>
<td>142</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS

## VIII. STRUCTURAL ASPECTS OF BETA LIPOPROTEINS
- Comparison of Human and Rat Beta Lipoproteins
- Cooperative Interactions in Beta Lipoproteins
- Abnormal Beta Lipoproteins

## IX. STEROID EFFECTS ON SERUM LIPOPROTEINS
- Etiology of Atherosclerosis
- Function of Cholesterol
- Sex Differences in the Subunit Structure of HDL
- Reciprocal Effect of Estrogens
- Comparison of Cortisone and Prednisone
- Clinical Effects of Free and Sulfate-Conjugated Estrienes

## X. LIPOPROTEIN LIPASE AND THE LIPOPROTEIN INSULIN ANTAGONIST
- Lipoprotein Lipase Activity
- Effect of Insulin, Ethanol, Catecholamines and Cyclic Adenylate Phospholipase A and Lipoprotein Structure
- Effects of Nicotinic Acid, Thyroxine, Atromid, Polyunsaturated Acids, Androsterone, Estrienes and Cholinesterase Inhibitors
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>X.</td>
<td></td>
</tr>
<tr>
<td>LIPOPROTEIN LIPASE AND THE LIPOPROTEIN INSULIN ANTAGONIST (continued)</td>
<td></td>
</tr>
<tr>
<td>Effects of Lipids on Platelet Aggregation and Fibrinolysis</td>
<td></td>
</tr>
<tr>
<td>Effects of Aldosterone Antagonists</td>
<td></td>
</tr>
<tr>
<td>Sequelae of Initial Myocardial Infarction</td>
<td></td>
</tr>
<tr>
<td>Comparison of Estrogen and Cortisol Effects on Platelet Aggregation and Glucose Tolerance</td>
<td></td>
</tr>
<tr>
<td>The Mechanism of Steroid Effects</td>
<td></td>
</tr>
<tr>
<td>Role of Phospholipids</td>
<td></td>
</tr>
<tr>
<td>Effects of Other Compounds</td>
<td></td>
</tr>
<tr>
<td>Role of Growth Hormone in Estrogen Effects</td>
<td></td>
</tr>
<tr>
<td>Phosphatidic Acid Synthesis</td>
<td>222</td>
</tr>
<tr>
<td>XI.</td>
<td></td>
</tr>
<tr>
<td>STEROID EFFECTS IN BREAST TUMOR THERAPY</td>
<td></td>
</tr>
<tr>
<td>Mechanism of Steroid Action</td>
<td></td>
</tr>
<tr>
<td>Results of Endocrine Ablation</td>
<td></td>
</tr>
<tr>
<td>Use of Steroid Discriminants</td>
<td></td>
</tr>
<tr>
<td>Function of Estriol</td>
<td></td>
</tr>
<tr>
<td>Effects of Thyroid and Growth Hormones on Steroid Metabolism</td>
<td></td>
</tr>
<tr>
<td>Structure-Function Studies on Anti-Tumor Compounds</td>
<td></td>
</tr>
<tr>
<td>Effects of Clavine and Morphine Alkaloids</td>
<td>273</td>
</tr>
<tr>
<td>Chapter</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>XII. MATERIALS AND METHODS</td>
<td></td>
</tr>
<tr>
<td>Chemicals</td>
<td></td>
</tr>
<tr>
<td>Synthesis of 6α-Fluoro-11β,17α,20β,21-Tetrahydroxy-1,4-Pregnadien-3-One</td>
<td></td>
</tr>
<tr>
<td>Thin-Layer Chromatography</td>
<td></td>
</tr>
<tr>
<td>The Assay of Insulin Inhibition</td>
<td></td>
</tr>
<tr>
<td>Preparation of Alloxan-Diabetic Adrenalectomized Rats</td>
<td></td>
</tr>
<tr>
<td>Experimental Procedure</td>
<td></td>
</tr>
<tr>
<td>Glucose Methodology</td>
<td></td>
</tr>
<tr>
<td>Handling of Radioactive Rats</td>
<td></td>
</tr>
<tr>
<td>Anesthesia</td>
<td></td>
</tr>
<tr>
<td>Metabolic Cage and Apparatus</td>
<td></td>
</tr>
<tr>
<td>Isolation of β-Lipoproteins</td>
<td></td>
</tr>
<tr>
<td>Infusion Solution</td>
<td></td>
</tr>
<tr>
<td>The Scintillation Counting of Barium Carbonate and Carbon Dioxide</td>
<td></td>
</tr>
<tr>
<td>Glycogen Analysis</td>
<td>330</td>
</tr>
</tbody>
</table>

| XIII. RESULTS | |
| Part I: Characterization of Endogenous 3-Hydroxy-17-Ketosteroids | |
| A Comparison of the Spectra of the Steroid Alcohols with those of the Corresponding o-Chlorobenzoates | |
| Anesthetic Effects on Blood Glucose and Insulin Hypoglycemia | |
TABLE OF CONTENTS

XIII. RESULTS (continued)

Part II:
The Measurement of CO₂ Specific Activity by Liquid Scintillation Efficiencies of Scintillation Solvents

Part III:
Comparison of Fluprednisolone and 20β-Hydroxyfluprednisolone Treatments Regression Statistics Sources of Random Variation.........................355

XIV. DISCUSSION

Definition of a Specific Glucocorticoid Effect
Explanation of Specificity Mechanistic Sequence in the Glucocorticoid Effect
Role of Platelet Aggregation in Diabetes Mellitus Relation to Multiple Sclerosis Role of Triglyceride Synthesis Effect of Anoxia Summary and Conclusion.................................394

BIBLIOGRAPHY..................................................403

SUPPLEMENTAL BIBLIOGRAPHY.................................506

xv
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>PERIODATE OXIDATION OF STEROID GLUCURONIDES</td>
<td>19</td>
</tr>
<tr>
<td>II.</td>
<td>SUGGESTED SYNTHESIS OF 4-CHLOROTHIOPHENE-3-CARBOXYLIC ACID CHLORIDE</td>
<td>27</td>
</tr>
<tr>
<td>III.</td>
<td>STRUCTURE OF GLYCYRRHIZIC ACID</td>
<td>90</td>
</tr>
<tr>
<td>IV.</td>
<td>D-GLYCERALDEHYDE-3-PHOSPHATE FORMATION VIA THE PENTOSE CYCLE</td>
<td>180</td>
</tr>
<tr>
<td>V.</td>
<td>STRUCTURE OF CHLORPROMAZINE</td>
<td>252</td>
</tr>
<tr>
<td>VI.</td>
<td>STRUCTURE OF SEROTONIN</td>
<td>256</td>
</tr>
<tr>
<td>VII.</td>
<td>STRUCTURE OF SANGUINARINE</td>
<td>297</td>
</tr>
<tr>
<td>VIII.</td>
<td>STRUCTURE OF 3-METHYLCHOLANTHRENE</td>
<td>301</td>
</tr>
<tr>
<td>IX.</td>
<td>STRUCTURE OF ERGONOVINE</td>
<td>312</td>
</tr>
<tr>
<td>X.</td>
<td>STRUCTURES OF AGROCLAVINE AND ELYMOCLAVINE</td>
<td>313</td>
</tr>
<tr>
<td>XI.</td>
<td>SUGGESTED SYNTHESIS OF 6,8-DIMETHYLERGOLAN-12-O1: Part I</td>
<td>320</td>
</tr>
<tr>
<td>XII.</td>
<td>SUGGESTED SYNTHESIS OF 6,8-DIMETHYLERGOLAN-12-O1: Part II</td>
<td>321</td>
</tr>
<tr>
<td>XIII.</td>
<td>METABOLIC CAGE AND APPARATUS</td>
<td>346</td>
</tr>
</tbody>
</table>

xvi
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>XIV.</td>
<td>CARBON DIOXIDE TRAPPING DEVICE</td>
</tr>
<tr>
<td>XV.</td>
<td>3α-HYDROXY-5α-ANDROSTAN-17-ONE</td>
</tr>
<tr>
<td>XVI.</td>
<td>3α-HYDROXY-5β-ANDROSTAN-17-ONE</td>
</tr>
<tr>
<td>XVII.</td>
<td>3β-HYDROXY-5α-ANDROSTAN-17-ONE</td>
</tr>
<tr>
<td>XVIII.</td>
<td>3β-HYDROXY-5β-ANDROSTAN-17-ONE</td>
</tr>
<tr>
<td>XIX.</td>
<td>3β-HYDROXY-5β-ANDROSTAN-17-ONE</td>
</tr>
<tr>
<td>XX.</td>
<td>3α-HYDROXY-5α-ANDROSTANE-11,17-DIONE</td>
</tr>
<tr>
<td>XXI.</td>
<td>3α-HYDROXY-5β-ANDROSTANE-11,17-DIONE</td>
</tr>
<tr>
<td>XXII.</td>
<td>3α-HYDROXY-5α-ANDROSTAN-17-ONE 3-o-CHLOROBENZOATE</td>
</tr>
<tr>
<td>XXIII.</td>
<td>3α-HYDROXY-5β-ANDROSTAN-17-ONE 3-o-CHLOROBENZOATE</td>
</tr>
<tr>
<td>XXIV.</td>
<td>3β-HYDROXY-5α-ANDROSTAN-17-ONE 3-o-CHLOROBENZOATE</td>
</tr>
<tr>
<td>XXV.</td>
<td>3β-HYDROXY-5β-ANDROSTAN-17-ONE 3-o-CHLOROBENZOATE</td>
</tr>
<tr>
<td>XXVI.</td>
<td>3α-HYDROXY-5α-ANDROSTANE-11,17-DIONE 3-o-CHLOROBENZOATE</td>
</tr>
<tr>
<td>XXVII.</td>
<td>3α-HYDROXY-5β-ANDROSTANE-11,17-DIONE 3-o-CHLOROBENZOATE</td>
</tr>
</tbody>
</table>

xvii
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>XXVIII.</td>
<td>EFFICIENCY VERSUS EXTERNAL STANDARD RATIO FOR QUATERNARY AMMONIUM CARBONATE FROM BARIUM CARBONATE</td>
<td>383</td>
</tr>
<tr>
<td>XXIX.</td>
<td>EFFICIENCY VERSUS EXTERNAL STANDARD RATIO FOR 0.5 MOLAR QUATERNARY AMMONIUM BICARBONATE IN TOLUENE</td>
<td>385</td>
</tr>
<tr>
<td>XXX.</td>
<td>GLUCOSE OXIDATION AFTER LIPOPROTEINS FROM FLUPREDNISOLONE AND 20β-HYDROXYFLUPREDNISOLONE TREATMENTS</td>
<td>391</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>INTRAVENOUS FLUID REPLACEMENT SOLUTION</td>
<td>86</td>
</tr>
<tr>
<td>II.</td>
<td>EFFECT OF 9α-FLUoro-11β,17α,21-Trihydroxy-16α-Methyl-1,4-Pregnadiene-3,20-Dione IN VITRO ON GLYCEROL-FFA BALANCE IN ADIPOSE TISSUE FROM FASTED RATS</td>
<td>124</td>
</tr>
<tr>
<td>III.</td>
<td>EFFECT OF BICARBONATE AND PHOSPHATE ON THE RATIO OF GLUCOSE CARBON 1 TO GLUCOSE CARBON 6 INCORPORATED INTO CARBON DIOXIDE, FATTY ACIDS, AND GLYCEROL BY ISOLATED EPIDIDYMYAL ADIPOSE TISSUE</td>
<td>147</td>
</tr>
<tr>
<td>IV.</td>
<td>EFFECT OF DIET AND HORMONES ON THE DISTRIBUTION OF GLUCOSE CARBON IN FAT PADS</td>
<td>175</td>
</tr>
<tr>
<td>V.</td>
<td>IN VITRO UTILIZATION OF CARBON-14-Labeled Glucose By Rat Diaphragm With 0.5 Units/ML Insulin</td>
<td>176</td>
</tr>
<tr>
<td>VI.</td>
<td>THE EFFECT OF CORTISOL ON GLUCOSE METABOLISM IN RAT EPIDIDYMAL ADIPOSE TISSUE IN VITRO</td>
<td>183</td>
</tr>
<tr>
<td>VII.</td>
<td>INFLUENCE OF HORMONES ON THE NICOTINAMIDE NUCLEOTIDE COENZYMES OF RAT EPIDIDYMAL FAT PAD</td>
<td>193</td>
</tr>
</tbody>
</table>

xix
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIII. EFFECT OF 20 DAYS OF GLUCOCORTICOID TREATMENT ON THE PLASMA LIPIDS OF THE RABBIT</td>
<td>212</td>
</tr>
<tr>
<td>IX. RELATIVE EFFECTIVENESS OF STEROIDS AGAINST BREAST CANCER</td>
<td>291</td>
</tr>
<tr>
<td>X. PERCENT DISTRIBUTION OF ADRENAL STEROIDS IN HUMAN PLASMA FRACTIONS VERSUS TIME AFTER AN INTRAVENOUS TRACER DOSE</td>
<td>306</td>
</tr>
<tr>
<td>XI. PHYSICAL PROPERTIES OF CLAVINE ALKALOIDS RELATED TO STEROIDS</td>
<td>314</td>
</tr>
<tr>
<td>XII. GLUCOCORTICOID AND PROGESTATIONAL ACTIVITIES OF SUBSTITUTED PROGESTERONES</td>
<td>328</td>
</tr>
<tr>
<td>XIII. CHEMICALS</td>
<td>331</td>
</tr>
<tr>
<td>XIV. CHEMICALS</td>
<td>332</td>
</tr>
<tr>
<td>XV. GLUCOSE INFUSION SOLUTION</td>
<td>350</td>
</tr>
<tr>
<td>XVI. MELTING POINTS AND POSITIVE SPECIFIC ROTATIONS OF MAJOR 17-KETOSTEROIDS AND THEIR ACETATES</td>
<td>356</td>
</tr>
<tr>
<td>XVII. INFRARED ABSORPTION MAXIMA OF STEROID KETONES</td>
<td>358</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
</tr>
<tr>
<td>XVIII.</td>
<td>INFRARED ABSORPTION CHARACTERISTICS OF 17-KETOSTEROIDS IN CARBON DISULFIDE SOLUTION</td>
</tr>
<tr>
<td>XIX.</td>
<td>INFRARED ABSORPTION CHARACTERISTICS OF 17-KETOSTEROID 3-o-CHLOROBENZOATES IN SOLUTION</td>
</tr>
<tr>
<td>XX.</td>
<td>NORMALIZED INFRARED ABSORPTION CHARACTERISTICS OF 17-KETOSTEROID 3-o-CHLOROBENZOATES IN SOLUTION</td>
</tr>
<tr>
<td>XXI.</td>
<td>EFFECT OF 2-CHLORO-1,1,2-TRIFLUOROETHYL ETHYL ETHER ON BLOOD GLUCOSE IN 4-HOUR FASTED RATS</td>
</tr>
<tr>
<td>XXII.</td>
<td>THE EFFECT OF PRETREATMENT WITH 2.1 MG OF THE ANESTHETIC STEROID 17α,21-DIHYDROXY-5α-PREGNANE-3,11,20-TRIONE 21-ACETATE ON THE ONSET OF HYPOGLYCEMIC DEATH FOLLOWING THE INJECTION OF 1 ML OF 44 PERCENT GLUCOSE AND 1 UNIT OF INSULIN INTO 6-HOUR FASTED ALLOXAN-DIABETIC-ADRENALECTOMIZED RATS</td>
</tr>
<tr>
<td>XXIII.</td>
<td>EFFICIENCY VERSUS EXTERNAL STANDARD RATIO FOR 0.5 MOLAR SOLUENE BICARBONATE IN TOLUENE CONTAINING 6.0 GRAMS/LITER DIPHENYLOXAZOLE</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>XXIV.</td>
<td>EFFICIENCIES OF SCINTILLATION SOLVENTS CONTAINING 6.0 GRAMS/LITER DIPHENYL-OXAZOLE AS PRIMARY FLUOR AS DETERMINED WITH HEXADECANE-1-14C.</td>
<td>387</td>
</tr>
<tr>
<td>XXV.</td>
<td>GLUCOSE OXIDATION AFTER LIPOPROTEINS FROM FLUPREDNISOLONE AND 20β-HYDROXY FLUPREDNISOLONE TREATMENTS</td>
<td>390</td>
</tr>
<tr>
<td>XXVI.</td>
<td>REGRESSION STATISTICS</td>
<td>392</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION TO DIABETES MELLITUS

Etiology of Beta Cell Exhaustion

The academic problem presented by diabetes mellitus is not the relative insulin deficiency but how it develops. The two most important expressions of this disease from this standpoint are "juvenile" diabetes and "maturity-onset" or adult diabetes. These two types of diabetes differ primarily in their rates of development. That juvenile diabetes is an accelerated form of adult diabetes is not unreasonable in view of the rapid rate of fetal growth.

What is the genetic basis of inherited insulin antagonism? In particular, what single gene might be responsible for it? What is the relationship of known diabetogenic factors to the genetic factor? The purpose of this dissertation is to explore the role of cortisol in insulin resistance and the possibility that a genetic defect in cortisol metabolism may lead to the overproduction of a circulating insulin antagonist.
Knowledge of the genetic defect in diabetes mellitus will not only aid in understanding the progression of the disease, but also will aid in its more accurate detection in the population before it is expressed clinically.

**The Relationship to Obesity, Cancer, and Vascular Disease**

Yalow and Berson (118) found that the response of insulin to glucose was delayed in obesity. This delay is apparently related to the high fasting insulin levels which are also found in obese subjects. The continuous output of insulin in the fasting state prevents the maximal storage of insulin granules in the \( \beta \) cells and abolishes the initial burst of insulin release in response to glucose.

Arendt and Pattee (19) performed combined insulin-glucose tolerance tests on obese subjects. They administered 0.1 unit of insulin per kilogram of body weight intravenously followed in 30 minutes by 0.8 grams of glucose per kilogram of body weight orally. In 32 obese subjects the response to this procedure was a fall in blood glucose to 50 percent of its fasting level at 90 minutes and then a maximum rise to 164 percent of fasting levels at 120 minutes. Glucose remained high at 180 minutes. In contrast, the normal response consisted of an initial fall in blood glucose to 38 percent of the fasting level followed by a maximum rise to 114 percent of the fasting level at 90 minutes.
Furthermore, the blood glucose level in control subjects fell below the fasting level before 180 minutes had elapsed. The obese group was clearly resistant to the administered insulin. In addition, their insulin response to glucose was clearly subnormal.

That this subnormal response is not due to lower serum insulin levels is illustrated in the work of Karam et al (562). These workers measured blood insulin levels at 30, 60, 90, and 120 minutes after oral and intravenous glucose administration. They found that obese diabetic patients had higher insulin levels at each interval compared to normal subjects. In addition, basal insulin levels were 30-146 microunits per ml in the obese group compared to 30-84 microunits per ml in the control group. These results show that the decreased glucose tolerance in obesity is not due to a decreased level of insulin in the blood. It is clear evidence for increased insulin antagonism in obesity. More than 51 percent of male diabetics and 59 percent of female diabetics are 20 percent heavier than ideal weight. In many cases a remission of diabetes follows weight loss.

Another interesting finding in this interrelationship has been reported by Yalow et al (1181). They found that prolonged fasting decreases the glucose tolerance of normal subjects but that it did not affect the glucose tolerance of
obese or diabetic obese subjects. These results indicate that obesity impairs the response to insulin by the same mechanism operative during fasting.

The Heredity and Detection of the Diabetic Genotype

Although the inheritance of abnormal glucose tolerance does not follow a simple recessive or dominant pattern, if diabetes mellitus is due to a single gene, its inheritance must follow one of these patterns. As noted earlier, insulin resistance will not produce abnormal glucose tolerance unless insulin output is deficient. Therefore, in the general population, the inheritance of the diabetic phenotype may be multi-factorial, including genes for insulin production and insulin destruction as well as for insulin resistance. Because of these modifying genes, the incidence of diabetes among the children of 0, 1, and 2 diabetic parents is 0.2, 1.6, and 12-14 percent respectively.

In more homogeneous populations containing more uniform modifying genes diabetes is inherited in dominant fashion. Pavel and Pierptea (817) studied a homogeneous population of 14,000 diabetics in Bucharest over a 26-year period. They observed a 27.7 percent incidence of diabetic descendants in 385 families in which both parents were diabetic. When this figure was corrected for the increasing life span of the descendants, the corrected incidence was 31.7 percent.
This percentage is less than the 50 percent incidence of heterozygotes expected for dominant inheritance. However, the difference could be due to an increased mortality among diabetic compared to non-diabetic fetuses. Vallance-Owen (1086) also found that the synalbumin insulin antagonist was inherited as an autosomal dominant but that overt diabetes developed in only 16 out of 58 individuals with inherited insulin antagonism. This percentage (27.7 percent) is similar to that noted above.

**Glucocorticoid-Glucose and Fucose Tolerance Tests**

The detection of the diabetic genotype is much more difficult than the detection of the diabetic phenotype. Although elevated fasting glucose is often indicative of diabetes mellitus, the results of the standard glucose tolerance test are less definitive. Fajans et al (303) have shown that standard glucose tolerance is randomly distributed in the population. Therefore, only a probability factor can be assigned from the results of this test.

In contrast to the random distribution of standard glucose tolerance, the cortisone-glucose tolerance test of Fajans and Conn (300) is somewhat more definitive in that 30 percent of the positive responders to this test later suffer marked deterioration in standard glucose tolerance due to the presence of the diabetic genotype. Since diabetes mellitus is
responsible for only one out of three positive cortisone-glucose tolerance tests, other factors besides the gene may impair glucose tolerance following cortisone. Three factors which may influence the cortisone-glucose tolerance test are: 1) the rate of cortisone metabolism; 2) hepatic glucose output in response to cortisone, and 3) peripheral glucose utilization in response to cortisone. Apparently, the gene for diabetes mellitus influences only the last factor.

Fajans and Conn (301) reported the details of the cortisone-glucose tolerance test. In the test approximately 8.0 mg/kg of cortisone acetate is administered to the subject 8.5 hours and 2.0 hours before the administration of 1.75 gm of glucose per kg of ideal weight. The long treatment period is noteworthy in reference to the mechanism of cortisone in this effect. When glucose is measured by the Somogyi-Nelson method, the 2-hour value should be less than 1.40 gm/l compared to 1.20 gm/l in the standard glucose tolerance test.

Fajans and Conn pointed out the utility of the cortisone-glucose tolerance test in deciding borderline results from the standard glucose tolerance test. They found that 90 percent of "probable" positive responders to the latter test to be positive responders to the cortisone-glucose tolerance test. They also observed that 28 percent of the relatives of diabetics gave positive cortisone-glucose tolerance tests,
but that only 4 percent of non-diabetics did so. This latter percentage is that expected from random error alone.

Prior to the cortisone-glucose tolerance test Thorn et al (1064) found that when a daily dose of 50 mg of prednisolone is withdrawn from normal and diabetic subjects, the latter continue to excrete glucose after 3 days while the non-diabetic subjects do not. This result suggests that glucocorticoid effects are prolonged in diabetics.

Duncan (259) has described an intravenous cortisone-glucose tolerance test in which 200 mgs of cortisone is administered 2 hours before glucose. He described the merits of plotting the glucose tolerance curve on semi-log paper. When the logarithm of the increase in blood glucose above the fasting level is plotted versus time, a straight line is obtained. A glucose disappearance rate can be calculated from the slope of this line. Approximately 83 percent of patients with mild diabetes but no non-diabetics have subnormal glucose disappearance rates. These results suggest that the severity of diabetes may be measured by the half-life of blood glucose. Thus, one may refer to 30-minute diabetics, 60-minute diabetics, etc. Furthermore, since half-lives are inversely proportional to glucose disappearance rates, relatively few equally spaced half-lives encompass a wide variation in the severity of diabetes. These
reports also indicate that glucocorticoids are involved in the mechanism of genetic diabetes. Berger et al (68) concluded that the mechanism of the cortisone-glucose tolerance test involved peripheral insulin antagonism. The fact that juvenile diabetes is often evident at birth may be an indication of the effect of insulin inhibitor concentration on the rate of β cell exhaustion.

The standard glucose tolerance test has two deficiencies: elevations in glucose stimulate insulin release and, secondly, most of the glucose is converted to glycogen so that the rate of glucose disappearance is not equal to the rate of glycolysis.

Pituitary basophilism results in an overproduction of ACTH in Cushing's Syndrome. Conn (103) has observed that 70 percent of these patients exhibit an insulin-resistant diabetes mellitus. Pfeiffer (831) found that both Cushing's Syndrome and acromegaly were preceded by long periods of impaired glucose tolerance associated with elevations in plasma insulin and an inverted circadian rhythm of ACTH secretion. On the other hand, insulin resistance due to insulin antibodies is not associated with the excretion of cortisol metabolites. The insulin requirement is not proportional to cortisol production even though cortisol output may be 4-23 fold elevated when the diabetes is uncontrolled. Rose
(903) has also demonstrated increased adrenocortical activity in alloxan-diabetic rats. The treatment of insulin resistance with excess insulin may actually increase the resistance. Somogyi (1003) noted that insulin hypoglycemia increases glucagon and glucocorticoid secretions which have hyperglycemic and anti-insulin effects.

What is needed is an extracellular indicator of the intracellular concentration of glucose-6-phosphate. This hexose phosphate is the precursor of several other hexoses which are incorporated into serum glycoproteins. The most convenient of glycoprotein hexoses to measure is fucose, 6-deoxy-L-galactose. The level of free serum fucose is negligible and it can be quantitated specifically in the presence of other reducing sugars by means of the Dische methylpentose reaction using cysteine and sulfuric acid. This colorimetric reaction at 400 nm is no more difficult than glucose measurement by ferricyanide reduction.

Fucose is incorporated into glycoproteins by a multi-step reaction sequence consisting of the conversion of glucose-6-phosphate to fructose-6-phosphate, then to mannose-1-phosphate, then to guanosine diphosphate-D-mannose. This nucleotide sugar is converted to guanosine diphosphate-L-fucose and subsequently attached to the completed protein chains of serum glycoproteins. Shaw et al (975) performed
the standard oral glucose tolerance test on a group of patients and classified all those whose one and two hour glucose concentrations were above 1.60 gm/l and 1.20 gm/l respectively as frank or overt diabetics. The cortisone-glucose tolerance tests was performed on the remaining patients and all positive responders were classified as subclinical or genetic diabetics. The remainder served as normal controls. When serum fucose levels during the standard glucose tolerance test were evaluated, the overt diabetics had two-hour fucose levels in excess of 0.75 gm/l, the subclinical diabetics had fucose levels in excess of 0.50 gm/l, and the controls all had fucose levels lower than 0.30 gm/l. Little overlap was observed between these groups, indicating that glycolytic inhibition is a good indication of genetic diabetes.

Navarete and Torres (776) reported the superiority of the synthetic glucocorticoid dexamethasone (9α-fluoro-11β, 16α, 17α, 21-tetrahydroxy-1, 4-pregnadiene-3, 20-dione) in glucocorticoid-glucose tolerance tests. They found 80 percent positive responses in prediabetics compared to 30 percent positive responses in the cortisone-glucose tolerance test. This is an important finding because it suggests that differences in the rates of the metabolism of synthetic and endogenous glucocorticoids may exist. In particular, a more rapid metabolism of cortisone in certain individuals
may prevent the expression of the diabetic gene. Lundback (G83) has also noted that individual variations in the response to cortisone or prednisolone were partly responsible for the false negative responses in the cortisone-glucose tolerance test.

These results suggest that the measurement of serum glycoprotein fucose during the dexamethasone-glucose tolerance test may provide almost complete detection of subclinical diabetes and, as such, may be of great utility in genetic studies of diabetes, including genetic counselling.

More importantly, these reports indicate glucocorticoids are involved in the expression of genetic diabetes. Berger et al (68) concluded that the mechanism of the cortisone-glucose tolerance test involved peripheral insulin antagonism. Furthermore, if juvenile diabetes is simply a double dose of insulin antagonism, the fact that juvenile diabetes is often evident at birth may be an indication of the effect of insulin-inhibitor concentration on the rate of β cell exhaustion. These same considerations also suggest that fetal glucocorticoid metabolism may be an important factor in the development of juvenile diabetes. The extent to which juvenile diabetes may be influenced by maternal glucocorticoid metabolism remains to be determined. Insulin resistance in tissues other than adipose tissue
may lead to obesity as long as the islet cells can respond with increased insulin output because andipose tissue is especially sensitive to insulin. Both the β lipoprotein insulin inhibitor and the synalbumin insulin antagonist are more active on muscle than on adipose tissue. In contrast, Antoniades (16) has demonstrated that bound insulin is more active on adipose tissue than on muscle. However, all of the insulin effects reported here have been demonstrated with unbound insulin in vitro. Therefore, the activity of bound insulin in the presence of excess of unbound insulin appears to be insignificant. Furthermore, an effect of glucocorticoids or other hormones on bound insulin has not been demonstrated.

Does diabetes mellitus like sickle cell anemia have survival value under certain conditions? When Eskimos subsist on a diet of lipid and protein with minimal carbohydrate, their incidence of overt diabetes is low. However, when they switch to a standard American diet, their incidence of diabetes is increased. Apparently, insulin resistance is relatively unimportant when dietary carbohydrate is low. As noted previously, an insulin antagonist which is active on muscle but not on adipose tissue promotes fat synthesis as long as islet cell insulin reserve can meet the demand. Accordingly, the gene for
diabetes mellitus may have been of value in enhancing fat formation from carbohydrates and have been of survival value during periods of seasonal abundance and scarcity. One of the factors contributing to the persistance of the gene has been that it is expressed late in life, usually after the reproductive years. In the past, few of the carriers of this gene lived long enough to develop the disease.

Problems in the Study of Cortisol Metabolism in Diabetes

Several studies of steroid metabolism in diabetes have been published. Chow and Barrous (181) in 1950 commented on the high ratio of unchanged cortisol to conjugated cortisol metabolites in the urine of diabetics. Subsequently, Garren and Cahill (360) reported diminished ring A reduction of cortisol, cortisone, and corticosterone as well as of the C-19 steroid 4-androstene-3, 17-dione in hepatic microsomes from alloxan-diabetic rats. Gold and Garren (382) and Charo-sal Gado et al (174) confirmed these findings and postulated that the reduced rate of testosterone inactivation in diabetic women contributed to the virilism observed in these patients.

Rifkin et al (884) have found that diabetic retinopathy and nephropathy are associated with a low excretion of adrenal androgens. It is therefore of interest that Lipsett (670) also found a low excretion of adrenal androgens in patients
with gonadal dysgenesis. Although a small amount of cortisol is found in normal urine, patients with gonadal dysgenesis may excrete up to 5 percent of administered cortisol unchanged. Forbes and Engel (323) have also noted a high incidence of gonadal dysgenesis in diabetics and their close relatives. Interestingly, Dilman et al (233) also attributed an observed lower incidence of endometrial carcinoma in diabetic women to hypothalamopituitary hypoactivity. This negative correlation between diabetes mellitus and malignancy has also been observed by others. Hudan (487) observed that the occurrence of malignant tumors in diabetic males was 87 percent of that in the total population; the corresponding figure for diabetic females was 37 percent. Although diabetes is equally prevalent among men and women, the ratio of male to female diabetic deaths observed by Malins et al (702), 0.60 in recent years, indicates that diabetes mellitus more adversely affects women despite the protection which it affords against cancer.

Although the above studies have ruled out a causative role of ring A reduced metabolites in diabetes mellitus, this does not imply that such steroids are devoid of antiglycolytic effects. The anesthetic activity of 21-hydroxy-5α-pregnane-3, 20-dione 21 succinate (Viadril) has been noted previously. Furthermore, Dundee (261) has recently
compared 3α-hydroxy-5α-pregnane-11, 20-dione and 3α, 21-di-
hydroxy-5α-pregnane-11, 20-dione 20 acetate (CT 1341) with
other intravenous anesthetics. Compounds of this type seem
to produce anesthesia by reducing the rate of glycolysis in
brain (398-399). The overall impression of these studies
is that glucocorticoids may have an especially effective
anti-glycolytic effect in brain and in malignant tissues
with high glycolytic rates.

Some preliminary experiments were conducted with the
aim of studying cortisol metabolism in diabetes. Basically,
one may expect to find higher levels of cortisol in the
plasma of diabetics. However, since the level of cortisol
may fluctuate, meaningful results can only be obtained if
the rate of metabolism of labeled cortisol is corrected
for the rate of cortisol production. If there is deficient
5β reduction of cortisol in diabetes, this will be reflected
in an increased ratio of 5α to 5β reduced urinary cortisol
metabolites.

The determination of cortisol metabolites in urine by
published procedures is lengthy because of the long period
of glucuronidase treatment which is necessary to hydrolyze
glucuronide conjugates. Furthermore, the large number of
closely related metabolites present often necessitates
multiple successive chromatographic steps in which the
separated metabolites must be located by non-destructive techniques. The problem of detecting ring A reduced cortisol metabolites during column chromatography can be solved by chromatographing ultraviolet-absorbing aromatic steroid esters. The choice of the particular ester for this use is based on several considerations. First, the esterifying reagent should form distinct steroid derivatives. Practically, this requirement translates into a reagent that will esterify only unhindered primary and secondary hydroxyl groups. Secondly, the derivative formed should be more soluble in carbon disulfide than their parent compounds to facilitate infra-red analysis in solution. Thirdly, the esterifying reagent should be capable of being conveniently labeled with an isotope that can be distinguished from both carbon-14 and tritium. In addition, a procedure using such a reagent should be designed to minimize the number of metabolites which must be separated and quantitated without reducing the metabolic information present in the metabolites. Although these studies are primarily concerned with 11-oxygenated steroid metabolites, the procedure described below is applicable to 11-deoxy steroids as well.

Generally, the first step in urinary steroid analysis begins with the collection of a 24-hour specimen. Secondly,
an aliquot of pooled urine from non-diabetics who have previously received an identical dose of glucocorticoid labeled with an indicator is added to both non-diabetic and diabetic urines. For example, if the metabolism of carbon-14 labeled cortisol is being studied in diabetics then pooled urine from diabetics given a comparable dose of tritiated cortisol will be added to each carbon-14 containing urine. If identical aliquots of indicator metabolites are added to the 24-hour specimens from control non-diabetics and diabetics such an addition then permits recovery corrections to be applied to each steroid metabolite measured. This procedure has the advantage that each indicator conjugate is similar to the conjugate which is to be measured so that it is a true indication of procedural losses.

The next step is to separate the urinary steroids and their conjugates from the bulk of the urine. This has been achieved by the absorption of both the free and conjugated steroids on diethylaminoethyl Sephadex anion exchange dextran gels. The absorbed steroids are washed with buffer to remove residual urine and then eluted from the gel with an aqueous base. Methanolic ammonia has been used, but other volatile organic amines such as triethylamine might also be used to elute the conjugated and non-conjugated steroids. The eluate from such a separation is
evaporated to dryness under reduced pressure and the residue is dissolved in water. A solution of sodium borohydride is then added which reduces steroid ketones over a period of several hours. Although many different ketone groups may react with this reagent, its primary function is to reduce the C-20 ketone to the corresponding C-20 hydroxyl group so that glucocorticoid metabolites with the dihydroxyacetone side chain will be measured along with their corresponding metabolites with glycerol side chains after the latter are converted to 17-ketosteroids by sodium metaperiodate treatment. Metaperiodate oxidation of vicinal glycols provides an alternate method for liberating steroids from their glucuronide conjugates. Essentially, the procedure oxidizes the glycerol side chain to a 17-ketosteroid and other glucuronides to formate esters which are readily hydrolyzed by weak bases. As in the enzymatic glucuronidase hydrolysis procedure, the metaperiodate procedure may give low yields when applied directly to diabetic urines containing high levels of glucose. However, this is not a problem if the conjugates are first isolated by column absorption as they are here. The chemical equations illustrating these reactions are shown in the Figure I.

Following oxidation and destruction of excess periodate the mixture is treated with sodium bicarbonate to saponify
FIGURE I

PERIODATE OXIDATION OF STEROID GLUCURONIDES

\[
\begin{align*}
&\text{COOH} \quad \text{H} \quad \text{O} \\
&\text{H} \quad \text{HO} \quad \text{H} \quad \text{OH} \quad \text{CH}_3 \quad \ldots \text{H} \\
+ 5 \text{IO}_4^- + 5 \text{IO}_3^- + \text{HCOOH} + 3 \text{HCOOH} \\
\end{align*}
\]

\[
\begin{align*}
&\text{COOH} \quad \text{O} \quad \text{H} \\
&\text{H} \quad \text{OH} \quad \text{OH} \quad \text{OH} \quad \text{CH}_2 \quad \ldots \text{OH} \\
+ 6 \text{IO}_4^- + 6 \text{IO}_3^- + \text{HCOOH} + 8 \text{HCOOH} + \text{HCHO} \\
\end{align*}
\]

\[
\begin{align*}
&\text{COOH} \quad \text{O} \quad \text{H} \\
&\text{H} \quad \text{OH} \quad \text{OH} \quad \text{OH} \quad \text{CH}_2 \quad \ldots \text{OH} \\
+ 5 \text{IO}_4^- + 5 \text{IO}_3^- + \text{HCOOH} + 4 \text{HCOOH} + \text{HCHO} \\
\end{align*}
\]
any formate esters produced. Treatment with sodium borohydride is then repeated to convert aldehyde groups arising from corticosterone and deoxycorticosterone to primary hydroxyl groups. Although newly formed 17-ketosteroids may also be reduced, such hydroxyl groups are later re-oxidized along with 11B-hydroxyl groups by chromium trioxide in pyridine. During these reactions all 17α-hydroxy steroids which differ only in their side chains are converted to the same compound. This conversion affords a real advantage in the diagnosis of virilizing adrenal hyperplasia in which both 5β-pregnane-3α,17α-20β-triol, characteristic of C-21 hydroxylase deficiency (salt-losing type) and 3α,17α-dihydroxy-5β-pregnane-20-one, characteristic of 11β-hydroxylase deficiency (hypertensive type) are converted to 5β-androstane-3α,17β-diol. This latter steroid is also formed in a negative metapyrone [2-methyl-1,2-bis(3-pyridyl)-1-propenone] test thereby indicating normal feedback control of adrenocorticotropic hormone production by the pituitary.

Following acidification and concentration under reduced pressure, the sulfate conjugates are solvolyzed by the procedure of Burstein and Lieberman (139). In this procedure, the sulfate conjugates are protonated by p-toluenesulfonic acid and transferred to the tetrahydrofuran phase. The low dielectric constant of this solvent discourages
the ionization of the conjugate and promotes the following reaction:

\[
\begin{align*}
\text{H}_2\text{O} & \quad \text{OH} \\
\text{O} & \quad \text{S=O} \quad \text{--+} \\
\text{O} & \quad \text{S=O} \quad \text{--+} \\
\text{H}_2\text{O} & \quad \text{OR} \\
\text{S=O} & \quad \text{ROH}
\end{align*}
\]

The fact that oxygen from water is not incorporated into the steroid product supports the above mechanism.

Following solvolysis, sodium hydroxide is added and the non-phenolic steroids are salted out in the tetrahydrofuran phase. This phase is then evaporated to dryness under nitrogen. The steroid mixture is then dissolved in pyridine and esterified with o-chlorobenzoyl chloride. The temperature and reaction time are controlled so that 11β-hydroxy and 17β-hydroxy groups present in the metabolites are not esterified. They will therefore be available for subsequent oxidation with chromium trioxide in pyridine. The conversion of 11β-hydroxyl and 17β-hydroxyl groups to their corresponding ketones reduces the total number of individual steroids in the complex mixture of urinary steroids, thus simplifying the subsequent chromatographic separation. Ketone groups in these positions also produce more definitive steroid spectra in the fingerprint region and aid in the separation of steroids from other steroids which cannot be converted to 11- and/or 17-ketosteroids, i.e.
11-deoxy and 17α-alkyl steroids.

Ellingboe et al (281) have described the use of Sephadex LH-20 in the separation of steroid ketones. Their work indicates the feasibility of partition chromatography using ultraviolet-transparent solvent systems such as dichloromethane-cyclooctane combined with ultraviolet detection and collection of steroid o-chlorobenzoates directly in liquid scintillation counting vials. Each fraction can then be heated to remove the dichloromethane which would otherwise quench the liquid scintillation process. Following this step a concentrated toluene solution of scintillation fluor could be added to the higher boiling cyclooctane solution of the steroid ester.

Although many gas-liquid chromatography procedures have been developed for steroids, these are not as useful as liquid partition chromatography when the recovery of the separated steroids is required. The principles involved in these liquid partition procedures have been described by Bush (144) and by Cartensen (161) and useful chromatographic separations have been reported by Katzenellenbogan et al (569) and by Wilson et al (1158). The following compounds derived from the metabolites of labeled corticosteone and cortisol are listed below in order of their elution from a straight-phase column:
**Major Derived Steroid Esters**

- 3α,11β-dihydroxy-18-oxo(11 18)-lactol-5β-androstane-17β,18-carbolectone 3-o-chlorobenzoate
- 5β-pregnane-3α,20β-diol di-o-chlorobenzoate
- 21-nor-5β-pregnane-3α,20-diol di-o-chlorobenzoate
- 3α-hydroxy-5α-androstane-11,17-dione o-chlorobenzoate
- 3α-hydroxy-5β-androstene-11,17-dione o-chlorobenzoate
- 3α-hydroxy-5α-androstane-17-one o-chlorobenzoate
- 3α-hydroxy-5β-androstane-17-one o-chlorobenzoate

In the method described so far for the separation of corticosterone and cortisol metabolites, the detection and collection of steroid peaks is less of a problem than their identification and quantification. The exact identity of the eluted steroids must be made by comparison with authentic compounds on the basis of chromatographic behavior and infrared spectra. Quantification is also dependent on identification since the number of o-chlorobenzoate groups per steroid molecule varies. Furthermore, synthesis must also be used to confirm the identity of the metabolites of synthetic glucocorticoids. The synthetic glucocorticoids most difficult to prepare are those reduced in ring A of the steroid nucleus. These derivatives, however, may be prepared using serum enzymes. Rongone et al. (809) have shown that ring A reduction of steroids takes place...
in the presence of serum, isocitrate, and NADPH.

Although the actual chromatographic separation of steroid \( o\)-chlorobenzoate esters has not been achieved, Ellingboe et al. have studied the factors which influence the separation of ketonic from non-ketonic C-19 steroids during molecular sieve chromatography. They found that ketonic steroids were eluted more rapidly due to the interaction of polar solvent molecules with the ketone which increases the solvated molecular weight and excludes it from gel pores. Although they used chloroform in their studies, a similar effect may be expected with dichloromethane which is more polar than chloroform. Cyclooctane may be used as the second component of the solvent system to adjust the partition coefficients of steroids between the polar Sephadex LH-20 stationary phase and the non-polar mobile phase in the straight phase separation.

Many procedures employing double isotope dilution for the analysis of urinary steroids have been devised. In these procedures accuracy is achieved through the use of indicator isotopic steroids which permit correction for incomplete recovery of steroid metabolites. Sensitivity, on the other hand, is achieved by stoichiometrically forming a steroid derivative with a reagent of accurately known specific activity. However, procedures using only carbon-14
and tritium isotopes cannot be used to measure the excretion of carbon-14 or tritium-labeled steroids. Either recovery or mass measurement must be sacrificed.

When $^{36}\text{Cl}$-o-chlorobenzoate esters are used to estimate mass, tritiated steroids can be used to estimate recovery of carbon-14 labeled steroids and vice-versa, i.e. a triple isotope dilution procedure can be employed. Chlorine-36 can be readily distinguished from both tritium and carbon-14 by liquid scintillation spectrometry. The energies of the $\beta$ emissions are 0.710, 0.018 and 0.156 Mev. respectively.

In addition, a simple route exists for the preparation of chlorine-36-labeled o-chlorobenzoic acid via the diazonium salt of anthranilic acid. The labeled acid can be converted to its acid chloride in refluxing thionyl chloride. Alternately, the tritiated or carbon-14-labeled acid chloride may be formed more directly from labeled o-nitrotoluene in a vapor phase reaction with thionyl chloride at 200-220°C as described by Meyer (738).

The use of the o-chlorobenzoyl-$^{36}\text{Cl}$ group to estimate steroid mass is limited in sensitivity by the maximum possible specific activity of chlorine-36, which, on the basis of its half-life of $3 \times 10^5$ years, is 0.3 percent of the maximum specific activity of the U-$^{14}\text{C}$-labeled acyl group and to only 0.0006 percent of the maximum specific activity
of the same o-chlorobenzoyl or acetyl group containing three tritium atoms. However, even the tritiated o-chlorobenzoyl group has only 7.5 percent of the maximum specific activity of a sulfur-35-containing acyl group such as the o-methylsulfonylbenzoyl-\(^{35}\)S group which contains only one sulfur-35 atom.

A three-step synthesis of o-methylsulfonylbenzoic acid from acetophenone and chlorosulfonic acid has been described by Weston and Suter (1135) and it appears to be well-suited for the preparation of the labeled compound. Although the sulfone is resistant to base and to chromium trioxide oxidation, it appears that o-methylsulfonylbenzoyl chloride may spontaneously cyclize in pyridine to 3(2)-thianapthenone-1,1-dioxide and therefore may not be useful as an esterifying reagent. An alternate sulfur-containing aromatic acid chloride is 4-chlorothiophene-3-carboxylic acid chloride. Whether this latter reagent is best prepared from fumaric or maleic acid is an interesting chemical question. A suggested synthesis from the less stable maleic acid is shown below.

It is not possible to distinguish the \(\beta\) emissions of carbon-14 (0.156 Mev.) from those of sulfur-32 (0.167 Mev.) efficiently. However, these isotopes may be distinguished by the 10.4 percent decay in sulfur-32 activity which occurs
SUGGESTED SYNTHESIS OF 4-CHLOROTHIOPHENE-3-CARBOXYLIC ACID CHLORIDE
when measurements of the total activity of both isotopes are made at exactly two-week intervals. This delay in the analytical procedure is relatively unimportant for the types of clinical determinations for which the triple isotope dilution procedure would be used. Furthermore, the delay is not necessary for the measurement of unlabeled steroid metabolites using $^{35}$S-4-chlorothiophene-3-carboxylic acid chloride and tritiated indicator steroids since radioactive tritium and sulfur can be readily distinguished. Thus, sulfur-labeled esters could be used routinely in both double and triple isotope dilution procedures.

Brooks et al. (121) have investigated the absorption chromatography of steroid benzoates on alumina. They formed the benzoyl derivatives by treatment of the alcohols with the acid chloride in pyridine for 18 hours at room temperature. Under these conditions 17β hydroxyl groups as well as the less hindered C-20 and C-3 hydroxyl groups were esterified. Esterification of the 17β hydroxyl group has also been observed with pentafluorobenzoyl chloride after 48 hours under similar conditions. Brooks et al. achieved separation of the benzoates by eluting them from the alumina with increasing proportions of benzene in petroleum ether. They observed that the separation of C-5 epimers of steroid benzoates was better in every case than
that obtained with the acetate esters.

In particular, the benzoyl esters appear to magnify the stereochemical difference between epimeric C-20 hydroxyl groups. Those differences are relatively small in the steroid alcohols and acetate esters. This effect of the C-20 benzoyl group occurs despite the free rotation about the bonds involved. Therefore, there is reason to expect that separations will be further improved by chromatographing ortho substituted benzoyl esters. In addition, the electron withdrawing chlorine substituent, increases the selectivity of the acid chloride for unhindered hydroxyl groups such as those in the C-3, C-17, and C-21 positions.

The separation of steroid derivatives by partition chromatography depends on their physiochemical differences relative to their similarities. Steroids epimeric at C-5 differ primarily in conformation and therefore may show their greatest differences in partition coefficient in rigid allicyclic solvents which interact simultaneously with portions of the A and B rings of these steroids. Examples of ultraviolet-transparent solvents that have been used for this purpose are cyclopentane, cyclohexane, and methylcyclohexane. Commercial decalin is a mixture of cis and trans isomers which may have opposing interactions with steroid solutes. Although cis decalin may be useful, it
must be separated from trans decalin which forms a hydroperoxide that may adversely affect steroid solutes and the liquid scintillation process.

Hayes et al (438) have reported the pulse heights of several aromatic and allicyclic solvents relative to toluene. They found that methylcyclohexane and dioxane produced 25 and 20 percent of the toluene pulse height respectively. However, their most significant finding was that dicyclohexyl had 48 percent of the toluene pulse height.

Although contamination of the dicyclohexyl (m.p. 2.5-3.0, b.p. 236.5-237.5) with phenylcyclohexane (m.p. 7.0-8.0, b.p. 235-236) formed during the catalytic hydrogenation of biphenyl could explain the results, the high pulse height obtained may be due to the greater rigidity of dicyclohexyl compared to methylcyclohexane.

The counting efficiencies of aromatic and non-aromatic liquid scintillation solvents increases as their viscosity increases. Cyclohexane is 3 times as viscous as n-hexane. Cyclooctane and decalin are 7 and 8 times as viscous as n-hexane respectively. The aromatic ether phenetole is over twice as viscous as toluene. A comparison of aromatic alcohols reveals that phenethyl alcohol is 50 percent more viscous than benzyl alcohol which, in turn, is over 16 times more viscous than n-hexane. Thus,
viscosity may explain why 2-phenoxyethanol is 86 percent as efficient as toluene as a scintillation solvent while benzyl alcohol is only 60 percent as efficient as toluene.

Non-bonded interactions contribute to the rigidity and viscosity of cyclooctane. Brown and Borkowski (123) found that 1-chloro-1-methyl-cyclooctane had a solvolysis rate in 80 percent ethanol at 25°C. that was greater than any other ring containing up to 15 methylene units. A cyclopentane analog, the next most active compound, was less than half as reactive.

More polar solvents of this type such as L-3,3-difluoro-p-menthane and 1,1-difluorocyclooctane may be prepared by the reaction of L-menthone and cyclooctanone with sulfur tetrafluoride (434) or carbonyl fluoride (308). Cyclooctanone may be prepared from cyclooctene by hydroboration followed by chromic acid oxidation (126).

Efforts designed to optimize the steroid partition chromatographic process by the use of selective solvent systems and stationary phases are justified (144, 161, 365). Many chromatographic procedures used in clinical determinations require many steps to separate steroid derivatives from labeled urinary impurities. No systematic study of the liquid partition chromatography of steroids has been made in spite of the availability of automated equipment.
adaptable to such a study. The criterion of adequate separation in the above processes is the presence of a constant isotope ratio across each elution peak.

Steroid partition chromatography may also provide a model in which to study the thermodynamic interactions of solvents and solutes, especially departures from non-ideal behavior. Those solvents, especially cyclic hydrocarbons, which can interact with steroid solutes in only a limited number of conformations, appear to afford a better chance of separation, but the theoretical and experimental basis of such an assumption has not been determined (144, 365). However, the partition chromatographic process described above may be optimized with the aid of infrared analytical techniques. For example, solutions of the 5α and 5β epimers of steroid benzoates dissolved in cyclooctane solutions containing various mole fractions of dichloromethane may be added to a given amount of the stationary phase support, Sephadex LH-20. After an equilibration period, the stationary phase can be separated by centrifugation and the composition change in the mobile phase determined by infrared spectrometry. Such a procedure could permit a determination of the optimal mole fraction of dichloromethane required for the separation. Furthermore, there appears to be no theoretical reason which would rule out the
separation of a large number of compounds in a single chromatographic step, provided that derivatives and solvent systems are chosen so that all the compounds to be separated have significantly different partition coefficients.
CHAPTER II

DIABETOGENIC FACTORS

**Human Placental Lactogen**

Normal pregnancy is accompanied by a two-fold increase in fasting insulin levels and a lower fasting blood glucose. Insulin response to intravenous glucose may be three times that postpartum (1206). Kalkhoff et al (283) also found decreased glucose tolerance during pregnancy, especially in diabetic women. Although the absolute values of plasma non-esterified fatty acids were significantly higher throughout the glucose tolerance test in diabetic subjects, the percent decrease from initial fasting levels was the same pre- and post-partum in normal and diabetic women despite more marked deterioration of glucose tolerance in the latter groups. Subclinical diabetes may be detected by the standard glucose tolerance test during pregnancy. The detection of latent diabetes is especially important because the fetus may develop hyperplastic islets as well as other abnormalities if the diabetes of
the mother is not properly managed. Conn and Fajans have estimated that the mother of a twelve-pound baby will develop overt diabetes in the next 25 years of her life.

Fitzgerald et al (320) noted that the incidence of diabetes increases with parity. Compared to nulliparous women, the incidence of diabetes in women with three children is twice as great, and in women with six children, six times as great.

Human placental lactogen and estriol appear to be responsible for the diabetogenic effect of pregnancy. Josimovich (550) attributed this effect of human placental lactogen to a potentiation of the effect of growth hormone. Samaan et al (929) also came to this conclusion. The exact nature of this potentiation is obscure. One interpretation is that the placental hormone can occupy some of the non-specific binding sites which are normally available to growth hormone, thereby rendering the secreted growth hormone more effective at specific sites.

Beck and Daughday (59) estimated that 290 mg per day of the placental hormone was produced near term. They also noted that the hormone impaired glucose tolerance even though it increased insulin response to glucose.

Growth Hormone

The overall effect of growth hormone is to stimulate
the formation of lean body mass at the expense of adipose tissue. Growth hormone promotes the secretion of insulin and glucagon, both of which promote the transfer of carbohydrate from the liver to the extrahepatic tissues. For example, the decreased levels of free fatty acids (FFA) following hypophysectomy have been implicated in the failure of the heart to accumulate glycogen as it normally does in fasting.

Young (1192) first indicated that the diabetogenic principle of the pituitary might be identical with growth hormone. Although growth hormone is not diabetogenic in normal rats, it is diabetogenic in animals with less pancreatic reserve. Dohan and Lukens (247) observed that insulin resistance was associated with diabetogenic doses of growth hormone but not with the permanent diabetes that followed the cessation of hormone administration. Similarly, Coggeshall and Root (185) noted that the diabetes occurring in 17 percent of patients with acromegaly was not marked by insulin resistance. However, they reported that 36 percent of all patients, more females than males, had some degree of glucosuria and that diabetes generally became overt 9.2 years after the diagnosis. Froesch et al (342) have also been able to obtain hyperglycemia without anabolic effects with bovine growth hormone in hypopituitary dwarfs.
It appears that the anti-insulin effect of growth hormone in man is independent of the anabolic effect so that separate regions of the protein may be responsible for each function. Reid (872) demonstrated that the diabetogenic activity of bovine growth hormone paralleled its growth activity during a series of structural modifications when the assays were conducted in rats. These results suggest that the anti-insulin effect of human growth hormone is in an invariant sequence in the hormone while the anabolic effect is in a more variable sequence.

De Bodo and Sinkoff (218) also found that growth hormone increased gluconeogenesis and insulin release from the pancreas. When an increased dose of 3.0 mg/kg/day of growth hormone is administered to normal dogs, they become diabetic in 5-7 days. De Bodo and Altzuler (221) observed that growth hormone was also diabetogenic in hypophysectomized dogs maintained with deoxycorticosterone. Low doses of growth hormone alleviated the insulin hypersensitivity of these animals. Glucocorticoids also partially reduced this hypersensitivity. The combination of glucocorticoids and growth hormone abolished the hypersensitivity. These workers also observed that the adrenalectomized dog was not as sensitive to insulin as the hypophysectomized dog, but that both glucocorticoids and
growth hormone were necessary to restore normal insulin sensitivity.

Luft et al (679) confirmed the ketogenic effect of growth hormone in hypophysectomized diabetic patients. And both Hunter and Greenwood (498) as well as Roth et al (912) reported that hypoglycemia is a potent stimulus to the production of somatotrophic releasing factor and growth hormone secretion. Patients with pituitary stalk section fail to respond to hypoglycemia with increased growth hormone secretion. The production of the releasing factor is inversely related to the level of blood glucose. Also, the inhibitory effect of glucose on the production of somatotrophic releasing factor can be blocked by 2-deoxyglucose, as can glucose-stimulated insulin release from the pancreas.

**Alloxan-Diabetes**

Alloxan (pyrimidinetetetrone) produces experimental diabetes by destroying the insulin-secreting cells of the pancreas. This effect was discovered by Dunn et al (263) and several reviews of the use of this drug have appeared (31, 679). Repeated doses of 100 mg/kg are also capable of destroying human islet tissue as demonstrated by Cann et al (192) in their description of the treatment of islet tumors. Blood glucose undergoes a triphasic change after alloxan is administered (479). First, there is a hyperglycemia
due to glycogenolysis in the liver. Secondly, a hypoglycemic phase occurs which is not due to the liberation of insulin from the pancreas. While uncertain, the cause of this hypoglycemia may be due to a block in gluconeogenesis in the liver. It is this hypoglycemic phase which damages the $\beta$ cells. Thirdly, permanent hyperglycemia ensues due to the destruction of the insulin-producing $\beta$ cells.

The effects of alloxan in hypophysectomized and adrenalectomized animals has been studied by Kirshbaum et al (593). They stressed the necessity of feeding these animals after the administration of alloxan to avoid deaths from hypoglycemia and of allowing at least 48 hours for the diabetic state to fully develop.

Interaction of Growth Hormone and Cortisol

Moody et al (756) showed that insulin increased the formation of glycogen in the absence of glucose, indicating that some of the glycogen was derived from glycolytic intermediates. Bornstein and Trehwella (95) noted that alloxan-diabetic rats had low levels of muscle glycogen which could be restored to normal by hypophysectomy or adrenalectomy. Thus, adrenal hormones increase glycogen formation in the non-diabetic animal but decrease it in the diabetic animal. Furthermore, they observed that insulin in vitro could not reverse the 50 percent drop in
glycogen levels in the muscles of diabetic rats, but that adrenalectomy could. On the other hand, Park and Daughaday (808) observed that the glycogen content of the hypophysectomized rat was unaffected by either growth hormone or adrenal cortical extract. However, hypophysectomized and adrenalectomized rats had higher glycogen levels than normal. These results suggest that growth hormone is necessary for the glucocorticoid effect on glycogen synthesis.

Russell (919) reported that pituitary extracts alone were sufficient to maintain muscle glycogen in fasting hypophysectomized animals but that adrenal cortical hormones were also required to maintain muscle glycogen in fed animals. These results may indicate that growth hormone increased basal glucose transport in the fasted animals but that growth hormone and glucocorticoids are both required to reduce glycolysis, increase glucose-6-phosphate concentration, and thereby increase glycogen formation.

Long and Lukens (672) reported similar findings in hypophysectomized-adrenalectomized-alloxan-diabetic cats. They demonstrated that the beneficial effects of hypophysectomy on survival were partly due to the loss of ACTH. The removal of the source of adrenal epinephrine by adrenomedullation did not protect the animals from ketosis. Although hypophysectomy and adrenalectomy did
not immediately improve carbohydrate oxidation, the animals who survived longest had the best glucose tolerance. Despite the absence of growth hormone, ACTH and glucocorticoids, the doubly-operated animals eventually utilized all their depot fat so that inanition rather than acidosis was the ultimate cause of death. These experiments emphasize the lipogenic effect of insulin.

Villee and Hastings (1103) also found that hypophysectomized rats had higher rates of glycogenesis compared to adrenalectomized rats. Growth hormone treatment, however, decreased glycogenolysis more than glycogenesis.

Luft (679) investigated the effect of growth hormone on the levels of pyruvate, lactate, and citrate during the intravenous glucose tolerance test in diabetes. He found that growth hormone elevated all three metabolites when it accentuated the diabetic-type glucose tolerance curve. Although growth hormone had no effect on fasting blood glucose, it did raise the concentration of free fatty acids and fasting insulin 3-fold. However, the drop in free fatty acids in response to glucose was not affected by growth hormone. These results suggest that the anti-insulin effect of growth hormone is not mediated by free fatty acids.

Renold et al (877) have shown that hormones affect the metabolism of glucose by the liver. Liver slices from
hypophysectomized rats have a rate of glucose uptake which is 80 percent of normal. On the other hand, liver slices from alloxan-diabetic rats assimilated glucose at 50 percent of the normal rate. Hypophysectomy of the alloxan-diabetic rats restored their hepatic glucose uptake to the level of that obtained with livers from hypophysectomized animals. From these experiments it can be concluded that insulin increases and pituitary factors decrease glucose uptake by the liver. Krahl (614) demonstrated that insulin in vitro could overcome the pituitary inhibition of hepatic glucose uptake. When 0.1 unit of insulin per milliter was added, the maximal rates of glucose uptake by liver slices from normal and hypophysectomized animals were the same. Hypophysectomy increases hepatic glucose uptake in spite of the fact that it also increases glucose-6-phosphase activity. Furthermore, Steiner and Williams (1028) found that the levels of glucose-6-phosphate in the livers of alloxan-diabetic rats was one-third normal. These reports merit further investigation. However, these hepatic effects are in agreement with the extrahepatic results of Russell (916) who found that the rate of glucose utilization in the hypophysectomized-eviscerated rat was 2 to 3-fold greater than that in eviscerated controls.

In addition to the effects of the adrenal and pituitary
hormones studied separately, many investigations have been carried out in which the combined effects of both types of hormones have been determined under identical conditions. Russell (915) found that anterior pituitary extract increased the amount of muscle glycogen in adrenalectomized rats without influencing the amount of liver glycogen. Also, the extract did not depress glucose oxidation in the adrenalectomized rat although it did cause this effect in intact rats. In contrast, adrenal extract did not increase muscle glycogen but it did slightly decrease glucose oxidation in adrenalectomized rats. These results show that the adrenal gland is necessary for the anti-insulin effect of growth hormone. The increased production of lactate and pyruvate following the administration of pituitary extracts to adrenalectomized rats is not sufficient to influence their glucose oxidation. In an attempt to assess the role of the liver in these effects, Russell (917) determined the effects of the extracts in hypophysectomized-eviscerated rats. She eviscerated fed rats which had been hypophysectomized for two weeks or adrenalectomized for 4-14 days prior to the experiment. When administered, anterior pituitary extract was given 24 hours before evisceration and cortical extract 1/2 hour before this procedure. She found that anterior pituitary extract alone as well as
adrenal extract alone were capable of reducing the amount of glucose which had to be infused to maintain the blood glucose of the hypophysectomized-eviscerated rat. No synergism was noted when both extracts were administered. The anterior pituitary extract, however, was more potent than the adrenal extract in maintaining muscle glycogen. The fact that the anterior pituitary extract did not exert its effect until one hour after its administration does not allow one to rule out the possibility that the effect was mediated by the adrenals and was due to ACTH in the crude pituitary extract. The absence of synergism may mean that the liver is required for the synergistic effect of pituitary and adrenal hormones (growth hormone and cortisol).

Deoxycorticosterone did not affect the glucose requirement of the hypophysectomized-eviscerated rat but it did reduce the glucose requirement of the adrenalectomized-eviscerated rat. It appears from these studies that deoxycorticosterone exerts an inhibitory influence on glucose utilization only when pituitary factors are present. Ventura and Selye (1092) also found that growth hormone was required for the inhibitory effect of deoxycorticosterone on the anti-inflammatory action of glucocorticoids. These results suggest that growth hormone may affect the metabolism of these steroids. In Russell's
experiments this effect could have required the presence of the liver since both hormones were administered prior to evisceration. However, she attributed the lower glucose requirement of the deoxycorticosterone-treated animals to their "better condition." McGarack et al (689) also found that the abnormal glucose tolerance curve seen in Addison's disease could be restored to normal by deoxycorticosterone acetate.

Russell (915, 919) further demonstrated that glucocorticoids were necessary for the glycogenic effect of growth hormone. She postulated that glucocorticoids inhibited the conversion of carbohydrate to fat thereby making it more available for glycogenesis. Heart muscle was found to be especially sensitive to the glycostatic effects of growth hormone. Heart glycogen is elevated in diabetes while the glycogen content of other tissues remains essentially normal. This elevation in cardiac glycogen is probably caused by the stress-induced increase in glucocorticoid levels. Furthermore, the well-known preference of the heart for the respiration of fatty acids, which is probably related to its rapid rate of acylcoenzyme A formation, may also be the basis for its increased glucocorticoid sensitivity.

Park (810) investigated the influence of the pituitary
on glucose metabolism in the isolated rat diaphragm from alloxan-diabetic rats. This muscle is thin enough so that diffusion is not rate-limiting for glucose metabolism. Park studied the effects of crude pituitary extracts and purified growth hormone in vivo and in vitro. The administration of 2 µg of crystalline growth hormone to alloxan-diabetic-hypophysectomized rats caused an initial drop in their blood glucose which was followed by a prolonged period of hyperglycemia. In vitro, purified growth hormone caused an increase in the glucose uptake of the isolated rat diaphragm muscle. The secondary in vivo elevation of blood glucose was also produced by injections of crude pituitary extracts. In fact, the crude extracts had a more rapid and direct effect compared to purified growth hormone. In addition, adrenal cortical extract was found to potentiate the effect of the pituitary hormone. Since these in vitro experiments were carried out in protein-free media, the results cannot be attributed to changes in insulin binding by serum proteins.

Spiro (1012) found that growth hormone is necessary for normal glucose transport in the liver. Hypophysectomy of alloxan-diabetic rats did not effect the rate of hepatic glucose phoshorylation but it did restore to normal the reduced rate of hepatic glucose uptake in the alloxan-diabetic rat. Goodman (303) reported that the intravenous
administration of growth hormone 30-45 minutes before sacrifice stimulated the uptake of xylose and α-aminoisobutyrate, a non-utilizable amino acid, by the diaphragm. He showed that this effect could be produced in vitro and that it was not secondary to lipolysis. If the hormone was injected 3 hours before sacrifice, the uptake of the sugar and the amino acid was not increased. In addition, the diaphragm was then refractory to the addition of growth hormone in vitro. The development of this refractory response was blocked in vitro by 10 µg/ml of actinomycin D. This indicates that DNA synthesis was required in order to block the stimulatory effect of growth hormone and raised the possibility that some of the effects of actinomycin D in vivo may be due to blockade of this growth hormone effect. It is important to note that these findings do not explain the secondary hyperglycemia obtained when hypophysectomized animals are treated with growth hormone.

Ottaway (802) showed that the stimulatory effect of growth hormone in vitro is inversely related to the fasting blood glucose of the hypophysectomized-diabetic rats from which the diaphragms are obtained. This is expected if the effect of growth hormone is primarily on glucose transport. Glucose transport becomes increasingly rate-limiting for glucose uptake as the blood glucose concentration is
Milman and Russell (749) reported that alloxan-diabetic rats were rendered hyperglycemic by the administration of 30 mg/kg of purified growth hormone while normal rats were not. However, they were if the treatment had been repeated 2 to 4 hours previously. They also exhibited decreased glucose tolerance, depressed respiratory quotients and glycogenesis. These results suggest that alloxan-diabetic rats are more sensitive to the effects of growth hormone.

Effects of Steroids and Related Compounds on the Pancreas

Lewis and co-workers (659) investigated the influence of 17β-hydroxy-4-androsten-3-one 17-propionate and 17β-hydroxy-17α-methyl-4-androsten-3-one on the incidence of diabetes in rats after subtotal pancreatectomy and also found that the androgens increased the incidence of diabetes. Hast (424) has recently reviewed these effects. While testosterone caused atrophy, estrogens generally exerted a protective or trophic effect. Deoxycorticosterone and progesterone had no effect. The estrogen effect could also be produced with 3,4-bis(p-hydroxyphenyl)-trans-3-hexene, a synthetic estrogen. This effect may account for the lower incidence of diabetes in pre-menopausal women and the more serious nature of the disease when it occurs in post menopausal women. Nonetheless, large doses
of estrogens are diabetogenic in spite of their beneficial effect on the pancreas.

James et al (523) found that 17β-hydroxy-17α-methyl-1,4-androstadiene-3-one lowered fasting glucose and impaired glucose tolerance by inhibiting ACTH secretion.

Arginine, leucine, and tris (hydroxymethyl)-aminomethane stimulate insulin release, but arginine also stimulates growth hormone release. This action of arginine can be used to test the pituitary reserve of growth hormone. The mechanism of action of the two amino acids on insulin release is different. The effect of leucine is counteracted by glucocorticoids, but that of arginine is not. Also, since leucine is a ketogenic amino acid, gluconeogenesis is not involved.

Houssay (481) compared the incidence of diabetes in 95 percent depancreatized male and female rats. He found that the incidence was three times greater in male rats. The administration of androgens to female rats also increased their incidence of diabetes. On the other hand, the administration of 50 µg/day of estradiol or 21-hydroxy-4-pregnane-3,11,20-trione in combination with 150 µg/day cortisone initially increased the incidence of diabetes, but this initial response was followed by hypertrophy of the pancreas with amelioration of the diabetes. Furthermore,
the glucocorticoids alone at 50 µg/day exerted just a protective effect. Therefore, physiological levels of glucocorticoids are not diabetogenic but elevated levels are diabetogenic.

Renold et al (879) demonstrated that the anti-insulin action of growth hormone and cortisol is not due to their effect on insulin release. Growth hormone and cortisol treatment corrected the weak and slow response of the β cells to glucose which is seen in the hypophysectomized dog. However, cortisol, as previously noted, also reduced the tendency of the insulin response to be prolonged when growth hormone alone was administered. Thus, cortisol actually prevents β cell exhaustion due to growth hormone. However, the ability of cortisol to oppose the stimulatory effect of growth hormone on insulin secretion is noteworthy because serotonin is believed to mediate the effect of growth hormone (986, 987).

Beck (58), Vermeulen et al (1098) and Starup et al (1022) have further indicated that 6-chloro-17α-hydroxyl-4, 6-pregnadiene-3,20-dione acetate (chlormadinone acetate) closely resembles glucocorticoids in its effects. This compound increased the insulin response to glucose while it decreased the glucose response to insulin. It should therefore serve as a model compound for future studies of
the anti-insulin effects of steroids. The stimulation of insulin release by progestins may explain the report of Gershberg et al (361) that carbohydrate tolerance was improved in women who had impaired glucose tolerance before treatment. It appears that the stimulation of insulin response by the progestin in these cases overrides the increase in insulin resistance also produced. However, the medication is still diabetogenic because it decreases glucose response to insulin and leads to pancreatic exhaustion.

Houssay (480) studied the effect of thyroid hormone on alloxan diabetes in the rat. He found that pretreatment with thyroid hormone, like estrogen treatment, hypertrophied the pancreas and made the rat more resistant to alloxan. On the other hand, thyroidectomy proved beneficial in alloxan-diabetic rats and thyroxine intensified their diabetes. This latter effect may be related to inhibition of lipoprotein lipase and glutamic dehydrogenase and stimulation of adipose tissue lipase produced by the hormone. These effects will be discussed in later chapters.

An endocrine question closely related to the action of estrogens concerns the physiological function of fetal estriol in pregnancy. On one hand, it is widely held that the formation of 3β-hydroxy-5-androsten-17-one by the fetal
adrenal and its subsequent 16α-hydroxylation by the fetal liver are good indications of fetal viability. On the other hand, the exact role of fetal estriol in the fetal or maternal economy has not been determined. Lemon (651) has postulated that high estriol levels during pregnancy may serve to protect maternal tissues from the tumorigenic potential of estradiol which is also elevated. However, other workers (716) have demonstrated increased estriol excretion in breast tumor patients thereby casting doubt on this hypothesis. The function of fetal estriol may be to decrease fetal insulin response to glucose. Diabetic women excrete a lower proportion of total urinary estrogens as estriol during pregnancy; they also tend to deliver larger infants. The unopposed action of insulin appears to be responsible for this effect. Furthermore, the infants of diabetic women tend to have hyperplastic islets. The decreased formation of estriol by the fetus may represent one of the early manifestations of the diabetic gene.

Lewis and co-workers (659) investigated the influence of 17β-hydroxy-4-androsten-3-one 17-propionate and 17β-hydroxy-17α-methyl-4-androsten-3-one on the incidence of diabetes in rats after subtotal pancreatectomy and also found that the androgens increased the incidence of
diabetes. Hast (213) has recently reviewed these effects. While testosterone caused atrophy, estrogens generally exerted a protective or trophic effect. Deoxycorticosterone and progesterone had no effect. The estrogen effect could also be produced with 3,4-bis(p-hydroxyphenyl)-trans-3-hexene, non-steroidal estrogen. This effect may account for the lower incidence of diabetes in pre-menopausal women and the more serious nature of the disease when it occurs in post-menopausal women. Notwithstanding, pharmacological doses of estrogens are diabetogenic so that this effect is not on the pancreas.

Lewis and McCullough (322) found that 17β-hydroxy-17α-methyl-4-androsten-3-one caused hepatic changes which decreased glucose tolerance in the rabbit by blocking glycogen formation. Also, the conjugation of bilirubin was inhibited producing reversible symptoms of hemolytic jaundice reversible when the steroid was withdrawn. These effects were not obtained in thyroidectomized animals suggesting that these steroids may affect the metabolism of thyroxine. James et al (251) found that 17β-hydroxy-17α-methyl-1,4-androstadien-3-one lowered fasting glucose and impaired glucose tolerance while inhibiting ACTH secretion.

Many efforts have been made to define the effects of estrogens on glucose metabolism. McKeins and Bell (691)
found that estrogen treatment stimulated glucose uptake by the isolated rat diaphragm. Ecktern and Villes (265) found that castration lowered the activities of glucose-6-phosphate dehydrogenase, aconitase and isocitrate dehydrogenase in rat uterus. The ablation had no effect on the tricarboxylic acid cycle, however.

McKeins and Bell (691) also found that the administration of 25 µg/day of 3,4-bis(p-hydroxyphenyl)-trans-3-hexene increased the activity of the pentose shunt in diaphragm muscle, adipose tissue and liver. At 100 µg/day no effect on insulin response was observed, but glucose metabolism via the pentose shunt was inhibited. The synthetic estrogen had no effect on the glucose metabolism of diaphragm in vitro at a concentration of 5 µg/ml.

Villee et al (1104) studied the effects of estradiol-17β on the transhydrogenation of pyridine nucleotides in estrogen dependent tissues. They concluded that estradiol may increase the rate of transhydrogenation and thereby provide NADP⁺ necessary for the utilization of glucose-6-phosphate via the pentose shunt and for the oxidation of isocitrate by mitochondrial threo-D₃s-Isocitrate: NADP oxidoreductase (decarboxylating) (EC 1.1.1.42). The transhydrogenase, NADPH: NAD oxidoreductase (EC 1.6.1.1) is distinct from 17β-hydroxysteroid dehydrogenase,
17β-hydroxysteroid: NAD 17β-oxidoreductase (EC 1.1.1.63) and from 17β-hydroxysteroid: NADP 17β-oxidoreductase (EC 1.1.1.64). It is possible that estradiol may catalyze transhydrogenation by acting as a coenzyme between these two enzymes. In contrast, Warren and Christ (1166) have demonstrated that a true estradiol-responsive transhydrogenase is present in human placenta. This enzyme combines with estradiol or estrone before pyridine nucleotides are bound. Also, the enzyme has one binding site which is specific for NAD⁺ and another site which can be occupied by either NADH or NADPH. Estradiol does not leave its binding site when it exerts its stimulatory effect on transhydrogenation. Apparently, the hormone functions more efficiently in this fashion rather than as a coenzyme. However, it is not clear whether estradiol undergoes oxidoreduction when it is bound to the enzyme and the effects of 17α-alkylated estrogens on the activity of this enzyme have not been reported.

The adverse effect of estrogens on glucose tolerance has rather broad structural specificity. Estradiol-17β-monobenzoate, bis-3,4-(p-hydroxyphenyl)-trans-3-hexene and 17α-ethinyl-17β-hydroxy-5(10)-estren-3-one also produced the effect.

Gershberg et al (361) reported that 46 percent of
their patients treated with norethynodrel and mestranol had elevated two-hour glucose levels during the standard glucose tolerance test. Similar results were found by Puchulu et al (853) who found that the incidence of abnormal glucose tolerance curves was increased from 11.1 percent to 66.7 percent if the phenolic C-3 hydroxyl group was methylated. Also, the incidence of abnormal tolerance was greater in patients who had a family history of diabetes. However, Gershberg et al (362) subsequently reported that the administration of 0.75 mg/day of mestanol improved the glucose tolerance of diabetic women. Triglyceride levels increased during mestranol treatment in both normal and diabetic women. These results suggest that the C-3 phenolic hydroxyl group of estrogens is not required for anti-insulin activity.

Spellacy and Carlson (1007, 1008) found both elevated glucose tolerance curves and elevated plasma insulin levels during the glucose tolerance test performed on patients receiving 9.85 mg 17α-ethinyl-17β-hydroxy-5(10)estren-3-one and 0.15 mg 17α-ethinyl-3-methoxy-1,3,5(10)-estriien-17β-ol daily. In contrast, when intravenous glucose tolerance tests were performed under similar conditions, Spellacy et al found normal glucose tolerance but two-fold increases in plasma insulin levels.
In similar experiments Yen and Vela (1187) reported no changes in either oral or intravenous glucose tolerance but noted 3-fold increases in serum growth hormone levels. Increased insulin resistance associated with estrogen-stimulated growth hormone release may be the mechanism behind this effect. Growth hormone secretion normally occurs in response to hypoglycemia and presumably leads to increased lipolysis of depot lipid. Apparently, insulin released in response to growth hormone does not exacerbate the hypoglycemia under these circumstances. This may be due to the simultaneous action of glucocorticoids. The failure of diabetic women to respond to estrogens with decreased glucose tolerance could mean that the anti-insulin effects of growth hormone are already manifest in these women.

Estrogens increase the hepatic synthesis of both thyroid binding globulin and corticosteroid-binding globulin. Indeed, Metcalf and Beaven (736) and Marks et al (715) observed 3-fold increases in transcortin during mestranol treatment. Furthermore, Goldman and Ovadia (384) confirmed that estrogens increase ACTH secretion in both rats and man. However, when estrogens increase ACTH secretion, they also increase transcortin synthesis so that most of the increased glucocorticoid output is bound in inactive form. Thus, estrogens permit ACTH levels to
remain elevated and to exert its effects on adenyl cyclase in target tissues. Cross-conjugated steroids such as 17β-hydroxy-17α-methyl-1,4-androstadien-3-one also have this effect (1160).

Plager and Matsui (837) investigated the effects of bound and unbound cortisol on the metabolism of glucose in slices of mouse ear in vitro. They found that 0.06 µg/ml of cortisol decreased the evolution of $^{14}$CO$_2$ from glucose-$^{14}$C more than from glucose-6-$^{14}$C or fructose-$^{14}$C. These results suggest that cortisol decreases glucose oxidation via the pentose shunt. Matsui and Plager also found that high doses of estrogens in vivo also depressed the oxidation of glucose by mouse ear strips. This result confirms the findings of Ingle (515) who demonstrated that the administration of 0.5 mg of estradiol per day to partially depancreatized rats produced glucosuria amounting to 4.0 grams per day. The mechanism of this effect may be similar to that which decreases glucose tolerance in women during estrogen therapy. Overell et al (804) were also able to obtain significant inhibitory effects on glucose uptake by mouse skin in vitro using 0.01 µg/ml of cortisol or prednisolone. The absence of the tricarboxylic acid cycle in mouse skin indicates that it is not involved in the steroid effect.
Slaunwhite and Sanberg (992) described transcortin, the glucocorticoid-binding protein of serum, and methods for its isolation. They showed that the level of transcortin was elevated during pregnancy. This elevation had two significant effects. First, it decreased the rate of metabolism of cortisol. Secondly, it removed non-specifically-bound cortisol from albumin binding sites enabling the albumin to bind more testosterone. Warren and Salhanick (1115) also described some of the properties of transcortin. They found that the strength of cortisol binding decreased with increasing temperature and advocated the comparison of diluted pathologic serum with control serum at 37°C to speed the attainment of equilibrium using equilibrium dialysis techniques.

Wira et al (1171) have pointed out that the criteria that must be met by a hormone receptor include hormone specificity, tissue specificity, high affinity and saturation. High-affinity steroid binding sites are generally specific sites. In the case of transcortin both the strength of binding as well as the total number of binding sites decrease with increasing temperature. However, even at constant temperature the equilibrium binding may be affected by cooperative interactions between binding protein subunits as protein concentration varies. Regardless of the
method chosen to separate free and bound steroids, the proportion graph of Balieu and Raynaud (52) in which the log of the fraction of steroid bound is plotted versus the total steroid concentration appears to be a simple and sensitive indicator of the heterogeneity of binding sites.

Scholtz and Huther (947) have measured the levels of cortisol and corticosterone in normal and diabetic women during pregnancy, labor and delivery. They found that diabetic women had 42 percent higher levels of cortisol and 63 percent higher levels of corticosterone during delivery. The higher levels observed suggest that decreased glucocorticoid metabolism in diabetic women is especially evident in times of stress.
CHAPTER III
INSULIN INHIBITORS

Human Insulin Antagonists

Insulin resistance in man is associated with abnormally high insulin requirements. Baird and Bornstein (38) noted that insulin-resistant diabetics were of two types. One type had no biologically active insulin in their sera and none could be isolated after a lipid solvent extraction procedure which permitted 85 percent recovery of added labeled insulin. On the other hand, the second type was characterized by serum insulin which, while not active in native serum, could be separated from an inhibitory factor by solvent extraction of the inhibitor. After separation, the insulin was active. Since the inhibitory factor was present in only one type of insulin-treated diabetic, it does not appear to be an insulin antibody. Rather, the insulin inhibitor in these patients appears to be a lipid. Furthermore, these observations do not eliminate an intracellular site of action for the lipid
inhibitor. The factors which cause the rise and decline of insulin resistance are important in the management of diabetes even though they are not well defined.

Plotz et al (841) noted that insulin-resistant diabetes occurred in 25 percent of all patients with Cushing's syndrome. Whether this is a direct glucocorticoid effect or is produced by the effect of the pituitary tumor on glucocorticoid metabolism is unclear.

Synalbumin In Vivo and In Vitro

Stadie et al (1015) studied the anti-insulin effect of growth hormone on rat diaphragm. They found that both pituitary and adrenal factors were necessary for the refractory response to insulin. Vallance-Owen et al (1085) studied the nature of an insulin antagonist which they found associated with human plasma albumin. They found that it was not due to albumin per se since it could be absorbed on a cellulose column leaving a non-inhibitory albumin fraction. The factor absorbed to the column was not identified. However, in assays of glucose uptake by rat diaphragm incubated in the presence of diluted diabetic serum, the dependency of the inhibitor on the pituitary was established.

Vallance-Owen studied the levels of the synalbumin insulin antagonist in the serum of normal, obese and adult diabetics. He found that the levels of the antagonist
were not different in the three cases when the whole serum was tested by the rat diaphragm assay. By itself this finding casts doubt on the in vivo significance of the antagonist. However, when the albumin fraction of the serum was isolated and tested separately, the amount of inhibitor bound to albumin was higher in obese and adult diabetics than in control groups. Of children who were synalbumin positive, 65 percent had at least one synalbumin-positive parent, suggesting the dominance of the trait.

The method used to assay the synalbumin insulin antagonist deserves comment. If the albumin fractions from normal and diabetic patients are prepared, both of these fractions inhibit the effects of 1 milliunit of insulin on the rat diaphragm. However, if the fractions are diluted 4-fold with buffer before the assay, the diabetic albumin retains its inhibitor effect while the normal albumin loses its inhibitory effect. The final albumin concentration in both cases is 1.25 percent. These characteristics appear to indicate that some reversibly bound factor is becoming unbound and diluted when the albumin is diluted. The denaturation of the albumin by heat renders the antagonist dialyzable even though the dialyzable fraction is partially inactivated by heat. Also, the active factor bound to albumin can be removed completely
by extraction with boiling ethanol. In contrast, chloroform and a mixture of isooctane and acetic acid, known to be effective in the extraction of fatty acids from albumin, did not remove the inhibitor from the albumin. Vallance-Owen also noted that the synalbumin inhibitor was inactive on adipose tissue. This finding led him to propose that diabetics become obese because they are diabetic.

Alp et al (9) purified the synalbumin antagonist 150-fold and separated it from albumin. As previously noted, they verified that the antagonist could be dialyzed free from 0.001 percent albumin but not from 0.2-3.0 percent albumin solutions. This finding supports the work of Vallance-Owen. However, Davidson and Goodner (215) were unable to show any physiological effects of the synalbumin antagonist in vivo. It did not affect plasma insulin, nor blood glucose, nor the disposal of a glucose load. It even had no effect in partially depancreatized rats. Therefore, the physiological significance of the antagonist remains to be determined.

Vargas et al (1089) reported that an inhibitor of glucose uptake in muscle was present in the $\alpha_2$ and $\beta$ globulin fractions of normal human plasma. Insulin activity was associated with the $\alpha_1$ globulin fraction. Taylor et al (1054) showed that growth hormone was required.
for the production of this inhibitor which reduced glucose uptake in the absence of insulin. Growth hormone alone was capable of producing the inhibitor in hypophysectomized rats. It was not removed by passage through a cellulose column. It was thought to be identical to the inhibitor of Baird and Bornstein.

The possible role of an insulin inhibitor in the etiology of diabetes has attracted the attention of many investigators. Park and Krahl (802) initiated studies of the effects of hormones on the glucose utilization of the isolated rat diaphragm. Using crude pituitary extract, they found that the injection of 3 mg/100 gm into intact rats three hours before sacrifice led to a depression of glucose uptake by the isolated diaphragm. This effect did not occur in hypophysectomized rats unless adrenal extract was also administered. The adrenal extract alone also intensified the anti-insulin effect in isolated diaphragms. These experimental results were obtained three hours post-injection. Krahl and Cori (612) further investigated the effect of adrenalectomy on glucose uptake of the diaphragm. They found that this operation increased the glucose uptake from 1.38 mg/gm/hr to 2.40 mg/gm/hr. In their work with alloxan-diabetic rats, these authors noted that the 6-hour fasting glucose levels of
the diabetic rats dropped from 300-800 mg/100 ml before adrenalectomy to 200-400 mg/100 ml after adrenalectomy. In later work, Krahl (613) studied the effects of crystalline growth hormone on this same system. He observed the following effects: 1) an increase in muscle glycogen, 2) an increase in glucose uptake, 3) a decrease in glycogenolysis, 4) a decrease in respiratory quotient, 5) a secondary decrease in the response to insulin which followed the initial stimulation. In addition, he measured the concentration of adenosine triphosphate in the diaphragms and found that it was the same in normal and diabetic diaphragms. Park et al (411) confirmed the biphasic nature of the growth hormone response on diaphragm muscle. The initial stimulation of glucose uptake could be obtained by the addition of growth hormone in vitro. The secondary inhibitory effect could be produced by the injection of 2 µg of growth hormone and the effect was potentiated by adrenal cortical extract.

**Inhibition by Beta Lipoproteins**

In 1953 Bornstein (98) reported that lipoproteins from diabetic rat serum could inhibit the glucose uptake of normal rat diaphragm. He isolated the lipoproteins both by ultracentrifugal flotation and by Method 10 of Cohn et al (188). He dialyzed the isolated lipoproteins against
Krebs-Henseleit bicarbonate buffer for 2-2.5 hours at 21°C and then used the lipoprotein in the glucose uptake test. He noted that dialysis was associated with instability of the lipoprotein inhibitor, as was storage at 0°C. The fraction appeared to be most stable when stored undiluted and undialyzed at 4-7°C. These observations indicate that some type of lipoprotein complex is involved. When he tested the lipoproteins thus obtained for their inhibitory effects on glucose uptake by rat diaphragm, he added the lipoprotein either to 4 percent albumin in buffer or to the lipoprotein-poor serum from which the lipoproteins had been removed. This latter serum had also been dialyzed. Bornstein reported that initially inhibitory diabetic serum became non-inhibitory after the removal of its lipoproteins. Addition of lipoprotein from normal diabetic serum to either normal or lipoprotein-free diabetic serum caused inhibition. The lipoproteins obtained from the serum of hypophysectomized-diabetic rats did not display inhibitory activity.

Bornstein noted that the β1-lipoprotein fraction obtained from normal human plasma was not inhibitory, while the same fraction III obtained from a patient rendered hypoglycemic by insulin was inhibitory. The stimulation of growth hormone release in hypoglycemia may explain this observation. However, this does not mean that the
lipoprotein bound factor was growth hormone. Normal rat dia-
phragms were used for these tests, and, as previously shown,
these are not sensitive to the direct effects of growth hor-
mone. In hypophysectomized-diabetic rats, Bornstein found
that glucocorticoids which are inactive alone enhance the
activity of growth hormone in producing the lipoprotein-bound
inhibitor. The injection of growth hormone and cortisone in-
to diabetic-hypophysectomized serum donors restored the in-
hibitory capacity of the serum. Injection of either hormone
alone was ineffective, the serum behaving as normal. These
hormones, 0.2 mg of growth hormones, and 0.1 mg of corti-
sone were injected interperitoneally for 2 days prior to
drawing blood. After the production of the inhibitor is
stimulated by the combined action of these two hormones,
it continues to be produced for at least one week. The in
vitro addition of growth hormones and cortisone, 0.1 mg/ml
and 0.01 mg/ml, respectively, did not significantly affect
glucose uptake during a one-hour incubation. The magni-
tudes of the in vivo treatments on glucose uptake are
large. The rate of glucose uptake by diabetic diaphragms
was 50 percent of normal and 38 percent of that found in
hypophysectomized diaphragm. The anti-insulin effect of
the lipoprotein-bound inhibitor was not completely over-
come by insulin in vitro. Glucose uptake was only 88
percent normal in the presence of 0.1 unit of insulin per ml. Therefore, although growth hormone and glucocorticoids may affect glucose transport, they also decrease glucose phosphorylation as well.

Krahl et al (615) carried out additional experiments on the β-lipoprotein-bound inhibitor discovered by Bornstein. They succeeded in isolating inhibitory lipoproteins from normal rat serum. Every part of their procedure was scrupulously standardized. The keys to the preparation of inhibitory lipoproteins from normal rat serum were speed and a reduction in the alcohol concentration used in Method 6 (187) for the isolation of lipoprotein fractions. The fractionation had to start within one hour after the blood was drawn. Inhibitory activity of fractions from diabetic rat sera was not correlated with protein-bound lipid or glucose levels of sera. The fact that the electrophoretic patterns of normal and inhibitory fractions were identical rules out the presence of fatty acids as the cause for the difference. Fractions prepared from fasting normal rats were just as inhibitory as fractions from diabetic rat sera. Non-inhibitory fractions could be obtained if the sera were kept at room temperature for 1 or more hours before fractionation, if the fraction was exposed to 15% ethanol solution for 30 minutes
or more, or if individual sera with protein-bound lipid values above 1100 mg/100 ml were included in the serum pool. The effect of the alcohol was probably on the lipoprotein rather than on the inhibitor itself. An alteration in the structure of the lipoprotein could prevent the binding of the inhibitor and its transport into rat diaphragm where it exerts its effects. The absence of the inhibitor from lipoprotein fractions with high lipid contents may indicate that the binding site for the inhibitor was blocked by the excess lipids. In contrast to this instability in solution, the inhibitory fractions could be stored as precipitated protein cakes for 4 days without loss of activity. The inhibitory activity of the lipoproteins could be enhanced by the intraperitoneal injection of the serum donors with 2 mg of growth hormone 24 hours prior to the experiment. That this effect is not secondary to lipolysis produced by growth hormone is indicated by the lack of an effect of ACTH and TSH hormones on the activity. The inhibitory activity of lipoprotein fraction could be destroyed by repeated freezing and thawing. The inhibitory activity of the amount of the inhibitor in 1 ml of fasted or diabetic rat serum could counteract 0.0003 units of insulin per ml. The significance of this finding can be judged when one considers that the normal insulin concentration
is approximately 0.0010 units/ml. Thus, the effect could be of physiological importance.

Whitney and Young (1141) confirmed the basic findings of Bornstein and Krahl on the hormonal dependence of the inhibitor. They found that the inhibitor did not increase in concentration in normal rats unless both growth hormone and cortisol were administered. The long term treatment which they described in order to see the effect may have been necessitated by the presence of the pituitary and adrenal in their animals. Essentially, they obtained an increase in the inhibition rather than an all or none effect. Their criterion was an inhibition of glucose uptake over a 90 minute incubation of the diaphragm at 37°C.

Kipnis and Stein (389) have reviewed insulin antagonism of all types and have put them into perspective, clarifying disputed points by some of their own experimental work. One of the first points they stressed was that the defective phosphorylation observed in diabetes did not imply a defect in the enzyme hexokinase. This will be discussed further in a later section of this dissertation. Secondly, glucose transport and utilization are decreased in the intact diaphragm of hypophysectomized rats whereas they are increased in the cut diaphragm. This statement does not explain the inhibition
produced by growth hormone and cortisol. However, Kipnis reported that growth hormone and cortisol injections into intact normal rats decreased the transport of glucose into rat diaphragm. He confirmed that purified albumin has a stimulatory effect on glucose uptake. Also, he was unable to verify the presence of bound insulin in serum and concluded that growth hormone did not release insulin from the pancreas. He observed that the sugar transport system is rate limiting at all times in the normal rat, even in the presence of insulin. This is not the case in the diabetic rat in which phosphorylation can be shown to be limiting after insulin administration corrects the defect in transport. Summarizing his findings, the defects in transport in alloxan-diabetic rats can be corrected rapidly by the addition of insulin in vitro. The defect in phosphorylation cannot be so corrected and limits glucose uptake in the presence of excess insulin. The mechanism of this decreased phosphorylating activity is unknown.

In a discussion of Kipnis' results, Bornstein confirmed that the lipoprotein inhibitor was not active on adipose tissue. The experiments of Bornstein showed that the factor bound to lipoproteins was neither cortisone nor growth hormone. It was immediately active on the diaphragm and did not require a latent period. One might suppose that the
factor was cortisol rather than cortisone. In general, it has been found that only 11-hydroxy glucocorticoids are effective in \textit{in vitro} systems in which protein synthesis mediates the glucocorticoid effect. Bornstein did not try cortisol in his experiments. However, Antoniades et al (15, 17) have shown that glucocorticoids are the steroids least strongly bound to the lipoprotein fraction of serum.

Randle et al (431) have shown that fatty acid oxidation leads to a decrease in glucose oxidation. For example, in the perfused rat heart, octanoate at 0.40 mg/ml inhibits glucose uptake 41 percent while 0.70 mg/ml \(\beta\)-hydroxybutyrate inhibits glucose uptake 50 percent. These changes are accompanied by an increase in the NADH/NAD\(^+\) ratio of mitochondria and an elevation in cytoplasmic citrate. These changes are somewhat similar to those observed during ethanol perfusion. The fact that oxygen consumption is not affected indicates that the oxidation of pyridine nucleotides is also unaffected by fatty acid respiration. In contrast, oxygen consumption is reduced by steroids when they produce similar changes in citrate and in the reduction potential of mitochondrial pyridine nucleotides.

Morgan et al (758) reported that glucose phosphorylation is the rate limiting reaction in glucose uptake by
the perfused rat heart at all glucose concentrations over 2.00 gm/l, even in the presence of 0.1 unit/ml of insulin.

Since insulin secretion is stimulated when glucose phosphorylation is inhibited, these results indicate that glycolytic inhibitors can be effective insulin antagonists under conditions similar to those encountered in diabetes in vivo.

**The Specificity of Glucocorticoid Effects**

Huisman (494) had observed that adrenalectomy or hypophysectomy increased the glucose uptake of the isolated rat diaphragm. He found that 0.5-2.5 mg/ml cortisone acetate in vitro decreased the glucose uptake of the isolated rat diaphragm 30-40 percent. Herman and Ramey (452) reported that 0.04 mg/ml cortisol inhibited glucose uptake of the diaphragm by 15 percent. At about the same time, Grossman and Ryder (205) reported that 0.3 mg/ml (8 x 10^{-4} M) cortisol decreased the glucose uptake of diaphragm by 32 percent. The effect was specific since 2 x 10^{-4} Molar cortisone was active while twice this concentration of corticosterone was inactive. Thus, the 17α-hydroxy group is required for this effect. This latter concentration of cortisone depressed glucose oxidation by 41 percent. These authors also reported that 1.63 µMolar deoxycorticosterone inhibited glycogen synthesis 31 percent and accelerated
glycogenolysis while having no effect on either glucose uptake or oxidation. These results with deoxycorticosterone have been confirmed by Verzar (577) and will be discussed in relation to glycogen metabolism. Rosenkrantz (455), in addition to confirming the observations of Grossman and Ryder on deoxycorticosterone, also reported that this steroid inhibited yeast hexokinase 60 percent at a concentration of 1.0 mg/ml. The interpretation of this finding is difficult because yeast does not contain steroid hormones or insulin.
Problems of Mineralcorticoid Therapy

At the present time it is not possible to define the specificity of the glucocorticoid effect on insulin antagonism by β-lipoprotein. However, some of the effects of cortisol on carbohydrate metabolism are non-specific in that they may also be produced by other steroids and non-steroids (12, 747, 1099).

In order to study glucocorticoid effects it is necessary to control endogenous cortisol levels. This is best done by adrenalectomy which, in addition, increases the sensitivity of the experimental animals to minute amounts of adrenal hormones. However, adrenalectomy also produces electrolyte imbalance which may indirectly affect carbohydrate metabolism. Therefore, since the amount of exogenously administered mineralcorticoid required to maintain electrolyte balance is greater than the amount endogenously secreted, the problem of mineralcorticoid therapy
is to obtain electrolyte balance without mineralcorticoid effects on carbohydrate metabolism.

Not all mineralcorticoid effects on carbohydrate metabolism are mediated by changes in electrolyte levels. Rather, some of these effects may be due to the interaction of the mineralcorticoids at glucocorticoid receptor sites. Therefore, the best mineralcorticoid for this application is that which interacts least at glucocorticoid receptor sites.

Grollman (413, 414) found that most of the deaths from adrenal insufficiency in rats occur about 18 days after adrenalectomy. Sabatini et al (922) have made a sub-microscopic study of the effect of the pituitary on the adrenal cortex of the rat. They found that cortical atrophy following hypophysectomy is due to shrinkage of the zona fasciculata and zona reticularis. In contrast, the zona glomerulosa remained normal two weeks after hypophysectomy.

ACTH

ACTH formed in the pituitary stimulates the production of glucocorticoids in the adrenal and can produce all glucocorticoid effects when administered to animals with normal adrenal glands. However, ACTH also has lipolytic effects which are not mediated by the adrenal glands. In Cushing's Syndrome there is an overproduction of ACTH due to hyperplasia of the basophil cells of the adenohypophysis.
In Addison's disease there is also an overproduction of ACTH, but this overproduction is secondary to the decreased cortisol production by the adrenal cortex causing decreased cortisol feedback of ACTH release. Cox and Hodges (203) also found that at least two weeks after adrenalectomy were required before the pituitary adapts itself to secrete and maintain an enormously increased output of ACTH in response to the complete absence of glucocorticoids.

Although potassium enhances glucose uptake and inhibits insulin release from the β cells of the pancreas, changes in potassium and other electrolyte effects may be avoided in the study of glucocorticoid effects by the use of synthetic glucocorticoids which have little or no effect on electrolytes (1195). Perhaps the most potent glucocorticoid which still shows low or negligible mineralcorticoid effects is 6α,9α-difluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione (Flumethasone). In this compound the 16α-methyl counteracts the salt retention produced by the 9α-fluorine substitution. A less potent glucocorticoid but one which still possesses minimal mineralcorticoid effects is 6α-fluoro-11β,17α,21-trihydroy-1,4-pregnadiene-3,20-dione (Fluprednisolone). In addition to the reduction in mineralcorticoid activity produced by the introduction of the double bond at C-1, the glucocorticoid potency is further enhanced by the 6α-fluorine substitution which electronically
interferes with the reduction of the double bond at C-4 (887). Interestingly, the higher potency of 6α-fluorinated derivatives compared to 6α-methylated derivatives indicates the superiority of the electronic block compared to the steric block with respect to the specific reductases involved.

The use of specific glucocorticoids, defined by their potency in glycogen deposition, along with specific mineralcorticoid therapy in control and experimental adrenalectomized rats simplifies the interpretation of the effects observed. But, these specific glucocorticoids may still have non-glucocorticoid effects even though they are devoid of mineralcorticoid effects. It is hoped that these distinctions will become clearer to the reader as the dissertation progresses.

In addition to their effect on hepatic glycogen deposition, specific glucocorticoids promote the mobilization of phospholipids from the liver while mineralcorticoids do not (654, 797, 1197). However, the latter steroids have a more potent hypercholesterolemic effect that is not generally appreciated, probably because mineralcorticoids are not normally in pathological excess (779).

**Adrenal Insufficiency and Diabetes Mellitus**

Adrenalectomized rats cannot be maintained for long periods simply by increasing their dietary sodium and
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Adrenal Insufficiency and Diabetes Mellitus

Adrenalectomized rats cannot be maintained for long periods simply by increasing their dietary sodium and
restricting their potassium. The pioneering work in this particular area was performed by Grollman (204). He demonstrated that completely adrenalectomized rats could not survive longer than approximately 18 days on sodium chloride therapy alone, but could survive if they were also given mineralcorticoids such as deoxycorticosterone. However, the failure to remove only 5 percent of the adrenal gland leads to the survival of the animals without mineralcorticoids due to the hypertrophy of the remaining tissue. Grollman also noted that young, untreated adrenalectomized animals survived for much shorter periods than adults, as did animals exposed to stress before adrenalectomy. Prolonged ether anesthesia during the adrenalectomy reduced the survival period from 14 to 7 days. Barbiturate anesthesia resulted in better survival rates than ether, but high-protein diets and post-operative stress reduced survival rates.

Grollman found that body weight loss was the most responsive non-chemical indicator of adrenal insufficiency in adrenalectomized rats. Other indicators have compensatory mechanisms which only break down during adrenal crisis. Body weight, however, may not be a specific index of adrenal insufficiency in alloxan-diabetic-adrenalectomized rats, because insulin deficiency itself causes weight
loss and protein depletion. The most sensitive chemical indicator of adrenal insufficiency is blood urea nitrogen (BUN). This is especially true when it is measured daily in each animal. The blood urea nitrogen will rise in adrenalectomized rats due to hemoconcentration at constant urea production and due to reduced urea clearance by the kidney as glomerular filtration rate falls. Diabetic-adrenalectomized rats will have a higher rate of urea production due to increased protein catabolism, especially if they are maintained on a high-protein, low-carbohydrate diet. However, the day to day changes in blood urea will still be a valid indicator of blood volume, the goal of mineralcorticoid therapy, despite the osmotic diuresis caused by glucosuria. Although insulin treatment is specifically indicated to control the hemoconcentration produced by glucosuria, insulin treatment was not used in these studies for reasons already discussed. Therefore, daily glucose determinations were not necessary.

The serum sodium levels in diabetic rats are somewhat low. Weisberg (1128) has shown that a reduction in serum sodium is a necessary consequence of the increased amounts of glucose and urea in blood. A rise of 100 mg/l in serum glucose will result in a sodium reduction of 0.28 meq/l while the same rise in urea nitrogen will decrease the
concentration of serum sodium by 1.8 meq/l. The relative amounts of glucose and urea which will displace 1 meq/l of sodium are 350 mg/l and 50 mg/l respectively. These results indicate that serum sodium levels can be altered by both urea and glucose.

The decreased ability of the adrenalectomized rat to excrete potassium is alleviated by the glucosuria of diabetes. The resulting high fluid intake effectively dilutes the elevated levels of potassium in the adrenalectomized rat. The same holds true for the elevated magnesium levels found in adrenalectomized rats. Potassium and magnesium levels in the non-diabetic-adrenalectomized rat are elevated approximately 42 percent and 23 percent respectively. Adrenalectomized rats, however, share with diabetic rats their tendency toward acidosis. The acidosis of adrenal insufficiency is due to the decreased reabsorption of sodium ion and increased reabsorption of potassium and hydrogen ions.

Electrolyte Therapy

Grollman (414) has shown that adequate replacement therapy of adrenalectomized rats cannot consist of electrolytes alone, although the reasons for this are obscure. The oral administration of 10 mg/kg daily deoxycorticosterone acetate admixed with the animal's food was found to result in adequate replacement therapy. The dose of mineralcorticoid
could be reduced if additional sodium chloride was also administered. McGarack et al. (689) showed in humans that the dose of mineralcorticoid was inversely related to the total dietary sodium. However, it should be noted that the correct therapy of Addison's disease requires the use of both mineralcorticoids and glucocorticoids.

An example from McGarack et al. (689) also illustrates the effects which mineralcorticoids can have on carbohydrate metabolism. They showed that normal glucose tolerance could be obtained in their Addisonian patients on a normal diet if 15-20 mg of deoxycorticosterone acetate was administered daily. Althauser et al. (12) have also shown that electrolyte balance in the rat is required for normal intestinal absorption of glucose. In adrenalectomized rats this function can be almost completely restored by the administration of 1 percent sodium chloride as drinking fluid.

In the treatment of alloxan-diabetic-adrenalectomized rats with varying degrees of glucosuria it is advantageous to administer the mineralcorticoid in the drinking fluid rather than admixed with the solid food for two reasons. First, since the bulk of dietary sodium is also in the drinking fluid, the dose of mineralcorticoid is in direct proportion to the filtered sodium load in the glomerulus.
secondly, the administration of the mineralcorticoid in the drinking fluid distributes the total dose throughout the day thereby making it more effective than the same total dose consumed mainly with solid food according to the nocturnal eating habits of the rat. The total dose of hormone administered is thereby reduced as are the dangers of hypertension, hypokalemia, paralysis, cardiac failure, and myocardial necrosis resulting from overdosage.

The administration of mineralcorticoids in drinking fluid, while eliminating the chance of infection associated with daily injections of the hormones, is not without problems. According to Grollman (414) a 200-gram rat requires approximately 2.0 milligrams of oral deoxycorticosterone acetate daily. While this requirement may be reduced if the hormone is administered in conjunction with 1 percent saline, the low solubility of deoxycorticosterone in this medium--approximately 17.0 mg/l--could supply about only one-third of the required dose.

This dilemma might be solved by the use of more potent mineralcorticoids. Bergstrom and Dodson (69) reported the synthesis of 9α-fluoro-21-hydroxy-4-pregnane-3,20-dione from corticosterone acetate. They treated the latter steroid with 70 percent hydrofluoric acid in anhydrous pyridine. Kagawa and Jacobs (553) bioassayed the main
product and found it to be 12-14 times as potent as deoxy-
corticosterone.

Aldosterone might also be used to maintain adrenalecto-
tomized rats in these studies. Although it is 15 times as
potent as deoxycorticosterone, Desaulles (230) has esti-
mated that it has 0.5 percent of the glucocorticoid potency
of cortisol. The ratio of mineralcorticoid to glucocorti-
coid activity in aldosterone might be increased by the in-
troduction of a 9α-fluorine atom. Barton and Beaton (49)
have described the conversion of corticosterone to aldos-
terone. In this transformation 11β-nitrites react photo-
chemically to yield C-18 or C-19 oximes which can be hy-
drolyzed to aldehydes. This reaction applied to 9α-fluoro-
11β,21-dihydroxy-4-pregnane-3,20-dione would yield 9α-flu-

Although intravenous fluid therapy introduces an addi-
tional variable in the maintenance of alloxan-diabetic-
adrenalectomized rats, it often saves a rat who has not
adapted to this state by increasing his saline intake.
Slow infusion of the following solution often corrects
the hemoconcentrations and acidosis of these animals in
this crisis.

When rats are first rendered alloxan-diabetic, they
tend to undergo a period of ketosis until their excess
<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>MEQ/L</th>
<th>GM/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>103.0</td>
<td>6.020</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>4.0</td>
<td>0.298</td>
</tr>
<tr>
<td>Calcium phosphate, dibasic</td>
<td>4.0</td>
<td>0.306</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>30.0</td>
<td>2.521</td>
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<tr>
<td>Glucose</td>
<td></td>
<td>1.000</td>
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<tr>
<td>Fructose</td>
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</tr>
</tbody>
</table>
lipid stores are depleted. This is especially true of larger rats. The oral hypoglycemic agent 1-phenethylbiguanide might be used to enable the animals to get through this period without resorting to the administration of insulin. This particular compound does not act by increasing insulin output but by another mechanism which has not been completely elucidated. Superficially, the effects of the compound resemble the effects of low concentrations of epinephrine on glucose metabolism, but differences no doubt exist. The point made here is that the use of this compound may enable the animals to get through a period of high mortality and thereby improve overall experimental efficiency.

Large doses of glucocorticoids, 17 mg/kg for 10 days, can produce glucosuria similar to that found in alloxan diabetes. This phenomenon is known as steroid diabetes (509, 510, 513, 517). Ingle et al (511) compared steroid diabetes with pancreatic diabetes in the rat and noted that glucocorticoid diabetes was resistant to insulin whereas pancreatic diabetes was not.

Diabetes Mellitus can also occur in patients with Addison's disease. This disease is characterized by adrenal insufficiency and its associated disturbances in mineral and carbohydrate metabolism. Thorn et al (1062) demonstrated impairment of the intestinal absorption of
glucose and an elevated respiratory quotient that is diminished by cortisol treatment. Althauser et al. (12) have shown that oral sodium chloride can partially restore the defect in the intestinal absorption of glucose. When diabetes complicates adrenal insufficiency, proper management of the patient becomes more complex (1061). If the severity of the diabetes corresponds to the degree of glucocorticoid deficiency, the patient can often be maintained by mineralcorticoids alone. In fact, adrenalectomy was used as one treatment for diabetes before insulin was discovered. However, if insulin is needed to control glucosuria, then glucocorticoids should be given as protection against hypoglycemia. Other considerations of the adrenal in diabetes have been reviewed (1063).

Excessive glucocorticoid treatment in Addison's disease may precipitate clinical diabetes (57), generally within 3 years. Carbohydrate and mineral balance are best maintained separately with cortisol and its 9α-fluoro derivative.

**Glycyrrhetic Acid**

If mineralcorticoids produce some glucocorticoid effects in adrenalectomized rats, what other means are available to maintain electrolyte balance in these animals? Louis and Cohn (678) isolated an electrolyte-active principle
from licorice extracts and used this compound to maintain adrenalectomized patients before deoxycorticosterone became readily available. The compound, glycyrrhetic acid, was isolated in the form of the ammonium salt of its 3-(2-O-β-D-glucuronopyranosyl)-α-D-glucuronopyranoside known as glycyrrhizin. This glycoside is intensely sweet and has been used as an artificial sweetener. The mineralcorticoid properties of the licorice glucuronoside are those of the parent compound glycyrrhetic acid, often termed glycyrrhetinic acid.

Glycyrrhetic acid is a molecule of great theoretical interest because it possesses anti-inflammatory and mineralcorticoid effects, but not glucocorticoid effects (318). The relationship between anti-inflammatory and glucocorticoid potency has been reviewed by Glenn et al (371). In general these two potencies parallel each other so that the reported separation of these effects in glycyrrhetic acid by Finney and Somers (318) should be further investigated.

In man the anti-inflammatory dose of licorice extract, which constitutes 6-14 percent of the dried rhizome and roots of the licorice plast, Glycyrrhiza glabra, is about 20-25 grams per day. Although this dose is large compared to anti-inflammatory steroids, treatment with licorice
Figure III

Structure of Glycyrrhizic Acid

3β-Hydroxy-11-Oxo-18β-Olean-12-En-30-Oic Acid

3-(2-O-β-D-Glucuronosyl)-α-D-Glucuronopyranoside
extract and carbonoxalane, the hemi-succinate ester of gly­
cyrrhetic acid, is preferred for the treatment of peptic ulcers which are adversely affected by anti-inflammatory steroids.

Not all commercial samples of glycyrrhetinic acid have anti-inflammatory activity because the active 18β form of the molecule with a cisD-E ring juncture may be converted to the more stable trans form during the acid hydrolysis of the glucuronoside glycyrrhizin. This transformation is readily explained by the allylic nature of the 18β hydrogen due to the presence of the conjugated double bond at C-12. Presumably the trans E ring cannot fit the receptor site for mineralcorticoid activity while the cis E ring can. The active β form of glycyrrhetinic acid crystal­
lized from alcohol has a melting point of 140°, [α]_D^{21} = +86° (c=0.1 in chloroform), while the properties of the inac­
tive α form are 335° and +98° respectively. The pure compound has an extinction coefficient of 12,200 at 242 nm in ethanol. Additional aspects of the stereochemistry of glycyrrhetinic acid have been reported by Beaton and Spring (55).

The role of the 11-ketone of glycyrrhetic acid in its mineral corticoid and anti-inflammatory activity has not been determined. However, the related compound, β amyrin,
which is identical except for the lack of the 11-ketone and C-30 carboxyl groups, also has anti-inflammatory activity equal to that of cortisol. Gupta et al (418) also found that α amyrin had only about one-half the anti-inflammatory activity of the β isomer.

The role of the C-11 carbonyl group in the mineralcorticoid activity of glycyrrhetic acid is unknown but could be determined by comparison with its 11-deoxy analog. Alternately, the mineralcorticoid activity of β amyrin and its C-11 carbonyl derivative could be compared. Such studies may clarify the function of the 11β-hydroxyl group in aldosterone despite its absence in the mineralcorticoid deoxycorticosterone. In addition, it is not known whether the C-11 ketone in glycyrrhetic acid is reduced to an 11β-hydroxyl group in vivo. However, it is known that the analogous reaction with glucocorticoids takes place primarily in the liver by the action of the enzyme 11β-hydroxysteroid dehydrogenase. This enzyme is inactive with 5β pregnene derivatives (486) but may or may not be blocked by the 4β-methyl group of glycyrrhetic acid.

Although previous paragraphs have indicated that it is feasible to maintain alloxan-diabetic-adrenalectomized rats by the administration of a mineralcorticoid in their drinking fluid, there are other reasons for not using
mineralcorticoids for this purpose. Verzar (1099) and Verzar and Wenner (1100) have shown that deoxycorticosterone decreases muscle glycogen by increasing glycogenolysis. Bartlett and Mac Kay (47) and Bartlett et al (48) have confirmed these findings in rat diaphragm muscle. Bacila and Baron (34) noted that deoxycorticosterone was equal to cortisol with respect to its inhibition of the anaerobic glycolysis of lymphocytes. Interestingly, while cortisol appeared to inhibit only the hexokinase reaction as observed also by Ilyin (506) and Ilyin and Titova (507), deoxycorticosterone appeared to inhibit other glycolytic reactions as well. Blecker (84) and Blecker and White (82, 83) have reviewed these effects. They found that 17β-hydroxy-17α-methyl-4-androsten-3-one was as potent as deoxycorticosterone while 17α-ethyl-17β-hydroxy-4-estren-3-one was three times more inhibitory to the glycolysis of lymphocytes.

Roberts and Nezamis (891) and Russell (917) also observed that deoxycorticosterone could produce some glucocorticoid effects in adrenalectomized rats especially when hepatic metabolism of the steroid is impaired. Glenn (372) similarly noted the local glucocorticoid effects of locally administered deoxycorticosterone. To the extent to which mineral corticoids produce glucocorticoid effects, mineralcorticoid maintenance of adrenalectomized rats may reduce
some glucocorticoid effects.

Disputes concerning the efficacy of glycyrrhetic acid in the maintenance of adrenalectomized subjects emphasize that this molecule has no glucocorticoid activity and cannot fulfill glucocorticoid requirements. Cristol et al. (297) reported that glycyrrhetic acid could maintain adrenalectomized rats and reduce their urinary sodium excretion. In addition, a dose of 300 mg/kg/day caused hypertension in rats similar to that caused by excess deoxycorticosterone. Hudson et al. (488) found that adrenalectomized cancer patients could be maintained on a regular diet and glycyrrhizin. The dose required was 100 times the subcutaneous maintenance dose of deoxycorticosterone. On the other hand, Elmadjian et al. (282) reported that two Addisonian-schizophrenic women could not be maintained by the administration of 56 mg/kg/day of ammonium glycyrrhizate in the absence of glucocorticoids. However, this dose of the compound reduced their cortisol requirement from 25 mg/day to 10 mg/day and maintained the patients in excellent electrolyte balance. These results indicate that glycyrrhetic acid has no glucocorticoid activity in the absence of glucocorticoids but that the compound may reduce the rate of metabolism of active glucocorticoids when they are also present.
Glucocorticoids may exert specific electrolyte effects. Mills and Thomas (747) found that cortisol and prednisolone, but not deoxycorticosterone stimulated the uptake of inorganic phosphate by muscle but not liver. These results suggest that the glucocorticoids increased the pool of glycolytic intermediates in muscle without affecting the pool size of these intermediates in liver. The difference in these tissues may be related to the presence of glucose-6-phosphatase in liver. A similar increase in muscle phosphorylated intermediates has been observed by others (759, 760).

Block et al (85) found that a sodium retaining factor could be found in the fetal adrenal gland. The intermediates 4-androstene-3,17-dione and 3β-hydroxy-5-androsten-17-one were also found. The compound 11β-hydroxy-4-androstene-3,17-dione, normally formed almost exclusively from cortisol in the adult, was produced earlier in development than cortisol. Although the latter 17-ketosteroid has the highest anabolic/androgenic ratio of any naturally occurring steroid (737), its effect on fetal carbohydrate metabolism has not been studied. The 11β-hydroxyl group reduces its androgenic activity to one-fifth that of androsterone. Rosenberg and Dorfman (906) also reported that the related steroid 9α-fluoro-11β-hydroxy-4-androstene-3,17-dione was also devoid of androgenic activity but that it did have
mineralcorticoid activity.

**Effects of Steroids on Blood Pressure**

At least part of the hypotensive effect of some steroids may represent an action on glucose utilization in vascular muscle. Sturtevant (1037) showed that 11β-hydroxy-4-androstene-3,17-dione was the most potent naturally occurring hypotensive steroid. It was capable of reducing the blood pressure of rats with metacortical hypertension 33-49 mm of mercury for 5.5 hours. In comparison, testosterone and 17β-hydroxy-4-androstene-3,11-dione reduced blood pressure by 18-22 mm and 4-androstene-3,17-dione and 4-androstene-3,11,17-trione reduced it by 9-18 mm. Most surprising was the finding that 11β,17β-dihydroxy-4-androstene-3-one was inactive. While these results indicate considerable steroid specificity with respect to hypotensive action, additional studies (1038) with synthetic hypotensive steroids based on 17β-hydroxy-4-estrene-3-one did not confirm such specificity. Sturtevant found that the parent compound as well as its 5(10) unsaturated derivative were inactive. Substitution in the 17α position with vinyl, allyl, or methyl groups resulted in inactive compounds, but the 17α-ethyl and 17α-propyl compounds were active. The most potent derivative was 17β-hydroxy-17α-propyl-5β-estren-3-one. Apparently, unsaturation at C-4 is not an absolute requirement for
physiological activity. Sturtevant did not test 11β-hydroxy-19-nor steroids but, judging from his earlier work, these compounds should be even more active. Such derivatives might be prepared by microbiological hydroxylation of estrenes or from androstenes by the technique of Barton and Beaton discussed in conjunction with the synthesis of mineralcorticoids. The finding that a long 17α alkyl group enhanced hypotensive activity indicates that the mechanism involved may be similar to that which operates with some progestational steroids. In the rabbit, 17α-(1-butynyl)-17β-hydroxy-4-estrene-3-one is 83 times as potent as 17α-ethinyl-17β-hydroxy-4-estrene-3-one by subcutaneous injection but only 63 percent as effective orally.

The influence of long 17α alkyl groups on hypotensive and progestational activity is reminiscent of Selye's (965) finding of increased anesthetic potency in a compound which he described as "21-ethylprogesterone" (21,25,26,27-tetranor-4-cholestone-3,20-dione) except that the alkyl group is in the β rather than in the α position. These results suggest that some C-18 progestens may have hypotensive effects but additional relations between the two activities remain to be investigated. However, the results indicate that hypotensive steroids are able to interfere with the action of catecholamines on
smooth muscle so that similar interference with the action of catecholamines may be found in other tissues.

The approach to mineralcorticoid replacement therapy taken in these experiments has been to administer an electrolyte-active compound in the drinking fluid of the experimental animals. Sodium glycyrrhetinate was the compound used. Although this compound contains a C-11 ketone, it possesses only mineralcorticoid and not glucocorticoid activity.

The differences between mineralcorticoid and glucocorticoids are generally appreciated, but the effects which these types of steroids may have in common are not. It seems that 11-deoxy steroids may meet the steric requirements of glucocorticoid sites but not vice versa. Glycyrrhetic acid is unique in that it has an oxygen at C-11 but shows only mineralcorticoid activity. It has been shown that the presence of the 11β-hydroxyl group alters the surface activity of the glucocorticoids relative to mineralcorticoids. It may also be possible that the C-30 carboxyl group in glycyrrhetic acid counteracts the surface effect of the 11β-hydroxyl group. In this regard it is noteworthy that surface activity was used in determining the configuration of the C-30 carboxyl group (55).

The concern about the possible interaction of
mineralcorticoids at glucocorticoid sites may seem inordinate to the reader. However, the basis of this concern is that such mineralcorticoid effects present in all experimental animals could mask similar effects produced by glucocorticoid derivatives. Unfortunately, while measures were taken to eliminate this problem, its actual magnitude was not determined.
CHAPTER V
GLUCOCORTICOIDs AND HEXOKINASE

Glycolysis in Muscle Cells and Erythrocytes

The amelioration of diabetes by hypophysectomy seen in the Houssay animal prompted further investigation into the effects of pituitary and adrenal extracts on the first reaction of glucose metabolism. Price et al (833) found that anterior pituitary extracts inhibited muscle hexokinase in vitro and that insulin reversed the inhibition in all cases. Stadie et al (1016) also reported that exposure of the rat diaphragm to pituitary extracts abolished its subsequent response to excess insulin. The factors involved in these observations have not been studied further. On the other hand, Price et al (852) found that crude adrenal cortical extract, but not crystalline cortisone, corticosterone or 21-hydroxy-4-pregnene-3,11,20-trione, inhibited muscle hexokinase. Essentially similar findings were reported by Sharma et al (974). These reports indicate that the identity of the active adrenal factor is
Bornstein (100) reported that β lipoproteins from normal rats were less inhibitory than β lipoproteins from diabetic rats towards muscle hexokinase. Although up to 27 percent inhibition was observed, other non-glycolytic enzymes were also inhibited. Insulin did not reverse the inhibition of the enzyme which was attributed to the phosphatidyl choline in β lipoprotein.

Bacila and Baron (34) have studied the effect of glucocorticoids on the anaerobic glycolysis of appendix lymphatic cells. Cortisol at a concentration of 50 µg/ml inhibited glycolysis 12 percent. At 100 µg/ml (2.8 x 10^{-4}) cortisol inhibited glycolysis 40 percent. Mouse diaphragm was inhibited 45 percent at a cortisol concentration of 50 µg/ml in a two-hour incubation in which the glucose concentration was 180 mg/ml. Cortisone acetate and deoxycorticosterone were as effective as cortisol in eliciting this effect. In contrast, the anaerobic glycolysis of acites tumor and lymphosarcoma were not affected at these steroid concentrations. These results may indicate that these tumors have lost their sensitivity to cortisol.

Human and rabbit erythrocyts glycolysis as well as yeast glycolysis were not affected by steroids under these conditions. A possible explanation for the resistance of
erythrocytes to these steroid effects is suggested by the work of Margraf et al (713). These workers found that red cells form glucocorticoid C-21 acetates and that 50 percent of circulating glucocorticoids are present in this form. The key role of red cell glycolysis in the generation of its major product, 2,3-diphospho-glycerate, and the role of the latter compound in hemoglobin function indicates that glycolytic inhibitors could adversely affect oxygen transport if their action in red cells was not prevented by acetylation. These acetate esters are rapidly hydrolyzed in plasma by pseudocholinesterase.

**Effects of Sulfhydryl Compounds and Reagents**

Besides alloxan, both dehydroascorbic acid (693) and uric acid (412) deplete insulin storage granules in the β cells. These compounds appear to exert their degranulation effect through reaction with glutathione. Indeed, alloxan has been used as an analytical reagent in the determination of glutathione. Degranulation is due to the reduction in glutathione levels. Lazarow (644) reported that glutathione increased the hyperglycemic response to growth hormone and glucocorticoids. However, he also noted that growth hormone and glucocorticoids suppressed glutathione levels before these hormones produced glucosuria. This observation is another indication that the
diabetogenic effects of these hormones are produced in the presence of elevated concentrations of insulin.

Bacila and Baron also observed an insulin-like effect of glutathione on the isolated rat diaphragm. They demonstrated that glutathione could prevent the inhibitory effect of cortisol on glucose metabolism and postulated that glutathione reacted with cortisol to form an inactive derivative. Although C-3 carbonyl group of dihydrosteroids had previously been shown to form thiazolidine derivatives with cysteine, this reaction is much less facile with conjugated C-3 steroid ketones.

The possibility that sulfhydryl compounds may exert an effect on the enzyme hexokinase has been investigated by several groups. Lazarus et al (645) studied the sulfhydryl groups of yeast hexokinase and found that the enzyme contained 8 sulfhydryl groups. Four of these reacted with methyl mercury iodide in the native enzyme with no loss in activity. However, four additional sulfhydryl groups were simultaneously exposed and their subsequent reaction with methyl mercury resulted in dissociation and inactivation of the enzyme. Similar conclusions were reached by Schulze and Colowick (955) in their experiments with methyl mercury nitrate in 5.4 M guanidine hydrochloride and with mercaptoethanol. These
findings indicate that simple mercaptide formation does not lead to inhibition of yeast hexokinase activity.

Ilyin (506) and Ilyin and Titova (507) performed some interesting experiments relative to the role of hexokinase sulfhydryl groups in an effect of glucocorticoids. When cortisone acetate was added to rabbit serum β lipoproteins, the lipoproteins inhibited yeast hexokinase. This effect could be reversed by 0.5 units of insulin in vitro. The reversal by insulin required the presence of free sulfhydryl groups, but inhibition by cortisol or cortisone acetate could still be demonstrated after the enzyme had reacted with N-ethylmaleimide or p-chloro-mercuribenzoate. Insulin prevented or reversed the binding of the steroid to the enzyme which remained active after treatment with sulfhydryl reagents. Titova (1067) reported that both oxytocin and vasopressin, which have disulfides similar to those in the A chain of insulin, could also reverse steroid inhibition of the enzyme. Insulin had no effect on yeast hexokinase activity in the absence of glucocorticoids. The effect appeared to be somewhat specific since insulin did not affect the free sulfhydryl groups of hemoglobin or the enzyme aldolase. Glucose-6-phosphate dehydrogenase, however, was also inhibited by the cortisone acetate-β lipoprotein complex.
The glucocorticoid effect on the enzyme hexokinase is also specific. The phosphorylation of glucose and mannose was inhibited, but that of fructose and 2-deoxyglucose was not. Zinc and silver cations prevented the inhibition of hexokinase by glucocorticoids as did N-ethylmaleimide. On the other hand, p-chloromercuribenzoate reversed glucocorticoid inhibition of the enzyme (506, 507, 1067). Thus, p-chloromercuribenzoate had the same effect as the hormone insulin. Although these results were obtained in a very heterogeneous system, i.e. rabbit β-lipoproteins, bovine insulin, and kinase from yeast which lacks both of the latter as well as glucocorticoids, the data validly indicate some of the pitfalls in work with hormones in vitro.

Properties of Hexokinase Isoenzymes

Schimke and Grosslard (744) studied the distribution of hexokinase isoenzymes in animal tissues. They also presented detailed kinetic and physiochemical data on these enzymes. Hexokinase isoenzymes are enumerated in the order in which they migrate during electrophoresis. This order is the same as the order in which the enzymes are eluted from the ion-exchanger diethylaminoethylcellulose by potassium chloride gradients. Type I hexokinase is found in brain and is the isoenzyme most stable at 45°C in the presence of glucose. Both types I and II hexokinase
are found in heart muscle and adipose tissue. The type II enzyme is least stable to heat. Type III hexokinase, which is found in liver, is most inhibited by high concentrations of glucose and least inhibited by glucose-6-phosphate.

Kidney contains all three hexokinase isoenzymes. These three low $k_m$ hexokinases have molecular weights of 96,000 and a similar pH optimum of 7.8-8.8. Glucose, mannose and glucosamine have the same $k_m$ value but that of fructose is 1000 times greater. However, these substrates and 2-deoxyglucose have the same $V_{max}$. Type IV glucokinase in liver is very heat labile. It is inactive with fructose and glucosamine but phosphorylates mannose and 2-deoxyglucose in addition to glucose. It has a molecular weight of 50,000 and a more acidic pH optimum of 7.5-8.5. Pilkis et al (836) have found that insulin is necessary for the induction of both glucokinase and hexokinase II. Hypophysectomy decreases hexokinase II but has no effect on glucokinase and this could explain why hypophysectomized rats lose muscle glycogen.

Hanson and Fromm (426) demonstrated that both types I and II hexokinase were present in skeletal muscle. Using D-fructose as substrate, these makers demonstrated that the mechanisms of the type I enzyme was sequential. In the sequential mechanism, the Michaelis constant of
the variable substrate remains the same or decreases as the concentration of the fixed substrate rises. Their proof of the sequential mechanism corrects their earlier claim that this enzyme followed a ping-pong mechanism. In this latter mechanism one of the products is released from the enzyme before the second substrate is bound.

On the other hand, type II hexokinase unlike type I hexokinase was found to be inhibited by mannose-6-phosphate and to possess kinetic properties similar to those of yeast hexokinase. Since adenosine-5'-triphosphate showed a mixed type of inhibition with respect to both glucose and adenosine-5'-triphosphate, the authors concluded that the mechanism of the enzyme was random, i.e. that the binding of either substrate influenced the $k_m$ for the other substrate.

Glucose-6-phosphate appears to be a mixed inhibitor of all rat hexokinase isoenzymes with respect to glucose. The decrease in the affinity of the enzymes for glucose produced by glucose-6-phosphate may decrease glucose transport by elevating the intracellular concentration of glucose which, in turn, decreases the rate of glucose transport by decreasing the concentration gradient for glucose. Yorke (1189) has observed in isolated rat diaphragm that dexamethasone simultaneously decreased the concentration of sn-3-glycerolphosphate and glucose transport at low steroid
concentrations that did not increase lipolysis. These findings could be explained by citrate inhibition of phosphofructokinase.

**Effect of Insulin on Isoenzymes**

It has been observed that insulin increases the proportion of type II hexokinase in adipose tissue while total hexokinase activity remains constant. The mechanism leading to this coordinate elevation in one isoenzyme and depression of another has not been studied.

Grossbard and Schimke (416) have examined the kinetics of rat hexokinase isoenzymes. Although the Michaelis constant of type II hexokinase for glucose if five times greater than that of the type I enzyme, the type II enzyme is about three times less sensitive to inhibition by complex of magnesium and adenosine-5'-diphosphate. MgATP inhibition of the rat isoenzymes appears to be more important that that produced by glucose-6-phosphate. It is sensitive to pH changes near pH 6.8. Bohnensack and Hofmann (88) have found that the Michaelis constant of the enzyme for glucose increases with increasing pH. Furthermore, the product inhibition of the enzyme seems to decrease with increasing pH. Noat *et al.* (787) have determined that the inhibitory effect of uncomplexed ATP under certain conditions may be due to an interaction with a yeast hexokinase-glucose complex and
that the inhibitor effect of excess magnesium under certain conditions may be due to the formation of a Mg$_2$ATP complex which decreases the concentration of the substrate MgATP. Such observations emphasize the importance of the proper ratio of magnesium to adenine nucleotides in the control of the activity of the enzyme.

Inhibition by MgADP may be more important in the control of the enzyme because it can be removed only by oxidative or substrate phosphorylation of the nucleotide. Since most of the studies with MgADP have not been carried out with an equivalent amount of inorganic phosphate, the full regulatory significance of MgADP inhibition of the enzyme remains to be determined. However, Blair (80) has simulated the hexokinase reaction with the aid of a computer and he concluded that magnesium ion may act as a feedback signal to the enzyme from the adenine nucleotide pool.

The interactions of magnesium and nucleotides is perhaps best seen in the complex kinetics of mitochondrial nucleoside diphosphate kinase which has been studied by Goffeau et al (378). They also demonstrated that the true substrates for this enzyme were magnesium-nucleotide complexes:

\[
\text{MgATP} + \text{MgXDP} = \text{MgADP} + \text{MgXTP}
\]
The complex MgADP was found to be fifty times more inhibitory to the enzyme than excess MgATP. From a study of the kinetic behavior of the enzyme, Goffeau et al. concluded that the mechanism was of the ping-pong type, i.e., a phosphorylated enzyme was an intermediate in the reaction. Furthermore, from differences in the pH dependencies of substrates and inhibitors of the reaction, these workers concluded that the enzyme had an allosteric site for MgXDP in addition to the substrate site and that the enzyme was a regulatory enzyme in metabolism. This conclusion was strengthened by the studies of Thompson and Atkinson (1058) who found that the activity of the enzyme was regulated by the energy charge of the adenine nucleotide pool. Klachko (395) has also postulated that the mechanism of action of insulin could be explained by its activation of the enzyme. Further studies are necessary.

The interpretation of the inhibition patterns of two substrate reactions is complex. However, if a reaction is ordered, a mixed or non-competitive inhibitor of the first substrate will usually appear to be a competitive inhibitor with respect to the second substrate. Hanson and Fromm (426) as well as Toews (1069) have studied the kinetics of rat skeletal muscle hexokinases.

Toews demonstrated that at pH 8.0 MgADP was a
non-competitive inhibitor of MgATP while glucose-6-phosphate was a mixed inhibitor of glucose and a non-competitive inhibitor of MgATP. Since a non-competitive inhibitor may be an uncompetitive inhibitor, these results are compatible with an ordered mechanism for rat skeletal muscle type II hexokinase in which MgATP adds first to the enzyme. It should be noted that the above results were observed at low MgADP levels. Kosow (609) noted that high levels of MgADP may produce mixed inhibition with respect to MgATP by blocking the release of glucose-6-phosphate may produce mixed inhibition with respect to MgATP by interacting at an allosteric site on the enzyme. These latter effects are similar to those which have been observed with nucleoside diphosphate kinase.

In contrast to the mechanism of rat type II hexokinase, Noat et al (785) in their study of yeast hexokinase noted that the reaction is ordered at pH 8.5 and that glucose adds first to the enzyme. Glucose-6-phosphate inhibition was found to be competitive with respect to glucose at an ionic strength of 0.30 but non-competitive with respect to MgATP. On the other hand, Fromm (343) has demonstrated mixed inhibition by glucose-6-phosphate with respect to glucose at pH 7.7, 0.05 ionic strength. Other workers (88) have confirmed that the non-competitive
component of this inhibition decreases with increasing pH. These results indicate that apparent enzyme mechanisms may change with pH.

The regulatory significance of the difference in mechanism between yeast and type II hexokinase is unclear. However, while glucose-6-phosphate is generally a competitive inhibitor of yeast hexokinase with respect to glucose, glucose-6-phosphate is a competitive inhibitor of the type II enzyme with respect to MgATP only at low MgADP levels. The main effect of MgADP inhibition in the muscle enzyme appears to be to change glucose-6-phosphate inhibition with respect to MgATP from a competitive to a mixed type.

Noat et al (786) obtained good agreement between calculated and experimentally produced curves of the progress of the hexokinase reaction versus time. They have used this curve to substantiate their conclusions regarding the mechanism of yeast hexokinase. A similar technique may be used for general isoenzyme analysis. It appears that if all isoenzymes of a given enzyme have the same mechanism, but different kinetic constants including product inhibition constants, the kinetic constants of the isoenzyme mixture will be a linear combination of the kinetic constant of the purified isoenzymes. Computer analysis of the reaction progress curve should then permit calculation of the isoenzyme composition. This approach
may be most successful with the five lactic dehydrogenase isoenzymes which are of no little clinical importance (956).

Type II hexokinase is very similar in structure to the other mammalian hexokinase isoenzymes with respect to molecular weight, number of subunits, pH optimum, and other kinetic parameters. It presumably contains both type I and type III protein subunits since it migrates between types I and III on electrophoresis. On this basis one would expect type II hexokinase to respond similarly to type III hexokinase under a variety of experimental conditions.

Shanygina (973) found that glucocorticoid treatment decreased heart hexokinase II 60 percent and liver hexokinase II by 73 percent. Although some compensatory increase in hexokinase I activity occurred in both tissues, the total hexokinase activity was reduced. Hexokinase II is also the isoenzyme which responds to insulin treatment which reverses the glucocorticoid effect. The effect of cortisol and its blockade by insulin are probably effects on the transcription of DNA and different from the in \textit{vitro} effects of these hormones on yeast hexokinase as described by Ilyin and Titova (507, 508, 1066, 1067).

Recently, effects of insulin on metabolism in the absence of glucose have been demonstrated (897). This implies
that insulin has effects other than its effects on glucose transport. In a study of the transport of 2-deoxyglucose in muscle, Kipnis and Cori (584) found that insulin \textit{in vitro} could further increase the accumulation of 2-deoxyglucose-6-phosphate after initial accumulation in the absence of insulin had ceased.
CHAPTER VI
GLUCOCORTICOIDS AND CYCLIC ADENYLATED

Effects on Triglyceride Synthesis and Lipolysis

Glucocorticoids inhibit the formation of glyceride glycerol in the absence of epinephrine but enhance its formation in the presence of epinephrine. These glucocorticoid effects may be mediated via citrate inhibition of the phosphofructokinase and pyruvate dehydrogenase reactions in the absence of cyclic adenylate. The abolition of the inhibitory effect of citrate on phosphofructokinase by cyclic adenylate, will also be considered. In addition, the mechanism by which glucocorticoids may affect citrate levels will be discussed.

As in any study of hormonal effects, observations must begin with those on intact animals. Levin and Farber (653) found that adrenalectomized rats could not mobilize fat to the liver during fasting because glucocorticoids are required for the lipolytic effects of growth hormone in fasting. Wilhelmi (1150) found that insulin was required
for lipogenesis from acetate and carbohydrate. However, cortisone administration inhibited lipogenesis from these sources. He noted that growth hormone treatment increased fatty acid oxidation and reduced the respiratory quotients in the animals and that this action of growth hormone required the administration of a small amount of cortisone which was ineffective when administered alone. At a constant level of administered growth hormone, glucosuria also remained constant throughout a 10-fold elevation of adrenocorticotrophin. On the other hand, at a constant level of administered adrenocorticotrophin, glucosuria was directly proportional to the dose of growth hormone. These results indicate that the glucocorticoid requirement is saturated by relatively small amounts of the hormone while the growth hormone requirement is not.

Welt and Wilhelmi (1133) found that growth hormone and adrenocorticotrophin decreased the incorporation of deuterium into the body fat of the rat while adrenalectomy had the opposite effect. The importance of such a decrease is indicated by the observation of Lukens (680) who pointed out that the normal rat converted 3 percent of its daily intake of carbohydrate to glycogen, 30 percent to fat, and 67 percent to carbon dioxide. This action of growth hormone and glucocorticoids may also be important in diabetes.
for, as noted by Chaikoff (166), lipogenesis from glucose was much more depressed than glycogen formation in this condition. Lukens noted that, although glucocorticoids and growth hormone depressed deuterium incorporation into fat, normal rats incorporated almost three times as much acetate as pyruvate into fatty acids as hypophysectomized rats indicating that inhibition of pyruvate dehydrogenase was a major effect of growth hormone and glucocorticoids in normal rats. In addition, the fact that adrenalectomized rats did not accumulate total body fat in spite of greater fat synthesis, indicates that adrenalectomy also increased fat oxidation. The mechanism whereby the adrenal inhibits fat oxidation is unknown but appears to be related to the catabolic effects of glucocorticoids on peripheral tissues leading to a sparing of body fat stores.

Effect of Growth Hormone on Fatty Acid Oxidation

Although glucocorticoids may decrease fat oxidation in vivo, growth hormone treatment leads to increased fatty acid oxidation. Greenbaum (407) observed that total body fat fell from 40 percent to 20 percent after growth hormone administration. Greenbaum and McLean (408) also observed increased hepatic fatty acid oxidation between 12 and 24 hours after growth hormone in rats, but noted that fatty acid oxidation was inhibited 6 hours after administration.
of the hormone. These findings may indicate that growth hormone does not directly increase fatty acid oxidation in the liver, but, rather, that the increased hepatic oxidation is secondary to increased lipolysis in the adipose tissue.

Swislocke and Szego (1043) also observed a reduction in serum free fatty acids in dogs within 30-60 minutes of growth hormone administration. In contrast, the increase in serum free fatty acids which was simultaneous with the increased fatty acid oxidation noted above was not observed until 12 hours due to the requirement of DNA and protein synthesis for the lipolytic response (407). Presumably, the protein synthesized under these conditions interacts with glucocorticoids and activates adipose tissue lipase. The early reduction of serum free fatty acids is believed to be due to a stimulation of insulin secretion by growth hormone.

**Influence of Dietary Carbohydrate**

Goodman (391) found that the effect of growth hormone on glucose metabolism in adipose tissue depends on the carbohydrate and fat content of the diet. On high-carbohydrate, low-fat diets, the effects of growth hormone in hypophysectomized rats is similar to the effects of growth hormone in normal rats. Serotonin released by growth hormone may function as a glucocorticoid under these conditions. Goodman (397) has also found that growth hormone inhibits glucose
oxidation and lipogenesis in fed hypophysectomized rats at concentrations which are not lipolytic. However, the fact that the rats used in these experiments had not been hypophysectomized by the author far enough in advance of the experiments to permit cortical atrophy does not eliminate a necessity for glucocorticoids in the effects of growth hormone. Growth hormone and glucocorticoids are ineffective in stimulating lipolysis when added in vitro to such a system unless the hypophysectomized rat has been treated with growth hormone for two days prior to the experiment.

Toxicity of Growth Hormone

The accelerating effect of growth hormone on fatty acid oxidation may not persist during chronic growth hormone administration. Winkler et al (1166) observed that this effect of the hormone could not be observed following 7 days of continuous treatment. De Bodo and Sinkoff (218) also observed this effect and further noted that growth hormone was toxic on prolonged administration. Glucocorticoids eliminated the toxicity and counteracted growth hormone-stimulated insulin release.

Hypophysectomy greatly reduces the lipolytic effect of epinephrine, abolishing the response at low epinephrine levels. Part of the effect of hypophysectomy has been attributed to the absence of ACTH-stimulated cortisol synthesis.
Cyclic adenylate produced in response to epinephrine activates phosphofructokinase and enhances the formation of glyceride glycerol. In the presence of epinephrine, adipose tissue from hypophysectomized rats esterifies 80 percent of the free fatty acids hydrolyzed during lipolysis compared to 70 percent in the presence of all pituitary hormones. Glucocorticoids alone enhance the effects of low epinephrine levels in hypophysectomized rats. However, the effects of high epinephrine levels are not enhanced. Furthermore, the fact that glucocorticoids exert no effects in hypophysectomized rats in the absence of epinephrine indicates that the effect of glucocorticoids under these conditions is due to an inhibition of the enzyme phosphodiesterase which has been described (969).

Role of Phosphodiesterase and Theophylline

Changes in phosphodiesterase activity cannot explain the effects of glucocorticoids in the presence of growth hormone because these effects occur with no change in the concentration of cyclic adenylate (395). Pain (299) noted that insulin, nicotinic acid, prostaglandin E₁ and 1-phenethylibiquanide inhibited the basal lipolysis of isolated fat cells as well as lipolysis caused by catecholamines, ACTH, growth hormone and glucocorticoids.

Weiss et al (1129) were among the first investigators to postulate a role for cyclic adenylate in adipose tissue
lipolysis. They also noted that the 1,3-dimethylated xanthine theophylline and adrenocorticotrophin had lipolytic effects. They reported that the maximal response to norepinephrine was only one-third that of theophylline suggesting that theophylline had lipolytic effects which were not mediated by cyclic adenylate. Compared with norepinephrine adrenocorticotrophin was 100 times as active on a molar basis. Epinephrine and adrenocorticotrophin activate adenyl cyclase by different mechanisms (146, 148).

The single phosphodiesterase enzyme which controls the degradation of cyclic adenylate is just as important as that which controls the formation of this nucleotide. Senft et al (968) noted that the activity of the enzyme was decreased in liver and adipose tissue of alloxan-diabetic rats thus explaining the increase in cyclic adenylate in these organs. Senft et al (969) observed that glucocorticoids also depressed the synthesis of phosphodiesterase by a mechanism that involved DNA and protein biosynthesis. Neither the action of insulin in increasing the synthesis of the enzyme nor that of glucocorticoids in decreasing enzyme synthesis can account for the lipolytic effects of theophylline which do not involve an elevation in cyclic adenylate. Bernbaumer et al (137) noted that neither growth hormone nor glucocorticoids alone directly affected enzyme
activity.

\textbf{\textit{N}^{6}-2'\textit{-Dibutryl Cyclic Adenylate}}

Many investigators have used the \textit{N}^{6}-2'\textit{-dibutryl derivative of cyclic adenylate in their experiments because of its more rapid penetration into cells}. They have assumed that all of the effects of the derivative are due to its conversion to cyclic adenylate. However, Solomon et al. (1002) have shown that the dibutyril derivative may have opposite effects. The dibutyril derivative has an inhibitory effect on glucose utilization whereas the unsubstituted nucleotide stimulates glucose utilization. The reason for this difference is not immediately apparent but the phenomenon should be kept in mind when interpreting the actions of the cyclic adenylate derivative.

Glucocorticoids have two distinct effects on adipose tissue both of which requires the action of growth hormone. The glucocorticoid effect which occurs at lowest concentration is only relatively specific in that it can also be produced by deoxycorticosterone (293). This effect is produced by the interaction of these steroids with a growth hormone-dependent protein. The steroid-protein complex then increases cytoplasmic citrate levels. Higher concentrations of glucocorticoids specifically interact with another growth hormone-dependent protein to increase
the activity of adipose tissue lipase. At 0.01 µg/ml dexamethasone, 9α-fluoro-11β,17α,21-trihydroxy-16α-methyl-1,4-pregnadiene-3,20-dione, has no significant effect on lipolysis but decreases palmitate-1-14C incorporation into triglycerides from 82 percent to 58 percent of the total fatty acids released by lipolysis (370, 371). Similar results were reported by Jeanrenaud and Renold (526) and are shown in Table II. Glucocorticoids alone in the absence of growth hormone also have effects on transcription in adipose tissue. For example, Senft et al (969) found that glucocorticoids depressed the biosynthesis of the enzyme phosphodiesterase in adipose tissue thereby enhancing the effects of theophylline, a phosphodiesterase inhibitor, on adipose tissue. Butcher et al (146) also showed that nicotinic acid could inhibit the enzyme while imidazole activated the enzyme, but the biological significance of these observations has not been determined.

Moskowitz and Fain (762) also showed that cycloheximide inhibits phosphodiesterase and increases cyclic adenylate. They found that growth hormone could also increase cyclic adenylate but only after a one-hour lag period in which DNA, RNA and protein synthesis was mandatory. However, their most surprising finding was that 1 mM theophylline stimulated lipolysis maximally so that
EFFECT OF 9α-FLUORO-11β,17α,21-TRIHYDROXY-16α-METHYL-1,4-PREGNADIENE-3,20-DIONE IN VITRO ON GLYCEROL-FFA BALANCE IN ADIPOSE TISSUE FROM FASTED RATS

<table>
<thead>
<tr>
<th>µmoles/gm of wet tissue wt</th>
<th>Total Lipolysis (Net Glycerol X 3)</th>
<th>Net FFA Production</th>
<th>FFA Re-Esterified</th>
<th>Re-Esterification Percent of Total Lipolysis</th>
</tr>
</thead>
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<td>Controls</td>
<td>30.6</td>
<td>22.0</td>
<td>8.6</td>
<td>28</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>33.5</td>
<td>30.5</td>
<td>3.0</td>
<td>9</td>
</tr>
<tr>
<td>0.1 µg/ml</td>
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</tbody>
</table>

From Jeanrenaud and Renold (526)

Incubations carried out for 4 hours in a Krebs-Ringer bicarbonate buffer containing 5 mM glucose.
it was not increased by growth hormone and glucocorticoids. Furthermore, this effect occurred with no elevation in cyclic adenylate. These results indicate that growth hormone and glucocorticoids do not increase lipolysis by increasing cyclic adenylate. Rather, these hormones produce an effect similar to that of theophylline which is also not active by virtue of its inhibition of phosphodiesterase. The mechanism whereby the combination of the growth hormone-dependent protein and glucocorticoid affects citrate levels has not been determined.

Munk (769) found that deoxycorticosterone in vitro could also reverse the effect of adrenalectomy but that it was less effective than either corticosterone or cortisol. Inhibitory effects of cortisol on the incorporation of acyl coenzyme A into triglycerides were observed at concentrations of $10^{-6}$ Molar during a 2.5 hour incubation. Fain et al. (299) demonstrated that the lipolytic effect of growth hormone appeared immediately if the glucocorticoid was added to adipose tissue which had previously been incubated for 2.5 hours with growth hormone. The fact that deoxycorticosterone can block triglyceride synthesis at higher concentration suggests that other steroids and non-steroids may also produce this effect by occupying glucocorticoid sites. Although glucocorticoids may have
the highest affinity for this site, it is not as specific a receptor site as that which leads to the stimulation of hepatic glycogenesis.

Hepatic Glucocorticoid Receptor Proteins

An example of a specific glucocorticoid effect is seen in the work of Beato et al (54) who have isolated a glucocorticoid receptor protein from liver which they have implicated in the transport of the cytoplasmic hormone to the nucleus where the glucocorticoid induction of gluconeogenic enzymes occurs. On the other hand, an example of a non-specific glucocorticoid effect is seen in the study of Dugle et al (510) on the diabetogenic action of steroids. They found that those steroids with keto groups at C-11 were more diabetogenic than 11β-hydroxy compounds. The compound 4-pregnene-3,11,20-trione was more diabetogenic than 11β-hydroxy-4-pregnene-3,20-dione. This observation is somewhat similar to observations that have been made concerning the anti-tumor potencies of steroids (369).

Effect of Triiodothyronine in Hypophysectomized Rats

In hypophysectomized rats, triiodothyronine has effects similar to growth hormone and glucocorticoids. It increases the sensitivity of adipose tissue to epinephrine after a three-hour incubation and to theophylline after a twelve-hour incubation (397). These findings indicate
that the hormone has at least two effects, one of which may be related to phosphodiesterase activity.

Norepinephrine is probably responsible for most of the glycerol release from adipose tissue. However, when the basal state is altered by epinephrine from the adrenal medulla, this catecholamine increases the synthesis of cyclic adenylate. Cyclic adenylate activates a specific lipase which non-stereo-specifically hydrolyzes one fatty acid from triglyceride substrates.

The degree to which glycolysis is coupled to pyruvate oxidation is determined by the lactic dehydrogenase isoenzyme composition in the tissue. Tight coupling occurs with LDH₁ in heart and the level of NADH is more strictly regulated than it is by LDH₅ in liver so that elevated levels of pyruvate and NADH prevail during gluconeogenesis.

Effects of Diabetes on Citrate and Pyruvate Levels

Dixit et al (238) have reported that a 2-3 fold elevation in muscle citrate is found in alloxan-diabetic rats. More importantly, they found a decreased rate of citrate oxidation by muscle but not by liver. These results may be due to the more rapid glucocorticoid metabolism of the liver in comparison to peripheral tissues. If true, the rate of glucocorticoid metabolism could influence diabetes
by this mechanism.

Henneman and Bunker (450) reported that human diabetics had higher levels of serum pyruvate due to a reduced capacity to oxidize pyruvate. This appears to be a direct consequence of the unopposed action of glucocorticoids since Haugaard and Haugaard (435) observed the same phenomena in alloxan-diabetic rats.

The degree of inhibition of pyruvate utilization relative to glycolysis is indicated by the percentage of blood glucose carbon which returns to the liver as glycerol or lactate to be incorporated into hepatic glycogen. This process has been termed recycling. Friedman et al (337) demonstrated that diabetic rats recycle 2.4 percent of their glucose compared to 1.2 percent for normal rats. This finding indicates that insulin decreases recycling. However, these same workers found that glucocorticoids did not affect recycling. This could be interpreted to mean that glucocorticoid-induced elevations in citrate inhibited glycolysis and pyruvate utilization to approximately the same extent.

Elevations in muscle citrate in diabetes have been demonstrated by Garland et al (358) and by Garland and Randle (359). Wieland and Wies (1147) have shown that a 3-fold elevation in hepatic acetyl coenzyme A occurs in
cortisol-treated diabetic rats compared to similar rats treated only with insulin. Cahill et al (151) reported that insulin decreased the conversion of glucose to glyceride glycerol 5-fold but that this conversion was increased by epinephrine. Epinephrine increases cyclic adenylate which decreases the $k_m$ of phosphofructokinase for fructose-6-phosphate. Apparently, this effect is sufficient to overcome the inhibition of phosphofructokinase by MgATP. In addition, the depletion of ATP produced by the activation of phosphofructokinase and hexokinase increases the conversion of triose phosphates to pyruvate.

Renold et al (878) and Phillips et al (833) reported that lipogenesis from pyruvate was reduced 9-fold while glucose utilization was reduced only 25 percent by cortisol in the presence of $10^{-4}$ Molar epinephrine. Deoxycorticosterone in vitro is 50 percent as effective as cortisol at a concentration of 30 µg/ml (769). Munk also found that lipogenesis was depressed in fat pads from adrenalectomized rats by the administration of 2.5 mg/kg cortisol before sacrifice.

Fain et al (299) reported that 0.004 µg/ml dexamethasone reduced the glucose uptake of the fat pad from 3.19 to 1.24 µmoles per 100 mg of tissue while pretreatment of the tissue with higher concentrations (0.032 µg/ml) for
1.5 hours resulted in up to 75 percent inhibition of glucose uptake. Goodman and Knobil (389) also noted that glucocorticoid treatment of hypophysectomized rats restored their lipolytic response to epinephrine but not to growth hormone. This finding is important because it indicates that glucocorticoids can enhance the effect of cyclic adenylate quite independently of its action with the growth hormone-dependent protein which leads to an increase in citrate levels. The effect of glucocorticoids on cyclic adenylate in the absence of growth hormone appears to be due to the induction of a glucocorticoid-dependent protein which inhibits the enzyme phosphodiesterase (969). This induction does not explain glucocorticoid effects in the absence of changes in the level of cyclic adenylate.

Yorke (1189) has found that glucose transport is inhibited by low glucocorticoid concentrations which are not lipolytic. Citrate inhibition of phosphofructokinase raises glucose-6-phosphate which, in turn, may raise the $k_m$ of hexokinase for glucose, thereby reducing enzyme activity and the glucose concentration gradient which partly determines glucose transport.

Control of Lipogenesis

Adipose tissue can respond to both the specific and non-specific effects of glucocorticoids. In 1951 Brady
et al (112) observed that cortisone treatment depressed both triglyceride and carbon dioxide formation from glucose. Landau and Katz (628) estimated that from 11-23 percent of glucose in adipose tissue was metabolized via the pentose phosphate shunt. However, Wies and Ball (1172) noted that most of the NADPH necessary for fatty acid synthesis in adipose tissue was formed in the tissue by the operation of the malate cycle:

\[
\text{Pyruvate} + \text{CO}_2 + \text{ATP} = \text{Oxalacetate} + \text{ADP} + P_i
\]

\[
\text{Oxalacetate} + H^+ + \text{NADH} = \text{Malate} + \text{NAD}^+
\]

\[
\text{Malate} + \text{NADP}^+ = \text{Pyruvate} + \text{CO}_2 + \text{NADPH}
\]

Sum: \[\text{NADH} + \text{NADP}^+ + \text{ATP} = \text{NAD}^+ + \text{NADPH} + \text{ADP} + P_i\]

The operation of this cycle explains the observations of Landau et al (629) that the amount of NADH generated during fatty acid synthesis was excessive while the rate of operation of the pentose shunt could not account for all of the NADPH required for synthesis. The role of pyruvate carboxylase in this cycle explains its presence in adipose tissue in the absence of gluconeogenesis. Furthermore, those factors which inhibit the pyruvate dehydrogenase reaction may increase the malate cycle.

Ball (40) determined that epinephrine could activate glycolysis in adipose tissue about 24 percent. As mentioned earlier this effect is due to an action of cyclic
adenylate on phosphofructokinase as well as to an elevation of long-chain acyl coenzyme A.

The anti-insulin effects of growth hormone and glucocorticoids occur in isolated adipose tissue and do not require the presence of β lipoprotein. However, the fact that growth hormone and glucocorticoids increase β lipoprotein insulin inhibition in vivo but not in vitro simply reflects the role of the liver in β lipoprotein synthesis. Therefore, β lipoproteins may transport the factor which mediates the citrate elevation caused by growth hormone and glucocorticoids in isolated tissues. Furthermore, the β lipoprotein insulin antagonist may be identical with the lysolecithin species described by Bolton et al (89) which increases the inhibitory effect of ADP on platelet metabolism.

Investigations of the hormonal dependence of the synalbumin insulin antagonist by Vallance-Owen et al (1084) indicate that this antagonist is also dependent on both growth hormone and glucocorticoids. Although the exact nature of the synalbumin antagonist is unknown, its reported properties are not inconsistent with those of a polar lipid. Lysolecithin is found in association with plasma albumin as is the synalbumin antagonist. If the synalbumin insulin antagonist is a lysolecithin dependent
on growth hormone and glucocorticoids, then another lysol-lecithin species bound to β lipoprotein may also be dependent on these two hormones.

Winternitz et al. (1170) showed that the effect of epinephrine on glycogenolysis and glycolysis was enhanced in adrenalectomized rats. Epinephrine produced a 62 percent fall in muscle glycogen in adrenalectomized rats compared to a 34 percent fall in normal rats. In addition, in the normal rat the muscle glycogen which disappeared could be accounted for as liver glycogen while that in the adrenalectomized rat could not. Thus, it appears that glucocorticoids also enhance the gluconeogenic process under these conditions. The extent of glycogenolysis in muscle compared to liver depends on the catecholamine used to elicit the effect. Isopropyl noradrenaline preferentially increases muscle glycogenolysis and has little or no effect on liver glycogen. Glycogenolysis in muscle implies increased glycolysis. The normal response of adrenalectomized rats to epinephrine could be restored by the administration of adrenal cortical extract 12 hours prior to the experiment or by the administration of cortisone for 24 hours beforehand. In diabetic rats, liver glycogen as well as muscle glycogen falls after epinephrine, suggesting that insulin is required for the formation of
liver glycogen from glucose synthesized in the liver. The enhanced effect of epinephrine was not seen in adrenalectomized-eviscerated rats and this may indicate that the liver produces a factor under the influence of glucocorticoids which increases the effect of epinephrine. This factor may or may not be identical to the anti-insulin factor discovered by Bornstein (98) which is transported to peripheral tissues as a lipoprotein complex. Winternitz has pointed out that the necessity for the liver in the production of the epinephrine opposing factor may partially explain Russell's (917) finding that there was no difference in the glucose tolerance of normal eviscerated and adrenalectomized-eviscerated rats maintained on deoxycorticosterone. It would also explain her finding that the increased sensitivity of the adrenalectomized rat to insulin is abolished by evisceration. If the primary effect of the hepatic factor is to decrease the levels of cyclic adenylate (290), it may be the same as that of the lyssolecithin reported by Bolton et al to combine with β lipoprotein and increase platelet aggregation.

Friedman et al (337) have found that glucocorticoids are required for the effect of hepatic cyclic adenylate on gluconeogenesis even though these steroids do not alter the concentration of the nucleotide. Bernbaumer et al
(137) have also shown that growth hormone and glucocorticoids either alone or in combination do not activate adenyl cyclase but they do enhance the action of cyclic adenylate already formed.

Scrutton and Utter (960) noted that glucagon and therefore cyclic adenylate enhanced pyruvate carboxylase activity in liver. Furthermore, Friedman et al (337) found that the rate of gluconeogenesis from lactate was enhanced by glucagon in livers from intact rats but not in livers from adrenalectomized rats. This response could be restored by the addition of glucocorticoids to the perfused liver. Meismann and Segal (730) have noted similar behavior in the effects of cyclic adenylate on the kinase which activates hepatic glycogen synthetase.

Although the induction of gluconeogenic enzymes is enhanced by glucocorticoids and opposed by growth hormone, not all of the effects of glucocorticoids on the liver can be blocked by inhibitors of DNA and protein synthesis. Long et al (676) found that doses of cortisol which have no effect on gluconeogenesis from protein have a marked inhibitory effect on glucose utilization in adrenalectomized-diabetic rats. These results suggest that the same glucocorticoid-dependent factor formed in the liver inhibits glycolysis there and in peripheral tissues.
One of the factors regulating gluconeogenesis is lipolysis. Williamson et al (1156, 1157) have pointed out that none of the steps in the conversion of fatty acids to acetyl coenzyme A or to acyl carnitine are rate-limiting for fatty acid oxidation. The ratio of NADH to NAD\(^+\) is increased by fatty acid oxidation and NADH is the main source of reducing equivalents for gluconeogenesis.

Vohainy et al (1107) have similarly commented on the failure of added carnitine to enhance palmitate oxidation in the perfused rat heart in vitro. On the other hand, carnitine reduced the rate of palmitate oxidation and increased the labeling of the phospholipid fraction. The reported enhancement of fatty acid oxidation by carnitine in isolated mitochondria may be related to the loss of endogenous carnitine during the preparation of the mitochondria.

**Effect of Ethanol on Gluconeogenesis**

Although gluconeogenesis is increased by an increase in the reduction potential of the pyridine nucleotides, excessive reduction potentials may actually inhibit gluconeogenesis as well as peripheral glucose utilization. In a study of ethanol-induced hypoglycemia Lochner et al (671) found that the cause was an inhibition of gluconeogenesis in spite of the fact that ethanol inhibited peripheral
glucose utilization 25 percent. Both the inhibition of glucose utilization as well as the decrease in gluconeogenesis may be attributed to an excessive reduction of pyridine nucleotides. Krebs (618) has shown that NADH depletes hepatic pyruvate by converting it to lactate thereby reducing the rate of the pyruvate carboxylase reaction.

The increase in the reduction potential of the pyridine nucleotides by ethanol depends on the preferential utilization of ethanol over other respiratory substrates. The conversion of ethanol to acetate does not require the utilization of adenosine-5'-triphosphate. On the contrary, NADH is generated in the alcohol dehydrogenase and aldehyde dehydrogenase steps. Thus, ethanol is oxidized in preference to other respiratory substrates because its oxidation is associated with a greater free energy decrease than the oxidation of other substrates.

**Effects of Clavine Alkaloids**

The fact that theophylline can produce all of the effects of growth hormone and glucocorticoids indicates the possibility that a similar endogenous compound may be involved in the effects of these hormones. Brek et al (116) reported that serotonin was lipolytic in vitro. Hotta et al (477) further demonstrated that dihydroergotamine enhanced lipolysis in isolated rat adipose tissue cells.
This effect is opposite to that expected from an adrenergic blocking agent. In related experiments Harvey et al (432) showed that the non-peptide ergot alkaloid ergonovine could block the hyperglycemic effect of epinephrine, although the dose required was about 40 times higher than that required for the same effect with the α-adrenergic blocking agent ergotamine. These results suggest that only non-peptide ergot alkaloids can block the effects of cyclic adenylylate. Fain (298) has also shown that the lipolytic effect of dihydroergotamine was potentiated by theophylline suggesting that the two drugs share a common receptor site.

Hotta et al (477) reported the lipolytic effects of dihydroergotamine on adipose tissue cells. They noted that the effect *in vitro* was similar to that observed *in vivo* and also noted that insulin could counteract the effect. Prior treatment with theophylline and/or growth hormone enhanced the effect which was similar to that of glucocorticoids in that dihydroergotamine had no effect on lipolysis produced by adrenocorticotropic, thyroid-stimulating hormone, or glucagon. These effects were confirmed by Nakano et al (775) and Fain (208). These results are unrelated to the blockade of adrenocorticorophin release by ergotamine observed by Levy and Raney (657) in adrenalectomized rats. Northrop and Parks (789) also reported lipolytic effects
obtained with growth hormone and glucocorticoids.

In contrast, Efendo and Ostman (271) found that theophylline did not increase the lipolytic effect of dexamethasone. They observed that theophylline blocked the conversion of glucose to glyceride glycerol without affecting the levels of cyclic adeny late. Butcher and Sutherland (145) have reported some properties of phosphodiesterase including its requirement for magnesium, stimulation by imidazole, and preferential inhibition by $10^{-2} \text{M}$ theophylline. The finding that theophylline blocks the conversion of glucose to glyceride glycerol could mean that theophylline has the same site of action as glucocorticoids, i.e. inhibition of triglyceride synthesis. This interpretation is born out by the finding that theophylline did not increase the lipolytic effect of glucocorticoids (397).

Bray and Goodman (115) also found that epinephrine increased glycolysis in adipose tissue independently of its lipolytic effect indicating that phosphofructokinase is affected by lower concentrations of cyclic adeny late than the levels required to activate adipose tissue lipase. On the other hand, Karl et al (564) found that when the synalbumin insulin antagonist acted on rat diaphragm, the concentration of sn-3-glycerophosphate was increased even
though glucose uptake was decreased. This indicates that the synalbumin antagonist inhibits the glycolysis of D-glyceraldehyde-3-phosphate.

Flatt (323) has emphasized that insulin-stimulated fatty acid synthesis is an energy-yielding process for adipose tissue cells. It is therefore limited by the capacity of the cell to utilize the energy which is produced. Because lipolysis in effect functions as an adenosine triphosphatase in the metabolism of the cell, the rates of fatty acid synthesis in the presence of epinephrine and insulin are greater than the rates in the absence of epinephrine. Furthermore, Flatt has shown that fatty acid synthesis is limited by the rate of triglyceride formation.

Rodbell (897) showed that insulin increased free fatty acid uptake by adipose tissue cells in the absence of glucose. Larner, however, detected no change in cyclic adenylate after insulin. These results suggest that insulin decreases lipolysis by a mechanism independent of cyclic adenylate. This effect of insulin may explain why adipose tissue is more sensitive to insulin than muscle (151).

**Anti-Lipolytic Effects of Oral Hypoglycemic Agents**

Brown and Stone (129) observed that tolbutamid [1-butyl-3-(p-tolylsulfonyl)urea] inhibits the lipolytic
effect of dexamethasone and growth hormone immediately and does not require a lag period. Likewise, Stone and Brown (1034) determined that phenformin (1-phenethylbiguanide) had an anti-lipolytic effect in the absence of glucose. The lipolytic effects of DL-arterenol, ACTH, glucagon, and theophylline were opposed by phenformin. These results suggest that tolbutamide directly opposes the anti-insulin effect of glucocorticoids but that phenformin blocks the action of cyclic adenylylate as one of its principle effects. Phenformin may also lead to inhibition of pyruvic dehydrogenase. Moorhouse et al (757) observed that the drug decreased pyruvate tolerance more in diabetics than in control subjects. These results suggest that phenformin may also enhance citrate inhibition of pyruvate dehydrogenase (721) in addition to its effects on cyclic adenylylate.
CHAPTER VII
METABOLIC EFFECTS OF GLUCOCORTICOIDS

The non-lipolytic effects of glucocorticoids and growth hormone on glucose metabolism are most important in the production of insulin resistance. Unfortunately, most previous investigators of the anti-insulin action of the hormones have studied them at lipolytic concentrations. A second complication of published investigations is that they have been done on adipose tissue in which the proportion of glucose metabolized via the Embden-Meyerhof path varies with hormone concentration.

Effects of Hormones on Glycolysis in Rat Diaphragm

The effects of insulin and of other hormones on glucose metabolism have been extensively studied with the isolated rat diaphragm preparation. This technique was introduced by Gemmill who found that insulin increased both glucose uptake and glycogen synthesis. Adrenal hormones have also been studied with this preparation. Epinephrine, the first known adrenal hormone, was
investigated by Cori et al (200). They found that epinephrine decreased glucose utilization. This effect was confirmed by Walaas and Walaas (1111) who found that 12 µg/ml epinephrine significantly decreased glucose uptake but significantly increased lactate output by the rat diaphragm preparation. Glycogenolysis also increased so that muscle glycogen was depleted within one hour. These results suggest that glucose-6-phosphate formed from glycogen glucose can inhibit glucose uptake by causing end-product inhibition of muscle hexokinase isoenzymes.

On the other hand, Ingle and Nezamis (518) found that the infusion of glucose and epinephrine into eviscerated rats had no effect on glucose tolerance. Furthermore, Bray and Goodman (115) found that epinephrine increases glucose uptake in adipose tissue and Williamson (1154) found the same effect in rat heart. These latter findings suggest that only high concentrations of epinephrine inhibit glucose uptake.

Ingle and Nezamis (514) also examined the effects of adrenalectomy on the glucose tolerance of the eviscerated rat. They found that adrenalectomy increased the uptake of glucose at low levels of glucose and insulin. On the other hand, at high levels of glucose and insulin, the adrenalectomized rats had higher blood glucose levels at
the end of the infusion. These results suggest that adrenal hormones enhance glycogen formation in extrahepatic tissues in the presence of insulin. One possible explanation of this effect is that adrenal hormones inhibit glycolysis and thereby increase the intracellular concentration of glucose-6-phosphate. Another possible explanation is that glucocorticoids increase glycogenesis by the kidney. However, other studies have shown that the kidneys release glucose into the blood only during hypoglycemia. Thus, the first explanation is more probable. Additional support for it is provided by Bowman and Deimer (107) who demonstrated that adrenalectomy increases the glycogenolytic effect of epinephrine.

Kipnis (585) confirmed these results with intact rat diaphragm. He noted that adrenalectomy did not affect glucose transport, but that it did increase glucose phosphorylation in the presence of insulin. Hypophysectomy, while it decreased basal glucose transport in the absence of insulin, also increased glucose phosphorylation in the presence of insulin. With the cut diaphragm, in contrast, hypophysectomy increased basal glucose transport but decreased glucose phosphorylation in response to insulin. Both adrenal and pituitary hormones decrease glucose phosphorylation in the intact rat.
Kipnis et al (586) also demonstrated intracellular free glucose in muscle stimulated with epinephrine. As discussed previously, glucose-6-phosphate inhibits muscle hexokinases. England and Randle (138) have shown that this mechanism may account for over 90 percent of the inhibition of these enzymes under certain conditions. However, this mechanism is less important in liver because it contains glucose-6-phosphatase. In addition, Ashmore et al (24) have reported that the concentration of glucose-6-phosphate in diabetic liver is one-third normal. These results suggest that the effect of growth hormone and glucocorticoids on glycolysis is more important in muscle and adipose tissue than in the liver. Ashmore et al (23) also noted that the rate of glucose phosphorylation was markedly depressed in liver slices from alloxan-diabetic rats.

**Effect of Bicarbonate**

Shaw and Stadie (977) first demonstrated the presence of two identical Embden-Meyerhof pathways in normal rat diaphragm. One of these is located on the cell surface, and the other is intracellular. The fact that insulin is effective in bicarbonate-phosphate buffer but not in phosphate buffer led to the discovery of these pathways. Only intracellular glycogen formation and glycolysis were increased by insulin. On the other hand extracellular
glycolysis was not responsive to insulin. Apparently, intracellular phosphofructokinase is inactive in the absence of bicarbonate according to these workers. Zahnd et al (1195) also found that the effect of insulin on glucose oxidation in adipose tissue was greater in the presence of bicarbonate (Table III). Most of these findings were confirmed by Landau and Sims (631, 985) using the intact rat diaphragm. They carried out all their experiments in bicarbonate buffer using either non-labeled or labeled glucose and glucose-6-phosphate. They were, therefore, unable to confirm or deny the effect of bicarbonate on intracellular phosphofructokinase. On the other hand, they did show that insulin has no effect on the conversion of added glucose-6-phosphate into glycogen or lactate. They also demonstrated that 11.6 percent of the total lactate produced in the absence of insulin was produced by the extracellular pathway. This percentage was 11.1 percent in the presence of insulin, but intracellular glycolysis was increased only 5 percent under these conditions. However, glycogen synthesis was increased 226 percent by 0.1 unit/ml insulin.

These results indicate that total glucose uptake is a poor measure of glycolysis, especially in the presence of insulin. Lactate and carbon dioxide production are better
TABLE III
EFFECT OF BICARBONATE AND PHOSPHATE ON THE RATIO OF GLUCOSE CARBON 1 TO GLUCOSE CARBON 6 INCORPORATED INTO CO₂, FATTY ACIDS AND GLYCEROL BY ISOLATED EPIDIDYMAL ADIPOSE TISSUE

<table>
<thead>
<tr>
<th></th>
<th>Oxidation to CO₂</th>
<th>Fatty Acids</th>
<th>Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bicarbonate</td>
<td>Kreb's Ringer Phosphate</td>
<td>Bicarbonate Phosphate</td>
</tr>
<tr>
<td>No Hormone</td>
<td>100.0</td>
<td>123.5</td>
<td>0.810</td>
</tr>
<tr>
<td>Insulin (0.1 U/ml)</td>
<td>957.2</td>
<td>246.3</td>
<td>3.89</td>
</tr>
<tr>
<td>Epinephrine 18 µg/ml</td>
<td>72.3</td>
<td>57.8</td>
<td>1.25</td>
</tr>
<tr>
<td>G. H. (100 µg/ml)</td>
<td>65.7</td>
<td>60.2</td>
<td>1.09</td>
</tr>
</tbody>
</table>

Zahnd et al (1195)
indicators. Also, the fact that the effect of insulin on glycolysis is relatively small means that even a slight inhibition of its effect may be consequential. The small increase in glycolysis in response to insulin could be secondary to insulin-stimulated glycogen formation. As the enzyme UTP: \( \alpha-\beta \)-glucose-1-phosphate uridyltransferase [EC 2.7.7.9] utilizes UTP, it is regenerated from ATP by nucleoside diphosphate kinase with depletion of the energy charge (one-half the number of pyrophosphate bonds) of the adenine nucleotide pool. Glycolysis increases when this occurs due to the action of control mechanisms which will be discussed later. Furthermore, physiological variations in \( pCO_2 \) may exert significant effects on glycolysis. However, the effects of bicarbonate buffers on glycolytic enzymes have not been thoroughly studied. One other point is clear. Since insulin in vitro can reverse the effect of the insulin-inhibitor dependent on growth hormone and glucocorticoids, it must affect only the intracellular glycolytic pathway.

In their authoritative work on the influence of the adrenal cortex on carbohydrate metabolism Long et al (673) described several fundamental actions of glucocorticoids. They found that in adrenalectomized animals "cortical extract produces a smaller proportion of glucose oxidation
and a larger deposition of the absorbed glucose as liver glycogen, while the muscle glycogen level is not increased."

In contrast to liver glycogen which can be increased in the fasted rat by glucocorticoid administration, muscle glycogen cannot be increased by glucocorticoids unless the animals are fed (676, 768).

Herring and Crans (453) noted that low doses of cortisol were much more effective in hypophysectomized rats in maintaining muscle glycogen than in maintaining liver glycogen. Villee and Hastings (1103) confirmed these findings using the isolated rat diaphragm and also demonstrated that part of the drop in muscle glycogen could be attributed to increased glucose utilization following adrenalectomy. The greater effect on muscle glycogen compared to liver glycogen indicates that gluconeogenesis is not involved in this effect.

Long et al (674) further studied the effect of cortisol on the glucose tolerance of the eviscerated rat. They found that the infusion of 0.2 mg of cortisol per hour at 2, 3 or 5 hours before glucose administration or during glucose administration did not decrease the glucose tolerance of the rat. However, these tests were performed without insulin so that glucose transport rather than glucose phosphorylation was limiting for glucose utilization.
Despite the fact that glucose phosphorylation is limiting under the conditions of the normal glucose tolerance test, results are often cited as evidence for the lack of effect of glucocorticoids on peripheral glucose utilization.

Effect on Peripheral Glucose Utilization

Evidence that glucocorticoids do depress the extrahepatic utilization of glucose has been provided by Boutwell and Niang (102). In experiments with mice they noted that the turnover time of glucose was increased 50 percent while the rate of glucose utilization was decreased to 65 percent of the control value 4-6 hours after the administration of 1 mg of cortisone. Also, Lecoco et al (648) administered a large priming dose of 33 mg of cortisol to dogs prior to the continuous infusion of 1.3 mg of cortisol per minute. They found that a 38 percent decrease in hepatic glucose output within 30 minutes with little change in blood glucose. Frawley (328) also showed that cortisol eliminated symptoms of hypoglycemia before any changes in blood glucose occurred.

Changes in Hepatic Glycolytic Intermediates

Lecoco et al (648) attributed the glycogen formation they observed to an increase in the hepatic concentration of glucose-6-phosphate, an activator of glycogen
synthesis. However, Hornbrook et al (476) observed a fall in hepatic glucose-6-phosphate and uridine diphosphate glucose after cortisol administration. Furthermore, the hepatic concentrations of a number of other glycolytic intermediates did not change after cortisol. Munk and Koritz (768) observed that an elevation in blood glucose always preceded the increase in liver glycogen by at least 30 minutes. It appears that glucose-6-phosphatase is also activated under these conditions (22).

One reason for these discrepancies may be the difficulty involved in measuring small significant changes in blood glucose. However, it appears that the activation of glycogen synthetase cannot explain the increased glucose output by the liver. This can only be explained by activation of glucose-6-phosphatase. Furthermore, the explanation that the initial increase in hepatic glycogen synthesis is not secondary to glucose-stimulated insulin release is supported by the fact that muscle glycogen rises 1 hour after both blood glucose and liver glycogen are elevated. If hepatic glycogen synthesis was caused by insulin, it should have coincided with insulin-stimulated muscle glycogen formation.

The Catabolic Effect of Cortisol

Long et al (676) also observed that glucose synthesis
was not a consequence of protein catabolism. During the first 4 hours following cortisol the total carbohydrate content of the fasted rat increased 250 mg/kg with no increase in blood urea nitrogen. The catabolic effect of glucocorticoids appears to be due to an inhibition of amino acid transport in peripheral tissues. Tilton and Torralla (1065) showed that the administration of cortisone acetate to adrenalectomized-eviscerated rats accelerated the rate of rise of plasma amino acids under all of the conditions which they studied. This finding has been confirmed in eviscerated-adrenalectomized-alloxan-diabetic rats by Smith and Long (997). Kostyo (610) showed that the effect of cortisol, corticosterone, deoxycorticosterone, and 17α,21-dihydroxy-4-pregnene-3,20-dione occurred after a three-hour induction period at $10^{-7}$ M concentration.

**Effects of Steroids on Glycogen**

Shortly after the total synthesis of the adrenal steroids the effects of these hormones on glycogen formation in rat diaphragm was determined. Bartlett et al (48) used labeled glucose to show that corticosterone was less potent than deoxycorticosterone at 10 μg/ml in inhibiting glycogen formation, thus confirming the earlier findings of Bartlett and MacKay (41). Verzar and Wenner (1099) further observed that 5-10 mg/100 ml deoxycorticosterone increased...
glycogenolysis while completely inhibiting glycogen synthesis caused by 10 milliunit/ml insulin. In a subsequent study Verzar and Wenner (1100) compared various hormones in the inhibition of glycogen synthesis. They found that the order of potency was 3,4-bis(p-hydroxyphenyl)-trans-3-hexene>progesterone>deoxycorticosterone>corticosterone. This order of hormones is not correlated with hormone action but is the order of decreasing polarity of the hormones, suggesting that they interact at a hydrophobic site.

**Control of Gluconeogenesis and Glycogenesis**

Seubert et al (970) found that gluconeogenesis does not require *de novo* enzyme biosynthesis. However, the action of pyruvate carbon dioxide ligase (ADP)(EC 6.4.1.1) and GTP: oxalacetate carboxy-lyase (transphosphorylating) (EC 4.1.1.32) require the expenditure of four moles of high energy phosphate in addition to those derived from glycolysis in order to reverse this process. Also, the activity of pyruvate kinase, ATP: pyruvate phosphotransferase (EC 2.7.1.40), is so much greater than that of the latter enzyme that no reversal of glycolysis could occur unless pyruvate kinase were inhibited. Shrago et al (982) found an elevation of phosphoenolpyruvate carboxykinase activity in alloxan-diabetes and following glucocorticoid treatment.
The low activity of fructose-1,6-diphosphate 1-phosphohydrolase (EC 3.1.3.11) in muscle prevents gluconeogenesis in this tissue. This enzyme is inhibited by high concentrations of its substrate and also by its product fructose-6-phosphate (752). This means that the enzyme cannot function unless phosphofructokinase is inhibited. Inhibition of phosphofructokinase is also important because hepatic pyruvate kinase is also activated by fructose-1,6-diphosphate. The required inhibition of this enzyme is produced by elevated levels of ATP and citrate. Martin and Silbert (721) found that citrate also inhibits pyruvate dehydrogenase. The effect was significant at 1 mM citrate and maximal at 12 mM citrate.

Larner investigated the mechanism by which insulin activates liver glycogen synthetase. He found that the activation of the enzyme by insulin is mediated by an inhibition of the protein kinase which phosphorylates the I form of the enzyme to the D form (636). Insulin decreases the affinity of Transferase I kinase for cyclic adenylate which increases the affinity of the kinase for ATP (637). Since the activity of the D form of the enzyme is dependent on the concentration of glucose-6-phosphate while the activity of the I form is not, a decrease in the phosphorylation of the I form increases the activity
of glycogen synthetase. Glucose-6-phosphate increases the affinity of glycogen synthetase D for its substrate uridine diphosphate glucose. However, the synthesis of uridine diphosphate glucose is not affected by this activation mechanism. Epinephrine increases the concentration of cyclic adenylate and thereby promotes the conversion of glycogen synthetase to its active D form. On the other hand, glucocorticoids may increase glycogen synthesis by interfering with the action of this nucleotide (204).

Vardanis (1088) found that both glucose-6-phosphate and inorganic phosphate activated mammalian liver glycogen synthetase by increasing the affinity of the enzyme for glycogen 50 fold. The concentration of inorganic phosphate required for half-maximal activation was 7.75 x 10^{-4} M, well within the physiological range. Steiner et al (1027) also determined that the enzyme could be activated by fructose-6-phosphate.

Stetten et al (1029) reported that the rate of oxidation of glucose to carbon dioxide was one-third normal in alloxan-diabetic rats. In more severely diabetic depancreatized dogs Feller (310) found the rate of glucose oxidation to be one-fifth normal. However, Chernick and Chaikoff (178) found that the oxidation of fructose and acetate to carbon dioxide was not impaired in the absence
of insulin, indicating that the block in glucose metabolism is at the phosphofructokinase reaction. This block is surmounted when fructose-1-phosphate is split to form D-glyceraldehyde-3-phosphate. In addition, Chaikoff (166) reported that lipogenesis from glucose was reduced to 10 percent of normal in the diabetic animal. These findings were confirmed by Feller (310) as well as by Stetten et al (1029) who concluded that lipogenesis from glucose was depressed more than glucose oxidation. Diabetic liver slices, for example, incorporate virtually no glucose into fatty acids compared to the 0.8 percent incorporated by normal liver slices.

The influence of insulin on the formation of pyruvate and glycogen in rat diaphragm has been studied by Villee et al (1104). They found that insulin increased the percentage of pyruvate formed from glucose more than it increased pyruvate oxidation. Diaphragms from adrenalectomized rats synthesized less glycogen than normal but more of it from glucose. The marked inhibitory effect of the pituitary on glycogen synthesis is shown in Table II. Adrenalectomy of diabetic rats restores glycolysis more than glycogenesis.

Cori (199) compared the rates of glycogen formation from glucose, fructose and galactose. Although glucose
was absorbed from the intestine twice as fast as fructose, both hexoses formed liver glycogen equally well and more rapidly than galactose. Galactose is probably a poor glycogen precursor because the conversion of uridine diphosphate galactose to uridine diphosphate glucose by UTP-glucose-4-epimerase (EC 5.1.3.2) depends on the concentration of NAD⁺. Peak glycogen values are obtained four hours after galactose administration. Cori also noted that 39 percent of fructose administered to rats was converted to liver glycogen compared to 17 percent in the case of glucose. These results indicate that fructose rather than glucose is more rapidly converted to liver glycogen.

Regulation of Fructolysis

The metabolism of fructose illustrates several important aspects of glycolytic control mechanisms. Fructose can be phosphorylated by all of the low \( k_m \) hexokinases, but its affinity for these enzymes is one-tenth of its affinity for a specific ATP: D-fructose 1-phosphotransferase (EC 2.7.1.3) in liver. This latter enzyme is as important as glucokinase in sucrose metabolism. In addition, the incorporation of fructose into glycogen is one-sixth normal in alloxan-diabetes (23). Since gluconeogenesis is increased in this disorder, this finding suggests that glycerolkinase or triokinase may be depressed
in this condition.

Liver, muscle and adipose tissue contain fructokinase isoenzymes. Hers and Kusaka (454, 455) have described some of the differences between the hepatic and muscle enzymes. The muscle isoenzyme has a low affinity for fructose compared to the hepatic isoenzyme Michaelis constant of $5 \times 10^{-4}$ Molar. However, the hepatic isoenzyme is exceptionally sensitive to competitive inhibition by ADP. This inhibition may involve the ribose moiety of the nucleotide. Gromora (415) has shown that the fructokinase present in adipose tissue decreases during fasting. This isoenzyme is probably different from the low $k_m$ forms found in intestinal mucosa and liver.

The fructose-1-phosphate formed by the above kinases has two fates. In muscle it is further phosphorylated by ATP: D-fructose-1-phosphate 6-phosphotransferase (EC 2.7.1) which has not been extensively studied since its discovery by Slein et al (995). In liver, fructose-1-phosphate is split to form D-glyceraldehyde and dihydroxyacetone phosphate by aldolase B, an isoenzyme of ketose-1-phosphate aldehyde lyase (EC 4.1.2.7). When saturated, this enzyme can split fructose-1-phosphate and fructose-1,6-diphosphate at the same rate. Liver aldolase B has both a lower $k_m$ and higher maximum velocity with fructose-
1-phosphate compared to the muscle isoenzyme. Aldolase A, however, has a maximum velocity with fructose-1,6-diphosphate which is over 12 times that of the hepatic enzyme. Phosphoglucomutase may also be an important regulatory enzyme because its product α-D-glucose-1,6-diphosphate, in addition to its catalysis of glucose-6-phosphate utilization via the phosphoglucoisomerase reaction, is equally potent as this glycolytic intermediate in causing end-product inhibition of hexokinase isoenzymes (609).

Fructose metabolism in liver can proceed by a pathway which bypasses the reaction catalyzed by the regulatory enzyme ATP: D-fructose-6-phosphate 1-phosphotransferase (EC 2.1.1.11) because liver contains glycerolkinase, ATP: D-glycerol-phosphotransferase (EC 2.7.1.30), and triokinase, ATP: D-glyceraldehyde 3-phosphotransferase (EC 2.7.1.28). In contrast, neither muscle nor adipose tissue contain significant amounts of these enzymes.

D-glyceraldehyde arising from peripheral fructose metabolism may be reduced to glycerol by alcohol dehydrogenase rather than being transported unchanged to the liver. Landau and Merlevede (626) found extensive labeling in positions 3 and 4 as well as in positions 1 and 6 of liver glycogen glucose following the administration of D-glyceraldehyde-3-\(_{14}^{14}\)C. However, their conclusion
that this could have resulted from the intermediate formation of glycerol does not appear to be justified in view of the asymmetric nature of glycerol phosphorylation. Glycerol formed in peripheral tissues may serve the function of lactate in transferring reducing equivalents from the pituitary to the liver.

**Effects on Sugar Transport**

Cori and Cori (200) compared the utilization of three hexoses in eviscerated rats in the presence and absence of insulin. They found that fructose was utilized 5.3 times as fast as glucose while mannose was utilized 4.3 times as fast in the absence of insulin. However, in the presence of insulin fructose and mannose disappeared at the same rate but only 38 percent as fast as glucose. The more rapid rates of fructose and mannose disappearance in the absence of insulin cannot be explained by the $k_m$ values of hexokinase for these hexoses. Since mannose and glucose are transported by different membrane carriers, insulin apparently decreases the $k_m$ of the carrier for glucose more than the $k_m$ of the carrier for mannose.

The use of fructose in the diet of the alloxan-diabetic rats has been reviewed by Sarett and Snipper (935). Since the liver converts fructose to glucose, they found no decrease in glucosuria when fructose constituted 60
percent of the diet. Only 5 percent of the dietary fructose was excreted as fructose. However, many of the rats fed fructose recovered from their diabetic symptoms and excreted less urine than the rats fed glucose. These results suggest that a significant fraction of the ingested fructose is metabolized directly by peripheral tissues in the absence of insulin via the fructokinase reaction which bypasses the citrate-inhibited phosphofructokinase reaction.

In rat heart Battaglia and Randle (50) showed that insulin stimulated the transport of glucose, mannose, D-3-O-methylglucose, 2-deoxyglucose, D-xylose, D-arabinose, D-lyxose and L-arabinose by one mechanism and the transport of fructose and galactose by another. Mackler and Guest (696) also showed that the increased utilization of fructose by diaphragm in the presence of insulin could be counteracted by glucose. This implies that fructose in the absence of glucose is phosphorylated mainly by hexokinase and only by fructokinase when hexokinase is saturated.

It has been shown that fructose re-feeding restored the glucose tolerance of the fasted rat more effectively than isocaloric sucrose re-feeding, despite the fact that the fructose re-fed rats had lower hepatic glucokinase
activity. This result indicates that the latter enzyme does not influence glucose tolerance in the rat.

Burch et al. (132) found that fructose feeding increased hepatic glucose and glucose-6-phosphate without increasing hepatic glycogen synthesis. They attributed the lack of glycogen synthesis to a decrease in uridine diphosphate glucose secondary to a decrease in inorganic phosphate due to its incorporation into fructose-1-phosphate. The activity of liver aldolase becomes rate-limiting for oxidative phosphorylation under these conditions. Therefore, fructose may be a tool for decreasing hepatic ATP. The possibility that fructose-1-phosphate directly inhibited UTP: α-D-glucose-1-phosphate uridyl transferase (EC 2.7.7.9) was not investigated.

Fructose is converted to glucose by the intestinal mucosa during its absorption. This transformation is catalyzed by ketose reductase and aldose reductase with sorbitol as an intermediate. This process results in the transhydrogenation of NADH to NADPH which is required for the synthesis of fatty acids from carbohydrate. The extent to which this process contributes to carbohydrate-induced lipemia following sucrose ingestion has not been determined.

Although Wick et al. (143) observed that fructose
was but poorly utilized in eviscerated rabbits in the presence of glucose, Miller et al (744) found that intravenous fructose disappeared faster than glucose in normal and diabetic subjects. Furthermore, Pearson and Rimer (820) observed that a patient unable to utilize glycogen because of phosphorylase deficiency (type V glycogen disease) could perform on a treadmill when plasma fructose was maintained at 0.10 mg/l. In contrast, a glucose level of 1.60 gm/l was required for this activity. These findings indicate that peripheral fructose utilization becomes more significant when glucose utilization is blocked. Therefore, the metabolic significance of fructokinase in peripheral tissues under normal conditions remains obscure.

Papper et al (806) studied the effect of cortisone on the fructose and glucose tolerance of normal man. An oral dose of 200 mg of cortisone acetate decreased glucose tolerance of normal men. An oral dose of 200 mg of cortisone acetate decreased glucose tolerance but did not impair fructose tolerance. This result suggests that most of the fructose is metabolized by the liver under the conditions of the test. On the other hand, Frawley et al (328) observed that glucocorticoids could reduce the disappearance of fructose from the serum at low glucose concentrations possibly because some fructose
is phosphorylated by hexokinase under these conditions. Frawley also noted that the cortisol effect could not be produced by corticosterone administration, indicating the necessity of the 17α-hydroxyl group in the effect of cortisol. Sprague et al (1013) also demonstrated that 11-dehydrocorticosterone had little effect on carbohydrate metabolism in Addison's disease.

The key role of triokinase and glycerolkinase in fructose metabolism suggests that fructose metabolism will be impaired when the activity of these kinases is reduced. For example, Glenn et al (370) reported that cortisol decreased the oxidation of glycerol to carbon dioxide. The extent to which the inhibition of these enzymes is due to the direct interaction of the dihydroxyacetone side chain of the glucocorticoid with the enzymes has not been determined. Nor has the effect of growth hormone in triokinase activity been reported.

Randle et al (861, 863) have attributed the reduced glycolysis of diabetic rat heart to the increased lipolysis which occurs in these tissues. They have termed their hypothesis the "glucose-fatty acid cycle." According to this hypothesis fatty acids are utilized in preference to glucose when they are present as respiratory substrates.
Lipolysis in muscle as well as in adipose tissue is increased by insulin deficiency. This effect is believed to be due to an increase in cyclic adenylate formation which is normally prevented by insulin. Since the rate at which fatty acids are oxidized is proportional to fatty acid concentration (341), lipolysis tends to increase the ratio of NADH to NAD$^+$ as well as the ratio of acyl coenzyme A to coenzyme A.

The ratio of NADH to NAD$^+$ in cytoplasm has been estimated from the ratio of lactate to pyruvate or from the ratio of sn-3-glycerolphosphate to dihydroxyacetone phosphate. Insulin deficiency reduces the lactate/pyruvate ratio to 54 percent of its control value in heart and to 74 percent of its control value in diaphragm. On the other hand, the ratio of sn-3-glycerolphosphate to dihydroxyacetone phosphate was reduced to 50 percent of its control value in heart and to 57 percent of its control value in diaphragm. In contrast, this ratio was unchanged in diabetic hepatic cytoplasm. These cytoplasmic ratio changes are opposite to those expected in mitochondria where fatty acid oxidation occurs.

Glycolysis is inhibited by citrate in extrahepatic tissues in diabetes even though the ratio of NADH to NAD$^+$ is decreased. Garland and Randle (359) have found that
that the citrate concentration was 53 µmoles/100 gm in diabetic rat heart compared to 22 µmoles/100 gm in normal rat heart. Garland et al (358) also determined that 1 mmolar citrate reduced the rate of the phosphofructokinase reaction by 23 percent in diabetic rat heart. These results were confirmed by Beatty and Peterson (56) who found that the excretion of citrate was increased in alloxan-diabetic and in depancreatized rats. Given the inhibition of phosphofructokinase by citrate in diabetes, the reversal of this inhibition by the reduction of citrate levels is a key to the mechanism of action of insulin. Although insulin may increase isocitrate oxidation in mitochondria, an action of insulin on isolated mitochondria has not been conclusively demonstrated. Also, since the NADH/NAD⁺ ratio is one of the factors which controls mitochondrial isocitrate oxidation, insulin may act by changing this ratio.

Garland and Randle (359) found that the ratio of acetyl coenzyme A to coenzyme A was increased 3-fold in diabetic rat heart. In addition, the free fatty acid concentration was increased 2.3-fold in diabetic heart and 1.8-fold in diabetic diaphragm. The output of glycerol was increased 2.3-fold in heart, 1.6-fold in diaphragm and 1.7-fold in adipose tissue. In muscle the
increase in the ratio of acetyl coenzyme A to coenzyme A leads to a 30 percent inhibition of pyruvate oxidation. However, in liver it leads to increased formation of ketone bodies.

Newsholme et al (781) also found that pyruvate perfusion inhibits glycolysis in muscle but not in liver. In muscle pyruvate inhibits the pyruvate kinase reaction. However, in liver pyruvate may enter the citric acid cycle as oxalacetate. This reaction is catalyzed by pyruvate: carbon-dioxide ligase (ADP)(EC 6.4.1.1) and is more endergonic than the entry of pyruvate into the cycle via the pyruvate dehydrogenase reaction.

The fatty acids released during lipolysis are activated to acyl coenzyme A derivatives. However, Garland and Randle (359) observed that the concentration of acyl coenzyme A in diabetic rat heart was only 66 percent of normal. This finding suggests that the incorporation of acyl coenzyme A into triglycerides is increased. Denton and Randle (227) have shown that the incorporation of sn-3-glycerolphosphate into glyceride glycerol increases in diabetic rat heart and epididymal adipose tissue as expected. However, the mechanism of this effect is obscure even though Denton and Randle indicated that it was dependent on growth hormone and glucocorticoids.
Lipogenesis and Ketosis

Denton and Halperin (228) studied the factors which control triglyceride formation and found that the rate did not correlate with either the concentration of sn-3-glycerol-phosphate nor with the concentration of acyl coenzyme A. Therefore, the mechanism whereby triglyceride synthesis is increased in diabetes has not been determined.

Jungas and Ball (551) have calculated that 40 percent of the oxygen consumption of fat cells from fed rats is related to the energy requirements of triglyceride synthesis. Triglyceride synthesis is virtually absent in 3-day fasted rats; inhibition of triglyceride synthesis thus is important to the conservation of glucose during fasting.

Flatt (323) has shown that fatty acid synthesis is increased in adipose tissue when triglyceride synthesis is increased because acyl coenzyme A inhibits acetyl coenzyme A carboxylase. Fatty acid synthesis in this tissue is limited by the ability of the cell to utilize approximately 1.8 moles of high energy phosphate per acetyl coenzyme A unit from glucose incorporated into fatty acids. Therefore, at any level of tissue respiration, the total ATP derived from fatty acid synthesis and fatty acid oxidation is constant. Consequently, the control of the rate
of fatty acid oxidation by control of the rate of acyl coenzyme A incorporation into triglycerides represents an important control point for glucocorticoid action.

Scow et al (957) studied ketosis in pancreatectomized rats. They found that either hypophysectomy or adrenalectomy abolished the ketosis. They also found that the administration of growth hormone alone to hypophysectomized-pancreatectomized rats had no effect on blood glucose or ketone bodies. On the other hand, cortisol alone at a dose of 2 mg/day killed 50 percent of the animals in 48 hours. Deoxycorticosterone had no effect under these conditions. The glucocorticoid also increased glycerides in the liver under these conditions. These results demonstrate that glucocorticoids alone accelerate ketosis and that growth hormone enhances this action.

Scow and Chernik (958) also showed that dexamethasone was very ketogenic in hypophysectomized-depancreatized rats but that growth hormone and glucocorticoids produced no change in body fat unless the rats were stressed. These results suggest that the major effect of glucocorticoids may be to enhance the effect of epinephrine. Growth hormone alone again had no effect but increased lipolysis in response to dexamethasone and theophylline. These results suggest that glucocorticoids
and theophylline share a common mechanism of action and that not all of the lipolytic effect of theophylline is due to its inhibition of phosphodiesterase. Fain (294) also observed that growth hormone alone was not lipolytic in adipose tissue but that it enhanced the effects of glucocorticoids. Uroguoti et al (1080) reached the same conclusion studying ketotic dogs.

The glycogenic effect of glucocorticoids depends upon their inhibition of pyruvic dehydrogenase, while its ketogenic effect is due to its enhancement of the lipolytic effect of cyclic adenylate. Renold et al (818) determined that cortisone depressed glucose uptake 25 percent while it depressed lipogenesis from pyruvate 89 percent. There are many other reports of decreased pyruvate tolerance following glucocorticoid administration (328, 450, 451). However, it is not clear whether an increase in the ratio of acetyl coenzyme A to coenzyme A is the mechanism whereby glucocorticoids inhibit pyruvic dehydrogenase.

Welt and Wilhelmi (1133) found that adrenalectomy increased hepatic lipogenesis. However, the inhibitory effect of glucocorticoids on hepatic lipogenesis may be masked by the mobilization of lipid to the liver from peripheral tissues. Hill and Drake (460) found an
increase in hepatic triglycerides but not phospholipids fol-
the administration of cortisone acetate. However, although
the enzyme which activates fatty acids to their acyl coen-
zyme A derivatives was increased 2-fold, triglyceride syn-
thesis was increased only 30 percent. These results sug-
gest that triglyceride synthesis is primarily inhibited by
glucocorticoids. Similarly, Hazzard et al (441) noted that
the rise in plasma triglycerides during estrogen therapy
was accompanied by a relative inhibition of triglyceride
synthesis.

The fact that adrenalectomy restores lipogenesis in
liver but not in isolated adipose tissue suggests that
glucocorticoids may influence enzymes unique to liver such
as glycerolkinase. Ashmore et al (25) found that gluco-
corticoids had no effect on the incorporation of glycerol
into glucose. On the other hand, Glenn et al (368) have
shown that glucocorticoids depress glycerol oxidation.
These results suggest that glycerol phosphorylation is not
rate-limiting for gluconeogenesis from glycerol.

In this discussion of the effects of glucocorticoids
on glycolytic regulation glycolytic oscillations have been
neglected. However, an understanding of such oscillations
appears essential for the complete understanding of gly-
colytic regulation. Betz and Chance (75) have summarized
their findings concerning the causes of glycolytic oscillations:

"If our hypothesis is true, the following points are responsible for the oscillations: (a) the limited activity of pyruvate decarboxylase, responsible for the accumulation of reduced pyridine nucleotide, pyruvate, fructose-1,6-diphosphate, dihydroxyacetone phosphate, and glyceraldehyde-3-phosphate; (b) the kinetics of phosphofructokinase, which is inhibited by its substrate, adenosine-5'-triphosphate; and activated by the products, fructose-1,6-diphosphate, and adenosine-5'-diphosphate; (c) the kinetics of glyceraldehyde-3-phosphate dehydrogenase, which is inhibited not only by its products, reduced pyridine nucleotide and 1,3-phosphoglycerate, but also by its substrate, blyceraldehyde-3-phosphate; (d) the intimate connection between different parts of the glycolytic pathway, by the adenosine-5'-triphosphate and reduced pyridine nucleotide feedback systems, which brings an accumulation of glyceraldehyde-3-phosphate just at the moment when glyceraldehyde-3-phosphate dehydrogenase is inhibited by its products, thus producing the inversion."
Effects of Hormones on Adipose Tissue

The best indications of the sites of hormone action have come from the study of the metabolism of specifically radiolabeled glucose in hormone-sensitive tissues such as adipose tissue. As a result of such studies the general site of action of several hormones can be outlined. Insulin, the hormone of primary interest in diabetes, appears to primarily increase fatty acid synthesis from pyruvate. All of its other actions appear to be secondary to its lipogenic effect. In accord with this proposed action those tissues in which lipogenesis is prominent are the tissues most sensitive to insulin. On the other hand, the heart is less affected by diabetes in comparison to other tissues because of its low rate of lipogenesis.

The main reactions affected by growth hormone, epinephrine, and glucocorticoids are less clear. Both growth hormone and epinephrine are lipolytic as well as antilipogenic. However, the action of these hormones may overlap to some extent. For example, insulin increases the lipolytic action of epinephrine while growth hormone and epinephrine may increase the formation of sn-3-glycerolphosphate from glucose.

Perhaps the most striking effect of insulin is the apparent increase in the efficiency of glucose utilization
that it produces. In particular, the low rate of glyceride glycerol synthesis necessary for the high rate of fatty acid synthesis indicates how much of the energy of glucose is wasted in glyceride glycerol synthesis in response to lipolytic hormones.

In fasted-refed rats the most marked effect of growth hormone is its depression of all phases of glucose metabolism. These effects are due to the increased utilization of endogenous triglycerides for oxidation. Also, growth hormone inhibits triglyceride synthesis under these conditions. In rats fed ad libitum growth hormone increases pyruvate oxidation less than insulin but increases triglyceride synthesis and citrate oxidation more than insulin. Additionally, growth hormone increases lipogenesis while epinephrine decreases lipogenesis. This latter effect of epinephrine is clearly opposite to that of insulin.

There appears to be a reasonable correlation between Kreb's cycle activity and glyceride glycerol synthesis. This indicates that when a molecule of D-glyceraldehyde-3-phosphate is used for triglyceride synthesis another molecule of pyruvate must be oxidized via the Kreb's cycle to keep the total oxidizable substrate approximately constant.

The low rate of glyceride glycerol synthesis plus the
### TABLE IV

EFFECT OF DIET AND HORMONES ON DISTRIBUTION OF GLUCOSE CARBON IN FAT PADS

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose Uptake</th>
<th>Pentose Cycle CO₂</th>
<th>Pyruvate Cycle CO₂</th>
<th>Kreb's Cycle CO₂</th>
<th>Total CO₂</th>
<th>Fatty Acid</th>
<th>Glyceride</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasted-Refed</td>
<td>460</td>
<td>59.8</td>
<td>101.2</td>
<td>13.8</td>
<td>174.8</td>
<td>193.2</td>
<td>32.2</td>
<td>59.8</td>
</tr>
<tr>
<td>Fed-ad libitum</td>
<td>100</td>
<td>8.0</td>
<td>13.0</td>
<td>8.0</td>
<td>29.0</td>
<td>21.0</td>
<td>20.0</td>
<td>30.0</td>
</tr>
</tbody>
</table>

**Relative Glucose Carbon Yield**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose Uptake</th>
<th>Percent Difference From Control Fasted-Refed Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>67.4</td>
<td>54.4</td>
</tr>
<tr>
<td>Growth Hormone</td>
<td>-28.2</td>
<td>-66.8</td>
</tr>
</tbody>
</table>

**Percent Difference From Control Fed Ad Libitum Rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucose Uptake</th>
<th>Percent Difference From Control Fed Ad Libitum Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>140</td>
<td>51.9</td>
</tr>
<tr>
<td>Growth Hormone</td>
<td>151</td>
<td>-55.6</td>
</tr>
</tbody>
</table>

From Katz et al (568)
TABLE V

IN VITRO UTILIZATION OF U-\(^{14}\)C-LABELED GLUCOSE BY RAT DIAPHRAGM WITH 0.5 UNIT/ML INSULIN

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
<th>Adrenal-ectomized</th>
<th>Diabetic Adrenal-ectomized</th>
<th>Hypophy-sectomized</th>
<th>Adrenal-ectomized/Hypophy-sectomized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Utilized (mg/gm/hr)</td>
<td>2.87</td>
<td>2.17</td>
<td>3.91</td>
<td>5.20</td>
<td>4.42</td>
<td>6.11</td>
</tr>
<tr>
<td>Utilized Glucose Metabolized to CO(_2) (mg/gm/hr)</td>
<td>0.22</td>
<td>0.11</td>
<td>0.29</td>
<td>0.18</td>
<td>0.27</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>7.7</td>
<td>5.1</td>
<td>7.4</td>
<td>3.5</td>
<td>6.1</td>
<td>7.2</td>
</tr>
<tr>
<td>Medium Glucose Metabolized to CO(_2) (percent)</td>
<td>16.3</td>
<td>9.2</td>
<td>25.3</td>
<td>15.9</td>
<td>28.5</td>
<td>33.9</td>
</tr>
<tr>
<td>Utilized Glucose Metabolized to Glycogen (mg/gm/hr)</td>
<td>0.62</td>
<td>0.61</td>
<td>0.54</td>
<td>0.71</td>
<td>1.71</td>
<td>2.23</td>
</tr>
<tr>
<td></td>
<td>21.6</td>
<td>28.1</td>
<td>13.8</td>
<td>13.6</td>
<td>38.7</td>
<td>36.5</td>
</tr>
<tr>
<td>Utilized Glucose Unaccounted for (mg/gm/hr)</td>
<td>2.03</td>
<td>1.45</td>
<td>3.03</td>
<td>4.31</td>
<td>2.44</td>
<td>3.44</td>
</tr>
</tbody>
</table>

From Villee and Hastings (1103)
low (25 percent) maximal contribution of the pentose cycle suggests that the concentration of triose phosphates is depressed due to their rapid utilization by D-glyceraldehyde-3-phosphate dehydrogenase and that the activity of the pentose cycle is closely tied to the activity of pyruvate dehydrogenase so that the inhibition of D-glyceraldehyde-3-phosphate dehydrogenase by NADH is not significant when its other product 1,3-diphosphoglycerate is depleted. Also, the additional ATP converted when glucose is metabolized via the pentose cycle and bypasses the exergonic phosphofructokinase reaction of the Embden-Meyerhof pathway is sufficient to convert one of the acetyl coenzyme A units derived from the hexose into malonyl coenzyme A.

The decrease in the pentose shunt in the refed rat treated with insulin is due to the diversion of glucose-6-phosphate to glycogen synthesis. The increase in glycolysis during glycogenesis provides the energy for that process through the formation of UTP via the nucleoside diphosphate kinase reaction. The high $k_m$ of D-glucose-6-phosphate: NADP oxidoreductase (EC 1.1.1.49) for its substrate reduces its activity when glucose-6-phosphate is converted to glycogen.

The activation of acetyl CoA carboxylase following insulin may also be responsible for the decrease in
cytoplasmic citrate via citrate cleavage to acetyl coenzyme A and oxalacetate.

In general, the higher the net rate of dihydroxyacetone phosphate utilization, either by its conversion to D-glyceraldehyde-3-phosphate or to sn-3-glycerolphosphate the lower the extent of equilibration expected in the triose phosphate. There is almost complete isotopic equilibration in the insulin-treated fat pad because most of the D-glyceraldehyde-3-phosphate is formed via the pentose cycle as shown in the accompanying diagram. The non-isotopic equilibration of triose phosphates in methods which assume equilibration results in a decrease in the ratio of glucose-1-\textsuperscript{14}C to glucose-6-\textsuperscript{14}C incorporated into acetyl CoA and an increase in the ratio of glucose-1-\textsuperscript{14}C to glucose-6-\textsuperscript{14}C incorporated into glyceride glycerol.

Using their method which corrects for the non-equilibrium of triose phosphates under various hormonal conditions, Katz et al. (568) obtained the data in Table IV relative to the effects of insulin, growth hormone and epinephrine on metabolism. In fasted-refed rats, which are similar to diabetic-refed rats, insulin increases pyruvate oxidation and fatty acid synthesis much more than glucose utilization. Isocitrate oxidation and glyceride glycerol synthesis, on the other hand, are
selectively decreased. In fed animals, these effects are even greater. The decrease in isocitrate oxidation can be explained by the elevation of NADH relative to NAD$^+$ produced by the pyruvate dehydrogenase reaction. However, the conversion of dihydroxyacetone phosphate to sn-3-glycerol phosphate is not increased, indicating that insulin increases the synthesis of glyceride fatty acids but not of glyceride glycerol.

Landau et al (629) have pointed out that although the activity of the enzyme D-glyceraldehyde-3-phosphate ketol-isomerase (EC 5.3.1.1) is not rate-limiting for glycolysis, the activity of the enzyme is not sufficient to completely equilibrate a label in C-3 of its substrates. This limitation is important in all studies with specifically-labeled glucose, especially in studies of glycolysis. For example, the percent of randomization in C-3 of the triose phosphates was 12 in control fat pads, 22 following insulin, 8 following growth hormone, 4 after thyrotrophin and 10 after epinephrine treatment.

Katz et al (568) investigated the effect of the triose phosphate isomerase reaction on the estimation of the pentose cycle contribution to glucose metabolism using glucose-1-$^{14}$C, glucose-6-$^{14}$C and glucose-U-$^{14}$C and measuring incorporation into glyceride glycerol and fatty acids.
FIGURE IV

D-GLYCERALDEHYDE-3-PHOSPHATE FORMATION VIA THE PENTOSE CYCLE

D-GLYCERALDEHYDE-3-PHOSPHATE FORMATION VIA THE PENTOSE CYCLE

$6 \text{NADP}^+ + 3 \text{H}_2\text{O} \rightarrow 3 \text{GLUCOSE-6-P}$

$3 \text{RIBULOSE-5-P} + 6 \text{NADPH} + 3 \text{ATP} + 3 \text{H}_2\text{O} \rightarrow$ (Glycolysis)

$\text{XYLOULOSE-5-P}$

$\text{RIBOSE-5P}$

$\text{GLYCOLALDEHYDE-3-P}$

$\text{SEZUHEPTULOSE-7-P}$

$\text{ERYTHROSE-4-P}$

$\text{XYLOULOSE-5-P}$

$\text{FRUCTOSE-6-P}$

$\text{FRUCTOSE-6-P}$

$\text{GLYCOLALDEHYDE-3-P}$

$2 \text{GLUCOSE-6-P}$
They compared their method using glucose-2-$^{14}$C to those which use only glucose-1-$^{14}$C and glucose-6-$^{14}$C and assume complete isotopic equilibration in D-glyceraldehyde-3-phosphate-3-$^{14}$C and dihydroxyacetone phosphate-1-$^{14}$C. In comparison to the glucose-2-$^{14}$C experiments in which isotope equilibration need not be assumed, the latter methods give good results in control and insulin-treated fat pads, but not in growth hormone and epinephrine-treated fat pads. The percent of glucose metabolized by the pentose pathway was 14 in control fat pads, 25 following insulin, 11 after either growth hormone or thyrotrophin and 7 after epinephrine-treatment.

The ketogenic effect of glucocorticoids appears to be due to an enhancement of the effects of cyclic adenylylate. DeBodo and Altszuler (219) demonstrated that hypophysectomized-adrenalectomized dogs were insensitive to epinephrine. Hypophysectomy also reduced gluconeogenesis by decreasing the supply of fatty acids for respiration. Senft et al (969) found that a glucocorticoid-dependent protein also decreased the activity of the enzyme phosphodiesterase in adipose tissue thereby decreasing the hydrolysis of cyclic adenylylate. The significance of this effect is uncertain because glucocorticoids enhance the effect of cyclic adenylylate without changing its concentration (307). Engel et al (285) noted that the barbiturate
nembutal markedly enhanced the ketogenic effect of ACTH. Whether this effect is due to an inhibition of phosphodiesterase activity has not been determined. However, such reports stress the necessity to control barbiturate anesthesia in glucocorticoid studies and to use other anesthetics when possible.

 Apparently, Kreb's cycle activity reaches a maximum in the presence of epinephrine but does not keep pace with lactate production in adipose tissue. The degree to which glycolysis is coupled to pyruvate oxidation is determined by the lactic dehydrogenase isoenzyme composition in the tissue. Tight coupling occurs with LDH$_1$ in heart and the level of NADH level is more strictly regulated than it is by LDH$_5$ in liver so that elevated levels of pyruvate and NADH prevail during gluconeogenesis.

 On the other hand, Denton and Randle (226) have demonstrated that the concentration of acyl coenzyme A is decreased in diabetic rat heart during the increased triglyceride synthesis which occurs when growth hormone and glucocorticoids increase lipolysis. This indicates that a mechanism other than lipolysis increases triglyceride synthesis during lipolysis.

 Leboeuf et al (647) (Table VI) found that cortisol
### TABLE VI

**THE EFFECTS OF CORTISOL ON GLUCOSE METABOLISM**

**BY RAT EPIDIDYMAL ADIPOSE TISSUE IN VITRO**

<table>
<thead>
<tr>
<th>Hormones Added</th>
<th>Total CO₂</th>
<th>Glyceride Glycerol</th>
<th>Fatty Acids</th>
<th>Glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (0.1 unit/ml)</td>
<td>386</td>
<td>37.6</td>
<td>534</td>
<td>385</td>
</tr>
<tr>
<td>Epinephrine (10⁻⁴ M)</td>
<td>185</td>
<td>175</td>
<td>54.7</td>
<td>73.1</td>
</tr>
<tr>
<td>Cortisol (10⁻⁴ M)</td>
<td>-18.1</td>
<td>-12.2</td>
<td>-22.4</td>
<td>-28.0</td>
</tr>
</tbody>
</table>

- **Percent Difference From Control**
- **Percent Difference From Hormone Minus Cortisol**

<table>
<thead>
<tr>
<th>Hormones Added</th>
<th>Total CO₂</th>
<th>Glyceride Glycerol</th>
<th>Fatty Acids</th>
<th>Glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin and Cortisol</td>
<td>3.68</td>
<td>4.97</td>
<td>8.10</td>
<td>-33.1</td>
</tr>
<tr>
<td>Epinephrine and Cortisol (Normal Rats)</td>
<td>-27.1</td>
<td>-22.0</td>
<td>-35.6</td>
<td></td>
</tr>
<tr>
<td>Epinephrine and Cortisol (Adrenalecto-mized Rats)</td>
<td>-41.3</td>
<td>-50.5</td>
<td>-4.48</td>
<td>-55.2</td>
</tr>
</tbody>
</table>

Lebœuf et al (647)
reduced glycogen synthesis 30% in the presence of insulin indicating that its effect is not on transport. Also, the decrease in glyceride glycerol synthesis by cortisol could be the basis of its ketogenic effect. Epinephrine, which increased glycogen formation, enhanced the inhibitory effect of cortisol. These results suggest that cortisol modifies the effects of cyclic adenylylate.

Jungas and Ball (551) found that 0.1 µg/ml epinephrine increased the output of free fatty acids and glycerol by adipose tissue. Insulin addition increased glycerol release 3-fold but decreased free fatty acid levels to low values. The oxygen consumption rose several fold during treatment. The effect of insulin in decreasing free fatty acid release and increasing glycerol release in the presence of epinephrine is dependent on glucose, although insulin itself in the absence of epinephrine is capable of suppressing lipolysis.

When glucose is the sole substrate of adipose tissue, it can furnish only 63 percent of the reduced nicotinamide adenine dinucleotide phosphate required for the observed conversion of glucose to fatty acids. The rest of the requirement must come from the oxidation of other substrates and through the transhydrogenation of NADH to NADPH. This finding is not unusual since Williamson and
Krebs have observed that glucose alone even in the presence of insulin can account for only 57 percent of the respiration of rat heart. In the absence of insulin the proportion falls to 24 percent. The preferred substrate for heart is acetoacetate which contributes 73-82 percent of the respiratory fuel in the absence of insulin and 51-64 percent in the presence of insulin. In contrast, liver can form but cannot oxidize acetoacetate, readily. The low utilization of acetoacetate by liver and its high utilization by heart has been attributed to the difference in Succinyl-CoA: 3-oxoacid CoA-transferase (EC 2.8.3.5) catalyzing the reaction:

\[
\begin{align*}
&\text{O} \quad \text{O} \\
&\text{O-CCH}_2\text{CH}_2\text{C-S-CoA} + \text{CH}_3\text{CCH}_2\text{C-O}^- \\
\implies &\text{O} \quad \text{O} \\
&\text{O-CCH}_2\text{CH}_2\text{C-O}^- + \text{CH}_3\text{CCH}_2\text{C-S-CoA}
\end{align*}
\]

The enzyme is normally present intramitochondrially in most tissues except liver. Heart has 2.5 times the intramitochondrial activity of skeletal muscle. In addition, heart muscle is unique in that it contains 20 times the extramitochondrial activity of skeletal muscle. The production of large amounts of succinate by this reaction is not without significance. Krebs has shown that succinate
is the preferred substrate of mitochondria. In the presence of succinate the oxidation of reduced nicotinamide adenine dinucleotide is decreased and the high energy phosphate associated with reduced NAD: (ubiquinone) oxidoreductase (EC 1.6.99.3) is reconverted to reduced NAD. This process has been termed reversed electron flow. With glucose in the absence of insulin, the heart preferentially utilizes its own energy stores in preference to the available glucose and this behavior persists even with insulin. These results tend to enforce the idea that the body treats glucose as if its only function were to provide the oxalacetate needed to catalyze the respiration of other substrates.

Flatt and Ball (322) have found that the rate limiting factor in the operation of the pentose cycle is the rate of regeneration of NADP+ from NADPH. According to this view it is the rate of fatty acid synthesis which regulates the operation of the pentose cycle and not vice versa as is commonly thought to be the case. Acetate metabolism provides the minimum amount of reducing equivalents not derived from the tricarboxylic cycle. Therefore, when acetate is provided as fuel, more of the cell's requirements for reducing equivalents is met by transhydrogenation from NADPH to NADH. This frees more NADP+ for the pentose cycle and as a result, almost all of the
glucose metabolized is metabolized via the pentose cycle and almost all of the glyceraldehyde-3-phosphate formed via this cycle is converted to fatty acids. Since glycolysis is more exergonic due to the phosphofructokinase reaction, some mechanism must operate to inhibit glycolysis. Citrate has been shown to be able to serve this function. When mitochondria are getting all their energy from acetate, the increase in the pentose cycle provides 95 percent of the reducing equivalents required for the observed acetate conversion to fatty acids. Flatt and Ball (322) found that acetate increases the pentose cycle 83 percent, the conversion of glucose to fatty acids by 16 percent, and total fatty acid synthesis by 60 percent. The increase in the pentose phosphate pathway that occurs on the addition of acetate illustrates some of the mechanisms which normally limit the operation of the cycle. Acetate enters the mitochondrion and is oxidized in preference to pyruvate because of the exergonic nature of the acetate CoA ligase (AMP)(EC 6.2.1.1) reaction:

\[
\begin{align*}
0 & \quad \text{O} \\
\text{CH}_3\text{C-O}^- + \text{CoA} + \text{ATP}^{-4} & \quad \text{CH}_3\text{C-S-CoA} + \text{AMP}^{-2} + \text{PP}^{-3}
\end{align*}
\]

(1)

Because acetate is oxidized in preference to pyruvate, the pyruvate is available for reduction in the cytoplasm by malic enzyme, L-malate: NADP oxidoreductase (decarboxylating)
(EC 1.1.1.40):

\[
\begin{align*}
\text{CH}_3\text{CO}^- + \text{NADPH} + \text{H}^+ + \text{HCO}_3^- & \rightarrow \text{CO}^- \\
\text{HO-C-H} + \text{NADP}^+ + \text{H}_2\text{O} & \\
\text{H-C-H} & \\
\text{CO}^- & \\
\text{O} & 
\end{align*}
\]

Although the equilibrium constant for the above reaction lies far to the left, the ratio of NADPH to NADP\(^+\) and the elevated pyruvate drives the reaction. The malate then diffuses into mitochondria where it undergoes conversion to oxalacetate by L-malate: NAD oxidoreductase (EC 1.1.1.37):

\[
\begin{align*}
\text{NAD}^+ + \text{HCH} \rightarrow \text{NADH} + \text{O} = \text{C} + \text{H}^+ \\
\text{HCH} & \\
\text{CO}^- & \\
\text{O} & 
\end{align*}
\]

The oxalacetate formed is condensed with acetyl CoA to citrate by citrate oxalacetate lyase (CoA-acetylating) (EC 4.1.3.7):

\[
\text{oxalacetate}^-^2 + \text{acetyl-S-CoA} \rightarrow \text{citrate}^-^3 + \text{CoA-SH} + \text{H}^+
\]

The citrate so produced diffuses out of the mitochondria and
is cleaved by ATP: citrate oxalacetate-lyase (CoA-acetylating and ATP-dephosphorylating) (EC 4.1.3.8):

\[
citrate^{-3} + ATP^{-4} + CoA =
\]

\[
oxalacetate^{-2} + acetyl-S-CoA + ADP^{-3} + P_i^{-2}
\]

(5)

The acetyl CoA produced is used for fatty acid synthesis in the cytoplasm and the oxalacetate is converted to phosphoenolpyruvate:

\[
H_2O + oxalacetate^{-2} + ITP^{-4} =
\]

\[
phosphoenolpyruvate^{-3} + HCO_3^{-1} + IDP^{-3} + H^+
\]

(6)

The phosphoenolpyruvate so produced is then available for the regeneration of inosine-5'-triphosphate and pyruvate by the reaction catalyzed by ATP: pyruvate phosphotransferase (EC 2.7.1.40):

\[
H^+ + phosphoenolpyruvate^{-3} + IDP^{-3} = pyruvate^{-1} + ITP^{-4}
\]

(7)

For every two cycles of reactions 1-4, one citrate molecule diffuses out of the mitochondria leaving behind a reduced NADH equivalent. This NADH equivalent was formed at the expense of NADPH in the cytoplasm and represents a transhydrogenation and transfer of reducing equivalents into the mitochondria.

The transfer of reduced NADH equivalents into mitochondria
normally occurs by reversal of reaction 3 in the cytoplasm, the oxalacetate required being provided by the L-aspartate: 2-oxoglutarate aminotransferase (EC 2.6.1.1) reaction:

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{CO}^- & \quad \text{CO}^- \\
\text{H}_3\text{N-C-H} & \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{C-O}^- & \quad \text{C-O}^- \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{O} & \quad \text{O} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{C-O}^- & \quad \text{C-O}^- \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{O} & \quad \text{O} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{C-O}^- & \quad \text{C-O}^- \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{O} & \quad \text{O} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{C-O}^- & \quad \text{C-O}^- \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{H-C-H} & \quad \text{H-C-H} \\
\text{O} & \quad \text{O} \\
\end{align*}
\]

(8)

After the malate and glutamate diffuse into the mitochondria, reaction 3 occurs as well as the reverse of reaction 8. The α-ketoglutarate and aspartate then diffuse out of the mitochondria into the cytoplasm to complete the cycle. Harding et al (427) found that adrenal steroids, testosterone propionate, and growth hormone were without effect on the above enzyme. They found, however, that glucocorticoids increased the activity of L-alanine: 2-oxoglutarate aminotransferase and that deoxycorticosterone depressed this enzyme. The other hormones were without effect.

**Role of Glutamic Dehydrogenase in Gluconeogenesis**

The mitochondrial enzyme L-glutamic: NAD(P) oxido-reductase (deaminating) (EC 1.4.1.3) must be considered as a regulatory enzyme essential for gluconeogenesis. This enzyme catalyzes the reaction:
\[ \text{H}_2\text{O} + \text{glutamate}^{-1} + \text{NAD(P)}^+ = \]

\[ \text{NH}_4^+ + 2\text{-ketoglutarate}^{-2} + \text{NAD(P)}\text{H} + \text{H}^+ \]  

During gluconeogenesis from amino acids there is a net production of glutamate by transaminase enzymes. This glutamate must be oxidized if gluconeogenesis is to proceed. Frieden (335) has reviewed some of the regulatory aspects of glutamic dehydrogenase. The active enzyme is composed of inactive subunits which associate in the presence of the amino acids L-methionine, L-leucine and L-isoleucine as well as NAD$^+$. At moderate concentrations of GTP, the enzyme is very sensitive to activation by ADP even though neither nucleotide is a reactant. The enzyme is also sensitive to product inhibition. Equimolar 2-ketoglutarate inhibits the enzyme 43 percent.

Although Yielding and Tomkins (1188) have described the non-specific inhibition of this enzyme by a number of steroids, no steroid was as inhibitory as thyroxine which was found by Winslow et al (1167) to inhibit the reaction 51 percent at $1.3 \times 10^{-5}$ Molar. However, the inhibitory action of thyroxine on the enzyme appears superficially to be contrary to the increased contribution of amino acids to basal metabolism in hyperthyroidism.

Williamson et al (1157) have shown that glutamate
exerts a catalytic influence on the transport of extramitochondrial NADH reducing equivalents into mitochondria. Therefore, when mitochondrial ADP rises glutamic dehydrogenase is activated leading to glutamate oxidation. On the other hand, if GTP is depressed during protein biosynthesis the effect of ADP on glutamate oxidation is attenuated. An increase in glutamate oxidation reduces the transport of extramitochondrial reducing equivalents into the mitochondrion. As a result glycolysis is reduced. Thus, glutamic dehydrogenase increases cytoplasmic NADH when it converts the amino acid glutamate to the oxalacetate precursor 2-ketoglutarate.

Villee (1105) reported that $10^{-6}$ Molar corticosterone increased NAD$^+$ reduction 75 percent indicates that the effect is not even relatively specific for glucocorticoids.

Krebs (617) has shown that the ratio of NAD$^+$ to NADH in diabetic liver is decreased extramitochondrially but increased intramitochondrially. The ratio fell from 7.25 to 2.08 in the cytoplasm and increased 7.3 to 10.8 in the mitochondrial matrix. The cytoplasm is thus in a more reduced state while the mitochondrial matrix is in a more oxidized state. This situation basically reflects a lack of transfer of reducing equivalent from the cytoplasm into the mitochondria.

The pyruvate that is not converted to acetyl CoA
TABLE VII

INFLUENCE OF HORMONES ON THE NICOTINAMIDE NUCLEOTIDE COENZYMES OF RAT EPIDIDYMAL FAT PAD

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NAD⁺</th>
<th>NADH</th>
<th>NADP⁺</th>
<th>NADPH</th>
<th>NAD⁺/NADH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.30</td>
<td>0.40</td>
<td>0.14</td>
<td>0.46</td>
<td>3.25</td>
</tr>
<tr>
<td>Hypophysectomized</td>
<td>1.14</td>
<td>0.29</td>
<td>0.17</td>
<td>0.25</td>
<td>3.93</td>
</tr>
<tr>
<td>Hypophysectomized + Growth Hormone</td>
<td>0.90</td>
<td>0.27</td>
<td>0.09</td>
<td>0.21</td>
<td>3.33</td>
</tr>
<tr>
<td>Control</td>
<td>0.75</td>
<td>0.21</td>
<td>-----</td>
<td>0.39</td>
<td>3.57</td>
</tr>
<tr>
<td>Alloxan-Diabetic</td>
<td>0.88</td>
<td>0.22</td>
<td>-----</td>
<td>0.13</td>
<td>4.00</td>
</tr>
</tbody>
</table>

McLean et al (693)
because of the inhibition of pyruvate dehydrogenase by the elevated ratio of acetyl CoA/CoA is converted to oxalacetate by pyruvate: carbon dioxide ligase (ADP)(EC 6.4.1.1):

\[
pyruvate^{-1} + \text{HCO}_3^{-1} + \text{ATP}^{-4} = \text{oxalacetate}^{-2} + \text{ADP}^{-3} + P_i^{-2} + \text{H}_3\text{O}^+ \tag{11}
\]

A portion of the oxalacetate formed in this reaction condenses with acetyl CoA to form citrate which diffuses out into the cytoplasm to inhibit phosphofructokinase. However, some of the oxalacetate is reduced to malate. The increase in mitochondrial malate effectively prevents the transfer of cytoplasmic reducing equivalents into the mitochondrion by abolishing the malate concentration gradient. As a result the hepatic ratio of NADH/NAD\(^+\) rises leading to gluconeogenesis. The malate concentration is increased so much that oxalacetate formation occurs in spite of the lower NADH/NAD\(^+\) ratio present in mitochondria. This is possible only because oxalacetate is also elevated. A possible reason for the failure of lipogenesis during gluconeogenesis is that higher concentrations of fructose-1, 6-diphosphate are required for lipogenesis than for gluconeogenesis. Plate et al (439) found that fructose-1, 6-diphosphate decreases the Michaelis constant of fatty acid synthetase for NADPH. Furthermore, low citrate
levels slightly increase the level of fructose-1,6-diphosphate and increase the activity of fructose diphosphatase. Higher levels of citrate further increase the concentration of the diphosphate which inhibits the diphosphatase and further channels glyceraldehyde-3-phosphate to glycerol glyceride formation.
CHAPTER VIII
STRUCTURAL ASPECTS OF LIPOPROTEINS

Comparison of Human and Rat Beta Lipoproteins

Both human and rat β lipoproteins have approximately the same molecular weight of about 2 million and both are composed of approximately 10 subunits. However, while these subunits are identical in the rat molecule, Shore and Shore (980) found three types of subunits in human β lipoprotein. The fact that delipidated rat β lipoproteins form insoluble aggregates less rapidly than human β lipoproteins means that protein-protein interactions which must be overcome during lipid binding are weaker in rat molecules. The function of the multiple peptide chains in the human molecule may be to provide cooperative interactions so that protein-protein interactions become weaker, and protein-lipid interactions stronger as more lipid is bound.

Cooperative Interactions in Beta Lipoproteins

In contrast to the findings of Gotto et al (402)
that no cooperative interactions between lipid and protein existed in \( \beta \) lipoprotein, Camejo et al. (157) found that the binding of phospholipids to apoalphalipoprotein exposed peptide bonds to the solvent with exchange of amide hydrogens. Phospholipids also appear to be important in insulin inhibition by \( \beta \) lipoprotein. The fact that phospholipases attack \( \beta \) lipoprotein indicate that the phospholipids are located on the surface of the molecule.

Granada and Scanu (405) have investigated the effects of lipid extraction on lipoprotein structure. Following the extraction procedure, two components of \( \beta \) lipoprotein with sedimentation constants of 10.9 S and 15.5 S were produced. Lipid extraction following succinylation of the available amino groups in the molecule resulted in a single 7.2 S subunit. This disassociation phenomena is similar to that reported by Shore and Shore (981) who found a molecular weight of \( 2.8-3.0 \times 10^6 \) for the intact \( \beta \) lipoprotein molecule and \( 6.4 \times 10^4 \) for each of the ten postulated subunits.

Gotto et al. (402) have described a technique for the preparation of delipidated \( \beta \) lipoprotein subunits free of detergent molecules. This procedure takes advantage of subtle differences in physical chemistry between dodecyl sulfate and decyl sulfate. Decyl sulfate can be reversibly
bound to the subunit while dodecyl sulfate cannot be completely removed by dialysis after lipid extraction.

Gotto et al. (403) further investigated the structure of the delipidated apolipoprotein by infrared analysis. They found the molecule to contain \( \beta \)-pleated sheet structure which did not change appreciably on delipidation. These findings are in basic agreement with those of Scanu (942) from circular dichroism measurements and indicate that there is little or no cooperative interaction between the apolipoprotein and its lipids. Camejo et al. (157) have, however, found that the delipidated high density lipoprotein molecules do have two unique characteristics. First, it was able to penetrate lipid films at pressures above the collapse pressure of the protein, and secondly, the protein itself could form a film even at low salt concentrations. This latter characteristic was ascribed to the absence of disulfide bridges in the molecule. These findings indicate that modification of the apolipoproteins by introduction of cross-links may provide some additional information about the function of the molecule (431, 462).

Chapman (172) and Colacicco (189) have described methods applicable to the interaction of apolipoproteins with lipid monolayers. These techniques generally involve
the introduction of the apolipoprotein under a lipid monolayer followed by observations of the changes produced in surface tension. This technique has provided more information than circular dichroism studies. A similar technique might be used to study the action of lipoprotein lipase. For example, the enzyme along with α apolipoprotein and acceptor albumin might be introduced into the bulk phase under the lipid substrate monolayer.

The possibility that polyunsaturated cholesterol esters may be involved in intermolecular bonding of lipoprotein subunits is indicated by Lindgren et al. (667) who found that dialysis of $S_f$ 0-20 β lipoproteins in the absence of chelating agents to prevent metal catalysis of air oxidation results in $S_f$ 0-2 fragments which resembled HDL₁. Authentic HDL₁ (immunochemical identification) has density properties similar to the β lipoproteins in rats and man but has not been further studied.

**Abnormal Beta Lipoproteins**

Alterations in β lipoprotein structure are not unique to the β lipoprotein insulin antagonist but are also found in other disorders of lipid metabolism. Switzer (1044) noted that an immunochemically distinct β lipoprotein, lipoprotein X, was formed in, and hence could not be used to test for the presence of, biliary obstruction.
Lipoprotein X contains lecithin as its main phospholipid component, only traces of triglycerides and cholesterol esters, but large amounts of free cholesterol. In contrast, Gardner et al (357) observed that serum cholesterol esters were reduced in viral hepatitis. Eder et al (268) were among the first to use percent cholesterol esterification as a chemical means of differentiating between viral hepatitis and obstructive jaundice.

In contrast Hezzard et al (442) noted that the abnormal β lipoprotein which migrates electrophoretically as a broad bend in Type III hyper-β-lipoproteinemia has an increased content of esterified cholesterol. Most of the individuals with this disorder develop Xanthoma tuberosum, characteristic cholesterol deposits in the skin, indicative of the rapid lethal progression of atherosclerosis. Type II hypercholesterolemia can be distinguished from the Type III disease by lipoprotein electrophoresis. However, individuals with Type II hypercholesterolemia may also be distinguished from Type III individuals by the presence of Xanthoma tendinosum instead of Xanthoma tuberosum. Unfortunately, the appearance of these subcutaneous cholesterol deposits is also indicative of comparable cholesterol deposition in arterial plaques.
**Etiology of Atherosclerosis**

Both dietary cholesterol and saturated fatty acids can contribute to the development of atherosclerosis in predisposed individuals. The main predisposition is an elevation in blood catecholamines due to environmental stress which increases not only fasting serum triglycerides but also post-prandial hyperlipemia. Afflicted individuals can be detected by their elevated catecholamine excretion when exposed to occupational stress (338). However, excretion patterns may be normal after the subject has been hospitalized.

The rise in norepinephrine secretion in such individuals frequently leads to an elevation in blood pressure so that the latter is associated with a three-fold increase in the chance of myocardial infarction (558). In another study Yater et al (1183) observed a 4-fold increased risk. It is not clear how hypertension
increases the risk but an effect of pressure on the disposition of lipids from \( \beta \) lipoproteins has not been excluded. Increased mechanical trauma to the vessel is also a possible explanation of the blood pressure effect. Elevations in epinephrine, on the other hand, may lead to enhanced platelet aggregation as well as to the previously described lipolytic effects. Past-prandial hyperlipemia may be responsible for the 20 percent reduction in coronary blood flow and the failure of collateral circulation in some cardiovascular patients. Friedman et al (338) have also observed the sludging of erythrocytes 3-9 hours following food ingestion in patients prone to atherosclerosis.

Rane et al (864) have shown that dietary factors alone could not account for the difference in atherosclerosis in two similar groups of East and West Finnish farmers indicating that diet alone is not the primary determinant of the disease. However, that high blood cholesterol levels accelerate the disease process is well known (558). In addition, \( \beta \) lipoprotein levels do not correlate better with the progression of the disease than do total serum cholesterol levels (380). It appears, however, that total cholesterol levels are related to the tendency for cholesterol to deposit in arterial fissures during the healing process which is constantly occurring.
platelet aggregates play an important role in this process because these lipid-rich cells are phagocytized by macrophages which are then converted to foam cells which are responsible for the release of cholesterol in the vicinity of the arterial lesion.

Although men have a higher total cholesterol and higher ratio of $\beta$ to $\alpha$ lipoprotein compared to women, recent studies have indicated that these differences cannot account completely for the sex differences in atherosclerosis. Rosenman and Friedman (910) have indicated that differences in behavior and occupational stress may be at least partly responsible. Women in the professions, for example, have a 5-fold increased incidence of atherosclerosis compared to women with less stressful occupations. However, these results do not rule out the importance of hormonal factors in the control of serum lipids. For example, atherosclerosis is more frequent among men with a mesomorphic or muscular physique. Such findings may indicate that both physical stature and aggressiveness may reflect patterns of testosterone production and target tissue responses and may provide a basis for the belief that atherosclerosis begins to develop in man at about the age of puberty.

Saturated fatty acids in the diet increase the
incidence of atherosclerosis by increasing the level of cholesterol. Apparently, there is a limit on the ratio of saturated to unsaturated fatty acids that may be present in β lipoprotein. Ways et al (1120) have suggested that excessive oxidation of linoleic acid may be an etiologic factor in acanthycytosis and pointed to their observation that erythrocyte linoleic acid was only 25 percent of normal in this disorder. Philips and Dodge (832) also confirmed the low levels of essential fatty acids in α-β-lipoproteinemia.

These results are puzzling because α lipoproteins normally transport essential fatty acids as cholesterol esters. In the genetic absence of α lipoproteins which occurs in Tangiers disease cholesterol esters accumulate in the reticuloendothelial system, but unesterified cholesterol is normal. Furthermore, low levels of carotene leading to pigmentary retinitis are observed even though the bulk of carotene is transported by β lipoprotein. Jepsco et al (529) also observed that a deficiency of cholesterol esters occurred in Xanthoma tuberosum characteristic of Type III hyper-β-lipoproteinemia in which an abnormally less dense lipoprotein is produced. The heterogeneous nature of this molecule is further indicated by the broad β band which it forms on electrophoresis. In contrast, high unesterified cholesterol levels characterize
Xanthoma tendinosum seen in Type II hyper-β-lipoproteinemia. These results indicate the importance of polyunsaturated fatty acids.

**Function of Cholesterol**

Saturated fatty acids may elevate serum cholesterol by forming lecithin-cholesterol complexes which increase the total number of cholesterol binding sites in the β lipoprotein molecule. An increase in total cholesterol and saturated lecithins, for example, is seen during high-dosage estrogen therapy. Recently, Ladbrooke et al (625) have shown that cholesterol forms a 1:1 molecular complex with dipalmitoyl phosphatidyl choline that abolishes the mesomorphic phase transition of the phospholipid. Apparently, cholesterol functions to reduce the random motion of the hydrocarbon tail of the saturated fatty acid. Polyunsaturated fatty acids, on the other hand, have greater rigidity than their saturated counterparts because of their double bonds. The presence of this increased rigidity is not disproven by the generally lower melting points of the polyunsaturated fatty acids because the crystal lattice energy, as evidenced by the heat of fusion, is lower for the polyunsaturated fatty acids.

The important role of polyunsaturated fatty acids in preventing the elevation of β lipoprotein levels is
seen in the results of a clinical study by Vergrassen et al (1094). They showed that when dietary fat was 50 percent of total calories, the isocaloric substitution of safflower oil for olive oil in the diet reduced cholesterol levels by 17 percent. Such an effect is equivalent to that obtained with hypocholesterolemic drugs such as 2-(p-chlorophenoxy)-2-methylpropionic acid and lipoidiastic steroids. Furthermore, Peifer and Holman (826) have shown that total unsaturation rather than essential unsaturation at carbons 12 and 15 determines the cholesterol-lowering effect of polyunsaturated fatty acids, indicating that physical properties are the main consideration. However, for other reasons related to platelet function, diets rich in the essential fatty acid linolenate are preferable to catalytically dehydrogenated polyunsaturated fatty acids for the prevention of myocardial infarction. Besides the mechanism outlined above, Lewis (660) has shown that polyunsaturated fatty acids also increase the conversion of cholesterol to bile acids and lower cholesterol levels by this means. On the other hand, patients with Type II hypercholesterolemia have been shown to be resistant to this latter effect.

Another puzzling aspect of lipid metabolism was reported by Setsuda and O'Kabe in reference to the low levels
of linoleic acid in diabetes. They reported that insulin, but not sulfonylurea drugs could restore normal levels of linoleate. Since the sulfonylureas stimulate insulin release, it appears that they also antagonize this effect of insulin.

The effects of glucocorticoids on serum lipids were discussed previously. Generally, these effects include an increase in phospholipids with lesser increases in cholesterol and triglycerides. These effects are generally favorable provided that the pancreas can produce sufficient insulin to overcome the lipolytic effects produced by the glucocorticoid. As discussed elsewhere in this dissertation, at least part of this glucocorticoid effect appears to be due to an action on the liver that is not dependent on protein biosynthesis.

Sex Differences in the Subunit Structure of HDL

In men $S_f^{O}$ 20-100 and 100-400 pre-β-lipoproteins show an upward trend in the third decade and even steeper upward trends in the fourth decade up to the fifth decade and then a decline occurs. In women the $S_f^{O}$ 100-400 pre-β-lipoproteins are approximately one-third the level in men in the third decade. The $S_f^{O}$ 20-100 pre-β-lipoproteins increase with age in women starting with the fifth decade. Between the third and fifth decades, mean HDL$_2$ α lipoproteins are
twice as high in women as in men. The other α lipoproteins, HDL₃ and HDL₁, do not change. These findings suggest that the estriene effect is not on the synthesis of the apo-α-lipoprotein subunit, but rather that it promotes the conversion of HDL₃ lipoprotein (M. W. 175,000) to HDL₂ lipoprotein (M. W. 360,000) by promoting a subunit rearrangement in which another high density apolipoprotein subunit of M. W. 40,000 and a phospholipid-cholesterol complex of 145,000 are added. Shore and Shore (981) have recently reviewed the structural aspects of the serum lipoproteins. They have noted that the peptide composition of HDL₂ is more heterogeneous than that of HDL₃. Whereas both lipoprotein species contain the two major carboxyl terminal glutamine and threonine peptides, HDL₂ also contains another peptide which is rich in serine, glutamic acid and glycine and which is believed to end in carboxy terminal alanine. All of these peptides contain methionine but the alanine containing peptide unique to HDL₂ is devoid of tryptophane. It appears that HDL₃ contains six polypeptide subunits of 15,000 molecular weight while HDL₂ contains eight. Although the exact role of the two major peptides is unknown, there presence in all high density lipoprotein species suggests that they form a dimer which then binds lipid as in the 40,000 M. W. fragment involved
Peefers and Blaton (825) have also identified four peptides in human HDL₂ by isoelectric focusing which have pI's of 9.16, 8.97, 6.36 and 5.38. The peptides which pI's of 8.97 and 6.36 correspond to the carboxy terminal threonine and glutamine peptides. The study of these peptides is complicated by the presence of polymorphic forms which separate on DEAE cellulose but electrophorese identically in polyacrylamide with 8 M urea.

**Reciprocal Effect of Estrogens**

Estriene effects on atherosclerosis offer the greatest hope for preventing the disorder by natural regulatory mechanisms. The terms androstane and estriene emphasize that these effects are distinct from those of endogenous hormones. Changes in β lipoprotein occur in a reciprocal fashion in response to steroid-induced α lipoprotein changes in 50 percent of human subjects. However, the net change in cholesterol levels will depend on the relative changes in lipoprotein species and their cholesterol contents. In man elevation in α lipoprotein is generally followed by a larger reduction in β lipoprotein so that total cholesterol levels are reduced. In the dog and especially in the rat, α lipoproteins predominate over β lipoprotein so that an elevation in α lipoprotein increases total cholesterol. The basic
effects of steroids on the lipoprotein distributions in both animals, however, is similar.

Studies by Bowers and Schally (105), Fillias (316), O'Key et al (794), Lyman et al (684) and Kritchersky et al (619) have shown that low-dosage estrogen therapy raises the level of cholesterol arachidonate in the rat. The incorporation of arachidonate and stearate into hepatic phospholipids is also increased. On the other hand, Aftergood et al (6) reported that the cholesterol esters of arachidonate and linoleate were markedly reduced and replaced by those of palmitoleate, oleate, and stearate when the human estrogen dose was administered. These effects appear to be secondary to effects on hepatic phospholipid synthesis. Ramey and Baron (858) observed that the estriene 3,17β-dihydroxy-1,3,5(10)-estriene-16-one increases the incorporation of acetate and inorganic phosphate into hepatic phospholipids.

The effect of glucocorticoids on serum lipids parallels its effects on lipid metabolism. Kobernich and More (602) showed that the administration of 20 mg of cortisone per day to rabbits elevated serum cholesterol and unesterified fatty acids at the time that steroid diabetes ensued. Brady et al (112) showed that the elevation in serum fatty acids was accompanied by reduced fatty acid synthesis from pyruvate
2-6 hours after glucocorticoid treatment when gluconeogenesis from pyruvate was increasing.

Adrenalectomized dogs maintained on deoxycorticosterone acetate show a 75 percent drop in total serum lipids over a three week period (238). However, this drop is not accompanied by changes in phospholipid concentration in the liver, kidney, small intestine, muscle or lung; and the half-life of plasma phospholipids is not altered. In contrast to these results in the dog Migeon (740) found that liver lipids increased in the adrenalectomized rat maintained on deoxycorticosterone acetate. Treatment with cortisone restored the liver lipids to normal but did not elevate serum cholesterol. The increased mobilization of lipid stores in the adrenalectomized rat was also reported by Tracht et al (1074). It is probably the result of the lipolytic activity of ACTH which increases after adrenalectomy. The suppression of ACTH release described by Levy and Ramey (657) will be discussed later in conjunction with the other effects of ergotamine.

Comparison of Cortisone and Prednisone

Furman et al (349) showed that the administration of 500 carbohydrate calories could prevent the elevation of cholesterol and phospholipids associated with the administration of 30 mg of prednisolone to men. It is not clear,
TABLE VIII
EFFECTS OF 20 DAYS OF GLUCOCORTICOID TREATMENT
ON THE PLASMA LIPIDS OF THE RABBIT

Percent Difference From Control Period

<table>
<thead>
<tr>
<th>Glucocorticoid Administered</th>
<th>Neutral Lipids</th>
<th>Total Phospholipids</th>
<th>Unesterified Cholesterol</th>
<th>Esterified Cholesterol</th>
<th>Total Lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>+53.5</td>
<td>+46.6</td>
<td>+100</td>
<td>-28.6</td>
<td>+47.5</td>
</tr>
<tr>
<td>Cortisone</td>
<td>+482</td>
<td>+89.3</td>
<td>+186</td>
<td>-29.3</td>
<td>+256</td>
</tr>
<tr>
<td>17α,21-dihydroxy-1,4-pregnadiene-3, 11,20-trione</td>
<td>+380</td>
<td>+126</td>
<td>+207</td>
<td>+14.3</td>
<td>+259</td>
</tr>
</tbody>
</table>

Adlersberg (4)
however, whether the suppression of free fatty acid mobilization was due to the glucose or to the insulin which it released. Lever et al (654) found that glucocorticoids increased a lipoprotein cholesterol and phospholipids to abnormally high values in man. Similar results were obtained by Adlersberg et al (4) in the rabbit and are shown in Table VIII. Especially prominent is the highly significant decrease in esterified cholesterol. Esterified cholesterol was increased by prednisone but decreased by cortisone. This difference may be related to the metabolism of the two steroids. The decreased esterification seen with endogenous glucocorticoids is similar to the effect produced by estrogens.

The metabolic effect of prednisone is almost the same as that of cortisone even though the total 17-ketosteroid excretion is depressed via the effect of the synthetic glucocorticoid on adrenocorticotropic hormone production. In addition, Howard et al (483) found that part of the effect of prednisolone in raising a lipoprotein concentration could be attributed to a decrease in adrenal androgen production.

Glucocorticoids seem to exert their effects on phospholipids. Allersberg et al (5) found that cortisol increased serum phospholipids 24 percent, cholesterol esters 23 percent, and cholesterol 19 percent. Triglycerides
were decreased by this hormone. Oliver and Boyd (797) and Lever et al (654) confirmed these findings. The latter group found increases in a lipoprotein phospholipids and cholesterol in 7 out of 8 patients. Unexpectedly, deoxycorticosterone acetate, when given at 2.5 percent of the dose of cortisone acetate, produced a 48 percent rise in a lipoprotein cholesterol and 10 percent decrease in β lipoprotein cholesterol in one-half the time. Despite the greater magnitude of the mineralcorticoid effect, the importance of the glucocorticoid effect is not minimized because the factors which regulate mineralcorticoid release are largely independent of those which regulate glucocorticoid secretion and vice versa. However, at least part of the effect of the adrenal gland on serum cholesterol is mediated by mineralcorticoids.

Clinical Effects of Free and Sulfate Conjugated Estrienes

Estrogens increase the output of the lipolytic hormone ACTH by the pituitary. Also, ACTH is sufficient to produce an increase in cortisol output by the adrenal cortex. However, the increased cortisol is not all in the free form and in fact is partially inactivated by being bound to transcortin which is also increased following estrogen treatment.

The decreased circulating linoleate seen during estrogen
therapy is partly attributable to enhanced mitochondrial chain elongation of this fatty acid to arachidonate (4w6). Furthermore, increased amounts of eicosatrienoic acid (4w9) are also formed from oleic acid, indicating that chain elongation may be increased generally (403). This process per se does not seem to be the cause of the insulin resistance seen during estrogen therapy, unless the substrate specificity of the enzymes which incorporate long-chain acyl coenzyme A derivatives into triglycerides is such that chain elongation results in a decreased rate of this reaction.

A basic difference exists between the effects of estrogens on serum cholesterol and the effects of glucocorticoids on hepatic enzyme induction. Steinberg (1025) has shown that growth hormone is required for the estrogen effect. In contrast, both Csanyi and Greengard (208) and Liberti et al (661) have shown that growth hormone suppresses glucocorticoid induction of tyrosine aminotransferase and tryptophane oxygenase. The suppression of cortisol-dependent enzyme induction by growth hormone is opposite to the synergistic anti-insulin action of these hormones.

Several attempts have been made to synthesize estriene steroids which would have an estrogen effect on serum lipids
but not the other effects associated with estrogen therapy. Such steroids have been termed "lipodiactic" steroids, and many compounds of this type are designed so that the ratio of lipid lowering effect to estrogenic effect is maximal. For example, Roussel (patent number 3,032,563) has introduced the steroid 16α-ethinyl-11β,16β-dihydroxy-1,4-androstadien-3-one. This compound possesses a number of the structural features usually associated with estrogens, anabolic steroids, and glucocorticoids. Particularly noteworthy is the displacement of the hydroxyl and ethinyl groups in ring D. Also, while the function of the 11-hydroxyl group in this compound has not been determined, its primary effect may be to decrease the conversion of the compound to the corresponding phenolic derivative (417).

Perhaps the most interesting lipodiactic steroid so far developed is 16,16-difluoro-17α-ethinyl-3-methoxy-1,3,5(10)-estrien-17β-ol. Except for the fluorine substitutions, this compound is identical to the estrogen which causes the most pronounced elevations in serum triglycerides. This close similarity in structure suggests that D ring binding is necessary for the triglyceride effect, while A ring binding facilitates the binding of D ring. Humber et al (496) have reported that this compound inhibits cholesterol synthesis 36 percent at 10⁻⁴ Molar.
Arnold et al (20) reported that the lipodiactic/estrogenic ratio for the steroid 17-\((3\text{-}\text{hydroxy-1-propynyl})\)-3-methoxy-1,3,5(10)-estriien-17β-ol was 1300 times that of equine estrogen sulfates and 3300 times that of estrone. The enhancement produced by the 17α side chain is especially noteworthy. A similar enhancement of anesthetic steroid effects was observed by Selye and is also found in progestational steroids.

Several clinical trials have outlined the effects of estrogen therapy on serum lipids. In 1957 Furman and Howard (349) studied the effects of estrone sulfate on α and β lipoproteins. In addition to the gynecomastia associated with the therapy, they noted that predictable changes in β lipoproteins could be produced in only about 56 percent of all their subjects. In 1962 these scientists reported similar findings in hyperlipemic subjects (350).

In 1962 Marmasten et al (717) reported the results of lipodiactic therapy following myocardial infarction. They found no correlation between serum lipids and survival. Furthermore, while equine estrogen sulfates significantly improved survival rates, no such improvement could be demonstrated for the non-conjugated steroids 17α-ethinyl-1,3,5(10)-estriene-3,17β-diol and 3-methoxy-16α-methyl-1,3,5(10)-estriene-16β,17β-diol (895). The conclusion of such studies
appears to be that only conjugated lipodiactic steroids are able to improve post-infarction survival by a mechanism unrelated to serum lipid levels. Further studies are necessary to confirm the conjugate effect.

Two additional lipodiactic steroids merit consideration from a structure-function standpoint. The first is the patented adrenal androgen analogue 17α-ethinyl-3-ethoxy-3,5-androstadiene-11β,17β-diol which is hydrolyzed in vivo to 17α-ethinyl-11β,17β-dihydroxy-4-androsten-3-one. This particular compound is similar to the endogenous cortisol metabolite 11β,17β-dihydroxy-4-androsten-3-one. In addition, this steroid is quite similar to the endogenous hypotensive steroids reported by Sturtevant (1037).

The second lipodiactic steroid that should be considered is that described by Gordon et al (400) as having good separation of lipid and estrogenic effects. The compound 2-methoxy-1,3,5(10)-estriene-3,16α,17β-triol is an endogenous steroid in man and its production is increased by thyroxine. Gallagher et al (355) found that the formation of this particular steroid was reduced in breast tumor patients. The relation between its lipodiactic effect and possible anti-tumor effect should be further investigated.

**Estrogen Conjugates**

The superiority of estrogen sulfates in lipodiactic
therapy may be explained by the finding of Fishman et al. (319) that these conjugates are intermediates in the formation of 2-methoxy estrogens. Therefore, although previous studies have been disappointing, these considerations suggest that 16,16-difluoro-17α-ethinyl-2-methoxy-1,3,5(10)-estrone-3,11β,17β-triol may merit clinical trial as a lipodiatetic drug.

Conjugated equine estrogens (Premarin) have generally been preferred to the use of non-conjugated estrogens due to their lower estrogenic side effects. Approximately 50 percent of myocardial infarction patients experience a maximal decrease in serum cholesterol of 10 percent during the administration of 4.0 mg/day of conjugated equine estrogens over a three-month period (1021). Those patients who do not respond to this dose may respond to the maximum dose of 10.0 mg/day. However, not all patients respond to the larger dose and there seems to be an increase in death rate from 2 to 8 percent associated with the maximum dose.

Oliver and Boyd (796) have demonstrated that 17α-ethinyl-1,3,5(10)-estrone-3,17β-diol at a dose of only 0.10 mg/day produces cholesterol lowering effects equivalent to 4.0 mg/day of conjugated estrogens. Moreover, 0.20 mg/day can lower cholesterol levels 25 percent in normal subjects and 35 percent in subjects with Type II hypercholesterolemia.
over a three-month period. These studies stress the difference in individual responses to drugs. Ideally, each patient should be titrated with the drug until side effects occur (895).

In 1963 Stamler et al (1021) further raised doubts about the efficacy of estrien therapy following myocardial infarction by showing that the commencement of therapy within three months following the infarction actually increased patient mortality. This occurred despite the fact that five-year mortality was reduced 50 percent. The early increase in deaths was one of the first indications of the risks associated with estrien therapy. While this finding has not been explained, it could have been due to enhanced platelet aggregation secondary to the estriene at a time when factors related to tissue damage were also actively promoting coagulation.

In order to compare the results of estrogen therapy with other treatments the data of Huninghak et al (497) may be cited as typical of long-term Atromid-S therapy. They reported that at least 50 percent of their patients experienced cholesterol reductions of at least 15 percent. If it is assumed that death rate is approximately proportional to cholesterol and that all other factors were similar, estriene therapy seems to be at least as beneficial
as other hyperlipidemic drugs.

Besides their effects on lipid biosynthesis, estrienes may also differ with respect to their effects on lipoprotein lipase activity. Keeping estriene inhibition of the enzyme to a minimum should be a goal in any series of structural alterations. This effect has apparently not been considered in the previous studies and may have contributed to individual variability if the lipids were not measured in the fasting state.
CHAPTER X

LIPOPROTEIN LIPASE AND THE LIPOPROTEIN INSULIN ANTAGONIST

Lipoprotein Lipase Activity: Effect of Insulin, Ethanol, Catecholamines and Cyclic Adenylate

Lipoprotein lipase may be involved in the degradation of the lipoprotein insulin antagonist. Lipoprotein lipase activity is decreased in diabetes (578). The sulfated polysaccharide heparin is required for enzyme activity and insulin may increase enzyme activity by increasing heparin synthesis by mast cells in the arterial wall. Schiller and Dorfman (943) have demonstrated decreased incorporation of radioactive sulfate into heparin in diabetes. The key role of heparin in the action of the enzyme was shown by Korn (606) who inactivated it by treatment with heparinase. Heparin is synthesized in lung, liver, muscle and intestine but has not been recovered from normal plasma; its presence there is due to its escape from perfused tissues.

Insulin may also affect the synthesis of the enzyme
protein. Kessler (578) found that diabetes reduced the heparin-releasable lipoprotein lipase from adipose tissue but increased the release of the enzyme from myocardial tissue. Kessler also investigated the activities of the myocardial and diaphragm enzymes during fasting where the activity increased and decreased respectively following glucose administration. The decrease in activity following glucose is probably related to the suppression of growth hormone release by glucose.

Mallov and Cera (703) found that heart lipoprotein lipase was increased by ethanol intoxication, catecholamines, and ether anesthesia. The ethanol stimulation of the enzyme did not occur in adrenalectomized rats or in puromycin-treated rats indicating that glucocorticoid-dependent protein synthesis may be required.

Wing and Robinson (1164, 1165) observed that serum lipoprotein lipase from fed animals had a shorter half-life than the enzyme from fasted animals. They also noted that actinomycin blockade of DNA synthesis produced an increase in the stable form of the enzyme. This effect of actinomycin could be reversed by catecholamines or adrenocorticotrophin, agents which increase cyclic adenylate. Huttunen and Steinberg (501) have shown that cyclic adenylate activates adipose tissue lipase via a phosphorylation
of the enzyme mediated by a specific protein kinase. A similar enzyme phosphorylation may be involved in the conversion of lipoprotein lipase to its unstable form. Catecholamine stimulation of this reaction may be of particular importance in carbohydrate-induced lipemia which is characterized by a primary elevation in $S_f 20-400$ pre-β-lipoproteins (7, 76).

**Phospholipase A and Lipoprotein Structure**

Although Dorzaki and Zieve (253) have shown that lipoprotein lipase is not stereospecific in its hydrolysis of triglycerides, they did show that the enzyme also possesses phospholipase A activity. Vogel and Bierman (1106) have confirmed the phospholipase activity in vivo. Dorzaki and Zieve studied this phospholipase activity in vitro and found that it was more susceptible to the micellar state of the substrate as influenced by salts and other agents than was the lipase activity of the enzyme. Chylomicrons contain phosphatidyl ethanolamine which is released as chylomicrons are degraded. If factors which prevent the degradation of this phospholipid are present, then its relative concentration in blood may increase under these conditions. Marinetti (714) studied the effect of phospholipase A action on β-lipoproteins. Although he found no change in molecular weight or in other molecular
parameters, the effect of phospholipid hydrolysis may be different when triglycerides are also being hydrolysed. Similarly, Banaszak and McDonald (41) reported that treatment of β-lipoprotein with proteolytic enzymes did not dramatically alter the clarity of lipoprotein solutions. Although it might be argued that some lipoprotein peptide bonds were buried and not exposed to the attack of the enzyme, these results do not rule out the importance of lipid-lipid interactions in holding lipoprotein subunits together. In contrast, the subunits of de-lipidated β-lipoprotein form insoluble aggregates.

**Effects of Nicotinic Acid, Thyroxine, Atromid, Polyunsaturated Acids, Androsterone Estrienes and Cholinesterase Inhibitors**

Many drugs affect the activity of lipoprotein lipase. Nicotinic acid, which inhibits adipose tissue lipase, was used by Molnar et al (753) in an attempt to reduce lipolysis in diabetes. Although the drug initially reduced free fatty acid levels, it also had a stimulatory effect on lipoprotein lipase which persisted at a drug level at which inhibition of adipose tissue lipase did not occur. The net effect of the drug was to raise fatty acid levels and to exacerbate the diabetes. Nicotinic acid also produces hypotension.

Porte et al (847) also found that thyroxine increased
lipoprotein lipase activity. However, the increase in myocardial metabolism also produced by the hormone may produce angina pectoris in patients with narrowed coronary arteries. Oliver and Boyd (798) have investigated the effect of D-thyroxine in the treatment of hypercholesterolemia. Although type III hypercholesterolemia was unaffected by the drug, a mean decrease of 16 percent in serum cholesterol was observed after 6 months of therapy with 7.5 mg of D-thyroxine per day. At this dose level 35 percent of the patients experienced angina. Furthermore, although no significant increases in basal metabolic rate were observed, most patients lost weight during therapy. An escape phenomena was noted with the drug, i.e. the drug became less effective with time. One possible explanation of this finding is that D-thyroxine displaces L-thyroxine from thyroid-binding globulin so that the hormone inhibits TSH release from the pituitary. An escape phenomena has also been observed with 2-(p-chlorophenoxy)-2-methyl-propionic acid (clofibrate) which may exert this effect by the same mechanism.

Atromid-S (clofibrate) is often prescribed for the treatment of type II hypercholesterolemia. However, Japson and James (530) reported that the drug produced only a transitory 9 percent reduction in cholesterol in this disorder. On the other hand, Hood et al (475) observed that the drug
caused a significant improvement in hypertriglyceridemia due to its activation of lipoprotein lipase. This result was best observed when activity was expressed per milligram of \(\beta\)-lipoprotein protein. The enzyme is normally associated with the lipoprotein and this fact has been used to advantage in its purification by floatation. Fielding (314) has purified the enzyme from rat plasma 500-fold. He observed that the optimum temperature of the enzyme was 37°C, the optimum pH 8.5, and that the enzyme lost 10-15 percent of its activity per day even at 0°C. The molecular weight of the enzyme was found to be 72,600. These results indicate that Atromid-S may be an effective drug for the treatment of hypertriglyceridemia but that D-thyroxine is a better treatment for type II hypercholesterolemia in those patients who do not experience angina.

Types I and V hypertriglyceridemia may represent metabolic defects in the activation of lipoprotein lipase. The proposed conversion of the stable form of the enzyme to the less stable form by the action of cyclic adenylate and the ability of glucocorticoids to enhance the action of the nucleotide may explain the reports by Bagdade et al. (35) of a reduction of lipoprotein lipase activity during glucocorticoid-induced lipemia. Similar decreases in enzyme activity may occur in patients with diabetic
retinopathy. Ianacone and Kornerup (503) reported elevations in serum lipids in this condition. A decrease in the enzymes sulfating heparin may be partly responsible. Yde (1186) found that these enzymes were reduced in diabetes. In fact, a negative correlation exists between the fasting levels of serum sulfation factor and serum glucose.

Rosenman et al (909) found that not all of the hypolipidemic effect of thyroxine could be explained by its activation of lipoprotein lipase. They found that thyroxine decreases serum cholesterol by increasing the rate of bile acid formation, even though it also increases the rate of cholesterol synthesis. Estrogens also increase bile acid formation but have a lesser effect on cholesterol synthesis. These results indicate that drug effects on the rate of bile acid formation can dominate effects on cholesterol synthesis and that drugs which can reduce the rate of the former process without affecting the latter may be valuable hypocholesterolemic agents. Polyunsaturated fatty acids also promote bile acid formation and they, in conjunction with estrienes may be the most effective treatment for type II hypercholesterolemia (659, 796, 1021, 1094). Dayton et al (217) have shown that when dietary fat constitutes 40 percent of calories a diet containing 38 percent of this fat as linoleic acid resulted in 48 deaths
over an 8-year study compared to 70 deaths for a diet containing only 10 percent of its fat as linoleic acid. This dietary treatment of a lipid disorder is similar to the dietary restriction of carbohydrate found by Kuo (622) to aid type IV hyperpre-β-lipoproteinemia.

Non-aromatic steroids may also affect lipoprotein lipase activity. Laron and Kowadlo (638) have demonstrated marked increases in plasma free fatty acids following the administration of 10 mg/kg of testosterone propionate to two-day fasted female rats. A 78 percent rise occurred in the treated animals versus a 5 percent rise in the controls. Although this rise was attributed to fat mobilization from depot lipid, it could not be produced in one day-fasted animals. Since β-lipoproteins begin to rise after approximately 48 hours of fasting due to lipolysis in adipose tissue, an activation of lipoprotein lipase by the steroid could also explain the findings.

Androsterone exerts a hypocholesterolemic effect in normal subjects that has not been explained (446). Significant reductions can be obtained by the intramuscular administration of 50 mg/kg of the steroid per day. In rats Abdell and Mosbach (1) have reported that 17β-hydroxy-17α-methyl-4-androsten-3-one and its metabolite 17α-methyl-5α-androstane-3β,17β-diol lowered serum cholesterol. The
effect of the metabolite appears to be similar to that observed with androsterone in man. However, the effect of the testosterone derivative in the rat is opposite to that observed in man because α-lipoproteins are the predominant cholesterol-bearing lipoproteins in the rat.

Although androsterone exerts a hypocholesterolemic effect in normal men, certain 17α-alkylated C-18 and C-19 steroids appear to exert this effect only in hypertriglyceridemic individuals. Sachs and Wolfman (923) demonstrated that 17β-hydroxy-17α-methyl-2-oxa-5α-androstan-3-one produced a 38 percent reduction in cholesterol, a 40 percent drop in phospholipid, and an 81 percent drop in triglycerides in subjects with hypertriglyceridemia. On the other hand, the same compound produced typical androgenic effects in normal individuals, namely, changes of +7 percent, -1 percent, and +65 percent in these same respective lipids. Furman and Howard (349) demonstrated the hypocholesterolemic effect of 17β-hydroxy-17α-methyl-4-androsten-3-one in type II hypercholesterolemia and Gluek et al (375) obtained similar effects with 17α-ethinyl-17β-hydroxy-4-estren-3-one in four patients with familial type V hypertriglyceridemia. In fact, a 2-10 fold reduction in plasma triglycerides was the major effect of the therapy. These reports represent a significant advance
in the treatment of this disorder. Acute abdominal pain is characteristic of type V hyperlipemia. Many of the symptoms resemble those of pancreatitis, including elevations of serum amylase and serum lipase normally used in the laboratory diagnosis of pancreatitis. However, the administration of heparin or the withholding of long-chain triglycerides from the diet lessens the abdominal pain associated with this disorder but not that associated with pancreatitis.

Hazzard et al (441) and Fabian et al (292) have shown that lipoprotein lipase is inhibited by aromatic steroids. Although estrienes inhibit lipoprotein lipase in normal and hyperlipidemic subjects, the mechanism of the effect is unknown. In contrast, Szensikowski et al (1045) have shown that eserine blocks 50 percent, and tetraisopropyl phosphoramide 93 percent of the post-absorptive lipolytic activity in the rat. The mechanism of this effect appears to be similar to that involved in the inhibition of the enzyme by protamine. The phosphoramidate may therefore be valuable in lipoprotein lipase studies.

**Effects of Lipids on Platelet Aggregation and Fibrinolysis**

Platelet aggregation is one of the initial events in myocardial infarction and β-lipoproteins have been shown to affect this process. While fatty acids can cause the reversible aggregation of platelets, the aggregate is real
and can momentarily block circulation in small capillaries. If this process leads to anoxic damage in the capillary, tissue thromboplastin is released into the circulation and some thrombin is formed. Since platelets are irreversibly aggregated by low levels of thrombin, those factors which control reversible platelet aggregation also control irreversible platelet aggregation.

The process of ADP-induced platelet aggregation may be viewed as a result of depressed cyclic adenylate levels in the platelet. As such it represents an effect of cyclic adenylate on cell surface charge that has not yet been reported for other types of cells. The mechanism whereby cyclic adenylate affects surface charge appears to be more closely related to electrolyte metabolism than to glucose metabolism.

Glucocorticoids also enhance platelet aggregation but the mechanism is not clear. Generally, platelet aggregates are formed in narrow pulmonary vessels following the administration of large doses of glucocorticoids. Apparently, platelet serotonin, which is released during the irreversible phase of platelet aggregation, causes the constriction of bronchioles and may enhance the anoxic effect of platelet aggregates.

High levels of free fatty acids in plasma promote
platelet aggregation. Hoak et al (465) found that both satu-
urated and polyunsaturated fatty acids had this effect.
However, whereas the polyunsaturated fatty acids produced
only microscopic aggregates, saturated fatty acids produced
visible clumps. According to Goloff et al (386) these ef-
facts are best seen in the vasculature of the wing of an
unanesthesized bat where as little as 0.01 cc of a 0.01 per-
cent suspension of sodium stearate produces vasoconstriction
and thrombogenic effects that cannot be duplicated by larger
doses of oleic or linoleic salts. This type of aggregation
is not inhibited by heparin and is different from irreversi-
ble platelet aggregation induced by thrombin. However,
Glynn et al (376) found that even reversible platelet ag-
gregation within the microcirculation could produce anoxic
damage in the arterioles and microcirculation.

Many cardiac patients have partially occluded coronary
arteries. These patients are often treated with heparin
to lessen the chance of intravascular clotting leading to
complete occlusion. Since lipoprotein lipase is activated
by this treatment, meals high in saturated fat may cause
abnormal increases in saturated free fatty acids which pro-
mote reversible platelet aggregation as described above.
This consideration emphasizes the role of diet in the con-
trol of atherosclerosis.
Kerr et al (573) found that platelet aggregation by saturated fatty acids was inhibited by the 18w3 fatty acid linolenate but not by the 18w6 fatty acid linoleate. Thompson (1059) found increased platelet adhesion in multiple sclerosis and noted that circulating cholesterol linoleate was depressed in the active stages of the disease. Since this ester is synthesized from a linoleate-containing lecithin, its depression in these stages might be explained by the presence of phospholipase A activity in the circulation of these patients. Bolton and Phillipson (92) presented evidence supporting the presence of an abnormal lysolecithin in multiple sclerosis that tends to support the above hypothesis. This lysolecithin increases in the chronic phases of the disease resulting in increased platelet sensitivity to ADP-induced aggregation. The abnormal lysolecithin is believed to be formed by the action of phospholipase A on an abnormal lecithin of neural origin. However, a similar lecithin species has been demonstrated in patients with vascular disease. Although the phospholipase A activity in vascular disease is released from erythrocytes by centrifugation, little is known about this enzyme nor about the enzyme active in multiple sclerosis. Platelet aggregation in response to ADP appears to be more frequent in venous regions of low turbulence so that these regions
are more susceptible to thrombosis.

Prostaglandin $\text{PGE}_1$ inhibits lipolysis and platelet aggregation. Karim and Sharma (563) found that ethanol could abolish uterine contractions produced by the oral administration of $\text{PGE}_2$ to gravid women at term. This finding suggests that a decrease in the cytoplasmic ratio of NADH to NAD$^+$ is one of the effects of the lipid. The possibility that prostaglandins exert some of their effects following their conversion to coenzyme A derivatives and the incorporation of the latter into phosphatidic acid and triglycerides has not been investigated.

The role of stress in atherosclerosis has already been discussed. Delong et al (225) showed that the whole blood clotting times of stressed rats was decreased relative to non-stressed controls. These results are consistent with the known affect of epinephrine on platelet aggregation. This action is not mediated by cyclic adenylate. Indeed, Marquis et al (719) have shown that cyclic adenylate decreases platelet aggregation in response to adenosine-5'-diphosphate, ADP. Therefore, any factor which decreases the cyclic nucleotide will increase platelet aggregation in response to ADP. While prostaglandin $\text{E}_1$ decreases the levels of cyclic adenylate in adipose tissue (146, 297), it increases the levels of the
cyclic nucleotide in platelets. In addition, it is interesting to note that although the prostaglandin is the most potent inhibitor of platelet aggregation yet discovered, it cannot be used therapeutically because of the hypotension which it also produces. This action appears to be opposite to the action which the prostaglandin has on uterine smooth muscle.

The arterial intima is continually being injured and repaired. The basis of the effect of serum lipids on thrombus formation is illustrated by the results of Bang et al (76). These workers found that chylomicrons and pre-β-lipoproteins were incorporated into the fibrin clot and that once incorporated the lipoprotein inhibited fibrinolysis of the thrombus.

The prostaglandins have an important role in modifying the actions of cyclic adenylate in adipose tissue. First, the mode of transport of prostaglandins does not seem to have been studied, especially with respect to their possible transport by lipoproteins. Bergstrom (70) noted that these lipids inhibited the lipolytic action of catecholamines but not their effects on heart or on glucose metabolism. Fain (297) reported that PGE₁ also inhibited the lipolytic action of growth hormone and glucocorticoids. Thus, prostaglandins may directly oppose the interaction of
glucocorticoids with the postulated growth hormone-dependent protein. However, PGE\textsubscript{1} may increase cyclic adenylate levels in some tissues. These results suggest that both PGE\textsubscript{1} and growth hormone along with glucocorticoids affect lipolysis independently of cyclic adenylate levels.

**Effects of Aldosterone Antagonists**

Glucocorticoids may act to decrease triglyceride synthesis. Danowski et al (212) noted that the aldosterone antagonist 3-(3-keto-7α-acetyltio-17β-hydroxy-4-androst-ten-17α-yl)-propionic acid gamma lactone, like glycyr rhetic acid, could enhance the inhibition of triglyceride synthesis by cortisol.

**Sequelle of Initial Myocardial Infarction**

The arterial damage resulting from an initial myocardial infarction results in the release of tissue thromboplastins and higher steady-state levels of plasma thrombin. Since thrombin preferentially accelerates platelet aggregation, an initial infarction increases the probability of a subsequent infarction. Thrombin acts by exposing platelet factor 3, a platelet membrane lipoprotein which contains phospholipids active in blood coagulation.

The elevation in β-lipoprotein which occurs following an infarction and persists for approximately two months thereafter appears to be due to an inhibition of lipoprotein
lipase activity that may be caused by the presence of heparin-neutralizing factors. However, the significance of the decrease in $S_f$ 12-20 lipoproteins following infarction, first reported by Dodds and Mills (246) remains obscure.

McDonald and Edgall (688) have verified the hypercoagulability of the blood of patients with angina pectoris or actual myocardial infarction due to the presence of tissue thromboplastin as mentioned above. Agents which increase platelet aggregation in response to ADP in vitro may also increase secondary platelet aggregation in response to ADP released from platelets primarily aggregated by long chain saturated fatty acids.

The main result of the increased ADP concentration in platelets is that processes which maintain the repulsive surface charge on the platelet are reduced so that platelets show increased adhesiveness to each other and to glass. This change in surface charge is reflected by changes in the electrophoretic mobility of the platelet.

French (330) has noted that low levels of thrombin preferentially accelerate platelet aggregation. After this initial aggregation which is an irreversible process, the platelets release a lipoprotein, platelet factor 3, which increases the rate of thrombin formation. It is this second
surge of thrombin formation which clots fibrinogen. However, although platelets are irreversibly aggregated, thrombus formation is still reversible at this stage. The irreversible stage in thrombus formation appears to be the absorption of certain plasma glycoproteins by the developing thrombus (131).

**Role of Phospholipids**

The increase in β-lipoprotein which follows myocardial infarction has been investigated by Hampton and Mitchell (425) and by Bolton et al (89). They found that the lipoprotein in this instance contained an abnormal lecithin which was converted to a lysolecithin by the action of an enzyme found to be of red cell origin. Although the exact nature of the abnormal lecithin has not been determined, it is believed to be a symmetrical lecithin containing only monoenolic or saturated fatty acids. A similar type of lecithin has been shown by Bolton et al (91) to be present in women taking estrogen-progestin combinations. The effect was attributed to the estrogen component because the progestin 6-chloro-17α-hydroxy-1,4-pregnadiene-3,20-dione 17 acetate was without effect when administered alone (843). The estrogen effect under these conditions appears to be due to an increase in lecithin saturation (423). An additional direct effect of estrogens on β-lipoprotein is not ruled
Indeed, Roberts and Szego (892) have reported the presence of covalently bound estrogen activity in the β-lipoproteins of human plasma. The estrogen activity was present in a hydrophilic esterified form equivalent to 18 µg/l of estriol.

Although the effects of estrogens on blood coagulation are complex, their enhancement of ADP-induced platelet aggregation appears to be due to an increase in the hepatic synthesis of monounsaturated lecithins. For example, Bolton et al (91) observed that platelet electrophoretic mobility, a measure of the repulsive negative charges between platelets, was increased by estrogen therapy. In addition, the platelets became much more sensitive to the reduction in their mobility normally caused by 0.05 µg/ml of ADP. For instance, an anurism of the left middle cerebral artery was observed in a patient whose platelet mobility was decreased 12 percent by 0.005 µg/ml ADP, the highest sensitivity observed during the study.

Hampton and Mitchell (425) have reported that the lecithin precursor of the platelet-active lysolecithin, although present in normal plasma, is increased 5-fold following myocardial infarction. The hydrolysis of this lecithin to the active lysolecithin was inhibited by cyanide and fluoroacetate anions. The phospholipase enzyme
responsible for the transformation seemed to be specific for lecithin bound to β-lipoprotein. Bolton et al (89) noted that the active lysolecithin was not formed if the lipoprotein had been frozen and thawed and thereby denatured. The phospholipase, therefore, shows substrate specificity similar to that shown by lipoprotein lipase. The function of the lipoprotein may be to bind the lecithin in an asymmetrical manner for Attwood et al (32) noted that asymmetrical micelles were more rapidly hydrolyzed by phospholipase A.

The phospholipase as well as its lysolecithin product are destroyed when incubated in plasma for 1 hour at room temperature. The lysolecithin appears to be destroyed by a separate lysolecithinase of wide distribution. On the other hand, the lecithinase is destroyed if the plasma comes in contact with glass. This latter inactivation does not appear to be caused by the proteolytic enzymes involved in blood coagulation which are surface-activated, but rather to an instability to glass surfaces similar to that observed with lipoprotein lipase (314). The conditions which affect the formation of the lysolecithin are also similar to those which have been reported for the β-lipoprotein insulin inhibitor by Bornstein (100).

Several questions related to the beneficial effects of estriene therapy on coronary thrombosis remain unanswered.
Although Ask-Upmark (29) and Tausk (1053) have reviewed pertinent findings relative to the relationship between estrogens and arterial damage, the fact that estrienes are not beneficial by virtue of their effects on serum lipoproteins nor by virtue of their effects on thrombus formation means that the actual basis of the long-term improvement in post-infarction survival is still unknown. Furthermore, the superiority of conjugated estrogens compared to non-conjugated estrogens in this type of therapy must be confirmed.

**Comparison of Estrogen and Cortisol Effects on Platelet Aggregation and Glucose Tolerance**

Egeberg and Owren (273) observed elevations in two hemostatic lipoproteins, platelet cofactor I and Factor V during estrogen therapy. Platelet cofactor I (Factor VIII) was also elevated in diabetic patients, patients with Cushing's disease, and during pregnancy. It is noteworthy that glucose intolerance has also been noted in each of these conditions.

Little is known about the biochemistry of Factor VIII. A deficiency of the activity of this factor produces Hemophilia A in males. It is known, however, that Factor VIII stimulates blood coagulation in response to tissue thromboplastins as well as in response to surface contact. Its action is closely related to that of another lipoprotein,
platelet factor 3. The complex of Factor VIII and platelet factor 3 has been termed "threone" and it accelerates the generation of thrombin by acting as a catalyst for the rapid interaction of other clotting factors leading to thrombin formation. Therefore, at least part of the estrogen effect on blood coagulation is due to an increased formation of Factor VIII, which increases the concentration of threone in blood. According to Hemker and Kahn (448), threone promotes the interaction of Factor VII with Factor IX as well as the interaction of Factors V and X.

Although the membrane lipoprotein platelet factor 3 is not required for the anti-insulin effect of β-lipoproteins, some of its properties will be described since they may be confused with the lysolecithin species which is believed to be required for insulin antagonism. The lipid portion of platelet factor 3 lipoprotein is active in certain coagulation tests such as the thromboplastin generation test and in the Stypven-time determination. The exact nature of this lipid portion of the molecule has not been determined. However, it is known that the active component is a phospholipid which can be formed from sn-1,2-dioleoyl-phosphatidyl ethanolamine (844). The active phospholipid is found in most crude cephalin preparations. Its in vivo significance is seen in the results of Marciniak et al (710)
who showed that the simultaneous injection of the cephalin fraction with autoprothrombin C (Factor X) was lethal while the injection of either factor alone was not lethal.

Platelet aggregation is prevented normally by the action of a membrane bound kinase which introduces negatively charged phosphate groups into the platelet membrane and thereby generates repulsive platelet charges. ATP is the phosphate donor for this reaction and the process is inhibited by ADP. This scheme was proposed by Salzman (928). However, the source of extracellular ATP for the reaction has not been specified. It appears that some mechanism exists by which intracellular ATP equivalents are transported through the membrane and subsequently converted to extracellular ATP because nucleotides do not penetrate the platelet membrane. Furthermore, the high levels of acid phosphatase contained in platelets may be involved in the removal of the membrane phosphate groups thereby necessitating their continuous replacement. Although ATP inhibits ADP-induced platelet aggregation, ATP does not inhibit platelet aggregation produced by thrombin. Both ADP and serotonin are known to be stored in platelet granules that are irreversibly disrupted during thrombin aggregation. This effect of thrombin on platelet granules may be related to its effect on platelet phospholipid
synthesis. Majewus et al (699) have shown that platelet phospholipid synthesis can be increased 7-fold by thrombin within 2 minutes and that this effect is accompanied by the reduction in the molecular weight of a platelet membrane protein from 190,000 to 107,000.

The Mechanism of Steroid Effects

Jederkin and White (527), White (1138), and Wade and Jones (1109) have reported that 6 x 10^{-4} Molar 3,4-bis(p-hydroxyphenyl)-trans-3-hexene,4,16-prenadiene-3,20-dione, progesterone, and 17a-ethyl-17β-hydroxy-4-estren-3-one increased mitochondrial ATP utilization and inhibited the rate of oxygen consumption. The first non-steroid was about 26 times as potent as the first steroid listed.

The chemical progesterone precursor, in turn, was about 3 times as potent as the latter two steroids which were approximately equal in effect, but 3 times as potent as deoxycorticosterone which was equal to 17β-hydroxy-17α-methyl-4-androsten-3-one in potency. Their data also indicate that the 3-fold difference in activity between the latter steroid and 17α-ethyl-17β-hydroxy-4-estren-3-one is due to the difference in the C-17 and not the C-10 substituents (492). The high activity of 4,16-pregnadien-3,20-dione also suggests that other flat polycyclic molecules such as 3-methylcholanthrene and ergolanes may also
be able to produce these effects. On a molar basis these inhibitory effects were greater than those of dicumarol, dinitrophenol, methylene blue, sodium azide or sodium cyanide. Essentially similar findings were reported by Jones and Wade (535) and by Hochster and Quastel (467).

Gordon et al (398, 399) attributed the increased utilization of extramitochondrial ATP to a steroid block in the reduction of mitochondrial cytochrome C by NADH. Since the steroids were effective in the presence of amytal (5-ethyl-5-isoamyl-barbituric acid) which inhibits electron transport at cytochrome C but did block reduction of coenzyme Q (ubiquinone), the steroids were believed to inhibit electron transfer between coenzyme Q and cytochrome b or between the latter and cytochrome C. Gordon et al (398) reached similar conclusions. Jedeikin and White (527) also found that 10^{-4} Molar deoxycorticosterone inhibited the oxidation of pyruvate and succinate and that NAD^{+} could overcome this inhibition.

Gallagher (352) also found that cortisol inhibited the oxidation of NADH. However, he found that the inhibition could be reversed by glutathione and acetyl coenzyme A and that the effect could be abolished by preparing mitochondria in water rather than in 0.25 Molar sucrose. These results emphasize the importance of mitochondrial integrity in the
effect of the steroids.

Blecher (84) further studied the effect of glucocorticoids on lymphatic cells and found that they inhibited glycolysis rather than glycogen formation. White and Makman (1139) reported that the effects of glucocorticoids on thymocytes required the presence of an energy source and intact cellular protein and nucleic acid synthetic capacities. These results suggest that the effects of glucocorticoids are much more specific than that of the other steroids mentioned above. That this specific glucocorticoid effect may be mediated by the \( \beta \)-lipoprotein glycolytic inhibitor is indicated by the findings of Hofert and White (468) demonstrating a major role of the liver in the systemic action of glucocorticoids in the involution of lymphoid cells.

Jones and Wakil (936) observed that NADH-cytochrome C reductase in microsomes has an absolute requirement for lysolactithin and lecithin. They postulated that these lipids combined to form micelles of distinctive structure. This effect is similar to that reported for the lipid portion of platelet factor 3. It was also reported that a similar type of micelle could be formed by 2-butyryl-3-oleyl-sn-1-glycerophosphoryl-choline. The possibility that certain steroids disrupt the structure of these micelles should be investigated.
Selye (965) first observed the anesthetic effects of steroids in mice. He found that female mice were much more sensitive to anesthesia than males. Using 135 gm mice, he found that as little as 5 mg of deoxycorticosterone or 15 mg of progesterone produced marked anesthesia in females. The anesthesia was further prolonged by the administration of 50 mg of androsterone. The male mice, however, were hardly affected by 40 mg of deoxycorticosterone. Winter (1169) confirmed these findings and showed that the anesthesia could be reversed by glucose.

Gordon (399) reported that 17α-ethyl-17β-hydroxy-4-androsten-3-one produced anesthesia while estradiol did not. However, Wade and Jones (1109) reported that estradiol was about as effective as 5β-pregnane-3α,20β-diol and testosterone. The use of non-hormonal steroids as anesthetics eliminates side effects such as salt-retention by deoxycorticosterone. For example, 21-hydroxy-5β-pregnene-3,20-dione 21-hemisuccinate has been used as a clinical anesthetic. The administration of 1.5 gm of the compound (Viadril) to men produces surgical anesthesia sometimes accompanied by profuse salivation which can be eliminated by atropine (dl-hyoscyamine), an anti-cholinergic belladonna alkaloid. In view of the fact that Viadril produces anesthesia more readily than analgesia, the substitution of
scopolamine (hyoscine), a parasympatholytic agent which is a precursor of atropine, for atropine, should depress the central nervous system and enable the effective dose and thrombophlebitis associated with the hemisuccinate ester to be reduced. Pedersen (823) reported that the administration of 0.5 mg of scopolamine to men produced sedation in 88.4 percent of them with no excitation or respiratory depression. Bradycardia, nausea and vomiting occurred in 20 percent. These results suggest that the combination of scopolamine and a more potent anesthetic which is water soluble may be a clinically useful agent in man. Scopolamine may also be used to treat the pyloric spasm which occurs in alloxan-diabetic rats, thus improving their survival.

The 11β-hydroxyl group dramatically changes the surface activity of steroids. Munk (766) found that cortisol laid on edge at the heptane-water interface while deoxycorticosterone and progesterone took up parallel positions. Weissman (1130) noted that cortisol counteracted the release of acid hydrolases from liver lysosomes by deoxycorticosterone and progesterone as well as the release of salts and glucose from synthetic micelles by the latter steroids. These findings suggest that glucocorticoids have specific surface effects.
Effects of Other Compounds

Mills and Roberts (745) as well as O'Brien (793) have shown that a number of clinically used drugs reduce the ADP-sensitivity of platelets. One of the more interesting is thorazine [2-chloro-10-(3-dimethylaminopropyl)-phenothiazine] which is also known as chlorpromazine. Several seemingly unrelated effects of this drug are of interest in light of the hyperglycemic effect of the drug. Reckless and Hopkin (869) demonstrated synergism in anesthetic effect of the phenothiazine and morphine; anesthetic steroids have also been described. Although chlorpromazine alone decreases respiratory rate, it also counteracts respiratory depression due to morphine (869); glucocorticoids have the same effect. Furthermore, the phenothiazine opposes the emetic effect of serotonin and apomorphine; both of the latter compounds have hyperglycemic effects (180, 987). Chlorpromazine also accelerates the clearing of hyperlipidemic serum (471) but inhibits ethanol metabolism (118).

While the above observations do not suggest a common mechanism, reports that an abnormal lecithin molecule transported by β-lipoprotein following its conversion to lysolecithin, increases platelet ADP-sensitivity, suggests that a specific lecithin may mediate glycolytic inhibition by steroids (573, 720, 928). Furthermore, since Hughes
and Tonks have shown that glucocorticoids enhance platelet aggregation (493), they may exert this effect by increasing the synthesis of the abnormal lecithin in the liver. Growth hormone or estrogens may also affect the synthesis of this lecithin species.

A platelet membrane lipoprotein, platelet cofactor 3, is exposed during irreversible platelet aggregation when it accelerates thrombin formation. This factor is described because it also contains an essential phosphatidyl ethanolamine derivative but is not concerned with reversible platelet aggregation. On the other hand, Factor XII, Hageman factor, which is a proteolytic enzyme responsible for the reduced clotting time of blood exposed to hydrophilic (glass) surfaces, has been shown by Harmison and Mammen (429) to increase platelet ADP-sensitivity. These workers also found that purified Hageman factor along with purified platelet cofactor 3 could activate purified prothrombin to thrombin.

Chlorpromazine, phentolamine (2-[N-(m-hydroxyphenyl)-p-toluidinomethyl]-imidazoline), and diphenylldiamine (2-diphenylmethoxy-N,N-dimethylethylamine) inhibit ADP-induced platelet aggregation (213, 1199). These drugs are very similar structurally. In addition to these platelet effects, Charaton and Bartlett produced hyperglycemia in dogs by the
FIGURE V

STRUCTURE OF CHLORPROMAZINE

CHLORPROMAZINE
administration of 50 mg of chlorpromazine. Dobkin et al (241) also produced hyperglycemia in man by the intravenous administration of 1.5 mg/kg of the drug. Furthermore, Hiles (457) noted that patients with a family history of diabetes were most prone to chlorpromazine hyperglycemia. The effect of chlorpromazine on glucose utilization is similar to that of cortisol.

Chakrelarte et al (167) found that the combination of the synthetic steroid 17a-ethyl-4-estren-17β-ol and the oral hypoglycemic agent phenethylbiquanide inhibited ADP-induced platelet aggregation. A reduction in platelet adhesiveness was produced in most patients by the administration of 50 mg of phenethylbiquanide and 4 mg of the steroid per day. Phenethylbiquanide inhibits oxidative phosphorylation, depresses cytoplasmic ATP, and therefore accelerates glycolysis by its anaerobic effect. However, if this were its only action, phenethylbiquanide would promote platelet aggregation. The inhibitory effect of this compound could be due to inhibition of platelet phosphodiesterase with resulting elevation in platelet cyclic adenylate which has been shown to have this effect. If the steroid also inhibits platelet aggregation by reducing cyclic adenylate levels, then it appears to have an effect opposite to that of glucocorticoids which enhance the effects of this
nucleotide. However, since platelets are capable of gluco-
neogenesis, the role of protein synthesis in these effects
cannot be determined from present data.

Zucher and Peterson (1198) reported that a single dose
of aspirin irreversibly reduced platelet aggregation in re-
sponse to epinephrine for the platelet life-span of 9-11
days. Hemostasis was still satisfactory, however. The
molecular reaction involved is believed to be the transfer
of the phenolic acetyl group of aspirin to a protein amino
group. Furthermore, al-Mondhiry et al (754) determined
that acetic anhydride could also produce the aspirin effect
whereas sodium acetate could not, even though total ace-
tate incorporation was about the same as with the anhydride.
Apparently, these workers did not distinguish between coval-
ent and non-covalent acetyl groups. Also, although these
workers were working with whole platelets, only one of many
acetylatable proteins is involved in the ADP-induced aggre-
gation.

In summary, the phenomenon of ADP-induced platelet ag-
gregation provides a model system in which the action of a
unique species of lysolecithin associated with β-lipopro-
tein may be studied. The identity of this lipoprotein fac-
tor with the insulin inhibitor cannot yet be made with cer-
tainty since the mechanism by which ADP exerts its effect
is not known. However, a reduction in platelet ATP is one of the effects of ADP so that some aspect of platelet metabolism is blocked by β-lipoprotein. If the insulin antagonist enhances platelet aggregation, then insulin should counteract its effect.

Sirek et al. (987) and Feldman and Lebowitz (309) further demonstrated that serotonin antagonists could block the hyperglycemic effect of serotonin. These latter workers also showed that the adrenergic blocking agent phentolamine also had this effect even though it did not block insulin release by epinephrine. In additional experiments Sirek et al. (987) showed that 2-bromolysergic acid diethylamide inhibited the rise in blood pressure following the transfusion of blood from normal dogs treated with growth hormone. These results suggest that growth hormone releases serotonin into the blood. The serotonin, in turn, releases adrenal epinephrine. In perhaps their most interesting report Sirek (986) showed that dihydroergotamine treatment abolished the early increase in glucose utilization following the injection of growth hormone into hypophysectomized dogs. This hypoglycemic effect of serotonin has been observed by others and may be due to the displacement of cortisol from some of its binding sites. In related experiments Levine et al. (656) found that serotonin at a
FIGURE VI
STRUCTURE OF SEROTONIN

SEROTONIN
concentration of $4 \times 10^{-4}$M produced glycogenolysis, hyperglycemia, and increased phosphorylase activity independently of the vasomotor effects of the amine on the liver. These effects could be blocked by the serotonin antagonist N1-methyl-ergonovine.

Adrenalectomized rats are more sensitive to serotonin, serotonin toxicity and intravenous serotonin hyperglycemia. Adrenalectomy also decreases blood serotonin, the induction of tryptophan pyrolase by serotonin, and the block in serotonin hyperglycemia by dihydroergotamine. Serotonin decreases thyroxine release and increases prolactin release. Serotonin induces hypertrophy of the mammary gland with hyperplasia of the ducts and lobular differentiation in most ovarectomized rats, but not in hypophysectomized rats. Serotonin decreases gluconeogenesis and lowers muscle glycogen but stimulates the growth of Walker carcinocarcinoma. Growth hormone increases the urinary excretion of 5-hydroxyindoleacetic acid.

Brand et al (113) reported that chlorpromazine could block the emetic effects of apomorphine, morphine, and dihydrogenated ergot alkaloids. Ansell and Marshall (14) reported that chlorpromazine inhibits phospholipid synthesis and Greig and Gibbons (11) have shown that the preservative effect which the phenothiazine has on red cells
in stored blood is due to the inhibition of a red cell lec-thinase by the drug. These results suggest that chlorpro-mazine may bind to alkaloid as well as to phospholipid bind-ing sites.

Gey and Pletscher (364) have shown that serotonin raises blood pyruvate and lactate levels suggesting that serotonin may interact with the growth hormone dependent protein which elevates cytoplasmic citrate levels.

Since ergot alkaloids and phenothiazines are not en-dogenous metabolites, if they are found to compete for some glucocorticoid-binding sites, this would indicate that some glucocorticoid effects are due to binding at non-specific sites and that certain drugs also exert their effects by binding to these sites. Furthermore, if ergot derivatives are found to have the highest binding affinities, the results may mean that related compounds could be impor-tant drugs capable of producing the non-specific but not the specific effects of glucocorticoids. The anti-inflam-matory effects and anti-glycolytic effects of the steroid are believed to be in this latter category.

In conclusion, the ability of β-lipoprotein lysolec-thin to enhance (89, 425) platelet aggregation by adeno-sine-5'-diphosphate may provide a convenient assay system with which to further study the phenomenon on a molecular
basis. With such an assay the factors which regulate the hepatic synthesis of the lecithin precursor of the lysolecithin may be studied. In particular, the roles of non-glucocorticoids, phenothiazines, serotonin, and serotonin antagonists in the effect can be determined. Furthermore, the hypothesis that such effects are related to a block in the conversion of diglycerides to triglycerides can be evaluated.

Role of Growth Hormone in Estrogen Effects

Steinberg (1025) has shown that the effects of estrogens on serum lipids in female hypophysectomized rats requires growth hormone. In addition, it appears that the deleterious effect of high-dosage estrogen therapy is also mediated by a growth hormone-dependent mechanism. Furthermore, both the effects of glucocorticoids on glucose uptake in adipose tissue (397) as well as the effects of this hormone on the production of the β-lipoprotein insulin inhibitor have been shown to be dependent upon growth hormone (98).

Bala et al (39) have shown that estrogens enhance growth hormone release while progestational steroids such as 17α-hydroxy-6α-methyl-4-pregnene-3,20-dione suppress growth hormone release in response to insulin or arginine. These results may indicate that the effects of the estrogen
and progestin components on growth hormone release may cancel each other to some extent, depending on their relative potencies and the ratio in which they are present in the medication. In many cases net effects on growth hormone levels are observed. Spellacy et al (1010, 1011) found elevations in growth hormone following the administration of 0.075 mg 17α-ethinyl-3-methoxy-1,3,5(10)-estrien-17β-ol and 5.0 mg 17α-ethinyl-17β-hydroxy-5(10)-estren-3-one. Since most combinations contain 0.05 mg of estrogen but 5.0 mg of progestin, the nature of the latter may determine the growth hormone effect of the various combinations. On the other hand, the sequential use of 0.10 mg of estrogen, double the above dose, may elevate growth hormone levels more frequently.

Several groups have reported the effects of estrogens and progestins in clinical studies. Svandborg and Vikrol (1041) reported that the percentage of lecithin increased while that of cholesterol and lysolecithin decreased following estradiol benzoate. The lipid pattern observed is somewhat similar to that which occurs in multiple sclerosis. Wynn et al (1179) showed that 31 percent of women taking estrogen-progestin combinations had fasting triglyceride levels higher than the upper limit of normal. Also, approximately, 15 percent of these same subjects had abnormal glucose tolerance tests. These changes were not
due to the progestin component because they could be pro-
duced by the estrogen component alone.

The most pronounced change in serum lipids with estro-
gen-progestin combinations is an approximately 160 percent
rise in the level of pre-β-lipoproteins. Other increases
found by Wynn et al (1179) expressed as the percentage of
pre-medication values include: triglycerides, 51.5; cho-
lesterol, 7.3; S_f 0-12 lipoproteins, 15.9; S_f 20-100 lipo-
proteins 56.2. Although no change occurred in S_f 12-20
lipoproteins, the atherosclerotic index was elevated 25
percent because of the pre-β-lipoprotein rise. The over-
all picture closely resembles that seen in type IV carbo-
hydrate-induced hyperpre-β-lipoproteinemia. The relatively
slight elevation in cholesterol cannot explain the in-
creased incidence of thrombophlebitis nor the changes in
glucose tolerance. Other studies, however, in which in-
dividual phospholipids were determined have helped to ex-
plain this result.

Brody et al (20) found that C-18 and C-21 progestins
had different effects in estrogen combinations. The C-21
progestins partially counteracted the estrogen-induced in-
crease in triglycerides as well as the decrease in phos-
phatidyl ethanolamine. These results suggest that C-21 pro-
gestins may inhibit the incorporation of sn-1,2-diglycerides
into triglycerides. Di Paola et al (35) have indicated that the 3-methyl ether of 17α-ethinyl-1,3,5(10) estriene-3,17β-diol more adversely affects glucose tolerance than the parent steroid, indicating that the C3-hydroxyl group is not required when administered for this effect.

Larsson-Cohn et al (640) studied the effects of the combination of 0.1 mg of the ether and 3 mg of 17-acetoxy-6-chloro-4,6-pregnadiene-3,20-dione on serum lipids. They found the following percent changes: triglycerides, +139; lecithin, +46; sphingomyelin, +12. Phosphatidyl ethanolamine was slightly increased, free fatty acids slightly reduced, and lysolecithin unchanged. These observations, however, do not permit a distinction between increased rates of lipid biosynthesis and decreased rates of lipid degradation.

The above effects on serum lipids are similar to those produced by glucocorticoids as observed by Sherbe (978) in women prior to adrenalectomy. Furthermore, the results suggest that the 11β and C-21 hydroxyl groups are not absolutely required for this effect even though they enhance it. Since the progestin component is usually present at 100 times the level of the estrogen component on a weight basis, effects that go unnoticed at physiologic progesterone levels may become significant at these higher levels.
Jeurand and Oliver (531) found that growth hormone treatment increased the saturation of serum triglycerides. Hagopian and Robinson (423) also found that estrogen treatment increased triglyceride saturation. This effect was found to occur during the synthesis of phosphatidic acid which has been shown to occur in mitochondria. Although triglyceride and phospholipid synthesis from diglycerides occurs extramitochondrially, factors regulating the transport of phosphatidic acid from mitochondria have not been completely elucidated. Greenbaum and Mc Lean (408) noted that diglycerides accumulate in the liver of rats following growth hormone treatment. Steinberg (1025) has also noted that growth hormone is required for estrogen-induced hypocholesterolemia in rats. These observations suggest that growth hormone and steroid effects on triglyceride synthesis occur extramitochondrially.

Phosphatidic Acid Synthesis

The enzymes of phosphatidic acid synthesis have been studied by several groups. O'Kuyama et al (795) found that oleic acid was esterified faster than stearic acid. Eloison et al (283) observed that 90 percent of palmitic acid was esterified at the sn-1 position while only 10 percent was esterified at the sn-2 position. Similar findings have been observed with oleic and palmitic acids. It thus
appears that phospholipid composition is a function of hepatic acyl coenzyme A concentrations.

Since the enzyme involved in the acylation of the sn-2 position is much more active than the enzyme acylating sn-1 position with palmitoyl coenzyme A. A similar situation also exists with respect to the enzymes catalyzing acyl transfer reactions with lysophosphatides. De Tomas and Renner (231) observed that unsaturated acyl coenzyme A was incorporated faster into lysophosphatidyl ethanolamine than into lyso-phosphatidyl choline. In addition, Akesson et al (8) noted that the enzyme which esterifies palmitoyl coenzyme A at sn-1 functions best with tetraenoic 2-acyl-sn-glycerol-3-phosphorylcholine and ethanolamines, incorporating 90 percent of added palmitate into these species within 5 seconds after its addition. Both the specificity in the formation of phosphatidic acid as well as the specificity in the acylation of lysophosphatides results in the formation of unsaturated phosphatidyl ethanolamine and the expense of saturated phosphatidyl cholines.

The further metabolism of phosphatidic acid does not appear to take place in mitochondria. The enzymes required for the addition of acyl coenzyme A to diglycerides appears to be in the cytoplasm while the enzymes which catalyze the formation of phospholipids from diglycerides are found in
hepatic microsomes. Recent studies by Reiser et al (876) indicate that triglycerides formed in the cytoplasm may serve as phospholipid precursors under certain conditions.

Besides being incorporated into phosphatidic acid, long-chain acyl coenzyme A derivatives may have several other fates. They may be converted to carnitine esters by an enzyme which has been found to be increased in the alloxan-diabetic rat. However, it should be noted that this reaction is not rate limiting for fatty acid oxidation (1107, 1156). If the fate of the acyl coenzyme A is to be oxidized, Huixtable and Wakil (463) have determined that the rate of this oxidation will decrease with increasing unsaturation except for linolenate, which is oxidized faster than linoleate. These studies also indicate that efficient incorporation of linoleyl coenzyme A into complex lipids is necessary to conserve this essential fatty acid from rapid oxidation.

Another possible fate of long-chain acyl coenzyme A is chain elongation. The chain elongation of saturated acyl coenzyme A such as palmitoyl coenzyme A occurs to a greater extent in females than in males (423). On the other hand, there is no difference in the chain elongation of linoleyl coenzyme A to arachidonyl coenzyme A. While these results suggest that separate enzymes may be involved in the chain
elongation of saturated and unsaturated coenzyme A derivatives with only the former under hormonal control, female rats do have higher serum arachidonate levels than males so that the degradation of arachidonate may also be hormonally controlled. This appears to be the case since unsaturation decreases fatty acid oxidation.

Not only is arachidonate less readily oxidized than other fatty acids, it is also less readily incorporated into the sn-2 position of phospholipids. Akesson (8) found that most of the label in linoleoyl coenzyme A was not incorporated unchanged or after conversion to arachidonyl coenzyme A, but that 60-80 percent of the linoleoyl label was incorporated as 18w6,9,12 octadecatrienoyl coenzyme A or as 20w6,9,12 eicosatrienoyl coenzyme A. This indicates that there is an optimal substrate unsaturation for this reaction (8).

In addition to the existence of optimal unsaturation in the formation of phosphatidic acid, there is also an optimal concentration of each acyl coenzyme A. Husbands and Lands (500) have shown that optimal incorporation occurs at 50 µM concentration with substrate inhibition at 60 µM. This finding suggests that an increase in acyl coenzyme A produced by lipolysis cannot increase triglyceride synthesis unless the formation of sn-3-glycerophosphatase is also
increased. However, an elevation in acyl coenzyme A renders these derivatives more available for oxidation, desaturation or chain elongation.

The tendency for estrogens to decrease phospholipid unsaturation is enhanced by stimulation of the methylation of phosphatidyl ethanolamine to phosphatidyl choline (684). Merkl and Lands (735) and Lands and Hart (633) have shown that hepatic enzymes catalyze exchange reactions between saturated diacylphosphoglycerides and unsaturated acyl coenzyme A. When the concentration of phosphatidyl ethanolamine is depressed by increased methylation, one of these enzymes can no longer catalyze the unsaturation of phosphatidyl ethanolamine prior to its methylation to phosphatidyl choline. Estrogens thus enhance the rate of synthesis of saturated lecithins. Ramey and Baron (858) also found that the estrogen analogue 17β-hydroxy-3-methoxy-1,3,5(10)-estriene-16-one increased the incorporation of inorganic phosphate and acetate into phospholipids. The extent to which estrogen-dependent chain elongation of saturated fatty acids depletes hepatic acetyl coenzyme A and regenerates coenzyme A remains to be determined.

The net decrease in esterified cholesterol during hyperestrogenic therapy is a direct consequence of the alterations in the composition of phosphatidyl choline which
donates the acyl group from its sn-2 position and of the rigid specificity of cholesterol: lecithin acyl transferase (373, 849) which has been found to produce cholesterol esters characteristic of the serum from which it is obtained within the limits of the composition of the lecithin substrates present.

As previously noted, estrogens promote the conversion of phosphatidyl ethanolamine to phosphatidyl cholines. Furthermore, phosphatidyl ethanolamines, in the course of their synthesis incorporate more highly unsaturated fatty acids into the sn-2 position and therefore, more highly saturated fatty acids in the sn-1 position. Therefore, the lysolecithins derived from these methylated phosphatidyl ethanolamines will also contain more highly unsaturated acids in the sn-1 position.

Regardless of the mechanism by which estrogens increase the methylation of phosphatidyl ethanolamine to phosphatidyl choline, the fact that ethanol administration which increases the NADH/NAD\(^+\) ratio also increases the methylation reaction, suggests that other agents which increase the reduction potential of the pyridine nucleotides will also increase the methylation reaction. Glucocorticoids have just that effect and may therefore produce changes in hepatic phospholipids similar to those produced by estrogens. For
example, an abnormal species of phosphatidyl choline is produced during high dosage estrogen therapy. This lecithin is converted to an abnormal lysolecithin which enhances platelet aggregation in response to adenosine-5'-diphosphate. This lysolecithin may function by specifically blocking the incorporation of long-chain saturated acyl coenzyme A into phosphatidic acid.

Cosgriff (202) has reported that glucocorticoid treatment enhances blood coagulation, but did not elucidate the mechanism by which this occurs. Therefore, an effect on phospholipid metabolism similar to that caused by estrogens has not been ruled out. On the other hand, glucocorticoids are known to promote the mobilization of phospholipids from the liver (1197). Furthermore, the effects of estrogens on the formation of high-density α-lipoproteins may be mediated by their effects on phospholipids. The cooperative nature of the interaction between phospholipids and high-density lipoproteins lends support to such a conclusion.

As noted earlier, any factor which increases the concentration of acyl coenzyme A will increase the ratio of NADH to NAD⁺. At present, the extent to which inhibition of the incorporation of these derivatives into triglycerides—a non-specific glucocorticoid effect—can account for this change is unknown as is the mechanism whereby
glucocorticoids decrease such incorporation.

In conclusion, the non-glucocorticoid effects of glucocorticoids have been reviewed with respect to their role in insulin antagonism and glycolytic inhibition. From considerations of the action of β-lipoprotein lecithins on platelet aggregation it appears that the same or similar lecithin molecule may, after its conversion to lysolecithin, affect the concentration of acyl coenzyme A in other cells by blocking the incorporation of these derivatives into phosphatidic acid.

Like estrogens, growth hormone also increases the percent saturation of triglycerides. However, the mechanism is somewhat different. As noted previously, both triglycerides and phospholipids are synthesized from diglycerides. However, the specificities of the enzymes involved are such that saturated diglycerides are directed towards triglyceride synthesis while unsaturated diglycerides are directed towards phospholipid synthesis via cytidine diphosphate choline and cytidine diphosphate ethanolamine. Growth hormone appears to exert its effects by decreasing the maximum velocity of triglyceride formation from saturated diglycerides.

Leal and Greenbaum (646) observed that growth hormone treatment depressed hepatic triglycerides 83 percent. Diglycerides, however, were increased 40 percent as was
phosphate incorporation into phospholipids. Acetate incorporation into phospholipids was decreased as a result of the increased diglyceride pool. Furthermore, because of the increased substrate pool and reduced maximal rate of triglyceride formation, more time is permitted for a better matching of the available substrates to the active site of the enzyme. These factors combine to increase triglyceride saturation. In addition, the increased pool size of phospholipids and the resulting exchange reactions which they undergo, further increase the saturation of the hepatic acyl coenzyme A pool from which triglycerides are synthesized.

Ethanol administration, which increases the reduction potential of the pyridine nucleotide pool, also has an effect on phospholipid synthesis. Fallon et al (304) reported that the administration of 20 percent ethanol increases the methylation of phosphatidyl ethanolamine, an effect also produced by estrogens. Furthermore, Medenhall et al (732) observed that ethanol administration, like growth hormone administration, increased triglyceride saturation, but not lecithin or cephalin saturation. These results confirm the specificity of the phospholipid synthesis. On the other hand, if the saturation of the diglyceride pool is increased, the same specificity may lead to a reduction in total phospholipid synthesis and to the
development of fatty infiltration of the liver.

Although estrogens may impair carbohydrate tolerance by increasing acyl coenzyme A concentration, this does not appear to be the mechanism by which estrogens affect glucose tolerance. Rather, the fact that estrogens produce the same effects as ethanol indicates that estrogens may increase lecithin unsaturation and decrease glucose tolerance by decreasing the oxidation of mitochondrial NADH.
CHAPTER XI
STEROID EFFECTS IN BREAST TUMOR THERAPY

Steroid hormones have long been employed in the treatment of breast cancer. Androgens, estrogens, progestins, and combinations of these hormones have been used without a complete understanding of how they exert their effects. The interpretation of the results of these treatments has been complicated because pharmacological steroid doses have been employed, because of the complex hormonal dependency of the tissue, and because of steroid metabolism elsewhere in the body.

Clinical findings in the hormonal treatment of breast cancer may be summarized as follows (76). First, tumors are generally more responsive to steroids if the tumor is hormone-dependent. This is the prime reason for early treatment. Secondly, low doses of estrogens promote tumor growth. The quantity of estrogens required for this effect may be produced in ovariectomized women from androgens formed in the adrenal gland. Thirdly, both oophorectomy
and adrenalectomy reduce estrogen production. Fourthly, and most importantly, high doses of estrogens have been shown to inhibit tumor growth more effectively than high doses of androgens. The fact that this latter estrogen effect is exactly opposite to its hormonal effect on the same tissue merits further research, especially because this disorder affects 5 percent of American women and because mortality from the disease has not decreased in recent decades. Furthermore, these steroid effects may be related to glucocorticoid effects.

**Mechanism of Steroid Action**

Pharmacological doses of estrogens inhibit mammary tumor growth more than similar doses of androgens. With both treatments there is maximal stimulation of the gland by estrogens. Treatment of an estrogen-dependent tumor with natural androgens which are converted to estrogens (417) is, in effect, a useless treatment because the basis of the androgen treatment is purported to be to counteract the stimulation of the hormone-dependent gland by low estrogen levels. This does not mean that androgens are completely ineffective in treating the disorder. Androgens may inhibit tumor growth by an anti-pituitary effect. However, the beneficial effects of androgens could be improved by the use of synthetic analogues which cannot be converted
to estrogens in vivo. This same reservation applies to glucocorticoids used to treat the disease.

The mechanisms described above cannot account for all of the greater effects of estrogens. There are several documented cases of both subjective and objective remissions of breast tumors with synthetic androgens in completely hypophysectomized patients (60). Since these patients were devoid of gonadotrophin secretion, the steroid effect was not mediated by the pituitary but must have been directly on the tumor tissue.

Experiments with synthetic steroids in the treatment of breast cancer in animals and women have indicated that the total effect observed is the summation of endocrine and non-endocrine effects. Endocrine effects include anabolic, anti-estrogenic, and anti-pituitary effects. Non-endocrine effects include a direct anti-glycolytic effect on the tumor. Thus, the most effective anti-tumor steroid for a given patient may depend on the relative hormonal dependence of the tumor which may change with time. Obviously, species differences in these influences may be considerable so that potency in the rat may differ substantially from that found in clinical trials. Still, there is reason to believe that the present cure rate of approximately 25 percent with steroid hormone therapy
could be doubled to 50 percent or higher by the judicious use of synthetic anti-tumor steroids which are not estrogen precursors.

In order to determine the non-endocrine anti-glycolytic effects of steroid hormones against breast tumors, studies would have to be carried out in hypophysectomized, oophorectomized, adrenalectomized tumor-bearing animals treated with growth hormone free of prolactin. Although such studies have not been reported, the structure-function relationships for anti-tumor activity may be discerned from studies already reported. Clinically, testosterone propionate has been used with favorable regressions in about 20 percent of patients who are less than 5 years postmenopausal. In patients who are more than 5 years postmenopausal, the stimulatory effect of physiological amounts of estrogens no longer counteracts the inhibitory effects of these compounds in pharmacological doses. It is important to note that these recommendations are based on the limited comparison of natural estrogens with testosterone propionate, an estrogen precursor. It is important, also, to recognize that most of the anti-tumor activity is pharmacological rather than hormonal. Therefore, although the net anti-tumor effects of estrogens is less than that of testosterone propionate in premenopausal women, synthetic
anti-tumor steroids can be made which are not estrogen precursors and which do not stimulate estrogen-dependent tumor tissue. Synthetic anti-tumor steroids have effects which are over 2.5 times that of testosterone propionate. This increase in potency does more than offset the 1.4 fold greater effect that natural estrogens have relative to natural androgens in patients who are more than 5 years post-menopausal. Thus synthetic anti-tumor steroids should emerge as the preferred hormonal treatment in all cases of breast cancer.

Results of Endocrine Ablation

The importance of estrogens in estrogen-dependent tumors and the production of adrenal androgens which are estrogen precursors has led to adrenalectomy as a form of therapy for breast cancer. Generally, oophorectomy is the first endocrine ablative procedure used following mastectomy. A favorable response to oophorectomy is obtained in 30 percent of pre-menopausal women. Approximately 40 percent of soft tissue, pleural, or skeletal metastases respond compared to 18 percent of liver, lung or CNS metastases. If the response to oophorectomy is favorable, this is an indication that the tumor is estrogen dependent and that the removal of adrenal estrogens may provide additional remission. Although adrenalectomy subsequent to oophorectomy does not
result in remission in all cases, it does not present the possibility of damage to optic and olfactory nerves associated with hypophysectomy.

**Use of Steroid Discriminants**

The adrenal gland also appears to be the source of anti-tumor activity which parallels the urinary excretion of 17-hydroxycorticoids as measured by the Porter-Silber reaction. Bulbrook (30, 130) recognized this and devised a discriminant function based on the excretion of 3α-hydroxy-5β-androstan-17-one relative to 17-hydroxycorticoids:

\[ 80 - 80 \times \text{mg 170HCS/24 hrs} + \mu \text{g 3 -hydroxy-5β-androsten-17-one/24 hrs} \]

When this discriminant is used, a number of greater than zero is associated with remission following adrenalectomy and vice-versa. In the initial study a positive discriminant was associated with remission 93 percent of the time and a negative discriminant with non-remission 85 percent of the time. Although the causative role of each discriminant factor need not be known for it to be valid, such a discriminant will best fit the population data from which it is derived.

Since the determination of Bulbrook's discriminant function involved the determination of 3α-hydroxy-5β-androstan-17-one by chromatographic techniques which were
unsuitable for many laboratories, other workers have devised discriminants that convey essentially the same information. Although inherently less specific because it measures groups of steroids, the simplest discriminant is the ratio of 11-deoxy-17-ketosteroids to 17 hydroxycorticoids excreted. If this ratio is less than 0.13 the discriminant is considered negative; if the ratio is greater than 0.16, the discriminant is considered positive. This determination is practical in the laboratory because the 11-deoxy-17-ketosteroids can be separated as a group by chromatography on Sephadex LH-20 and quantitated by the Zimmerman reaction. It should be noted here that this same information can be obtained after the conversion of 17 hydroxycorticoids to C-19 steroids by a method already described in detail.

To understand why these discriminant functions accurately predict the response to adrenalectomy in premenopausal patients it is helpful to assume that anti-tumor activity is associated with 17 hydroxycorticoids and that ring A reduced C-19 steroids of adrenal origin excreted in the urine reflect the formation of the estrogen precursor 4-androstene-3,17-dione by the adrenal gland. According to these assumptions a high production of estrogen precursors relative to anti-tumor steroids is associated with a favorable response to adrenalectomy. On the other hand,
a low production of estrogen precursors relative to anti-tumor steroids is associated with an unfavorable response to adrenalectomy. Empirically, the anti-tumor activity of the adrenal is associated with the excretion of 17-hydroxycorticoids while the excretion of 11-deoxy-17-ketosteroids provides an index of the adrenal as a source of estrogens.

The pituitary gland is necessary for the induction of mammary cancer by carcinogenic hydrocarbons such as 3-methylcholanthrene. Presumably the carcinogen can only cause a mutation when mitosis is stimulated by pituitary hormones. Alternately, the carcinogen may interact with a receptor protein produced in response to growth hormone or prolactin. This finding indicates that the response to carcinogens depends on growth hormone, prolactin, or both.

Peerlman (818) reported that cancerous persons excrete significantly less 17-ketosteroids than non-cancerous persons. Moreover, Sokal et al (1000) noted that patients with malignant conditions generally show marked increases in 17-ketosteroid excretion during the administration of 300 mg of cortisone acetate per day. A clear demarcation with little overlap was observed in cancerous and non-cancerous subjects. These results suggest that reduced glucocorticoid production with increased conversion of glucocorticoids to 17-ketosteroids is characteristic of
malignant states.

Function of Estriol

Lemon (552) has recently examined the relationship between breast cancer and estriol excretion. He found subnormal excretion of estriol relative to estrone and estradiol in fibrocystic disease, in fibrocystic disease complicating mammary cancer, and in premenopausal and postmenopausal mammary cancer patients. Hyperthyroidism was the only non-neoplastic syndrome with a similar excretion pattern, but up to one-fifth of euthyroid premenopausal women without mammary disease had similar excretion patterns. Marmaston et al (717) also measured estriol excretion in breast cancer patients and found that both pre- and postmenopausal patients had significantly increased estriol excretion. These results do not support an anti-tumor effect of estriol.

Saxema and Emerson (941) have found that estrogens produced in the adrenal gland may exacerbate mammary tumors. Postmenopausal mammary tumor patients who improved following adrenalectomy excreted 0.88 µg estrone per day before and 3.36 µg estrone per day (340 ± 196%) after ACTH treatment. On the other hand, the negative responders excreted 1.51 µg estrone per day before and 2.25 µg estrone per day (66 ± 38%) after ACTH. Apparently, adrenal estrogen synthesis is more sensitive to ACTH in the patients who respond
to adrenalectomy even though they have lower basal estrogen excretion. This may indicate that the responders have lower endogenous ACTH secretion and also lower glucocorticoid levels. These findings further suggest that determination of the change in the ratio of total urinary estrogens to total urinary 17-hydroxycorticoids after ACTH treatment may provide a more precise predictor of the response to adrenalectomy. These determinations are available in most laboratories via the Kober and Porter-Silber reactions respectively. The optimal age for response to adrenalectomy following oophorectomy is 50-59 with a remission rate of 56 percent compared to a 29 percent remission rate for the 40-49 age group.

Gallagher et al (355) also reported that male breast cancer is associated with increased estriol production. The rise in estriol excretion in this disorder is associated with myxedema and a reduced formation of 2-methoxy estriol. This latter steroid has been shown to be the natural steroid which has the highest lipodiatctic/estrogenic ratio. Decreased production of this steroid in hypothyroidism may explain the elevation of β-lipoproteins in this disorder. In female breast cancer, Barclay et al (45) have confirmed that the pre-β-lipoproteins are decreased secondary to a drop in high-density lipoprotein
phospholipid. Despite these changes a marked drop in plasma triglycerides is the most significant change in plasma lipids.

Effects of Thyroid and Growth Hormones on Steroid Metabolism

Liechty et al (666) and Bulbrook et al (130) have reported that the incidence of breast cancer is greater in hypothyroid than in euthyroid women. The reason for this difference may be related to the effects of thyroxine on glucocorticoid metabolism.

Bradlow et al (111) found that triiodothyronine increased the activities of mitochondrial 4,5α-reductases for a number of steroids thereby increasing the excretion of androsterone and decreasing the excretion of 3α-hydroxy-5β-androstan-17-one, its 5β diastereoisomer. In contrast, thyroid hormone has no effect on the 4,5β-reductases in the cytoplasm. The decreased excretion of 3α-hydroxy-5β-androstan-17-one simply reflects the relative competition of the mitochondrial and cytoplasmic enzymes for available 4-androstene-3,17-dione. Yates et al (1184) have also found that thyroxine increases the conversion of cortisone to 17α,21-dihydroxy-5α-pregnene-3,11,20-trione by rat liver mitochondria.

Gallagher et al (355) reported that the reduction of 11β-hydroxy-4-androsten-3,17-dione to 11β-hydroxy-5α-androstane-3,17-dione was increased by thyroid hormone. The more rapid formation of the 5α metabolite resulted in decreased formation
of 3α,11β-dihydroxy-4-androsten-17-one. In myxedema, the conversion of cortisol to 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one is doubled and its conversion to 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione is halved. The reverse changes are seen in hyperthyroidism. The formation of 3α,11β,17α,21-tetrahydroxy-5α-pregnane-20-one was unaffected by thyroid hormone. Although Koerner and Hellman (604) reported that thyroxine administration reduced the activity of 11β-hydroxy dehydrogenase in rat liver but not in rat kidney, thyroid hormone treatment results in an overall increase in 11-keto glucocorticoid metabolites due to the peripheral metabolism of the steroid (447).

In myxedemia, the increase in 5α steroid reduction and decrease in 11β-hydroxy dehydrogenase activity results in increased formation of 3α,11β-dihydroxy-4-androstan-17-one and its 3β-epimer in approximately equal amounts (355). The 3β-epimer is similar to 3β,11β-dihydroxy-5α-androstan-17-one. Kemp et al (575) found that this latter steroid was somewhat unusual in that it formed an 11 monoacetate preferentially when esterified with acetic anhydride and pyridine at room temperature. This behavior is very different from that of other 11β-hydroxylated steroids which form such a derivative only when heated. The possible role of the allylic alcohol in the hyper-β-lipoproteinemia of
myxedema has not been determined.

Triiodothyronine promotes the conversion of cortisol to cortisone as well as the 5α reduction of cortisone. The hormone, however, does not promote the 5α reduction of cortisol. Other steroids may exert the same effect in different ways. For example, Schriefers et al (953) reported that 17β-hydroxy-1-methyl-1-androsten-3-one decreased both adrenal weight and corticosterone production rate. In contrast, 17α-ethinyl-17β-hydroxy-4-estren-3-one decreased corticosterone production without affecting adrenal weight.

Not all of the effects of hypophysectomy on the 5α reduction of steroids can be attributed to the lack of thyroid hormone. Forchielli et al (327) found that the 5α reductase for androstene-3,17-dione could be restored to 50 percent of prehypophysectomy levels by the administration of 10 I U/day of adrenocorticotrophic hormone or by 10 µg/day of growth hormone. These results suggest that this reduction may be catalyzed by two distinct enzymes under different hormonal control.

There has been some controversy about the identity of human growth hormone and human prolactin. Apparently, these two hormones are very similar so that they cannot be readily separated. However, that the two hormones may be distinct entities is suggested by the lack of a constant ratio of
growth hormone to prolactin activities in different preparations. Furthermore, Brauman et al (114) used tissue cultures from human anterior hypophysis removed from hypothalamic influences to show that prolactin secretion could increase while growth hormone secretion decreased and vice versa on the addition of hypothalamic extracts, indicating distinct hormones.

The exact relationship between growth hormone and prolactin is especially important in the treatment of breast cancer by pituitary stalk section. This procedure decreases the output of growth hormone but increases the output of prolactin. Since prolactin has a stimulatory effect on breast tumors, complete hypophysectomy is preferable to pituitary stalk section.

In the intact rat prolactin may play an important role in the induction of mammary tumors by estrogens. Low concentrations of estrogens increase prolactin secretion. Prolactin, in turn, stimulates progesterone production by the corpus luteum, and the progesterone, in turn, further stimulates prolactin output so that a state of pseudopregnancy is produced. During this state DNA synthesis is greatly increased in the mammary gland and this event alone may increase the possibility of gene mutation. Indeed, a number of enzymes are induced during the development of lactation
and these inductions may provide a good biochemical model for the study of the interaction of many hormones in the regulation of metabolism. For example, Howanitz and Levy (482) observed large increases in citrate cleavage enzyme and similar increases have been observed in the enzymes of the pentose cycle following parturition. The interaction of growth hormone, insulin, ACTH, glucocorticoids, estrogens and prolactin in the metabolism of the gland should provide more metabolic insight than similar studies of adipose tissue have provided.

As mentioned previously, the formation of some cortisol metabolites is influenced by many other hormones so that the level of the metabolite actually excreted may be an indication of the hormone output that effects the production of these metabolites. For example, the excretion of 3α,11β-dihydroxy-5β-androstan-17-one is increased in myxedema not only because of the reduced conversion of cortisol to cortisone, but also because the 5α reductases for these glucocorticoids are also decreased. The close association of this steroid with myxedema and of the latter with cancer incidence may explain the results of Dobrin et al (242-245) who reported that levels of this steroid had prognostic value in breast cancer (242) and in cancer of the prostate (818). If abnormally elevated levels of this steroid
are found in the course of urinary steroid excretion studies, they should be followed up with more modern and definitive thyroid function studies and with specific prostatic acid phosphatase determinations.

Another type of steroid metabolite characteristic of myxedema is the allylic alcohol 3α,11β-dihydroxy-4-androsten-17-one. In contrast to the reduction of the double bond, the reduction of the 3 ketone is not diminished by hypothyroidism. Ringold (887) has shown that the major metabolites of 6α-fluoro-17β-hydroxy-4-androsten-3-one are 6α-fluoro-5β-androstane-3,17-dione and 6α-fluoro-4-androstene-3α,17β-diol. The 6α-fluorine decreases 5α enzymatic reduction as well as the rate of oxidation of the 17β hydroxyl group.

The previous considerations of the effects of estrogens and progestins on mammary tumor induction may not apply to established tumors. For example, Crawley and Mac Donald (205) obtained a 35 percent remission rate in the treatment of mammary carcinoma in postmenopausal women with estrogens alone. However, in estrogen-resistant patients, the simultaneous administration of 17α-hydroxyprogesterone caproate increased their remission rate to 50 percent. The additional benefit produced by 17α-hydroxyprogesterone may be due to inhibition of growth hormone release which has been demonstrated in acromegalics but inconsistently in normal subjects.
(627). However, as noted previously, therapy with estrogens or estrogen precursors is inexcusable in view of the availability of synthetic steroids which are just as effective but are not estrogen precursors.

Recently, Lemon (650) has advocated the use of synthetic glucocorticoids such as prednisone as an alternative to adrenalectomy for the treatment of estrogen-sensitive breast tumors in oophorectomized patients. Prior oophorectomy is required because glucocorticoids stimulate the release of follicle-stimulating hormone which increases estrogen production. In addition, prednisone suppresses the release of ACTH and the production of endogenous cortisol which is a better estrogen precursor than prednisone. Since prednisone also suppresses thyroid function, triiodothyronine supplementation was employed. Calcium salts were administered to counteract the catabolic effect of the glucocorticoid on bone. However, if these were the only mechanisms by which the glucocorticoid acted, the treatment would be no better than oophorectomy alone. In contrast, Lemon found that 48 percent of his patients benefited from prednisone therapy even though one-third of these had previously failed to respond to cortisone. This percentage is high compared to the 23 percent remission rate of bone metastases produced by androgens or estrogens and
compared to the 44 percent remission of soft tissue involvement produced by estrogens. Symptoms of Cushing's disease including weight gain, truncal obesity, peptic ulcer and osteoporesis were prominent side-effects of this medication.

Structure-Function Studies on Anti-Tumor Compounds

The prednisone remission rates are high compared to the figure of 25 percent reported for 17α-hydroxy-6α-methyl-4-pregnene-3,20-dione and the figure of 26 percent reported for 9α-fluoro-11β,17α-dihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione. However, since results for other compounds such as 6-chloro-9α-fluoro-17α-hydroxy-1,4-pregnadiene-3,11,20-trione, which should have higher activity on the basis of previous considerations, were not reported, an assessment of the role of the C-21 hydroxyl group in these effects cannot be made. However, Lemon noted that the glucocorticoids seemed to have an inherent anti-tumor activity.

Glenn et al (368) have determined the relative anti-tumor and androgenic activities of several synthetic anti-tumor steroids. Using their data which is recorded in Table IX and assuming that the factors by which structural modifications enhance activity can be multiplied, it is possible to calculate that the approximate anti-tumor activity of 17β-hydroxy-2α-methyl-5α-estrane-3,11-dione would be 2.2 times that of 11β-hydroxy-5α-estrane-3,11-dione which,
TABLE IX

RELATIVE EFFECTIVENESS OF STEROIDS AGAINST BREAST CANCER

<table>
<thead>
<tr>
<th>Steroid Activities Expressed as Percentage of Testosterone Propionate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>9α-fluoro-17β-hydroxy-17α-methyl-4-androstene-3,11-dione</td>
<td>255</td>
</tr>
<tr>
<td>A-nortestosterone propionate</td>
<td>131</td>
</tr>
<tr>
<td>2α-fluoro-17β-hydroxy-4-androsten-3-one 17 propionate</td>
<td>53</td>
</tr>
<tr>
<td>11β,17β-dihydroxy-17 -methyl-4-androsten-3-one</td>
<td>39</td>
</tr>
<tr>
<td>11β,17β-dihydroxy-17 -methyl-1,4-androstadien-3-one</td>
<td>30</td>
</tr>
<tr>
<td>17β-hydroxy-17α-methyl-4,6-androstadien-3-one</td>
<td>20</td>
</tr>
<tr>
<td>17α-ethinyl-17β-hydroxy-4-androsten-3-one</td>
<td>0</td>
</tr>
<tr>
<td>17β-hydroxy-6β-methyl-5α-androstan-3-one propionate</td>
<td>205</td>
</tr>
<tr>
<td>9α-fluoro-11β, 17β-dihydroxy-17α-methyl-5β-androstan-3-one</td>
<td>119</td>
</tr>
<tr>
<td>17β-hydroxy-17α-methyl-5α-androstane-3,11-dione</td>
<td>89</td>
</tr>
<tr>
<td>5α-androstan-17β-ol propionate</td>
<td>32</td>
</tr>
<tr>
<td>17β-hydroxy-17α-methyl-5β-androstan-3-one</td>
<td>0</td>
</tr>
<tr>
<td>17α-ethinyl-17β-hydroxy-4-estren-3-one</td>
<td>96</td>
</tr>
<tr>
<td>17β-hydroxy-17α-methyl-4-estren-3-one</td>
<td>76</td>
</tr>
<tr>
<td>17α-ethinyl-17β-hydroxy-5(10)-estren-3-one</td>
<td>65</td>
</tr>
<tr>
<td>17β-hydroxy-2α-methyl-4-estrene-3-one</td>
<td>41</td>
</tr>
</tbody>
</table>

Segaloff (962)
in turn, is twice that of testosterone propionate. Studies by Gallagher et al (355) suggest that the estradiol metabolite 3-hydroxy-2-methoxy-1,3,5(10)-estren-17-one which is formed in response to thyroid hormone may be an active anti-tumor agent.

Segaloff (962) has reported the oral activities of several anti-tumor steroids which are presented in Table IX. These data indicate that A-nortestosterone propionate is more active than testosterone propionate and that the 9α-chloro-11β,17β-dihydroxy-17α-methyl-4-androstene-3-one has the same anti-tumor activity as its 9α-fluorine analog but only one-tenth of its androgenic activity. Also, 6α-fluorination reduces anti-tumor activity even though Kincl and Dorfman (581) found that this substitution increased pituitary gonadotrophin inhibitory activity.

Rooks et al (902) showed that the introduction of an 11-ketone enhanced the potency of 17β-hydroxy-2α,17α-dimethyl-1, (5α)-androstane-3-one in inhibiting the incorporation of glycine into rat tumor protein. A similar inhibition of the incorporation of glycine into protein was also observed by Bornstein (98) in his work on lipoprotein insulin antagonism.

Anti-tumor activity is also distinct from anabolic activity. The latter activity appears to require both the
18 methyl group and a pi type molecular orbital at position 2 or 3 in the steroid nucleus. Wolf et al (1173) demonstrated that 2α,3α-epoxy-5α-androstan-17β-ol has the same anabolic effect as testosterone propionate, while 2α,3α-epithio-5α-androstan-17β-ol is a potent anti-estrogen as well. Kincl and Dorfman (582) reported that 2(5α)-androsten-17β-ol was 60 percent more myotropic and 60 percent less androgenic than testosterone. The dichloroacetate was its most potent ester. Later, Nutting et al (791) confirmed these findings and, in addition, showed that the 1α-methyl group further increased its oral anabolic-androgenic ratio. Edgren (270) extended these findings and demonstrated that ethyl groups at the 13β and 17α positions of 4-gonen-3-one greatly increased its anabolic activity but not its androgenic activity. The 17β-hydroxyl group lowered the anabolic-androgenic ratio. Also, androgenic activity appeared to be associated with a certain conformation of the non-conjugated 3-ketone that was favored by the presence of the 19-methyl group. Fang et al (305) have isolated a receptor protein for androgens which has a higher affinity for 5α-androstan-3,17-dione than it has for testosterone. Although the 6β methyl group markedly enhances activity, the reduction in activity by 6α-fluorine substitution and the abolishment of activity by the 6α-chlorine
substitution indicates that steric factors are important at this position in the steroid nucleus. In contrast to these steric effects the presence of a vinylic chlorine in the C-6 position as in 6-chloro-17-hydroxy-4,6-pregnadiene-3,20-dione acetate may actually enhance anti-tumor activity.

Rooks et al (902) also found that the 6α methyl as well as 6α-fluorine substitutions reduced anti-tumor activity in the steroid 17β-hydroxy-2α,17α-dimethyl-5α-androstan-3-one while unsaturation at carbon 1 increased activity. On the other hand, both 11β-hydroxy-6α-methyl-4-pregnane-3,20-dione as well as 9α-fluoro-11β-17α-dihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione 17 acetate are active against testosterone propionate-resistant tumors. In these compounds the 6α-methyl group reduces the salt retention caused by the 9α-fluorine substitution.

Glenn et al (367) found that 17β-hydroxy-2α-methyl-5α-androstene-3,11-dione was a potent anti-tumor steroid. They showed that the shift of the C-19-methyl group to the 2α position enhanced activity. Also, the 11β-hydroxy derivative of this compound was somewhat less active than the 11-ketone but was preferred because of its lesser side effects. Segaloff (962) also found that 11-ketones were more active than the corresponding 11β-hydroxy compounds while the reverse is true for anti-glycolytic effects (293).
The influence of methyl substitution in ring A on anti-tumor activity has also been investigated. De Marco et al. (224) found that 4,17β-dihydroxy-17α-methyl-4-estren-3-one was over twice as active as testosterone in inhibiting the growth of Ehrlick carcinoma in rats. Furthermore, both 17β-hydroxy-2α-methyl-5α-androstan-3-one and 17β-hydroxy-4α-methyl-5α-androstan-3-one are highly active against mammary tumors (902). Although an analogous methyl group is present in all ergot alkaloids, anti-tumor activity in these compounds has not been thoroughly studied.

Kim et al. (980) have described an anti-tumor triterpene from Wallenia yunguenis (Myrsinaceae) which was active against Walker 256 I M carcinosarcoma. This compound, known as myrsine-saponin (3β,27α-dihydroxyolean-12-ene-28-oic acid), has a structure similar to glycyrrhetinic acid which has been shown to have anti-inflammatory but not glucocorticoid effects (318, 418). The C-27α hydroxyl group in this compound is in a position similar to that of the N1 nitrogen in cla-vine alkaloids.

Other factors which may influence the potency of anti-tumor steroids are their solubilities in physiological fluids and their conjugation rates prior to excretion. Protein binding, especially as influenced by other drugs or endogenous metabolites, is also an important factor in activity.
Since Glenn et al (371) and Frawley (328) have shown that glucocorticoids inhibit pyruvate oxidation, it has been assumed that inhibition of glucose utilization is secondary to the inhibition of pyruvate oxidation. The basis of the block in pyruvate oxidation is the increase in long-chain fatty acyl coenzyme A which promotes fatty acid oxidation and an increase in the ratio of acetyl coenzyme A to free coenzyme A. The increase in acetyl coenzyme A not only inhibits pyruvate dehydrogenase but also increases the formation of cytoplasmic citrate which blocks glycolysis at the phosphofructokinase step.

Besides glucocorticoids, other non-steroid compounds have also been shown to inhibit pyruvate oxidation. Sarker (936) found that the alkaloid sanguinarine produced by Sanguinaria canadensis inhibited pyruvate oxidation by pigeon brain mitochondria 35 percent at a concentration of $4.1 \times 10^{-5} \text{M}$. Sublethal doses have been used to produce experimental glaucoma in animals. Furthermore, the compound is also a potent oxytoxic, 0.5 mg/kg produces prompt uterine contractions.

The inhibition of pyruvate oxidation by sanguinarine can be abolished by prior addition of mercaptoethanol to the suspension of pigeon brain mitochondria. A covalent sanguinarine derivative was formed but was not identified.
FIGURE VII
STRUCTURE OF SANGUINARINE

SANGUINARINE
Although sanguinarine contains pentavalent nitrogen, the extensive conjugation delocalizes the charge in the nucleus of the molecule. Furthermore, the base exists in two polymorphic crystalline forms and its quaternary salts are reddish in color. These properties suggest that labeled sanguinarine may find use in the affinity labeling of non-specific glucocorticoid sites of the type under discussion here. The thiol effect mentioned above is similar to that reported to occur in the glucocorticoid inhibition of yeast hexokinase (507, 508). In the latter case, treatment of the enzyme with N-ethylmaleimide prevents the reversal of glucocorticoid inhibition by insulin in vitro.

Hartwell (433) has reported that sanguinarine and its analogue chelerythrine have a necrotizing activity against sarcoma 37 in mice. The specificity of this effect has not been investigated but there are reports that Cherokee Indians used the bloodroot (Sanguinaria canadensis) for the treatment of warts and polyps as early as 1817 and for the treatment of breast cancer as early as 1857.

The total synthesis of sanguinarine has not been described and the best source of the compound is probably argemone oil as described by Sarkar (936).

The enhanced anti-mammary tumor activities of 6α-methylated progesterones appears to be due to the effects which
these compounds have on the pituitary. Glenn et al (368) concluded that both adrenal and gonadal functions were markedly inhibited by 6α-methylated steroids. This inhibition occurred in spite of the fact that the compounds had little or no effect on bone growth. These findings are similar to the results of a comparative study reported by Laron and Pertzelan (639). In fact, the suppressive effects of 6α-methylated hormones on pituitary function were so great that they persisted for approximately two weeks after the cessation of steroid administration. Liddle (663) observed that the 6α-methyl substitution increased the activity of cortisol and 11β-hydroxy-4-pregnene-3,20-dione, while it decreased the activity of 9α-fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione. In agreement with previous studies Liddle found that 11β-hydroxy-4-pregnene-3,20-dione increased the excretion of 17-hydroxycorticoids perhaps by competing with cortisol for feedback inhibition receptors which regulate ACTH release in the pituitary. Kleeman et al (596) also observed that the non-glucocorticoid 9α-fluoro-11β,17α-dihydroxy-6α-methyl-1,4-pregnadiene-3,20-dione had no effect on nitrogen excretion or eosinophiles but that it suppressed ACTH secretion and exacerbated diabetes at a dose of 30-80 mg per day. Lipsett and Bergenstal (669) treated Addisonian patients with 9α-fluoro-11β,17α-dihydroxy-6α-methyl-1,
4-pregnadiene-3,20-dione and found that the steroid had life-maintaining activity equal to that of cortisol.

In contrast to the adverse effects on the induction of mammary tumors, carcinogenic hydrocarbons have been shown to enhance the effects of anti-tumor steroids. Both Huggins et al. (492) as well as Glenn et al. (366) reported that 3-methyl-cholanthrene acted synergistically with anti-tumor steroids. Furthermore, Huggins et al. found that only those steroids which promoted the growth of the normal mammary gland acted synergistically with 3-methylcholanthrene. Alone, the latter compound was catabolic. Its structure is shown in Figure VIII.

A 17α-alkyl group may enhance anti-tumor activity in a rather specific manner. Whereas progestational activity is enhanced by 17α-(1-butynyl) groups, saturated alkyl groups larger than ethyl and ethinyl groups reduce anti-tumor activity. For example, 17α-ethyl-17β-hydroxy-4-estren-3-one is a powerful inhibitor of tumor growth while its 17α-ethinyl analog actually promotes tumor growth. The 17α-propyl group abolished activity. These findings may be related to those of Drill and Riegel (256) who found that 17α-ethinyl-17β-hydroxy-4-estrene-3-one had only 1 percent of the anabolic activity of the 17α-ethyl analog. The marked dependency of anti-tumor activity on the nature of the
FIGURE VIII

STRUCTURE OF 3-METHYLCHOLANTHRENE

3-METHYLCHOLANTHRENE
17α-alkyl group suggests that the ethyl group can conform by free rotation to the requirements of the receptor but that the more rigid ethinyl and larger propyl groups cannot.

At this point it may be helpful to clarify the effect of steroid substitutions on androgenic, anabolic, anti-pituitary and anti-tumor activity. The steroid 17β-hydroxy-6β-methyl-4-androsten-3-one has the highest reported androgenic/anabolic ratio. The C-3 carbonyl oxygen, C-4 double bond, and C-19 methyl groups increase androgenic activity. In contrast, the 17α-ethyl group reduces androgenic activity 6-fold, and the C-11 carbonyl oxygen 5-fold. On the other hand, the androstane derivative with the highest anti-pituitary/androgenic activity is a 2-formyl-2(5α)-androsten-17S-ol (582). The steroid with the highest anabolic/androgenic activity ratio is 17α-ethyl-17β-hydroxy-1(5α)-estrene-3,11-dione. The 17α-ethyl group increases anabolic activity and decreases androgenic activity. Unsaturated 17α-alkyl groups, such as the 17α-ethinyl group, decrease both activities.

The evaluation of anti-tumor steroids in vitro may not reflect therapeutic results because of the absence of the effect of the steroid on the pituitary. Inhibition of the release of pituitary hormones, especially prolactin and growth hormone, is important in anti-tumor activity. Pituitary inhibition would result in decreased prolactin output.
Since breast tumors in adrenalectomized-oophorectomized patients may be dependent on pituitary prolactin, reduction of prolactin secretion, in addition to glycolytic inhibition, appears to be the main action of anti-tumor steroids in these patients. In contrast, tumors which reactivate following an initial remission in response to steroids may be less dependent on prolactin so that steroids with a higher ratio of anti-glycolytic to anti-prolactin activity may be more effective against these reactivated tumors.

Although hypophysectomy eliminates prolactin output, growth hormone secretion, which appears to be important in the anti-glycolytic activity of steroids, is also abolished. The fact that hypophysectomy removes both tumor-stimulating as well as tumor-inhibiting factors may explain the lack of an effect of hypophysectomy in these patients. The recent availability of human growth hormone for substitution therapy may lead to increased use of hypophysectomy to eliminate prolactin output.

Dorfman (251) has shown that the efficacy of anti-tumor steroids is not related solely to pituitary inhibition. Dorfman's results are limited by the fact that he evaluated the parental activity of only those derivatives known to be orally active. He found that 17β-hydroxy-17α-methyl-4-androstene-3,11-dione was the most potent anti-tumor steroid.
investigated even though it has no anti-estrogen activity. From other studies it appears that 4-chloro-17α-hydroxy-2α-methyl-19-nor-4-pregnene-3,11-20-trione 17-acetate may have higher activity since some anti-tumor activity is sacrificed in order to obtain oral activity.

The 17α-alkyl group is not the only structural modification that enhances oral activity by blocking the oxidation of the 17β-hydroxyl group. For example, 17β-hydroxy-2α-methyl-1, (5α)-androst-3-one (stenbolone) has been patented as a potent, orally active anabolic agent. The finding that structural modifications in ring A influence enzymatic reactions in ring D and vice-versa indicates that the overall structure of the molecule and its metabolic reactions must be considered in the design of steroid drugs. Such findings raise the possibility of eliminating the hepatic changes associated with 17α-alkylated steroids.

Dorfman (251) has demonstrated that anti-estrogen activity as measured by the action of the compound in inhibiting the effects of estrogens on mouse uterus is not required for anti-tumor activity. For example, 17β-hydroxy-17α-methyl-1,4-androstadiene-3,11-dione was very active against testosterone-propionate-resistant tumors yet had no detectable anti-estrogen effect. Other C-4 unsaturated steroids shared this property. On the other hand,
5α-reduction resulted in compounds which had high anti-estrogen activity in addition to undiminished anti-tumor activity. Therefore, the structural requirements for anti-tumor activity are less stringent than those for anti-estrogen activity. Fluorine substitution in the 9α position increases anti-estrogen activity (1090) while 2α-fluorine substitution increases anti-pituitary activity (582).

Antoniades et al (15) have shown that human plasma fractions II and III which contain the β-lipoprotein and β- and γ-globulins respectively, preferentially bind the unconjugated steroids corticosterone, 11β-hydroxy-4-androstene-3,17-dione, and cortisol in that order (Table X). However, since most of the glucocorticoids in plasma are specifically bound by transcortin in fraction IV-I, the β-lipoprotein fraction is the fraction which contains most if not all of the 11β-hydroxy-4-androstene-3,17-dione in plasma.

Sandberg and Slaunwhite (931) further investigated the metabolism of this steroid and found that it existed in two pools which were cleared with half-lives of 30 and 80 minutes. Conjugates of the steroid, although rapidly formed, were slowly cleared. Approximately 61 percent of the excreted radioactivity from the steroid was excreted in conjugated form and this fraction had a half-life of 98 minutes. Furthermore, 14 percent of the conjugate fraction
TABLE X
PERCENT DISTRIBUTION OF ADRENAL STEROIDS IN HUMAN PLASMA FRACTIONS VERSUS TIME AFTER AN INTRAVENOUS TRACER DOSE

<table>
<thead>
<tr>
<th>Time Interval</th>
<th>Plasma Fraction (Method 6)</th>
<th>11β-Hydroxy-4-Andro-11β,17a,21-Trihydroxy-Stene-3,17-Dione</th>
<th>11β,21-Dihydroxy-4-Pregnene-3,20-Dione</th>
<th>11β,17α,21-Trihydroxy-4-Pregnene-3,20-Dione</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10 Minutes</td>
<td>20 Minutes</td>
<td>120 Minutes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Conjugated</td>
<td>Non-Conjugated</td>
<td>Conjugated</td>
</tr>
<tr>
<td>I</td>
<td></td>
<td>6.4</td>
<td>5.4</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.7</td>
</tr>
<tr>
<td>II + III</td>
<td></td>
<td>16.0</td>
<td>39.1</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14.4</td>
</tr>
<tr>
<td>IV-1</td>
<td></td>
<td>6.5</td>
<td>13.00</td>
<td>52.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27.6</td>
</tr>
<tr>
<td>IV-4</td>
<td></td>
<td>11.2</td>
<td>4.4</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.6</td>
</tr>
<tr>
<td>V</td>
<td></td>
<td>60.0</td>
<td>38.1</td>
<td>31.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>49.7</td>
</tr>
</tbody>
</table>

Antoniades et al (15)
could not be accounted for as either sulfate or glucuronide conjugates. This 14 percent, however, could be hydrolyzed with hot acid. These results suggest that at least one glucocorticoid metabolite may be associated with \( \beta \)-lipoprotein and may form unusual conjugates. Both the identity of the conjugated steroids and their role in insulin inhibition remain to be determined.

The steroid 11\( \beta \)-hydroxy-4-androstene-3,17-dione is formed from cortisol in a reaction that is promoted by estrogens (222). Also, thyroid hormone appears to increase the conversion of this steroid to 17\( \beta \)-hydroxy-5\( \alpha \)-androstene-3,11-dione by promoting its 5\( \alpha \)-reduction and 11\( \beta \)-hydroxy dehydrogenation (111). All of the intermediates in this process have been isolated from rat urine by Schubert and Wehrberger (954). In euthyroid subjects 11\( \beta \)-hydroxy-4-androstene-3,17-dione is metabolized as follows: 60 percent is converted to 3\( \alpha \),11\( \beta \)-dihydroxy-5\( \alpha \)-androstan-17-one; 10 percent is converted to 3\( \alpha \),11\( \beta \)-dihydroxy-5\( \beta \)-androstan-17-one; 6 percent to 3\( \alpha \),11\( \beta \)-dihydroxy-5\( \beta \)-androstan-17-one; and 3 percent to 3\( \alpha \)-hydroxy-5\( \alpha \)-androstene-11,17-dione. In myxedema, more 3\( \alpha \)-hydroxy-5\( \beta \)-androstene-11,17-dione is formed from 4-androstene-3,11,17-trione, indicating that the 5\( \alpha \)-reductase for the latter steroid as well as that for its 11\( \beta \)-hydroxy analog is also depressed. The extent
to which any of these alterations are involved in the hyper-β-lipoproteinemia of myxedema is unknown.

Distinct enzymes appear to be involved in the metabolism of each steroid. Tomkin (1070) observed that the enzyme which catalyzed the 5β reduction of cortisone to be inactive with cortisol and 11β-hydroxy-4-androstene-3,17-dione. The introduction of a double bond at C-1 completely blocks the reduction of cortisol but not the reduction of cortisone. This unsaturation, however, does not block the conversion of prednisolone to prednisone. This latter reaction is enhanced by 4-androstene-3,11,17-trione in which the 11α hydrogen of cortisol is transferred to the 11α position of the trione (257, 1194). The double bond in position 1 is reduced in vivo (1078), but it may be reduced after the double bond in the 4 position in some cases. Thus, the presence of the 1 double bond may enhance androgenicity in the androstene series while it decreases sodium retention in the pregnane series. Also, Slaunwhite and Sandberg (990) have found that the presence of the 1 double bond in glucocorticoids decreases their conversion to 17 ketosteroids. Similarly, Langecker (634) found that substituents in ring A influenced the rate of oxidation of the 17β-hydroxyl group in the androstene series. In order of decreasing rates of oxidation, the order is testosterone,
17β-hydroxy-5α-androstan-3-one, 17β-hydroxy-1,(5α)-androstan-3-one, and 17β-hydroxy-1-methyl-1,(5α)-androstan-3-one. In addition, Ringold (888) has pointed out that the opposite situation may occur, i.e. substituents in the 16 position can markedly alter the rate of oxidation of the 3α-hydroxy group by 3α-hydroxysteroid dehydrogenase. Clearly, these examples indicate that caution should be observed in extrapolating the metabolism of one steroid to that of another. Further complicating steroid metabolism are the effects which one steroid may have on the metabolism of another steroid. For example, Hagen and Troop (422) found that testosterone depressed the ring A reduction while accelerating the C-20 reduction of cortisone in both males and females. Cortisone acetate, on the other hand, depressed ring A reduction of cortisone only in males while it depressed C-20 reduction in both males and females. With cortisol Deckx et al (222) obtained more C-20 reduction in males and relatively more 4,5α reduction and 17-ketosteroid formation with females. Yates et al (1184) confirmed these findings, showing that female rats had from 3 to 10 times the rate of ring A reduction of cortisol as males had. The investigators Schriefers and Wassmuth (951) have shown that most of the increase in ring A reduction in females for cortisone is of the 4,5α type. Balieu and Maurais-Jaurvis
(51), however, reported that the ratio of $5\alpha$ to $5\beta$ reduction products of 17-ketosteroids depended on the oxidation of the substituent at position 17. Thus, the ratio was higher in women with the $17\beta$-hydroxy derivatives and higher in men with the $17$-keto derivatives. If one assumes that the same enzymes carry out the transformations in either sex, then the sexes must differ in the ratio of the enzymes which act on the $17\beta$ hydroxyl and 17 keto steroids. The rate of ring A reduction is important because this is the rate-limiting reaction in steroid metabolism (952). Female rat liver has also been shown to convert more cortisol to $11\beta$-hydroxy-4-androstene-3,17-dione (222).

Although it is possible to determine the extent to which any of these steroids bind to purified $\beta$-lipoprotein using the technique of equilibrium dialysis, it is unlikely that these studies alone will uncover the mechanism of anti-insulin effect of the lipoproteins. Bornstein (98) found that the addition of cortisone to lipoproteins in vitro did not result in insulin antagonism like that produced when cortisone was injected into the rat before blood lipoproteins were isolated.

Effects of Clavine and Morphine Alkaloids

Clavine alkaloids are formed by the fungus *Claviceps purpurea* which is parasitic on Elymus mollis (rye) or on
pennisetium typhohordeum. The hormone-like potency of d-lysergic acid diethylamide has prompted research on these alkaloids in an effort to find other active compounds. For example, ergonovine, more correctly termed ergometrine [N-(a-(hydroxymethyl)-ethyl)-d-lysergamide], is derived from the naturally occurring compound ergotamine. Ergotamine has the same nucleus as ergonovine (d-lysergic acid), but also has a cyclic polypeptide side chain which is derived from the amino acids alanine, phenylalanine, and proline. The polypeptide side chain is required for α-adrenergic blocking activity which is characteristic of ergotamine and dihydroergotamine. Thus, ergotamine will block epi-nephrine-induced contractions of the isolated rabbit uterus, but ergonovine will not. The C-9 double bond enhances the vasoconstrictive and oxytocic actions of ergonovine. This double bond may be shifted to C-8 as in argoclavine with retention of both properties. The structures of ergonovine and argoclavine are shown on the following page (948, 1019). In addition, the structure of elymoclavine is shown because it is also an important naturally occurring clavine alkaloid.

Yanai and Nagasawa (1182) have shown that 2-brominated ergot alkaloids suppress mammary lobulo-alveolar formation and decrease the frequency of mammary pre-cancerous
FIGURE IX
STRUCTURE OF ERGONOVINE

ERGONOVINE
FIGURE X

STRUCTURES OF AGROCLAVINE AND ELYMOCLAVINE

\[ R = CH_3 \quad \text{AGROCLAVINE} \]

\[ R = CH_2OH \quad \text{ELYMOCLAVINE} \]
TABLE XI

PHYSICAL PROPERTIES OF CLAVINE ALKALOIDS RELATED TO STEROIDS

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Configuration</th>
<th>Melting Point</th>
<th>Specific Rotation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agroclavine</td>
<td>(5α), 8--Methyl-8-Ene</td>
<td>205-206</td>
<td>-182</td>
</tr>
<tr>
<td>Costaclavine</td>
<td>(5β), 8α-Methyl</td>
<td>182</td>
<td>+44</td>
</tr>
<tr>
<td>Elymoclavine</td>
<td>(5α), 8--Hydroxymethyl-8-Ene</td>
<td>248-252</td>
<td>-59</td>
</tr>
<tr>
<td>Festuclavine</td>
<td>(5α), 8α-Methyl</td>
<td>203-204</td>
<td>-90</td>
</tr>
<tr>
<td>Isolysergine</td>
<td>(5α), 8β-Methyl-9-Ene</td>
<td>134-137</td>
<td>+203</td>
</tr>
<tr>
<td>Lysergine</td>
<td>(5α), 8α-Methyl-9-Ene</td>
<td>288-289</td>
<td>+64</td>
</tr>
<tr>
<td>Lysergene</td>
<td>(5α), 8--Methylene-9-Ene</td>
<td>244-245</td>
<td>+407</td>
</tr>
<tr>
<td>Pyroclavine</td>
<td>(5α), 8β-Methyl</td>
<td>243-244</td>
<td>-109</td>
</tr>
</tbody>
</table>

Ultraviolet Absorption Maxima (nm)

- Unconjugated........... 225, 284, 293,
- Conjugated............. 225, 240, 284, 293, 315,
- Lysergene............... 225, 243, 263, 335
hyperplastic aleovolar nodules by decreasing prolactin output from the pituitary. Knew-Hseung et al (599) have also suggested that ergocornine directly blocks estrogen-stimulated prolactin release. These effects resemble the inhibition of prolactin release by high levels of estrogens.

The enhancement of anti-tumor activity by 4-methylation and 2α-formylation in the steroid series has been previously noted (251, 902). The analogous modifications in the clavine alkaloids are N⁶-methylation (present in all ergolanes) and 8α-formylation. Another important modification is the introduction of bromine in the C-2 position. Savine (939) found that the 2-brominated derivative of d-lysergic acid diethylamide was a more potent antagonist of serotonin and catecholamines even though it was devoid of vasoconstrictive and psychomimetic effects. Berde (66) has reviewed the comparative effects of these compounds. These effects are of interest because Gay and Pletscher (364) have reported that serotonin elevates blood pyruvate and lactate when administered systemically.

Elymoclavine, 8-hydroxymethyl-8-ergolene, or its N¹-methyl or 2-bromo derivative may show some anti-tumor activity. This should be increased by the oxidation of the primary hydroxyl group to the aldehyde with dicycloheximide and dimethylsulfoxide yielding 8-formyl-6-methyl-8-ergolene:
Elymoclavine is 8-hydroxymethyl-6-methyl-8-ergolane. The corresponding saturated derivative might be prepared by the hydroboration of agroclavine. Brown et al (124, 125) have described the conversion of α-pinene to β-pinene in 54 percent yield via hydroboration. This result suggests that the endocyclic C-8 double bond of agroclavine may be hydroborated with diisocampheylborane, and the product either oxidized to 8α-hydroxymethyl-6-ergolane or hydrolyzed with acetic acid to festuclavine. The same compound might also be made from dihydroergotamine following hydrolysis and reduction of the carboxyl group.

The preparation of ergonovine from ergotamine via the hydrazide is accompanied by racemization to isolysergic acid hydrazide due to the acidic hydrogen at C-8. The possibility of preventing this racemization by protecting the
c-9 double bond does not seem to have been investigated. This double bond might be protected by the formation of its cis glycol with hydrogen peroxide using osmium tetroxide as catalyst. Following the formation and reaction of hydrazide intermediates the double bond can be regenerated by the procedure of Corey and Winter (197). This procedure involves the reaction of the glycol with N,N'-thiocarbonylimidazole to form the cyclic thiocarbonate. The carbonate is then treated with trimethyl phosphate to regenerate the double bond with the formation of trimethylthiophosphate and carbon dioxide.

Kornfield et al (608) have described the total synthesis of 2,3-dihydro-6-methyl-9-ergolen-8-one [9-keto-7-methyl-4,5,5α,6,6α,7,8,9-octahydroindolo-(4,3-f,g)-guinoline]. The keto group in the above compound may be replaced by a methylene group by the use of the Wittig reagent methylene diphenylphosphorane. The ethyl reagent has been used to introduce the side chain into 17 ketosteroids. The resulting lysergic derivative can be converted to the 8α-methyl or 8α-hydroxymethyl compound by the method of Brown et al (124). This, in turn, can be directly converted to the C-12 hydroxy indole with potassium nitrodisulfonate as described by Stadler et al (1020). The corresponding C-12 hydroxy ergot alkaloids are not found in nature because the enzyme
involved in the initial biosynthetic step cannot utilize 5-hydroxytryptophan in place of tryptophan.

On the other hand, Jacobs and Gould (520) have reported the total synthesis of racemic 6,8-dimethylergolane by a method that can be modified to permit the synthesis of the 12-hydroxy derivative. However, they did not compare the particular diastereomer which they isolated with authentic festuclavine and the optical rotation which they reported indicates that their product was contaminated by other diastereomers. Esterification of the C-12 hydroxyl group with an optically active acid such as that derived from β-pinene by hydroboration offers the possibility of asymmetric induction in the subsequent reductive steps.

Ergot alkaloids have been shown to block hepatic glycogenolysis in response to cyclic adenylate (432). Ergonovine may act as a glucocorticoid analog in adrenalectomized rats because it is able to block ACTH release. Rezabeck et al (882) have shown that 8α-cyanomethyl-6-methyl-ergolane has hormone-like activity. When administered at a dose of 1 mg/kg daily, the compound prevented conception. When administered at 10 mg/kg daily, the compound caused abortion up to the 7th day after copulation. The structure of the alkaloid is similar to 2α-cyano-17β-hydroxy-4,4,17α-trimethyl-5-androsten-3-one, an inhibitor
of pituitary function and steroidogenesis in the rat (582). Related ergolanes may also be active against mammary tumors.

Structure-function studies have indicated that other modifications besides 2-bromination may decrease the action of clavine alkaloids on smooth muscle. In particular, methylation of the adjacent N-1 nitrogen significantly reduces the oxytoxic and vasoconstrictive actions of these compounds. The best known compound of this type is Sansert [N-(hydroxy-methyl)propyl]-l-methyl-d(+)-lysergamide. This compound has found application in the treatment of migraine headache (983).

Migraine headache is believed to be caused by a genetic deficiency of monamine oxidase in the brain which results in the accumulation of tyramine and 5-hydroxyindoleacetic acid formed from serotonin. The vasodilation produced by the latter compound induces the headache. Sansert is believed to act by inhibiting the formation of 5-hydroxyindoleacetic acid and it does not share the vasoconstrictive properties of other clavine alkaloids (66).

Although the treatment of migraine with Sansert is effective, the treatment can produce a condition known as retroperitoneal fibrosis in about 1 out of 10,000 patients taking the drug. Retroperitoneal fibrosis is a disease in which pulmonary and aortic complications resemble those seen in rheumatoid disorders. This is not surprising
FIGURE XI

SUGGESTED SYNTHESIS OF 6,8-DIMETHYLERGOLAN-12-OL

PART I

SYNTHESIS OF 6,8-DIMETHYLERGOLAN-12-OL
FIGURE XII

SUGGESTED SYNTHESIS OF 6,8-DIMETHYLERGOLAN-12-ol

PART II

SYNTHESIS OF 6,8-DIMETHYLERGOLAN-12-OL (CON'T)
since serotonin is believed to be involved in the inflammation reaction. Graham (404) has also reported endocardial lesions resembling those found in the carcinoid syndrome. Although these changes are partially reversed when the drug is withdrawn, some changes persist.

Changes of particular interest during retroperitoneal fibrosis include thrombophlebitis and fibrotic obstruction in the great vessels and lymphatics as well as in the ureters and bowel. These early lesions involve the infiltration of fat by lymphocytes followed by plasma cells and then fibrosis. These changes can lead to pleural thickening in the lung causing friction rub. The great vessel changes appear grossly similar to the changes that take place during atherosclerosis.

A similar type of cystic degeneration and its relation to mammary cancer has been described by Davis et al (216). They showed that women with cystic disease have 26 times more cancer of the breast than do women in general. Moreover, biopsy tissue removed during surgery revealed an 18 percent incidence of malignant degeneration. The chance of developing lesions in the breast remaining after partial mastectomy is about 20 percent. For this reason, a complete mastectomy is often done initially. The important point is that the superficially normal
breast removed during the complete operation affords an opportunity to study those biochemical and histological changes associated with the precancerous state. The fact that ergot alkaloids can produce similar and somewhat reversible changes similar to those found in cystic disease suggests that the alkaloid may be mimicking the effect of an endogenous metabolite.

Other similarities appear to exist between ergot alkaloids and steroids. Zellwige et al (1196) have described the teratogenic effect of lysergic acid diethylamide in a single case of unilateral fibular aplastic syndrome in a child born to parents who had taken the drug. Chromatid breaks were observed in the white cells of mother, father and child. The relationship between these breaks and the production of retroperitoneal fibrosis during migraine therapy with Sansert \([N-(\text{hydroxymethyl})-\text{propyl}-1-\text{methyl-}
\text{d}(+)\text{-lysergamide}]\) is not clear.

Takano et al (1047) observed more fetal malformations in pregnant rabbits given \(1\ \text{mg/kg}\) chlormadinone acetate than in those given 1 percent mestranol in norethisterone. Also, Carr (160) has described a higher incidence of spontaneous abortions with progestins due to the formation of triploid chromosomes. However, only 2.5 percent of XO abnormalities came to term as female infants with Turner's
syndrome.

While these and other findings may possibly explain the recent withdrawal of chlormadinone acetate from the market, genetic effects do not appear to be involved in the anti-insulin effects of these compounds.

Bergen et al (67) also found that glucocorticoids may compete with lysergic acid diethylamide for some binding sites. Cortisol, cortisone, corticosterone, deoxycorticosterone and progesterone at $10^{-7}$ Molar inhibited the metabolism of the alkaloid by 88, 88, 73, 62 and 20 percent respectively.

Morphine may also share some receptor sites with serotonin, glucocorticoids and estrogens. Gaddum and Picarelli (351) described two types of serotonin receptors in intestine. The M receptors could be blocked by morphine and were mainly found in nerves. The D receptors, on the other hand, were blocked by dihydroergotamine and were found mainly in muscle. The anesthetic, muscle-relaxant, and respiratory depression produced by morphine may be due to inhibition of serotonin.

Pee Ping and Walsh (824) found that morphine increased glucose uptake in diaphragms from normal rats but decreased glucose uptake in diaphragms from morphinized rats. Cortisol antagonized the effect of morphine. On the other hand,
morphine antagonized cortisol-stimulated respiration but enhanced the effect of cortisol on glycogenesis.

In further studies Ng and Walsh (783) found that either epinephrine or cortisol reduced glucose uptake by rat diaphragm. When epinephrine was added first, the secondary addition of cortisol increased glucose uptake. However, if the diaphragms were from chronically morphinized rats, the addition of epinephrine had no effect and cortisol increased glucose uptake. This effect of cortisol alone was abolished if epinephrine was also added. In addition, these workers (784) found that the block in glucose uptake by epinephrine in normal diaphragm was restored to morphinized diaphragm in the presence of morphine. These results indicate that cortisol and morphine have similar effects on normal and morphinized diaphragms respectively. Further analysis of the results suggests that morphine antagonizes the inhibitory effect of cortisol upon glucose transport. Also, most glucocorticoid sites in morphinized tissue, including that of the growth hormone-dependent protein which acts as a glucocorticoid receptor and leads to citrate elevation, is occupied by morphine in morphinized tissues. Furthermore, the morphine-protein complex appears to be more effective than the glucocorticoid-protein complex in increasing cytoplasmic citrate. Therefore, when cortisol
displaces morphine, citrate is lowered and glucose uptake is increased. These results suggest that citrate elevation in response to morphine is dependent on growth hormone and may be abolished by hypophysectomy.

Levy and Ramey (657) have shown that ergotamine, like morphine, inhibits ACTH release and fat mobilization in adrenalectomized rats. On the other hand, Harvey et al. (432) showed that ergonovine inhibits epinephrine-induced hyperglycemia and that dihydroergotamine treatment actually increased hepatic glucose uptake in response to cyclic adenylate. These results suggest that clavine alkaloids block the effects of cyclic adenylate on phosphorylase b kinase and on transferase I kinase. These latter effects are directly opposite to the enhancement of cyclic adenylate effects by cortisol. Therefore, it appears that clavine alkaloids block the effects of cortisol at specific receptor sites but not at relatively non-specific sites.

Anti-tumor activity appears to be closely related to but distinct from progestational activity. Rooks et al. (902) have observed that 9α-fluoro-6α-methyl-11β,17α-dihydroxy-4-pregnene-3,20-dione 17-acetate caused little or no inhibition of glycine uptake in fibroadenoma in rats. Glenn et al. (368) made similar observations with the corresponding C-1 unsaturated analog but also observed that it
produced a 73 percent inhibition of a testosterone propionate-resistant fibroadenoma. Dorfman (251) observed an 87 percent inhibition of an adenocarcinoma with the C-1 unsaturated analog. He further noted that 11β-hydroxy-6α-methyl-4-pregnene-3,20-dione caused a 48 percent inhibition of fibroadenoma while Glenn et al (368) reported that 17α-hydroxy-6α-methyl-4-pregnene-3,20-dione 17 acetate inhibited fibroadenoma potently but actually stimulated a testosterone propionate-resistant fibroadenoma. In summary, these findings indicate that an oxygen at carbon 11 is required for the inhibition of testosterone propionate-resistant tumors. Apparently, the oxygen at C-11 blocks the conversion of testosterone to estradiol by these resistant tumors. The 9α-fluorine substitution abolishes inhibition of fibroadenomas. The fact that the 2α-methyl substitution abolishes progestational activity suggests that 17-acetoxyla-methyl-19-nor-4-pregnene-3,11,20-trione may show anti-tumor activity. The metabolism of 2α-methyl steroids, especially C-11 oxygenated glucocorticoids, has been discussed by Bush and Mahesh (142).

As shown in Table XII, the steroid 9α-bromo-4-pregnene-3,11,20-trione is a C-11 oxygenated steroid with a high ratio of progestational to glucocorticoid activity. It is noteworthy that Huggins et al (490) found that this
TABLE XII

GLUCOCORTICOID AND PROGESTATIONAL ACTIVITIES OF SUBSTITUTED PROGESTERONES

<table>
<thead>
<tr>
<th>Substituted Progesterone</th>
<th>Progestational*</th>
<th>Glucocorticoid**</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>9α-Br, 11-OH</td>
<td>0.12-0.18</td>
<td>0.08</td>
<td>1.5-2.3</td>
</tr>
<tr>
<td>9α-Cl, 11-OH</td>
<td>0.06</td>
<td>0.35</td>
<td>0.16</td>
</tr>
<tr>
<td>9α-Fl, 11-OH</td>
<td>0.18-0.30</td>
<td>0.85</td>
<td>0.22-0.36</td>
</tr>
<tr>
<td>9α-Br, 11-=O</td>
<td>0.18-0.30</td>
<td>0.03</td>
<td>6.0-10.0</td>
</tr>
<tr>
<td>9α-Cl, 11-=O</td>
<td>0.06</td>
<td>0.15</td>
<td>0.60</td>
</tr>
<tr>
<td>9α-Fl, 11-=O</td>
<td>0.12-0.18</td>
<td>1.10</td>
<td>0.10-0.16</td>
</tr>
<tr>
<td>9α-Br-11β-OH-17α-OCOCH₃***</td>
<td>1.75-3.50</td>
<td>5.0****</td>
<td></td>
</tr>
<tr>
<td>6α-CH₃, 17α-OCOCH₃</td>
<td>10</td>
<td>20 ****</td>
<td></td>
</tr>
<tr>
<td>17α-OCOCH₃</td>
<td>0.07-0.15</td>
<td>0.2****</td>
<td></td>
</tr>
</tbody>
</table>

*Oral Clauberg Assay. 17α-ethinyl-17β-hydroxy-4-estren-3-one = 1.0

Miyake and Rooks (751)

**Liver Glycogen Deposition. Cortisol = 1.0

Fried and Borman (333)

***Bergström and Nicholson (68)

****Subcutaneous Clauberg Assay. Progesterone = 1.0
steroid markedly enhanced the anti-tumor effects of estrogens as well as the carcinogenic and catabolic effects of 3-methylcholanthrene. These results are in agreement with clinical studies in which 17α-hydroxyprogesterone caproate was found to enhance the anti-tumor activity of pharmacological doses of estrogens (490). It appears that estrogens should always be administered in combination with progestins for maximal anti-tumor activity.

In summary, it appears that the anti-tumor effect of 9α-bromo-4-pregnene-3,11,20-trione is greater in combination with estrogens. While it is unknown whether C-19 progestins are also synergistic with estrogens, it appears that the function of the C-11 oxygen is not to prevent the progestin from acting as an estrogen precursor. Finally, further work will be needed to elucidate the mechanism of the direct anti-tumor effects of steroids which are independent of other hormones.
CHAPTER XII
MATERIALS AND METHODS

Tables XIII and XIV list the major chemicals used according to their sources. Other chemicals used but not specifically listed were of reagent grade. Solutions were prepared in deionized, glass-distilled water. The melting points listed were determined between cover glasses using a Fisher melting point apparatus and are uncorrected.

Since one of the main purposes of this work was to compare the effects of fluprednisolone and 20β-hydroxy-fluprednisolone, it was necessary for the author to synthesize the latter compound from the former. Norymberski and Woods (790) have described a technique for the selective reduction of steroid C-20 carbonyl groups which employs a 1.5 molar excess of sodium borohydride at 0°C for 1 hour. Their technique was employed to synthesize the 6α-fluoro-11β,17α,20β,21-tetrahydroxy-1,4-pregnadien-3-one used in this study. The reduced steroid was separated from fluprednisolone by preparative thin-layer chromatography.
## TABLE XIII
### CHEMICALS

**Abbott Laboratories**

Penthrane<sup>R</sup> (methoxy flurane) - 2,2-dichloro-1,1-difluoroethyl methyl ether,  
List No. 6864

**Amersham/Searle**

D-Glucose-6-<sup>14</sup>C, 52.4 mCi/mMole, Number CFA 351, Lot 10  
Hexadecane-1-<sup>14</sup>C, 2220 dpm/mg

**Cal-Atomic**

D-Glucose-6-<sup>14</sup>C, 10 mCi/mMole, Lot 78101

**International Chemical Company**

4-androstene-3,11,17-trione, Lot 439211  
3α-hydroxy-5α-androstene-11,17-dione, Lot 19β-0330  
3β-hydroxy-5β-androstene-11,17-dione, Lot 19β-1580  
3β-hydroxy-5α-androsten-17-one, Lot 57β-0480  
3β-hydroxy-11-oxoolean-12-ene-30-oic acid (glycyrrhetic acid),  
mp 155-161°C  
21-hydroxy-4-pregnene-3,20-dione acetate, Lot 4097, mp 137°C  
11β,17α,20α,21-tetrahydroxy-4-pregnene-3-one 21 acetate, Lot 023199

**Mann Research Laboratories**

17α,21-dihydroxy-5β-pregnene-3,11,20-trione, Lot T207  
11β,17α,21-trihydroxy-4-pregnene-3,20-dione, Lot T3305  
11β,17α,21-trihydroxy-5β-pregnene-3,20-dione, Lot T1828
TABLE XIV
CHEMICALS

Nutritional Biochemical Company

Diabetic Rat Diet, Lot TD-68580, Composition:

3% Sodium-free salt mixture
5% Safflower oil
1% Vitamin fortification mixture, complete
55% Casein, high protein
5% Non-nutritive fiber
21% D-fructose
10% Sodium succinate .6 H₂O

Sigma Chemical Company

17α,21-dihydroxy-5β-pregnene-3,11,20-trione 21 acetate, Lot 95β-2390-1
3α-hydroxy-5α-androstan-17-one, Lot 28β-2430
3α-hydroxy-5β-androstan-17-one, 98%, Lot 127β-2500
3β-hydroxy-5β-androsten-17-one, Lot 97β-0330
3β-hydroxy-5-androsten-17-one, Lot 18β-3030

Upjohn Pharmaceuticals

6α-fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione
(fluprednisolone), Lot WA 701, m p 206-208°C, acetate,
m p 235-238°C
on 20 cm x 20 cm glass plates coated with silica gel G-254 and fluorescent indicator (Brinkman Instruments). Several solvent systems were satisfactory for this purpose. Among these were the upper phase of tertiary amyl alcohol, anisole, water (190/600/10, R_f 0.12 and R_f 0.42 respectively); the system 1,8-epoxy-p-menthane, n-propanol, water (100/20/5, R_f 0.23 and R_f 0.59 respectively) and the system methylcyclohexane, n-propanol-water (75/25/5) where the separation was equivalent. It should be noted that this latter solvent system is similar to the ternary minimum boiling azeotrope of methylcyclohexane, ethanol, water (66/24/10, b p 66°C). The reduced 20β-hydroxyl derivative was detected by its quenching of the fluorescence of the phosphor in the silica gel. Therefore, if reduction of the conjugated C-3 ketone occurred, the resulting compound would not have been detected. As expected, the more polar 20β-hydroxyl derivative migrated slower than the parent steroid. Zones which contained the steroids were located under ultraviolet light and scraped from the plate into a beaker containing methanol. After thorough mixing, the methanol was passed through a fine sintered glass filter and flash evaporated to dryness under reduced pressure. Recrystallized 20β-hydroxyfluprednisolone had a melting point of 188-192°C compared to 206-208°C for fluprednisolone. The diacetate of the
reduced derivative was also prepared with acetic anhydride and pyridine and it had a melting point of 210°C. As far as the author could determine, the melting points for these reduced derivatives have not been reported.

While on the subject of steroid chromatography, it should be noted that cortisol and fluprednisolone migrate together in such solvent systems at t-amyl alcohol, chlorobenzene, water (150/590/10, \( R_f \) 0.18), 95 percent ethanol-water saturated with cyclohexane (\( R_f \) 0.227); in 95 percent ethanol-water, benzotrifluoride water (115,360,25, \( R_f \) 0.226), and in cyclopentanol, dibromomethane water (10/10/1, \( R_f \) 0.81). Thus, these steroids and possibly their metabolites would be most difficult to separate in studies of the metabolism of the labeled hormones. Other considerations suggest that these steroids may be separated as C-21 esters in the ternary azeotrope of cyclooctane, n-propanol-water.

Infrared spectra were recorded using a double-beam Perkin-Elmer 337 grating spectrophotometer. The resolution obtained with this instrument is better than that in spectra found in the literature so that direct comparisons are not possible. The steroid spectra were recorded using redistilled carbon disulfide (odorless) as solvent in 2.0 mm sodium chloride cavity cells manufactured by Barnes Engineering, Inc. Solvent evaporation was excessive when the
flexible plastic stoppers supplied with these cells were used. They were replaced with solid teflon stoppers ground from a teflon rod with the aid of a pencil sharpener. However, since excessive sealing pressure on the stoppers resulted in the splitting of the cavity cells, solvent evaporation during the recording of spectra had to be routinely monitored and sealing pressure increased slightly if necessary.

Infrared spectra were recorded on transmittance paper so that the transmittance of the solvent could be subtracted from that of the solvent plus solute in the longer path length cell. That is, the blank cell did not completely compensate for the infrared absorption of the solvent. Still, there were negative absorbance regions due to the displacement of solvent by solute in regions of high solvent absorption. The same set of cells was used for all spectra in the 1300-400 cm\(^{-1}\) region. The cells cleaned with methylene chloride and carbon disulfide and air-dried before use. The scanning time was 72 minutes and the slit program was one-tenth normal slit width. Melting points were determined between cover glasses on a Fisher melting point apparatus and are uncorrected. The solution spectroscopy of steroid esters was chosen because it eliminates hydrogen bonding and polymorphism associated with the spectra of solid
hydroxylic steroids. Although steroid acetates are suitable for this purpose, steroid benzoates have better chromatographic properties (121). Furthermore, the ultraviolet absorption of the benzoyl group facilitates the collection of chromatographically separated steroid peaks.

Krahl et al (365) have demonstrated the effect of the rat β-lipoprotein insulin antagonist in vitro on rat diaphragm but no one has yet evaluated its action in vivo. A very sensitive in vivo assay of insulin in fasting alloxan-diabetic adrenalectomized rats has been described by Bornstein and Trewhella (95). However, it is a two-point assay based on the drop in blood glucose produced by insulin in exactly one hour at 37°C. Insulin antagonism counteracts the drop in blood glucose. In order to overcome the limitations of the two-point assay, multipoint assays have been devised in which the specific activity of expired CO₂ is measured at intervals during the infusion of labeled glucose (220, 311, 1029). Since the specific activity of expired CO₂ approaches that of the infused glucose in approximately 4 hours, all measurements must be made in this interval. Demonstration of glucocorticoid-dependent insulin inhibition by lipoproteins in vivo is essential to the concept of inherited insulin antagonism in diabetes mellitus.
In these experiments β-lipoproteins isolated from the sera of steroid-treated donor rats are administered to recipient rats and the effect of the lipoprotein on the oxidation of glucose-6-$^{14}$C to carbon dioxide is measured. Steroid contamination was minimized by withholding steroid treatment for 6 hours before lipoprotein isolation as well as by the washing step applied to the precipitated lipoprotein during its isolation.

Male Sprague-Dawley rats weighing approximately 175 grams were obtained locally. The following procedure was used to produce alloxan-diabetes. First, the rats were fasted about 24 hours to deplete hepatic glycogen. Secondly, a solution of 65 mg/ml of alloxan monohydrate in saline was injected intravenously at a dose of 1.0 ml/kg. The animals were then given food ad libitum. Approximately two-thirds of the rats developed a 24-hour fasting glucose in excess of 2.0 gm/l within 2 days after alloxan administration. However, one-half of the diabetic animals died and one-third of the original number of rats did not become diabetic according to the above criterion. Insulin and electrolyte therapy were not used because such treatment would have increased the variation between rats. All rats having fasting glucose levels less than 2.0 gm/l were recycled and subsequently received 1.5 ml/kg of alloxan.
The wide variation in the response of individual rats to the standard dose of alloxan remains unexplained. However, the dose of 65 mg/kg appeared optimal with respect to the yield of viable alloxan-diabetic rats.

Acidosis was associated with obesity in the alloxan-diabetic rats. Also, growth was self-limiting in the diabetic rats in that it tended to exacerbate the insulin deficiency. However, those alloxan-diabetic rats that remained alive for at least one week were adrenalectomized under pentobarbital anesthesia. An abdominal approach was used in which both kidneys and adrenals were clearly visible. This approach insured complete adrenalectomy. All rats were injected with 10 mg/kg of 6a-fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione to prevent hypoglycemia during the immediate postoperative period. Also, the rats were transferred to a low glucose diet high in fructose and protein with glycyrrhetinic acid in saline as drinking fluid. This diet is described in detail on page 332.

Adrenalectomy produces an approximate reduction of 50 percent in serum glucose. That is, the severity of diabetes is reduced by this amount. Afterwards it is possible to further reduce insulin secretory capacity by administering additional alloxan to raise the glucose levels to pre-adrenalectomy levels. Those adrenalectomized rats
who had fasting glucose levels less than 400% mg prior to adrenalectomy were again given the standard dose of 65 mg/kg of alloxan intravenously. Then the fasting serum glucose was again determined to select those rats having 6-hour fasting glucose levels of at least 200% mg when maintained on the low-glucose diet. These rats could not be fasted for more than 6 hours without risk of death from spontaneous hypoglycemia. This procedure is listed stepwise on the following pages. It results in the preparation of severely diabetic rats without insulin treatment.

**Experimental Procedure**

1. Obtain 180-gram male Sprague-Dawley rats locally and maintain them on a standard laboratory diet for 2 days.
2. Fast all rats for 24 hours, then inject 65 mg/kg of alloxan monohydrate in saline into the tail vein.
3. Feed all rats ad libitum for 2 days.
4. Fast all rats again for 24 hours.
5. Anesthetize the rats with 20 mg/kg sodium pentobarbital and obtain 400 µl samples of tail blood.
6. Measure serum glucose with a glucose oxidase method.
7. Reject as non-diabetic all rats with serum glucose less than 2.0 gm/l.
8. Feed the remaining diabetic rats ad libitum for at least 1 week.

10. Inject adrenalectomized rats with 10 mg/kg fluprednisolone post-operatively and then transfer them to the low glucose diet and the saline-glycyrrhetinate drinking fluid.

11. Allow at least 1 week for recovery from adrenalectomy.

12. Fast rats having pre-adrenalectomy glucose values between 2.0 and 4.0 gm/l for 6 hours and then inject 65 mg/kg of alloxan monohydrate as previously described.

13. Feed all rats ad libitum for 2 days.

14. Fast all rats for 6 hours and measure serum glucose as previously described.

15. Reject as non-diabetic all rats having serum glucose levels less than 2.0 gm/l.

16. Group the diabetic rats in pairs on the basis of weight and fasting serum glucose. Designate the heavier of the pair the donor rat and the lighter the recipient rat.

17. Inject the donor rat with 10 mg/kg of either 6α-fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione or 6α-fluoro-11β,17α,20β,21-tetrahydroxy-1,4-pregnadiene-3-one in propylene glycol subcutaneously at 48, 24 and 6 hours before exsanguination via the abdominal aorta.

18. Fast the donor rat for 6 hours prior to exsanguination.
19. Exsanguinate the donor rats under methoxyflurane anesthesia. At the same time begin to fast the recipient rat for 6 hours.

20. Transfer the blood from a 10 cc syringe to a 25 ml plastic International centrifuge (PR-2) tube and centrifuge for 10 minutes at 8,000 x g at 0°C using the high speed attachment and head number 298.

21. Remove the serum from the clot with a clean calibrated syringe and needle.

22. Precipitate the serum β-lipoproteins with dextran sulfate and calcium chloride as described in the text.

23. Wash the β-lipoprotein precipitate with calcium acetate.

24. Dissolve the precipitate in 1.0 ml of tris(hydroxy-methyl)-aminomethane-EDTA buffer, pH 9.1 that has been saturated with blue dextran.

25. Equilibrate Bio-Gel DM-100 (a polyacrylamide sieving gel containing diethylaminoethyl anion exchange groups) with isotonic tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.40 at 37°C and pack it into a column 2.0 x 30 cm.

26. Apply the blue lipoprotein solution in a narrow zone at the top of the above column and elute it with the isotonic buffer which has a pH of 7.8 at 0-4°C.

27. Collect 0.5 ml fractions and pool the 4 fractions.
having the highest concentration of blue dextran and hence of the \( \beta \)-lipoprotein which is excluded from the gel with it.

28. Inject 2.0 ml of the \( \beta \)-lipoprotein solution intraperitoneally into the recipient rat anesthetized with penthrane.

29. Inject the recipient rat with a priming dose of 50 \( \mu \)Ci/kg of glucose-6-\( ^{14} \)C. Then follow with a constant infusion of insulin and glucose-6-\( ^{14} \)C (0.05 \( \mu \)Ci/mmole) as described in the text.

30. Collect 5 samples of \( ^{14} \)CO\(_2\) of 45-minutes duration each directly in scintillation vials as described in the text.

31. Terminate the experiment after 225 minutes in one of two ways: 1) Place the rat in a cage under a laboratory hood for 1 week so that the rat may return to background radioactivity levels prior to receiving lipoproteins from rats treated with other steroids; 2) Following the second experiment with the recipient rat place him in liquid nitrogen in preparation for liver and muscle glycogen analysis.

32. Measure the specific activity of the expired \( ^{14} \)CO\(_2\) with a liquid scintillation counter using an open window.
33. Plot the specific activity of the $^{14}$CO$_2$ versus time and fit the best straight line to these points which also passes through the origin. The slope of this regression line is then taken as a measure of the rate of glucose oxidation. The details of the statistical treatment have been described by Sokal and Rohlf (1001).

Serum glucose was determined with a commercial glucose oxidase reagent (Glucostat Regular, Worthington Biochemical Corporation). The lyophilized reagent was reconstituted in 30 ml of 0.10 M tris(hydroxymethyl)aminomethane-maleate buffer, pH 6.9, instead of 83 ml of water in order to provide a greater excess of enzyme for glucose determinations in the diabetic range. This added buffer strength necessitated the use of twice the volume of 5.0 N HCl to stop the reaction. Also, the reaction time was reduced from the recommended 15 minutes to 5-10 minutes to increase the linearity of the reaction. The glucose oxidase-peroxidase reagent was stored frozen and it was filtered through Whatman 50 filter paper immediately before use to remove pigment. Glucose standards were preserved with benzoic acid and the actual determinations were carried out at ambient temperature using an ultramicro technique. The reagent described above loses activity during prolonged use at ambient temperature.

When the method described in step 31 is used for
reducing the radioactivity of expired CO$_2$ to background levels. Arrangements should be made to insure that the hood ventilating system is not shut down during the procedure. Ideally, the radioactive rat should be maintained in a large metabolic cage. The room air could first be bubbled through sodium hydroxide and then through a constant humidity solution in order to maintain a comfortable environment for the rat during the week. This would result in the production of about 225 grams of barium carbonate for disposal. The disadvantage of this procedure is that it requires a separate metabolic cage for each rat to avoid cross contamination. In the absence of the necessary equipment the author thought that it would be safer to vent the $^{14}$CO$_2$ directly into the atmosphere rather than into the sewage system where it may have come into contact with acid without the benefit of a hood.

Rats were anesthetized with the inhalation anesthetic 2,2-dichloro-1,1-difluoroethyl methyl ether (Penthrane, methoxyflurane) or with 2-chloro-1,1,2-trifluoroethyl ethyl ether. Poznak and Artersio (850) compared several derivatives of 1,1-difluoroethyl methyl ether as anesthetic agents and found that the 2,2-dichloro compound was both a potent anesthetic as well as non-flammable in the concentrations required for anesthesia. The vapor pressure
of the anesthetic in inspired air was regulated by bubbling room air first through sodium hydroxide to absorb carbon dioxide and then through a gas trap containing the anesthetic at a temperature approaching -21.1°C which was maintained by surrounding the trap with frozen sodium chloride eutectic (23.3 percent NaCl). Although the use of a single temperature limits precise control of the depth of anesthesia, this can be accomplished by controlling the flow rate of carbon-dioxide-free air through the vaporization trap. Faster flow rates reduce the concentration of the anesthetic agent in inspired air (Figure XIII).

In early experiments the β-lipoprotein fraction of rat serum was isolated by a modification of the ethanol precipitation technique described by Krahl et al (306). The modification was that manipulations other than centrifugation at -5°C were conducted at 0°C. The precipitation reagent was composed of the following ingredients:

38 ml absolute ethanol
0.6 ml of 0.8 M HOAc-NaOAc buffer, pH 4.0
4.0 ml of 0.06 M NaCl
0.42 ml of 1.0 M HOAc
H₂O to 200 ml

The precipitation reagent was cooled to 0°C. Approximately 2.0 ml of donor rat serum was slowly added with continuous
FIGURE XIII

METABOLIC CAGE AND APPARATUS
stirring to 4 volumes of precipitation reagent. The pH of the resulting solution was adjusted to pH 6.2 at 0°C with the aid of standard buffers at this temperature, a micro pH electrode and the pH 4.0 acetate buffer described above. The lipoprotein precipitate was centrifuged for 5 minutes at 8,000 x g in 7.0 ml polycarbonate centrifuge tubes in the number 296 head and high-speed attachment of the PR-2 International refrigerated centrifuge. The precipitated lipoprotein was dissolved in isotonic tris(hydroxymethyl) aminomethane-HCl buffer, pH 7.4 at 37°C and administered to recipient rats. This buffer was 0.162 M in amine and 0.135 M in HCl. In some instances the final lipoprotein precipitate was not completely soluble suggesting that some denaturation had occurred.

In later experiments reported here the β-lipoprotein fraction was isolated by dextran sulfate precipitation according to the technique of Stokes et al (1032). Each ml of donor serum was mixed with 4.84 mg of solid CaCl₂·2H₂O, 0.04 ml of 1 percent dextran sulfate (Pharmacia), and saturated tris(hydroxymethyl)aminomethane to bring the pH to 0°C to 9.0. The precipitated lipoprotein was centrifuged as previously described and then washed with 0.004 M CaCl₂·2H₂O solution and recentrifuged. Several crystals of blue dextran were then added to the precipitate followed
by 1.0 ml of 0.4847 M tris(hydroxymethyl)aminomethane (58.783 gm/l)-0.0810 M ethylenediaminetetraacetic acid (3.946 gm/l) buffer, pH 9.2. The suspended precipitate and crystals were shaken in the cold room for approximately 30 minutes and then centrifuged as previously described. The resulting solution, saturated with blue dextran marker, was applied to an ion exchange-gel exclusion column consisting of diethylaminoethyl groups on a polyacrylamide matrix (Bio-gel DM-100). This gel excludes protein having molecular weights in excess of 100,000. The column, 20 mm x 300 mm, was equilibrated with the isotonic tris-HCl buffer, pH 7.8 at 0°C (pH 7.40 at 37°C) described above. Both the blue dextran and the β-lipoprotein were excluded from the gel and emerged from the column with the void volume of approximately 16.6 ml. The dextran sulfate and EDTA anions were exchanged for chloride ions while chelated calcium and protein contaminants entered the gel. The four bluest 0.5 ml eluant fractions were pooled and administered to recipient rats. Since the concentration of blue dextran and tris buffer administered to each recipient rat was the same, these agents should not affect the results. The dextran sulfate precipitation procedure is superior to the ethanol precipitation procedure because it avoids solvent denaturation and removes low molecular weight phospholipases which may be present.
However, it employs high pH, 9.2, and may result in the loss of dializable lipoprotein constituents during passage through the gel exclusion column. These potential limitations have not been evaluated but should be kept in mind when the technique is used.

Following the administration of the β-lipoprotein fraction from the steroid-treated donor rats to the anesthetized recipient rat, a continuous infusion of glucose and insulin was initiated. The composition of this solution is shown in Table XV. The specific activity of the glucose infusion solution was measured by counting a weighed sample of glucose 2,5-dichloro-phenylhydrazone, m p 152-152.5 (lit 160°C) which was prepared as described by Mandl and Newberg (708). The derivative was formed in anhydrous n-propanol and recrystallized from 1,1,2-trichloroethane. For example, a solution diluted to contain 0.05 µCi/mmole according to manufacturer's specifications was found to contain 0.0676 µCi/mmole by the above technique. The discrepancy between calculated and experimentally determined glucose infusion rates is attributed to the viscosity of the 27 percent glucose solution.

An ethanolic solution of glucose-6-14C was diluted to contain 5.0 µCi/ml and used as such to prime the body glucose pool of the rat. This solution was administered
### TABLE XV

**GLUCOSE INFUSION SOLUTION**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Infusion Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>272.11 mg/l</td>
<td>83.3 mg/hr</td>
</tr>
<tr>
<td>Sodium heparin</td>
<td>0.01 mg/l</td>
<td>0.003 mg/hr</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>0.54 mg/l</td>
<td>0.17 mg/hr</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.02 mg/l</td>
<td>0.14 units/hr</td>
</tr>
</tbody>
</table>

Infusion Rate = 0.3 ml/hr
to the rat at a dose of 1.0 ml/kg in order to raise the specific activity of the body glucose pool to that of the glucose infusion solution, i.e. 0.05 µCi/mmole. At this level of radioactivity approximately 10.1 µCi of radioactivity was required per 200 gm rat.

In preliminary experiments the respiratory carbon dioxide was isolated as barium carbonate and counted at infinite thickness (20 mg/cm²) in an end window planchet counter. These experiments indicated that the specific activity of the carbon dioxide reached a plateau after about 4 hours of infusion. In another series of experiments an attempt was made to increase counting efficiency and to by-pass the plating procedure associated with planchet counting by converting barium carbonate to quaternary ammonium carbonate in toluene prior to homogeneous liquid scintillation counting. Finely divided barium carbonate was mechanically shaken for 24 hours at ambient temperature with quaternary ammonium tosylate in toluene for reaction according to the equation:

$$\text{BaCO}_3 + 2 \text{R}_4\text{N RSO}_3^- = \text{Ba}(\text{RSO}_3^-)_2 + (\text{R}_4\text{N})_2\text{CO}_3$$

The fact that carbonate is isolated as its barium salt is somewhat disadvantageous because the barium salts of organic sulfonates are among the most soluble sulfonate salts. Since the equilibrium in the above reaction depends on the
relative solubilities of barium carbonate and barium sulfonate, the forward reaction may be enhanced by using sulfonates which form barium salts that are relatively insoluble in toluene, i.e. barium methanesulfonate. In order to be competitive to the direct trapping of carbon dioxide in quaternary ammonium hydroxide, a method described below, the above reaction would have to proceed almost to completion. The solubility of cesium carbonate in 2-propanol (.025 M) was determined in order to test the feasibility of related reactions employing different metal carbonates.

All steroid comparisons are based on the direct trapping of respiratory carbon dioxide in a 0.5 molar solution of quaternary ammonium hydroxide in toluene. A special gas trapping device was constructed which permitted the use of standard liquid scintillation vials. Its construction is shown in Figure XIV. Since the device was constructed of several pieces, it was necessary to use vacuum grease on crevices and threads to prevent gas leaks. With the exception of the teflon coupling a similar single-piece device may be constructed entirely of glass thus greatly reducing the possibility of leakage.

Collection vials were changed at 45-minute intervals up to 225 minutes. Then the anesthetized rat was removed from the metabolic cage and either placed in a cage under
FIGURE XIV

CARBON DIOXIDE TRAPPING DEVICE

FROM METABOLIC GAGE

NUMBER 00 1-HOLE STOPPER

POLYETHYLENE T-JOINT

INVERTED TOP SCREW CAP TEST TUBE

NUMBER 5 1-HOLE STOPPER

24 MM TEFOLON COUPLING

8MM GAS DISPERSION TUBE

10 ML 0.5 M QUARTERNARY AMMONIUM HYDROXIDE IN TOLUENE FLUOR

STANDARD SCINTILLATION VIAL

CO₂ TRAPPING DEVICE
a fume hood to allow expired radioactivity to return to background levels or placed in liquid nitrogen for subsequent muscle and hepatic glycogen analysis following the second lipoprotein treatment administered to the rat. This protocol allowed both steroid treatments to be compared in the same rat. Following freezing in liquid nitrogen the rat carcasses were stored at 120°C in a deep freeze. For glycogen analysis the carcasses were removed and warmed in a cold room at 0°C, the limbs were removed and the digits and skin were discarded in the appropriate manner with the rest of the radioactive carcass with the exception of the liver. The resulting mass of tissue was weighed and digested in hot KOH. When cooled, the digestion mixture was decanted from the bones and filtered through alkali-resistant fiber glass. The bones were then dried and their weight was subtracted from the total weight of the tissue. The glycogen was then precipitated with ethanol according to the technique of Good et al (367). However, despite the administration of glucose and insulin, only traces of glycogen were found in some experiments.
CHAPTER XIII

RESULTS

Part I: Infrared Studies

For convenience in confirming the identity of commercial steroids, the melting points and specific rotations of some endogenous 3-hydroxy-17-ketosteroids and their acetates can be found in Table XVI. Note that these parameters are not very definitive. Exceptions include the low melting point and high specific rotation of 3α-hydroxy-5β-androstan-17-one, the high melting point of its 11β-hydroxyl derivative, and the high specific rotation of the latter's dehydration product. In contrast, 3β-hydroxy-5α-androsten-17-one has a low specific rotation. In general, 5α-epimerization and ester formation lower specific rotation with the exception of uncertain entries which are underlined.

The infrared spectra of these 3-hydroxy-17-ketosteroids are more definitive. Note that, from the data of Jones et al (538-547) in Table XVII, C-11, C-17, and
<table>
<thead>
<tr>
<th>17-Ketosteroid</th>
<th>Alcohol</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>3α-hydroxy-5α-androstan-17-one</td>
<td>184-185, 94.6°</td>
<td>160-161, 77°</td>
</tr>
<tr>
<td>3α-hydroxy-5β-androstan-17-one</td>
<td>143-144, 111°</td>
<td>151-155, 98.4°</td>
</tr>
<tr>
<td>3α-hydroxy-9(11), (5α)-androstan-17-one</td>
<td>187-188, 136°</td>
<td>190-192, 135°</td>
</tr>
<tr>
<td>3α-hydroxy-9(11), (5β)-androstan-17-one</td>
<td>169-170, 151°</td>
<td>86-88</td>
</tr>
<tr>
<td>3α,11β-dihydroxy-5α-androstan-17-one</td>
<td>199-200, 98.4°</td>
<td>240-242</td>
</tr>
<tr>
<td>3α,11β-dihydroxy-5β-androstan-17-one</td>
<td>237-238, 96°</td>
<td></td>
</tr>
<tr>
<td>3α-hydroxy-5α-androstane-11,17-dione</td>
<td>153-155, 127°</td>
<td>184-185, 113°</td>
</tr>
<tr>
<td>3α-hydroxy-5β-androstane-11,17-dione</td>
<td>188-189, 95.8°</td>
<td>163-164, 145°</td>
</tr>
<tr>
<td>3β-hydroxy-5α-androstan-17-one</td>
<td>178-179, 87.5°</td>
<td>96-97, 68°</td>
</tr>
<tr>
<td>3β-hydroxy-5β-androstan-17-one</td>
<td>152-153, 11°</td>
<td>171-172, 3.9°</td>
</tr>
<tr>
<td>3β-hydroxy-5β-androstan-17-one</td>
<td>154-155, 89°</td>
<td>157-158, 82°</td>
</tr>
<tr>
<td>Ketone</td>
<td>(cm⁻¹) (CEI₄)</td>
<td>(cm⁻¹) (CHCl₃)</td>
</tr>
<tr>
<td>------------------------------</td>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>C-3</td>
<td>1717-1713</td>
<td>1711-1706</td>
</tr>
<tr>
<td>Δ¹-C-3</td>
<td>1680-1684</td>
<td>1670</td>
</tr>
<tr>
<td>Δ⁴-C-3</td>
<td>1674-1678</td>
<td>1666</td>
</tr>
<tr>
<td>Δ¹,₄-C-3</td>
<td>1666-1663</td>
<td></td>
</tr>
<tr>
<td>Δ⁴,₆-C-3</td>
<td>1669-1666</td>
<td></td>
</tr>
<tr>
<td>C-11</td>
<td>1712-1706</td>
<td>1705-1698</td>
</tr>
<tr>
<td>C-17</td>
<td>1745-1743</td>
<td>1736-1733</td>
</tr>
<tr>
<td>C-20</td>
<td>1709-1706</td>
<td>1707-1694</td>
</tr>
<tr>
<td>Δ¹₆-C-20</td>
<td>1666-1670</td>
<td>1652-1662</td>
</tr>
<tr>
<td>17α-OH-C-20</td>
<td>1706-1710</td>
<td>1698-1702</td>
</tr>
<tr>
<td>17α-OH-C-20-21-OAc</td>
<td>1708, 1693</td>
<td>1723-1728</td>
</tr>
<tr>
<td>C-20-21-OAc</td>
<td></td>
<td>1720-1727</td>
</tr>
<tr>
<td>C-3,11</td>
<td></td>
<td>1713</td>
</tr>
<tr>
<td>Δ¹-C-3,17</td>
<td>1719, 1745</td>
<td></td>
</tr>
<tr>
<td>C-3,20</td>
<td>1719, 1710</td>
<td></td>
</tr>
<tr>
<td>C-11,17</td>
<td>1719-1714, 1754</td>
<td></td>
</tr>
<tr>
<td>C-11,20</td>
<td>1713</td>
<td>1711</td>
</tr>
<tr>
<td>17α-OH-C-3,11,20</td>
<td></td>
<td>1705, 1685</td>
</tr>
</tbody>
</table>

Jones et al (538 - 547)
C-11,17 ketones can be readily distinguished. However, C-3 acetate esters in chloroform absorbing at 1739-1735 cm\(^{-1}\) mask C-17 ketones absorbing at 1736-1733 cm\(^{-1}\). Likewise, C-3 benzoates in chloroform absorbing at 1724-1717 cm\(^{-1}\) mask C-11 ketones in 17-ketosteroids absorbing at 1719-1714 cm\(^{-1}\). Unfortunately, the absorption shift of C-3 o-chlorobenzoates to lower frequencies is not quite large enough to eliminate this masking.

To test the feasibility of the infrared analysis of mixtures of these 17-ketosteroids, a unique absorption peak was found which characterized each compound in the mixture. Then the absorption coefficient of each compound at each wavelength was determined. It was assumed that the absorbance at all other frequencies is proportional to its absorbance at its unique frequency. However, it should be noted that all compounds absorb at the unique frequency of any particular compound. It is possible to consider the observed spectrum of a mixture of these pure compounds as a linear summation of the spectra of the individual compounds. That is, the absorbance at each unique frequency of the spectrum of the mixture is given by:

\[ A_f = bB_f + cC_f + dD_f + dE_f + \ldots \]

where the lower case letter represent the proportion of each compound in the mixture. When the total number of
FIGURE XV

3α-HYDROXY-5α-ANDROSTAN-17-ONE

3α-Hydroxy-5α-androstan-17-one m.p. 182.5
FREQUENCY (CM⁻¹) 2.0 mg./ml., 2.0 mm.
FIGURE XVI

3α-HYDROXY-5β-ANDROSTAN-17-ONE

3α-Hydroxy-5β-androstan-17-one m.p. 150.5°, FREQUENCY (CM⁻¹) 151.0, 2.0 mg./ml., 2.0 mm
FIGURE XVII

3β-HYDROXY-5α-ANDROSTAN-17-ONE

3β-Hydroxy-5α-androstane-17-one m.p. 169-170
FREQUENCY (CM⁻¹) 2.0 mg./ml., 2.0 mm.
FIGURE XVIII

3β-HYDROXY-5β-ANDROSTAN-17-ONE

3β-Hydroxy-5β-androstane-17-one m.p. 152.5°F

FREQUENCY (cm⁻¹) 353.5, 2.0 mg./ml., 2.0 mm.
FIGURE XIX

3β-HYDROXY-5-ANDROSTEN-17-ONE

TRANSMITTANCE (%)

FREQUENCY (CM⁻¹) 2.0 mg./ml., 2.0 mm.

3β-Hydroxy-5-androst-17-one m.p. 149
FIGURE XX

3α-HYDROXY-5α-ANDROSTANE-11,17-DIONE

TRANSMITTANCE (%)

100
80
60
40
20
0

1300 1200 1100 1000 900 800 700 600

3α-Hydroxy-5α-androstan-11,17-dione m.p. 157
FREQUENCY [cm⁻¹] 2.0 mg./ml., 2.0 mm.
FIGURE XXI

3α-HYDROXY-5β-ANDROSTANE-11,17-DIONE

\[
\text{TRANSMITTANCE (\%)}
\]

\[
\text{FREQUENCY (C} \text{M}^{-1})
\]

3α-Hydroxy-5β-androstane-11,17-dione m.p. 185

2.0 mg./ml., 2.0 mm.
### TABLE XVIII

**INFRARED ABSORPTION CHARACTERISTICS OF 17-KETOSTEROIDS**

**IN CARBON DISULFIDE SOLUTION**

<table>
<thead>
<tr>
<th>17-Ketosteroid</th>
<th>Extinction Coefficients (0.2%, 0.2 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency cm(^{-1}) 1058 1034 994 988 955 932 802</td>
</tr>
<tr>
<td>3α-hydroxy-5α-androstan-17-one</td>
<td>0.049 0.027 0.301 0.061 0.003 0.028 0.007</td>
</tr>
<tr>
<td>3α-hydroxy-5β-androstan-17-one</td>
<td>0.220 0.270 0.115 0.088 0.022 0.015 0.000</td>
</tr>
<tr>
<td>3β-hydroxy-5α-androstan-17-one</td>
<td>0.079 0.387 0.042 0.042 0.022 0.044 0.009</td>
</tr>
<tr>
<td>3β-hydroxy-5β-androstan-17-one</td>
<td>0.099 0.027 0.028 0.032 0.085 0.009 0.007</td>
</tr>
<tr>
<td>3β-hydroxy-5-androstan-17-one</td>
<td>0.069 0.076 0.062 0.042 0.036 0.022 0.066</td>
</tr>
<tr>
<td>3α-hydroxy-5α-androstane-11,17-dione</td>
<td>0.024 0.062 0.081 0.237 0.015 0.015 0.012</td>
</tr>
<tr>
<td>3α-hydroxy-5β-androstane-11,17-dione</td>
<td>0.046 0.155 0.054 0.016 0.024 0.073 0.012</td>
</tr>
</tbody>
</table>
compounds in a mixture is unknown, the criteria for including another compound in the summation is that its inclusion decreases the deviation of the summated from the observed spectrum.

While only obviously unique frequencies have been treated here, each compound has a definite absorption at each frequency. Using computerized methods, the summation process may be repeated at 1 cm\(^{-1}\) intervals or less, depending on the slit width program of the instrument. Such a procedure would necessitate the direct recording of infrared spectra on magnetic tape or disc.

The infrared results in Table XVIII indicate that there is little difficulty in distinguishing pairs of C-5 epimeric steroid alcohols. Note that the absorbences which are underlined are not necessarily at the best frequencies for this particular purpose. The resolution of the spectrometer is a key factor in the analysis of complex mixtures of these steroids at the selected frequencies. For example, the absorption of 3\(\alpha\)-hydroxy-5\(\beta\)-androstan-17-one at 1034 cm\(^{-1}\) occurs as a trough between two peaks and may decrease with better resolution thus making this frequency more specific for 3\(\beta\)-hydroxy-5\(\alpha\)-androstan-17-one.

Although it was necessary to scan the entire fingerprint region in carbon disulfide in order to determine
characteristic frequencies, the fact that these frequencies are between 1058 and 802 cm\(^{-1}\) means that less volatile solvents which produce greater concentration accuracy can be used if they have an infrared window in the region. Bromoform, which has an absorbence relative to air of less than 0.36 between 1050 and 900 cm\(^{-1}\) in a 2.0 mm cell is almost adequate for this purpose. Deuterated bromoform should also be investigated in this use. It is possible that the 802 cm\(^{-1}\) frequency may be replaced by another frequency which is masked in the 835-900 cm\(^{-1}\) region of carbon disulfide absorption. The melting points that were determined for 3\(\alpha\)-hydroxy-5\(\beta\)-androstan-17-one and 3\(\beta\)-hydroxy-5\(\alpha\)-androstan-17-one are somewhat lower than previously reported values suggesting contamination by 3\(\beta\)-hydroxy-5-androstan-17-one. However, the infrared analysis does not support that explanation.

The infrared spectra of the corresponding o-chlorobenzoates are much less distinctive. This is expected since all these derivatives share the absorptions of the o-chlorobenzoate group including peaks at 1280, 1240 and 745 cm\(^{-1}\). However, characteristic frequencies do exist for the 11-deoxy derivatives which are markedly different from the characteristic frequencies in the corresponding steroid alcohols. The frequency (700 cm\(^{-1}\)) appears to
FIGURE XXII

3α-HYDROXY-5α-ANDROSTAN-17-ONE 3-O-CHLOROBENZOATE

Androsterone 3-o-chlorobenzoate m.p. 151.5° FREQUENCY (cm⁻¹) 152.5, 2.0 mg./ml., 2.0 cm.
FIGURE XXIII

3α-HYDROXY-5β-ANDROSTAN-17-ONE 3-O-CHLOROBENZOATE

3α-Hydroxy-5β-androstan-17-one 3-o-chlorobenzoate m.p. 123-125

FREQUENCY (cm⁻¹) 2.0 mg./ml., 2.0 mm.

TRANSMITTANCE (%)
FIGURE XXIV

3β-HYDROXY-5α-ANDROSTAN-17-ONE 3-o-CHLOROBENZOATE
FIGURE XXV

3β-HYDROXY-5β-ANDROSTAN-17-ONE 3-α-CHLOROBENZOATE

TRANSMITTANCE (%)

1300 1200 1100 1000 900 800 700 600 FREQUENCY (CM⁻¹) 2.0 mg./ml., 2.0 mm.

3β-Hydroxy-5β-androstane-17-one 3-α-chlorobenzoate m.p. 230
FIGURE XXVI

3α-HYDROXY-5α-ANDROSTANE-11,17-DIONE 3-o-CHLOROBENZOATE
FIGURE XXVII

3α-HYDROXY-5β-ANDROSTANE-11,17-DIONE 3-O-CHLOROBENZOATE
be associated with the C-3 hydrogen. This vibration is influenced by the conformation of the ester group as well as by the nature of the A-B ring juncture and the presence of the C-11 ketone. This is hard to explain and may be further investigated by comparison with the spectra of the meta- and para-chloro-substituted benzoyl esters.

The C-11 oxygenated steroid esters seem to be devoid of characteristic frequencies. However, these frequencies may be present in a region of solvent absorption as discussed previously. This disadvantage is partially offset by the fact that C-11 ketonic and non-ketonic steroids are much more easily separated as C-3 esters rather than alcohols. The wide melting point range of 3α-hydroxy-5β-androstan-17-one 3-o-chlorobenzoate confirms the presence of impurities noted in the alcohol which are difficult to separate chromatographically. Also noteworthy is the fact that the ester of 3β-hydroxy-5α-androstan-17-one had a melting point close to that of the parent steroid. The uncorrected extinction coefficients of the 17-ketosteroid 3-o-chlorobenzoates are shown in Table XIX. The same data has been recalculated to correct for differences in concentration caused by the evaporation of carbon disulfide. This was done by assuming that the absorbance at 1050 cm⁻¹ was 0.300 in the spectrum of all esters. The main effect
<table>
<thead>
<tr>
<th>3-α-Chlorobenzoate</th>
<th>Extinction Coefficients (0.2%, 0.2 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency cm⁻¹</td>
</tr>
<tr>
<td>3α-hydroxy-5α-androstan-17-one</td>
<td>0.059</td>
</tr>
<tr>
<td>3α-hydroxy-5β-androstan-17-one</td>
<td>0.056</td>
</tr>
<tr>
<td>3β-hydroxy-5α-androstan-17-one</td>
<td>0.046</td>
</tr>
<tr>
<td>3β-hydroxy-5β-androstan-17-one</td>
<td>0.079</td>
</tr>
<tr>
<td>3β-hydroxy-5-androstan-17-one</td>
<td><strong>0.176</strong></td>
</tr>
<tr>
<td>3α-hydroxy-5α-androstane-11,17-dione</td>
<td>0.052</td>
</tr>
<tr>
<td>3α-hydroxy-5β-androstane-11,17-dione</td>
<td>0.038</td>
</tr>
</tbody>
</table>
### TABLE XX

**INFRARED ABSORPTION CHARACTERISTICS OF 17-KETOSTEROID 3-o-CHLOROBENZOATES IN SOLUTION NORMALIZED TO AN EXTINCTION COEFFICIENT OF 0.300 AT 1050 cm$^{-1}$**

<table>
<thead>
<tr>
<th>3-o-Chlorobenzoate</th>
<th>Extinction Coefficients (0.2%, 0.2 cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency cm$^{-1}$</td>
</tr>
<tr>
<td>3α-hydroxy-5α-androstan-17-one</td>
<td>0.055</td>
</tr>
<tr>
<td>3α-hydroxy-5β-androstan-17-one</td>
<td>0.052</td>
</tr>
<tr>
<td>3β-hydroxy-5α-androstan-17-one</td>
<td>0.046</td>
</tr>
<tr>
<td>3β-hydroxy-5β-androstan-17-one</td>
<td>0.051</td>
</tr>
<tr>
<td>3β-hydroxy-5-androstan-17-one</td>
<td><strong>0.186</strong></td>
</tr>
<tr>
<td>3α-hydroxy-5α-androstane-11,17-dione</td>
<td>0.057</td>
</tr>
<tr>
<td>3α-hydroxy-5β-androstane-11,17-dione</td>
<td>0.062</td>
</tr>
</tbody>
</table>
of this approximation is to reduce the extinction coefficients of 3α-hydroxy-5β-androstan-17-one. Otherwise, the qualitative interpretation of the data is the same. The corrected data is shown in Table XX.

Part II: Rat Maintenance

The second important experimental result is also qualitative. It was found possible to maintain alloxan-diabetic adrenalectomized rats on the specially compounded diet shown on page 332 of Chapter XII. This diet was administered in conjunction with a saturated solution of sodium glycyrrhizinate in saline at pH 8.0. The solubility of sodium glycyrrhizinate in 0.9 percent saline was found to be 0.213 gm/l at this pH. Rats maintained on the solid low-carbohydrate diet remained hyperglycemic. No deficiencies were noted in the diet during prolonged feeding. The diet was palatable to the rats and it served its purpose of maintaining healthy diabetic-adrenalectomized animals in the absence of insulin injections.

A minor result of these investigations is the verification of the effect of the halogenated anesthetic 2-chloro-1,1,2-trifluoroethyl ethyl ether on fasting serum glucose. Although it is important to be aware of this effect, it does not appear to be an important factor in these experiments because they were carried out during
TABLE XXI

The Effect of 2-Chloro-1,1-2-trifluoroethyl Ethyl Ether on Blood Glucose of Fasting (4 Hours) Rats.

<table>
<thead>
<tr>
<th>Non-anesthetized</th>
<th>Anesthetized</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.10 gm/l</td>
<td>1.31 gm/l</td>
</tr>
<tr>
<td>0.99 gm/l</td>
<td>1.13 gm/l</td>
</tr>
<tr>
<td>0.93 gm/l</td>
<td>1.20 gm/l</td>
</tr>
<tr>
<td>1.01 gm/l</td>
<td>Means</td>
</tr>
<tr>
<td></td>
<td>1.21 gm/l</td>
</tr>
</tbody>
</table>
an infusion of glucose at a level of approximately 500 mg per 100 ml. Furthermore, the effect of the anesthetic was constant in all experimental treatments so that it should not be a factor in differences between treatments.

Although the focus of the experimental approach has been on the transport of steroid-dependent anti-insulin factors by 8-lipoproteins, some experiments were also performed to test direct steroid effects. Of course, glucocorticoids cannot be tested directly because of their gluconeogenic effects on the liver. However, other steroids may be tested for their effect on glucose oxidation. Perhaps the best way to do this is to administer the steroid treatment to a diabetic-adrenalectomized rat prior to the administration of labeled glucose and the measurement of glucose oxidation in a metabolic cage.

A somewhat simpler approach consists of the measurement of the effects of such steroids on the survival time of adrenalectomized rats following the administration of glucose and excess insulin. The results from this type of study are shown in Table XXII. Although the results indicate that so-called anesthetic steroids have anti-insulin effects, the variable length of time between the onset of hyperglycemic convulsions and hypoglycemic death made the latter event unsatisfactory as an experimental
The Effect of Pretreatment with 2.1 mg of the Anesthetic Steroid 17α,21-Dihydroxy-5β-Pregnane-3,11,20-Trione 21-Acetate on the Onset of Hypoglycemic Death Following the Injection of 1 ml of 44 Percent Glucose and 1 unit of Insulin Into 6-Hour Fasted Alloxan-Diabetic Adrenalectomized Rats.

<table>
<thead>
<tr>
<th>Survival Times (Minutes)</th>
<th>Control</th>
<th>Steroid-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>195</td>
<td>309</td>
</tr>
<tr>
<td></td>
<td>284</td>
<td>410</td>
</tr>
<tr>
<td></td>
<td>193</td>
<td>387</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>223</td>
<td>Means 369</td>
</tr>
</tbody>
</table>
Part II: Research on the Measurement of CO₂ Specific Activity by Liquid Scintillation Techniques

A method was designed for the homogeneous liquid scintillation counting of barium carbonate as quaternary ammonium carbonate following its reaction with quaternary ammonium tosylate. This method was found to be less sensitive than the measurement of relative specific activity by planchet counting at infinite thickness. The cause of the reduced sensitivity was not the efficiency of liquid scintillation counting which was high, but the low percentage conversion of the reaction forming quaternary ammonium carbonate. Solubility considerations suggest that the percentage conversion may be increased by reaction with quaternary ammonium mesylate. The plot of efficiency versus external standard ratio employing quaternary ammonium tosylate is shown in Figure XXVIII.

The method of determination of specific activity of CO₂ actually used in these experiments is based on the direct trapping of the gas by a quaternary ammonium hydroxide dissolved in toluene. The specially designed and constructed apparatus used by the author for this purpose is shown in Figure XIV, page 353. The efficiency plot for this method of counting is shown in Figure XXIX. Since the
FIGURE XXVIII

EFFICIENCY VERSUS EXTERNAL STANDARD RATIO FOR QUATERNARY AMMONIUM CARBONATE DERIVED FROM BARIUM CARBONATE

COUNTING EFFICIENCY VERSUS EXTERNAL STANDARD RATIO FOR QUATERNARY AMMONIUM CARBONATE DERIVED FROM BARIUM CARBONATE
TABLE XXIII

EFFICIENCY VERSUS EXTERNAL STANDARD RATIO FOR 0.5 MOLAR QUATERNARY AMMONIUM BICARBONATE IN TOLUENE CONTAINING 6.0 GRAMS/LITER 2,5-DIPHENYLOXAZOLE

<table>
<thead>
<tr>
<th>External Standard Ratio</th>
<th>Hexadecane-1(^{14})C Specific Activity</th>
<th>Vial Weight</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.323</td>
<td>2965 cpm</td>
<td>14278.8 mg</td>
<td>89.0%</td>
</tr>
<tr>
<td>+Hexadecane-1(^{14})C</td>
<td></td>
<td>14278.8 mg</td>
<td></td>
</tr>
<tr>
<td>-Hexadecane-1(^{14})C</td>
<td></td>
<td>14277.3 mg</td>
<td></td>
</tr>
<tr>
<td>Net</td>
<td></td>
<td>2965.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.5 mg x 2220 dpm/mg = 3330 dpm</td>
<td></td>
</tr>
<tr>
<td>0.360</td>
<td>6122 cpm</td>
<td>16765.8 mg</td>
<td>95.1%</td>
</tr>
<tr>
<td>+Hexadecane-1(^{14})C</td>
<td></td>
<td>16765.8 mg</td>
<td></td>
</tr>
<tr>
<td>-Hexadecane-1(^{14})C</td>
<td></td>
<td>16762.9 mg</td>
<td></td>
</tr>
<tr>
<td>Net</td>
<td></td>
<td>6122.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.9 mg x 2220 dpm/mg = 6438 dpm</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE XXIX

EFFICIENCY VERSUS EXTERNAL STANDARD RATIO FOR
0.5 MOLAR QUATERNARY AMMONIUM BICARBONATE IN TOLUENE

EFFICIENCY VERSUS EXTERNAL STANDARD RATIO FOR 0.5
MOLAR QUATERNARY AMMONIUM BICARBONATE IN TOLUENE
CONTAINING 6.0 GRAMS/LITER 2,5-DIPHENYLOXAZOLE
combining capacity of the organic base must be saturated in order to calculate specific activities by this method, the low efficiency of CO$_2$ collection (approximately 50 percent) and the low rate of CO$_2$ production by the rat combine to limit the frequency of specific activity measurements.

Another liquid scintillation method for the determination of specific activity was devised with the hope of increasing the efficiency of CO$_2$ collection. However, the method was not tested because it requires temperature-controlled counting. In the method an aqueous cesium hydroxide solution is saturated with CO$_2$. Cesium bicarbonate and primary fluor are then precipitated by the addition of 2-phenoxyethanol saturated with primary fluor, thereby forming a four-phase invariant system at constant temperature and pressure. After equilibration below ambient temperature, the organic phenoxyethanol phase containing cesium bicarbonate, water and primary fluor should remain homogeneous for counting at ambient temperature. Studies were carried out to determine the comparative counting efficiencies of a number of organic solvents. As seen in Table XXIV, the efficiency of phenoxyethanol is 76.7 percent, or approximately 85 percent of the efficiency of toluene. The same solvent phenoxyethanol was also used to determine the specific activity of the glucose
**TABLE XXIV**

**EFFICIENCIES OF SCINTILLATION SOLVENTS CONTAINING 6.0 GMS/LITER DIPHENYLOXAZOLE AS PRIMARY FLUOR AS DETERMINED WITH HEXADECANE-1-C\(^{14}\) (2220 DPM/MG)**

<table>
<thead>
<tr>
<th>Activity (cpm)</th>
<th>Hexadecane</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Toluene</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spike ...35126.6</td>
<td>16674.4 mg</td>
<td>35,028.6 cpm = 89.1%</td>
</tr>
<tr>
<td>Bkg...........98.0</td>
<td>16656.7 mg</td>
<td></td>
</tr>
<tr>
<td>Net...........35028.6</td>
<td>17.7 mg x 2220 dpm/mg = 39,284.0 dpm</td>
<td></td>
</tr>
<tr>
<td><strong>Benzyl Alcohol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spike ....8747.1</td>
<td>16485.6 mg</td>
<td>8,878.0 cpm = 54.0%</td>
</tr>
<tr>
<td>Bkg...........69.1</td>
<td>16478.2 mg</td>
<td></td>
</tr>
<tr>
<td>Net...........8878.0</td>
<td>7.4 mg x 2220 dpm/mg = 16,428.0 dpm</td>
<td></td>
</tr>
<tr>
<td><strong>Phenoxyethanol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spike ....9784.5</td>
<td>16472.8 mg</td>
<td>9,709.0 cpm = 76.7%</td>
</tr>
<tr>
<td>Bkg...........75.2</td>
<td>16467.1 mg</td>
<td></td>
</tr>
<tr>
<td>Net...........9709.3</td>
<td>5.7 mg x 2220 dpm/mg = 12,654.0 dpm</td>
<td></td>
</tr>
<tr>
<td><strong>Anisole</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spike ....13096.5</td>
<td>17081.8 mg</td>
<td>12,967.0 cpm = 89.9%</td>
</tr>
<tr>
<td>Bkg...........129.4</td>
<td>17075.3 mg</td>
<td></td>
</tr>
<tr>
<td>Net...........12967.1</td>
<td>6.5 mg x 2220 dpm/mg = 14,430.0 dpm</td>
<td></td>
</tr>
<tr>
<td><strong>Dioxane</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spike ....3863.1</td>
<td>16666.4 mg</td>
<td>3,789.0 cpm = 24.0%</td>
</tr>
<tr>
<td>Bkg...........73.5</td>
<td>16659.3 mg</td>
<td></td>
</tr>
<tr>
<td>Net...........3789.6</td>
<td>7.1 mg x 2220 dpm/mg = 15,762.0 dpm</td>
<td></td>
</tr>
</tbody>
</table>
infusion solution. Glucose was isolated from the solution and counted as its 2,5-dichloro phenylhydrazone. It is appropriate to note that labeled 2,5-dichloro phenylhydrazine may be synthesized from chlorine-36 labeled p-dichlorobenzene of known specific activity so that glucose specific activity may be determined solely from radioactivity measurements. An additional advantage of 2,5-dichloro phenylhydrazones over nitrophenylhydrazones as derivatives is that they are not colored and therefore they are less quenching to the liquid scintillation process. The specific activity of glycogen glucose can also be determined by this method following hydrolysis with sulfuric acid and neutralization with bar- rium carbonate.

Despite the fact that excess insulin and glucose were infused in these experiments and despite the fact that all limb muscles were taken for the isolation of muscle glyco- gen, sufficient muscle glycogen could not be recovered from enough rats to make valid comparisons between steroid treatments. It is doubtful that significant muscle glyco- genolysis occurred during freezing in liquid nitrogen be- cause one would expect the limbs to freeze rapidly.

Part III: Effects of Steroid Treatments

Results from the best executed series of comparisons of fluprednisolone and 20β-hydroxy fluprednisolone treatments
is shown in Table XXV and plotted in Figure XXX. In these experiments the effects of β-lipoproteins from rats receiving each of the steroid treatments were determined in different recipient rats because only a limited number of experiments were completed by the proposed method in which a single recipient rat received β-lipoproteins from both types of steroid-treated rats. It appears that the second use of a recipient is more critical than the first in that the rat is more susceptible to respiratory depression and respiratory arrest.

The slope of the regression line for each set of observations was determined by the regression analysis in Table XXVI. The assumption that the regression line passes through the origin is theoretically correct and provides more meaningful regression slopes than those obtained by treating the origin as just another data point. The statistical treatment was simplified by treating the independent variable time in units of 45 minutes. However, the slope, b, so obtained must be divided by 45; hence the b/45 term in Table XXVI. Random variations in the rates of glucose oxidation were greater than those due to steroid treatments. The greatest variation was uncontrolled respiratory depression and anoxia produced by the anesthetic and the deficits of carbon dioxide and oxygen in the inspired air.
TABLE XXV

GLUCOSE OXIDATION AFTER LIPOPROTEINS FROM FLUPREDNISOLONE
AND 20\(^\beta\)-HYDROXYFLUPREDNISOLONE TREATMENTS

<table>
<thead>
<tr>
<th>Regression Line Symbol</th>
<th>Experimental Weight GMS</th>
<th>Fasting Glucose GMS/L</th>
<th>Counts Per Minute Per Micromole CO(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>45 Min</td>
</tr>
<tr>
<td><strong>6a-fluoro-11(^\beta),17a,20(^\beta),21-tetrahydroxy-1,4-pregnadiene-3-one</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>330</td>
<td>1.96</td>
<td>39.1</td>
</tr>
<tr>
<td>B</td>
<td>329</td>
<td>1.96</td>
<td>76.7</td>
</tr>
<tr>
<td>E</td>
<td>358</td>
<td>4.88</td>
<td>14.8</td>
</tr>
<tr>
<td>F</td>
<td>429</td>
<td>2.02</td>
<td>16.2</td>
</tr>
<tr>
<td><strong>6a-fluoro-11(^\beta),17a,21-trihydroxy-1,4-pregnadiene-3,20-dione</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>323</td>
<td>3.16</td>
<td>25.0</td>
</tr>
<tr>
<td>D</td>
<td>401</td>
<td>2.99</td>
<td>12.8</td>
</tr>
<tr>
<td>G</td>
<td>386</td>
<td>4.67</td>
<td>14.3</td>
</tr>
<tr>
<td>H</td>
<td>314</td>
<td>3.69</td>
<td>15.0</td>
</tr>
</tbody>
</table>
FIGURE XXX

GLUCOSE OXIDATION AFTER LIPOPROTEINS FROM FLUPREDNISOLONE AND 20β-HYDROXYFLUPREDNISOLONE TREATMENTS

FIGURE XI GLUCOSE OXIDATION AFTER LIPOPROTEINS FROM FLUPREDNISOLONE AND 20β-HYDROXYFLUPREDNISOLONE TREATMENTS

COUNTS PER MINUTE PER MICROMOLE CO₂

- - FLUPREDNISOLONE

- - 20β-HYDROXYFLUPREDNISOLONE

MINUTES
TABLE XXVI

REGRESSION STATISTICS

\[ S = \text{Specific Activity}, \quad T = \text{Time}, \quad I = \text{Interval Time} \]

\[ I = T/45, \quad S = bI, \quad \Xi I = 6, \quad \Xi I^2 = 14, \quad (\Xi I^2)/4 = 9, \quad [\Xi I^2 - (\Xi I)^2/4] = 5 \]

<table>
<thead>
<tr>
<th>Regression</th>
<th>( \Xi S )</th>
<th>( \Xi S^2 )</th>
<th>( \Xi IS )</th>
<th>( [\Xi S^2 - (\Xi IS)^2/\Xi I^2] )</th>
<th>( [\Xi IS - \Xi I \Xi S/4] )</th>
<th>( b/45 )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>6a-fluoro-11\beta,17\alpha,20\beta,21-tetrahydroxy-1,4-pregnadiene-3-one</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>725.4</td>
<td>526,205.16</td>
<td>1,829.6</td>
<td>287,094.74</td>
<td>741.50</td>
<td>2.904</td>
</tr>
<tr>
<td>B</td>
<td>617.6</td>
<td>180,277.50</td>
<td>1,547.7</td>
<td>9,178.26</td>
<td>459.60</td>
<td>2.456</td>
</tr>
<tr>
<td>E</td>
<td>255.5</td>
<td>33,080.81</td>
<td>646.5</td>
<td>30,095.37</td>
<td>263.25</td>
<td>1.026</td>
</tr>
<tr>
<td>F</td>
<td>241.0</td>
<td>34,567.14</td>
<td>546.5</td>
<td>13,324.12</td>
<td>185.00</td>
<td>0.867</td>
</tr>
<tr>
<td></td>
<td></td>
<td>339,602.49</td>
<td>1649.35</td>
<td></td>
<td>1.813 \pm 1.019</td>
<td></td>
</tr>
</tbody>
</table>

| **6a-fluoro-11\beta,17\alpha,21-trihydroxy-1,4-pregnadiene-3,20-dione** | | | | | | |
| C | 372.7 | 62,725.77 | 923.0 | 2,588.00 | 363.95 | 1.465 |
| D | 281.1 | 41,287.13 | 734.2 | 2,783.58 | 312.55 | 1.165 |
| G | 168.9 | 13,088.19 | 422.4 | 343.78 | 169.05 | 0.672 |
| H | 142.4 | 9,214.00 | 354.4 | 242.67 | 140.80 | 0.563 |
|  |  | 5,958.03 | 986.35 | | 0.966 \pm 0.424 |  |

**Totals:** 2804.6 900,445.70 7,004.3 345,560.52 2635.70

Critical Sum of Squares = \( F_{0.90}(7.7) \times \frac{\Xi d^2_{st}}{3} = 2.78 \times 345,560.52/3 = 320,219.42 \)

Sum of Squares Among All Slopes = 345,560.52 - \((2635.70)^2/8 \times 5 = 171,877.66 \)

Since 171,877.66 < 320,219.42, the slopes are not significantly different at the 90 percent confidence level. In contrast, a Student's t value of 1.535 for the difference between the mean slopes of steroid treatments is greater than \( t_{0.90(16)} \).
This variation and that in infusion rates could be eliminated by the use of a mechanical respirator and positive displacement infusion pump.

In order to demonstrate significant differences between steroid treatments the specific activity of the glucose administered to the rat will have to be increased 5-10 fold and more frequent specific activity measurements will have to be made. Provided that 20β-hydroxyfluprednisolone does not stimulate glucose oxidation, then the demonstration that the rate of glucose oxidation is lower with the fluprednisolone treatment than with the 20β-hydroxyfluprednisolone treatment proves that the effect of fluprednisolone is significant in vivo. The possibility that 20β-hydroxyfluprednisolone does stimulate glucose oxidation may be eliminated by comparing both steroid treatments with the basal glucose oxidation rate. To do this one must be able to predict the latter portions of the specific activity versus time curve from measurements of its initial portion. Each recipient rat could be subjected to a control period and then to an experimental period after lipoprotein administration. Differences between the experimentally determined and calculated specific activity curves could then be observed.
CHAPTER XIV
DISCUSSION

The purpose of this dissertation is to evaluate insulin inhibition by glucocorticoids in vivo. It includes an evaluation of the specificity of the effect. For the purposes of this discussion the author defines a specific glucocorticoid effect as one which requires all of the functional groups present in cortisol. If insulin inhibition by glucocorticoids is non-specific, then 20β-hydroxy glucocorticoids should produce insulin antagonism in vivo. On the other hand, if insulin inhibition by glucocorticoids is specific, it should not be produced by the 20β-hydroxy glucocorticoids in vivo because these metabolites are formed irreversibly from glucocorticoids in the rat (486) and thus are not precursors of glucocorticoids.

If the anti-insulin effect of fluprednisolone is significantly greater than that of 20β-hydroxy fluprednisolone, what are possible explanations of this specificity? One explanation is that the C-20 ketone interacts with a
receptor protein through anil formation. It is unlikely that differences in hydrogen bonding between the C-20 ketone and C-20 hydroxyl group could account for the differences in the observed effects. On the other hand, the C-21 deoxy derivative which may be formed from the C-21 mesylate, could be used to evaluate the effect of hydrogen bonding on the glucocorticoid effect. For example, Munk (766) has shown that the 11β-hydroxyl group markedly alters the surface activity of steroids.

The following tentative sequence of events is thought to occur during glycolytic inhibition by glucocorticoids. First, the glucocorticoid combines with a growth hormone dependent protein and the resulting steroid-protein complex causes a decrease in triglyceride synthesis which leads to an increase in the synthesis of saturated lecithins which are transported to peripheral tissues by β-lipoproteins. Enzymes of red cell origin hydrolyze the lecithin to an active lyssolecithin species which inhibits oxidative phosphorylation in mitochondria perhaps by displacing the essential phospholipids of cytochrome C. Thus, the specificity of the phospholipid effect is due mainly to the protein and not to any special properties of the lyssolecithin itself. However, since the mitochondria contain an enzyme which normally converts the lyssolecithin species back to lecithin,
the lysolecithin which affects the activity of the mitochondria is probably resistant to this conversion and derived from non-mitochondrial species of lecithin (633).

The hypothesis that these steroid effects are caused by species of \( \beta \)-lipoprotein lysolecithin can be investigated by correlating \( \beta \)-lipoprotein lysolecithin composition with the magnitude of inhibition of glucose oxidation. Lysolecithin species may be characterized following hydrolysis with lysolecithinase and gas chromatography of the methyl esters of the resulting fatty acids.

Since \( \beta \)-lipoproteins are synthesized in the liver, and since the liver has been implicated in the anti-insulin effect of glucocorticoids, the possibility that the time course of the synthesis of the inhibitor parallels the induction of gluconeogenic enzymes such as tryptophane pyrrolase and tyrosine transaminase (208, 661) should be determined. Experiments with actinomycin D may not decide whether protein synthesis is required for the anti-insulin effect because this inhibitor of DNA synthesis may also block the synthesis of the \( \beta \)-lipoprotein necessary to transport the inhibitor. On the other hand, considerations in preceding chapters suggest that growth hormone is required for the anti-insulin effect of glucocorticoids because it is necessary for the production of a particular receptor.
protein for the glucocorticoid. This particular receptor protein might be detected by comparing the hepatic cortisol binding proteins of hypophysectomized rats before and after growth hormone treatment.

The specificity of the insulin antagonism by glucocorticoids is its most interesting and critical aspect. As previously noted, many polycyclic compounds such as estrogens and chlorpromazine produce hyperglycemic effects which appear to be mechanistically similar to that of glucocorticoids. Therefore, the possibility that must be eliminated is that pharmacological doses of these synthetic compounds exert their hyperglycemic effect by interacting with the growth hormone dependent receptor protein.

Serotonin is a hyperglycemic compound released in response to growth hormone treatment. Since the hyperglycemic effect of serotonin is caused by its release of adrenal epinephrine, the effect of serotonin should not be important in adrenalectomized rats. However, the effect of serotonin and serotonin antagonists may be significant modifiers of insulin inhibition by endogenous glucocorticoids in intact animals. For example, some serotonin antagonists can suppress ACTH release in addition to displacing serotonin from its binding sites.

The mechanism of the enhanced platelet aggregation
which is found in diabetes mellitus and which contributes to diabetic retinopathy by blocking blood flow in the stagnant regions of the venous circulation of the retina may be similar to the mechanism of glycolytic inhibition by glucocorticoids. For example, it is known that platelet glycolysis is necessary to maintain the repulsive electrical charges between platelets. Furthermore, platelets are capable of triglyceride and phospholipid synthesis so that they are susceptible to factors which affect these processes. The importance of platelet function in the pathology of diabetes mellitus is evident from the fact that 60 percent of patients with diabetes for more than 10 years have retinal hemorrhages and from the fact that 69.4 percent with diabetes for over 15 years die of atherosclerosis.

Fructose overcomes glycolytic inhibition by glucocorticoids in liver and adipose tissue. Although fructolysis bypasses the inhibition of phosphofructokinase by citrate in insulin deficient tissues, it is not clear how fructose reverses the inhibition of lipogenesis which is the cause of the elevated citrate levels. It appears that fructose or fructose-1-phosphate has a direct effect on triglyceride synthesis in mitochondria which counteracts the inhibition of this process postulated to be produced by a complex of glucocorticoids with a growth hormone-dependent
protein. Thus, the effect of fructose is similar to that of insulin under these circumstances. However, the presence of fructose or its esters in mitochondria has not been reported.

In multiple sclerosis the phospholipase A which forms the β-lipoprotein lysolecithin species that enhances platelet aggregation is released from damaged nervous tissue (92). The phospholipase A activity of lipoprotein lipase appears to be more specific in that it is not responsible for the formation of the insulin-inhibitory lysolecithin species. Indeed, since insulin resistance is associated with diabetic lipemia, the insulin inhibitory lysolecithin species may block the activity of lipoprotein lipase. Apparently, this reduction in lipoprotein lipase activity obscures the inhibition of triglyceride synthesis in diabetes. Hill et al (460) have shown that triglyceride synthesis is impaired by glucocorticoids even though the activities of triglyceride-synthesizing enzymes in tissue extracts is increased (439). Hazzard et al (441) also noted a relative inhibition of triglyceride synthesis in estrogen-induced hypertriglyceridemia which suggests that estrogens may interact with the growth hormone-dependent receptor protein for glucocorticoids. The presence of lysolecithinase activity in plasma (848) may explain the instability of the β-lipoprotein insulin inhibitor which was reported by
The anoxia suggested by the low recoveries of glycogen is probably due to respiratory depression produced by the anesthetic methoxyflurane. This respiratory depression is accentuated by the absence of carbon dioxide, a respiratory stimulant, in the inspired air. Methoxyflurane, unlike nitrous oxide, does not contribute to anoxia by displacing oxygen because its partial pressure is only 10 mm Hg at 6.27°C and approximately 5 mm Hg at the temperature of -21.1°C used in these experiments. Respiratory depression leading to anoxia might be counteracted by a slight reduction in the pH of the glucose infusion solution. Alternatively, diethyl ether, which is a respiratory stimulant, may be used as a substitute anesthetic with exactly the same apparatus.

Restatement of the Problem

The general problem treated was the role of cortisol in Diabetes Mellitus. The particular aspect studied was the evaluation in vivo of insulin antagonists transported by rat β-lipoproteins in response to the glucocorticoid 6α-fluoro-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione. The specificity of the steroid effect was also evaluated by comparison with its derivative 6α-fluoro-11β,17α,20β,21-tetrahydroxy-1,4-pregnadiene-3-one.
Restatement of the Approach

\( \beta \)-lipoproteins were isolated from the sera of steroid-treated donors and administered to recipient rats and the effect of the lipoprotein on the oxidation of glucose-6-\( ^{14} \)C to carbon dioxide was measured. The assumptions made were:

1) that the rate of glycolysis was the main determinant in the rate of \( ^{14} \)CO\(_2\) formation from glucose-6-\( ^{14} \)C; 2) that the \( ^{14} \)CO\(_2\) was mainly formed in extra hepatic tissues; 3) that the proportion of glucose metabolized directly to \( ^{14} \)CO\(_2\) is the same in all experiments, and 4) that gluconeogenesis is not a factor in these experiments.

Results

Provided that the reduced steroid 6\( \alpha \)-fluoro-11\( \beta \),17\( \alpha \), 20\( \alpha \),21-tetrahydroxy-1,4-pregnadiene-3-one does not stimulate glucose oxidation, the rate of glucose oxidation was lower with lipoproteins from rats treated with 6\( \alpha \)-fluoro-11\( \beta \),17\( \alpha \), 21-trihydroxy-1,4-pregnadiene-3,20-dione. However, this difference was not statistically significant.

Discussion

An approach to the evaluation of insulin antagonism in vivo has been developed and its limitations have been determined. The frequency with which specific activity measurements could be made was the limiting factor in these experiments. The absolute specificity of the steroid effect
cannot be determined until the absolute effect of the steroid on glucose oxidation is determined with respect to a non-treatment control. The insulin inhibitor appears to be a species of lysolecithin which inhibits but does not uncouple oxidative phosphorylation leading to lower levels of ATP as a function of NADH concentration, or, conversely, to higher levels of NADH as a function of ATP concentration.
BIBLIOGRAPHY


175. Chenoweth, M. B., "Physiological and Biochemical Responses to Methoxyflurane Anesthesia," in Experimental Animal Anesthesiology, D. C. Sawyer (Editor), USAF School of Aerospace Medicine, 1965, pp. 185-206


533. Jones, H. B., Gofman, J. W., Lindgren, F. T., Lyon, T. P.,
Graham, D. M., Strisower, B. and Nichols, A. V., "Lipo-

535. Jones, H. W. and Wade, R., "The Effect of Progesterone on the
Rate of Phosphate Release from Adenosine Triphosphates

536. Jones, P. D. and Wakil, S. J., "A Requirement for Phospholi-
pids by the Microsomal Reduced Diphosphopyridine Nucleo-
tide Cytochrome C Reductase," J. Biol. Chem. 242, 5267-
73 (1967).

537. Jones, R. N., "The Characterization of Sterol Hormones by Ul-
Res. 2, 3-29 (1948).

538. Jones, R. N., Williams, V. Z., Whalen, M. J. and Dobriner, K.,
"Steroid Metabolism IV. Characterization of Carbonyl
and other Functional Groups in Steroids by Infrared Spec-

539. Jones, R. N. and Dobriner, K., "Infrared Spectrometry Applied
to Steroid Structure and Metabolism," Vitamins and Hor-
mones VII, 293-364 (1949).

540. Jones, R. N., Humphries, P., Herling, F. and Dobriner, K.,
"Studies in Steroid Metabolism, X. The Effects of Ster-
eochemical Configuration at Position 3 and 5 on the Infra-
red Spectra of 3-Acetoxy Steroids," J. Am. Chem. Soc. 73
3215-20 (1951).


   V. The Penetration and Phosphorylation of 2-Deoxyglucose
   in the Rat Diaphragm," J. Biol. Chem. 234, 171-77 1959

588. Kipnis, D. M. and Cori, C. F., "Studies of Tissue Permeable-
   ty, The Penetration and Phosphorylation of 2-Deoxyglucose
   235, 3070-75 (1960).

589. Kipnis, D. M. and Stein, M. T., "Insulin Antagonism; Funda-
   15, 156-91 (1964).

590. Kipnis, D. M., "Growth Hormone and Insulin Antagonism," in
   On the Nature and Treatment of Diabetes, B. S. Leibel
   and G. A. Wrenshall (Editors), Excerpta Medica Foundation,
   New York, 1965, pp 258-73

591. Kipnis, D. M., "Insulin Antagonism and Diabetes Mellitus,"in
   Diabetes--Proceedings of the Sixth Congress of the
   International Diabetes Federation," Excerpta Medica
   Foundation, Amsterdam, 1969, pp 257-72

   Acid d-hydroxybutylamide 2.--A Synthetic Oxytocic II.
   (1944).

593. Kirshbaum, A., Lemen, J. W. and Molander, D., "Relation of
   Adrenal Gland and Hypophysis to Blood Sugar Levels Fol-


694. MacDonald, B. S., Sykes, P. J., Adhikary, P. M. and Harkness, R. A., "The Identification of 17a-Hydroxy-17-methyl-1,4-androstadien-3-one as a Metabolite of the Anabolic Steroid Drug 17B-Hydroxy-17a-Methyl-1,4-Androstadien-3-one in Man," Steroids 18, 753-66 (1972).


734. Mercier-Bodard, C., Alfsen, A., Balieu, E. E., "Sex-Steroid Binding Plasma Protein (SBP)," in Karolinska Symposia on Research Methods in Reproductive Endocrinology, Suppl. 147, 2nd Symposium, Steroid Assay by Protein Binding, E. Diczfalusy (Editor), Bogliykkerel Forum, Copenhagen, 1971, pp. 204-21


529


973. Shanygina, K. I., "Hexokinase Isozymes in Rat Tissues and the Regulation of their Activity by Hormones," Biochem (Rus) 36, 301-05 (1971).


<table>
<thead>
<tr>
<th>No.</th>
<th>Reference</th>
</tr>
</thead>
</table>


"Fasting Serum Triglycerides, Cholesterol, and Lipoprotein Levels During Oral-Contraceptive Therapy,"


SUPPLEMENTAL BIBLIOGRAPHY


The dissertation submitted by Theodore S. Musiala has been read and approved by five members of the faculty of the Loyola University of Chicago.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

January 12, 1973
Date

Signature of Adviser