Advances in
CLINICAL CHEMISTRY
VOLUME 26

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Edited by
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Quadretek Associates, Inc.
Cedar Grove, New Jersey

VOLUME 26 • 1987

ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
Orlando San Diego New York Austin
Boston London Sydney Tokyo Toronto

ACADEMIC PRESS, INC.
Orlando, Florida 32887

United Kingdom Edition published by
ACADEMIC PRESS INC. (LONDON) LTD.
24–28 Oval Road, London NW1 7DX

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 58-12341

ISBN 0–12–010326–5

PRINTED IN THE UNITED STATES OF AMERICA

87 88 89 90 9 8 7 6 5 4 3 2 1

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PREFACE

The field of clinical chemistry continues to expand and diversify. In keeping with the present editorial philosophy of this series, several new areas of interest are included in Volume 26. These include specially written chapters on the Thymus, Neurochemistry, Pulmonary Biochemistry, Nonenzymatically Glycosylated Proteins, and Sulfohydrolases. The Editorial Board is pleased that its previous volumes have received favorable reviewer response. We also acknowledge the responsiveness of our readership. Future contributions will continue to reflect the interests expressed so graciously.

I wish to express my gratitude to an Editorial Board which is diligent, capable, and extremely cooperative. In addition, my thanks are offered to the contributors of this volume for their efforts and enthusiasm. Finally, I'd like to acknowledge the patience of my wife, Joanne, and my family. My special best wishes go to my son, Paul, for his courage and determination.

HERBERT E. SPIEGEL

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NONENZYMATICALLY GLYCOSYLATED PROTEINS

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1. Introduction

1.1. Historical Identification

It has been known for over 25 years that human and other mammalian red cells normally contain hemoglobins other than adult (A_0), fetal (F), and A_2. These included the A_1 minor hemoglobins with fast electrophoretic mobility (A7, C11). Views on their constitution and properties were tentative, but an increase in fast-moving hemoglobins (FMH) was found in four diabetics on the oral hypoglycemic drug tolbutamide. It was considered to be a hemoglobin–tolbutamide reaction, analogous to the hemoglobin–glutathione aging component (H16). Schroeder and Holmquist (S18) in 1968 summed up the knowledge at that time concerning hemoglobin A_{1c}—a normal and constant component of the red cell, comprising some 5–7% of total red cell hemoglobin in humans and the most abundant of the minor hemoglobins. They identified an aldehyde or ketone link to the N-terminal amino acid of the β-hemoglobin chain, through a Schiff base reaction, and considered its properties consistent with those of a long-chain aliphatic lipid. In the same year, Rahbar (R1) reported that hemoglobin A_{1c} exhibited an appreciable increase in diabetic patients, to approximately double the values found in normal subjects. Shortly afterward came proof that a hexose of undefined identity was linked to the N-terminal valine of β chains in Hb A_{1c} (B21). Intensive studies over the following 10 years disclosed that Hb A_{1c} results from a nonenzymatic glycosylation of Hb A_0 (M11, S46) and that other hemoglobin species (embryonic, fetal, A_2) and variant hemoglobins undergo a similar process. Subsequently, it became evident that a wide variety of

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proteins, depending mainly on reactive valyl and lysyl residues in their amino acid composition, underwent a variable degree of glycosylation.

Various aspects of the process of glycosylation and its clinical correlates from the diagnostic viewpoint (B1, G13, J7, N6, R2) and implications in the pathophysiology of complications in diabetes mellitus and other diseases (B12, B33, C6, P9) will be examined and reviewed.

1.2. Clinical Aspects

Nonenzymatic glycosylated proteins have become of interest to the clinician and clinical chemist from two aspects; first, in the assay for glycosylated hemoglobin, first described in 1971 by Trivelli *et al.* (T8), and subsequently for serum albumin (D6, D22, G26) as probes of control in glycemic states, with particular reference to diabetes mellitus. To date, these tests form the principal applications in routine diagnostic laboratories. Second, there are experimental studies and intense speculations whether glycosylations of proteins, and particularly long-lived proteins, contribute to the manifold tissue complications that develop in the chronic diabetic state. As a corollary is the consideration whether periodic lifelong monitoring of glycosylated hemoglobin (GHb) and plasma albumin (GA1b) with improved glycemic control will diminish the incidence and severity of these complications. There is also the consideration whether glycosylation of long-lived proteins (lens crystallins, collagens, etc.) forms part of the aging process.

2. Nonenzymatic Glycosylation of Proteins

The reaction of amino acids, chiefly lysine and valine, with sugars having reducing groups has been known since 1912 (M2, M3) and is familiar to food chemists and technologists as the "Maillard" or "browning" reaction. There is an extensive literature on the chemistry of the Maillard reaction, and detailed reviews have appeared periodically (C4, E3, K1, L6, R6). The three stages of the sugar–amino acid condensation reaction were clarified over 25 years ago by Hodge (H12, H13). The first phase involves a condensation between a free amino group from an amino acid (or a protein) and the aldehyde group of a reducing sugar (e.g., glucose), resulting in a Schiff base (aldimine) and a molecule of water. Subsequent cyclization and isomerization under acidic conditions results in a 1-amino-1-deoxy-2-ketose Amadori (ketoamine) rearrangement. Aldopentoses are more reactive than aldohexoses, which are more so than disaccharides.

The second step involves the removal of amino groups from the reducing sugar complex with subsequent dehydration, cyclization, degradation, or

amine condensation. At food storage temperatures, hydroxymethylfurfural, reductones, dehydroreductones, and α-dicarbonyls are formed. At higher temperatures during food processing, dehydration of α-amino acids to the next lower aldehyde occurs, with pyrroles, pyrazines, and sugar degradation products being formed. Such compounds produce the aromas of cooked foods. The third and final phase of the Maillard reaction results from polymerization reactions of the second-step compounds, yielding soluble and insoluble brown melanoidin pigments.

While the aldimine and ketoamine stages had been identified in living biologic tissues, it is only in the past 5 years that attention has been directed to the possibilities of advanced Maillard reactions and the formation of post-Amadori rearrangement products (B33, P13).

2.1. Chemical Aspects

One has to reflect on the situation that while glycosylation of food amino acids (and proteins) was a well-known phenomenon in the 1950s and 1960s, it was not until the identification of the increased glycosylated hemoglobin in diabetics in 1971 (T8) that it was realized that the nonenzymatic glycosylation of hemoglobin was a universal phenomenon in all biological species possessing this protein. Only in the past 8 years has it been established that nonenzymatic protein glycosylation is a feature common to most biological systems.

Another indication of the relevance of the Maillard reaction to human nutrition and metabolism was the finding of urinary loss of bound amino acids after infusion of autoclaved sugar (glucose or fructose)—amino acid or peptide solutions. Nonutilizable amino acids were detected in the blood and urine (S38, S39). After these and other reports, parenteral solutions were sterilized by nonheat methods.

Little attention has been devoted to the relation of late browning reactions to human metabolism. However, in considering long-lived proteins in man (e.g., lens crystallins, collagens, glomerular basement membrane), it is apparent that such reactions may be relevant to aging processes.

Red cell glucose, galactose, and their phosphorylated derivatives react with the terminal valine of the α- and β-hemoglobin chains and with valyl, lysyl, and other amino acids with a free ϵ-NH_2 group in nonterminus sites of the hemoglobin molecule to form glycosylated and galactosylated, and so on, hemoglobins. A labile and reversible Schiff base [aldimine, e.g., β-*N*-valyl-1-deoxyglucose (glucosylvaline)] is formed. This then undergoes a time-related Amadori rearrangement (H13) to form a relatively irreversible 1-deoxy-2-ketose hemoglobin adduct [ketoamine, β-*N*-valyl-1-deoxyfructose (generically termed fructosamine)]. This is depicted in Fig. 1. The terminal

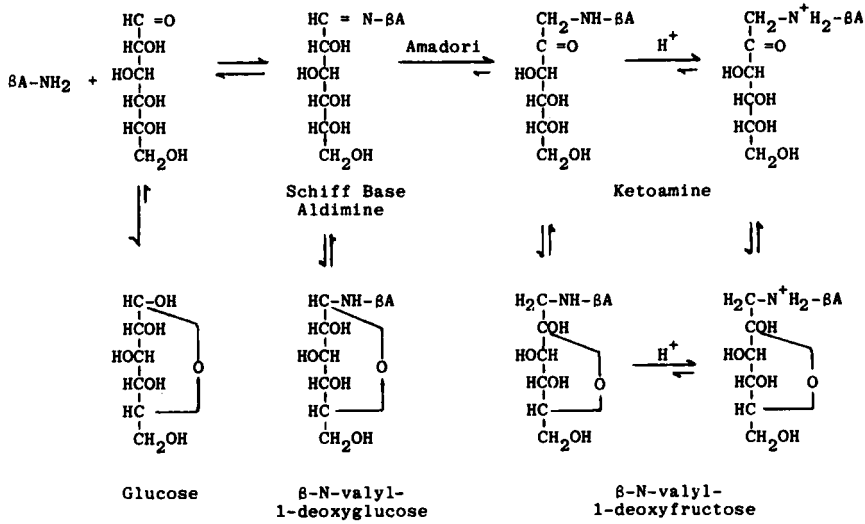

FIG. 1. Formation of hemoglobin A_{1c}. The configuration of the reacting glucose is mainly in the ring form (F5). The adduct illustrated here is with the terminal β-valine of adult hemoglobin. The same reaction occurs with reacting valine and lysine (and possibly arginine and alanine) residues in peptides and proteins. Similar reactions occur with a variety of sugars. (See Table 1.) Modified from Bunn (B39).

valines are more reactive than the ϵ-amino groups due to their lower p*K*, making them more efficient nucleophiles for the initial aldimine adduct formation (B40, B42). The subsequent ketoamine decreases the isoelectric point (p*I*) of the protein and permits the separation of Hb A_{1c} (a β-valine adduct displacing diphosphoglycerate) as a distinct entity by various methodologies. The α-valine is less reactive, possibly due to the presence of diphosphoglycerate binding to β-Val 1 and a lesser p*K* decrease, so that the hemoglobin adduct (A_{1d3}) is only slightly faster moving than A_0 (A2, A7). This probably corresponds to the observation of various authors that the leading edge of A_0 contains carbohydrate and results from the inadequate resolution by their electrophoretic and chromatographic methods. Aldohexose adducts with lysine residues in hemoglobin do not produce a change in charge relations; hence chromatography at pH 7 does not separate lysine adducts, since these and unaltered ϵ-lysines are completely positively charged.

Following the study of hemoglobin (B41), glycosylation has been demonstrated for many proteins, ranging from those with short (e.g., albumin) to very long (viz., lens crystallins, elastin, collagens) biological half-lives.

The term glycosylation (more rarely, glucosylation) has been used almost universally to reflect the nonenzymatic adduct of the aldohexose glucose

with free-NH_2 groups in amino acids, present as such or in peptides and proteins. Glucosyl valine and glycohemoglobin have also been used to describe the ketoamine products formed. Roth (R14) has stated that the correct and recommended designation is glycated protein; glycosylation is retained here because of its very wide acceptance.

2.1.1. *Structure of Glycosylated Hemoglobins*

By purification, chemical transformations, and subjection to tritiated borohydride reduction, isolated hemoglobin fractions were characterized; confirmation was provided by incubation *in vitro* of pure Hb A_0 with labeled sugars and markers. The minor components of human adult hemoglobin (A_0) and that of miscellaneous animal species have been separated by chromatographic and electrophoretic techniques into faster eluting or moving A_{1a1}, A_{1a2}, A_{1b}, and A_{1c}. Thus A_{1a1} proved to have the N-terminal β-valine linked to fructose 1,6-diphosphate, and possibly other red cell phosphates such as ADP or ATP (H5); A_{1a2} with glucose 6-phosphate (H5, M11, S46); A_{1b} with a sugar as yet not definitively identified; and A_{1c} with glucose (K15). Using large columns with Bio-Rex cation-exchange chromatography at 4°C and an exponentially increasing sodium phosphate gradient, Abraham *et al.* (A1, A2) have identified the following array of hemoglobins, all containing a protein-bound ketoamine, separable and eluting in the following order: A_{1a1}, A_{1a2}, A_{1b1}, A_{1b2}, A_{1b3}, A_{1c}, A_{1d1}, A_{1d2}, and A_{1d3}. Hemoglobin A_{1c} was present in largest concentration, some 5% of total hemoglobin, with Hb A_{1d3} next in frequency at about 40% of the Hb A_{1c} levels; both were increased some 75% in diabetic subjects. Thus Hb A_{1c} values provide the best indicator for the glycosylation of adult hemoglobin. The extent of glycosylation of Hb A_{1c} is conditioned by the concentration and time duration of glucose exposure to form a stable ketoamine, the kinetics of the aldimine and ketoamine processes, and the life span of the red cell containing the particular hemoglobin molecule (H6a).

After purified hemoglobin has been incubated with [^{14}C]glucose *in vitro* or human hemoglobin has been purified from red cells and incubated with [^{3}H]$NaBH_4$, studies of the structure of the product have indicated that specific valines and lysines in both the α- and β-hemoglobin chains undergo glycosylation (B39, B42). Apart from the main residues, β-Val 1 and α-Val 1, these are α-Lys 7, α-Lys 16, α-Lys 40, α-Lys 61, β-Lys 8, β-Lys 17, and β-Lys 66. One or more sites may be glycosylated (A2, B39). Examination of the Perutz model of the hemoglobin molecule reveals that the nonterminal lysine residues tend not to be on the exposed surface of the globin chain. They would, therefore, exert a minor influence, when glycosylated, on the electrical charge relations with the surrounding fluid milieu. Hence glycosylated Hb A_0 migrates chromatographically and electrophoretically close to Hb A_0.

Normal hemoglobins other than Hb A_0 also undergo glycosylation. This became apparent when estimations of glycosylated hemoglobin by different methods were compared, particularly in respect of column chromatographic and photometric determinations (e.g., B42). Thus human Hb A_2 (T2) and Hb F (C5) and mouse embryonic hemoglobin (M4) have all been shown to undergo glycosylation. Glycosylation of human hemoglobin and other proteins in the embryo and fetus may thus be of major clinical importance to the fetus in maternal diabetes.

Similarly, glycosylation of variant hemoglobins C, D, E, G, and S has been reported (A2, A5, B10, K18, T3). The glycosylated hemoglobins of these variants all exhibit chromatographic and electrophoretic mobilities different and clearly separable from the parent hemoglobin. It is reasonable to conclude that all hemoglobins form adducts with reacting sugars. Relevance to interpretation of assay results and functional aspects will be dealt with in Sections 3.5 and 5.3.

Myoglobin, with an analogous structure and function to hemoglobin, is also glycosylated (B9). Purified horse and dog skeletal and heart muscle myoglobin was found to have 1.4–2.0% glycosylation. Since the 153-amino acid myoglobin has no terminal valine, it is presumed that the lysine residues undergo glycosylation; more recently four separate samples of human myoglobin were found to average 2.3% glycosylation (B13).

2.1.2. *Glycosylation of Other Proteins*

Because of simplicity in isolation and examination, glycosylated total serum (plasma) proteins have been estimated (K7, M12, Y1). Nevertheless, the assay of glycosylated serum albumin is preferable, since it is a homogeneous entity and has a fairly specific biologic half-life of some 20 days. Glycosylation of horse serum albumin was reported in 1956 (M21). However, it was not until the phenomenon of human hemoglobin glycosylation was well accepted that glycosylated serum albumin was found in 1979 to be a normal constituent of human blood and also elevated in diabetic specimens (D6, D22, D23, G26, N10). With the processing of human blood for very pure albumin for intravenous therapy in burns and during surgery, for example, a number of simple techniques are available for the preparation of pure undenatured human serum albumin (H10). These are readily applicable to the separation of serum albumin and its glycosylated species. Lysine residues 199 and 525, with low pK_a, are the principal sites of glycosylation (B6, G6, S23). Of particular interest is that Lys 199 is the sole binding site for acetylsalicylic acid (aspirin); at one-tenth the molar concentration of glucose, acetylsalicylate will produce a 50% block in albumin glycosylation. Other plasma proteins could be studied [e.g., glycosylated transferrin with a half-

life of 8 days (K3) and glycosylated ceruloplasmin ($t^{1/2}$, 4 days)] by radial immunodiffusion ("Partigen," Behring).

Glycosylation of fibrinogen and fibrin has been shown both *in vitro* and *in vivo* (B9, B30, C1, M16). In coagulation, factor XIII, acting as a transglutaminase, stabilizes the fibrin clot by forming intermolecular cross-links between glutamine and lysine residues (F3) of adjacent fibrin monomers. The fibrin clot is thus destabilized by glycosylation, which takes place at the lysine residues.

Glycosylation of lipoproteins appears to occur preferentially for the low-density (LDL) forms (G15, H6a, S5, S9, S40, S41), but has also been reported for high-density lipoprotein, HDL (W16).

Glycosylation of the red cell membrane proteins (B2, H6a, M25, S10), including spectrin (M13), has been studied in relation to the deformability of the erythrocyte membrane. Glycosylation of the membrane proteins of platelets, leukocytes, lymphocytes, and macrophages would be relevant to functional aspects.

Glycosylation of the lens crystallin proteins (A11, C8, C9, H6a, M5, M31, P4, S45) is of considerable interest because of the long-lived nature of the α, β, and γ crystallins, the molecular aggregation and decreased solubility of the glycosylated products, the possibility of further reactions leading to "browning" effects (M28, M29), and their relevance to cataract formation and other complications in diabetes and aging.

Collagen is long-lived and the most abundant protein in humans; as the fibrillar protein of connective tissues, it accounts for the main mass of skin, hair, bones, tendons, and blood vessels. Its structural and functional properties depend on intra- and intermolecular cross-linking of collagen fibrils, mediated by the presence of lysine and hydroxylysine residues. Glycosylation of collagen leads to interference with cross-linking produced by deamination (H2, K17, R8, R9, S13, S14). In view of its particular importance in human disease, the glomerular basement membrane collagen and the effect of its glycosylation merit separate consideration (C14, M15, S12). Because of easy access, glycosylated hair collagen has been studied (P1).

Glycosylation of myelin protein elements in the peripheral and central nervous system (V5) may have fundamental implications for nerve conduction and central nervous system functions.

Glycosylation of insulin has been shown *in vitro* (D21). The only other peptide hormone with any significant number of valine and lysine residues is the parathyroid hormone. However, with the short half-life of these hormones in the circulation and tissues, any degree of stable ketoamine formation will be slight. With regard to nonenzymatic glycosylation of enzymes, this has received little attention as yet.

Urinary excretion of glycosylated amino acids and peptides has been

monitored (B29). Amino acid analysis of NaB^3H_4-reduced hydrolysates indicated that glycosylated lysines formed 67–86% of the ninhydrin-positive glycosylated amino acids and peptides in urine; the mean level in 13 diabetic patients was 1.5 times greater than that from 9 normal volunteers.

Nonenzymatic galactosylation of human serum albumin (U2, U3) and LDL (S4) has been demonstrated both *in vitro* and after isolation from human subjects with galactosemia.

2.2. Biochemical Aspects

2.2.1. *In Vitro Biosynthesis*

Haney and Bunn (H5) and others subsequently (B41, S24) prepared human Hb A_0 by chromatography and purified it free from other hemoglobins and modified products. Incubation with glucose and other sugars (mannose, lactose, galactose) for varying times and sugar concentrations showed that these reacted with Hb A_0 to form Hb A_{1c}, identical with naturally occurring Hb A_{1c} isolated from red cells of normal and diabetic subjects (K15, M11, S46). Previously, the amino acid sequences of Hb A_0 and A_{1c} had been shown to be identical (S18), with the latter having a low-molecular-weight $NaBH_4$-reducible Schiff base moiety, at the β-NH_2 (valine) terminus (B21). The precise nature of the adduct was shown to be glucose (B40), as confirmed by Koenig *et al.* (K15). They isolated the reducing sugars by mild acid hydrolysis and identified them as glucose and its epimer mannose in a 3 : 1 ratio. The nature of the sugar–protein link in Hb A_{1c} was determined by treatment with NaB^3H_4 and oxidation with periodate. If linked as an aldimine (Schiff base), then tritiated formaldehyde should have been found; instead, virtually all radioactivity was recovered as tritiated formic acid. This indicated that glucose reacted initially with the β-Val 1 terminus to form an aldimine linkage and then underwent an Amadori rearrangement to form the more stable ketoamine compound. This was identified as 1-deoxy-1-(*N*-valyl) fructose (K15) and is present in a ring (probably chair) configuration (F5).

Incubation of purified Hb A_0 (and other hemoglobins) with [U-^{14}C]glucose, [1-^{14}C]glucose, and [2-^{14}C]glucose confirmed these conclusions. The nonenzymatic nature of the process was indicated by the fact that red cell hemolysates and pure Hb A_0, incubated with glucose or labeled glucose, formed Hb A_{1c} at identical rates, and that L-[^{14}C]glucose and D-[^{14}C]glucose reacted similarly. Bunn *et al.* (B42), incubating pure Hb A_0 with [^{14}C2-^{3}H]glucose, used the ^{3}H : ^{14}C ratio in Hb A_{1c} to measure accurately the Amadori rearrangement to the more stable ketoamine. In the Amadori rearrangement the tritium would be lost from carbon two in the sugar protein adduct. After 6 days, some 60% of the newly synthesized Hb A_{1c} had undergone the Amadori rearrangement and an estimated 91% after 22 days.

Hemoglobin A_{1c} increased with time of incubation, glucose concentration (5–500 m*M*), and temperatures (4–37°C) in intact red cells (S36). Low temperatures permitted prolonged incubations with minimal hemolysis. Incubation in 500 m*M* glucose at 4°C resulted initially in a linear increase of Hb A_{1c} until, at 6 weeks, it comprised 50% of total hemoglobin. Increments appeared to attain a plateau, but interpretation may be complicated by increasing hemolysis in the incubates.

Studies were conducted in 40% red cell suspensions in autologous human plasma containing 5–50 m*M* repurified glucose (corresponding to concentrations that might be expected in diabetic bloods) and other pure sugars [1-*O*-methyl-α-D-pyranoside, fructose, mannose, galactose, maltose, lactose (Sigma)]. Incubations were at 37°C in a 20% O_2–80% N_2 atmosphere with automatic regulation of suspension pH at 7.4, for periods up to 56 hours. With particular attention to experimental conditions, there was less than 4% hemolysis and 1.5% methemoglobin at the end period. Ketoamine formation was slight for the nonreducing sugar, methyl glucoside, and the ketohexose fructose (due to steric hindrance in aldimine formation). Galactose the four-carbon epimer of glucose, was more reactive than glucose itself, with the two-carbon epimer mannose intermediate. Lactose produced two to three times the amount of ketoamine compared with glucose, with maltose slightly more reactive than glucose itself. The results are summarized in Table 1. Examination of molecular models of sugars in the ring or chair form, with reference to the terminal Val–His dipeptide in the hemoglobin β chain, indicated structural constraints providing a more favorable steric disposition of the OH groups and H atoms for galactose relative to mannose and glucose.

TABLE 1
In Vitro INCUBATION OF RED CELL SUSPENSION WITH SUGARS

Sugars			Hb A_{1c} (%)				
Glucose (m*M*)	Other (m*M*)	Incubation (hr)	0	24	32	48	56
0		5.48 ±0.18					
5				5.2	5.4	5.9	5.8
25				6.0	6.8	7.2	7.3
50				6.6	7.0	7.5	7.9
0	Methyl glucoside, 50			5.2	5.4	5.0	—
0	Fructose, 50			5.0	4.7	5.2	—
0	Galactose, 25			6.8	8.2	10.5	11.4
0	Galactose, 50			8.4	10.4	13.0	13.6
0	Mannose, 50			11.1	13.8	15.0	15.6
0	Lactose, 50			—	11.2	—	15.0
0	Maltose, 50			5.6	—	5.8	6.1

In addition to free and phosphorylated glucose conditioning the biosynthesis of glycosylated hemoglobins, glucose can be transferred from membrane glycoconjugates to the hemoglobin of isolated young red blood cells (G11). While of considerable theoretical interest, the amount of glycosylated hemoglobin formed by this and similar processes must be of minor practical importance.

The biokinetics of the formation of the ketoamine adduct Hb A_{1c} through a labile and reversible aldimine form (T7) has received intensive study in recent years (A3, B40, H8, M33, M35, S48, W5). The initial rate for the aldimine condensation is very similar in several reports (H8, M33, S48, W5). The formation of the final ketoamine was considered to be irreversible (B40), and Graf *et al.* (G21), finding a 50% conversion of hemoglobin A to A_{1c} *in vitro*, projected a saturable system of 23% glycosylation *in vivo* for severe states of glycemia. However, prolonged saline incubation of pure Hb A_{1c} revealed progressive decrease in Hb A_{1c} concentration with concomitant formation and increase of Hb A (M33, M35). The degradation of Hb A_{1c} to the aldimine was some 12% the rate of its formation. Thus, a steady-state Hb A_{1c} concentration would be reached in 3 to 4 weeks after a change in blood glucose level; this finds confirmation in clinical investigative experience.

In 1979 it was shown that human albumin (HSA), isolated and purified from serum of normal and diabetic subjects, exhibited some 8% and 16% glycosylation, respectively (D6, D22, G26, M12). The spectrophotometric assay gave somewhat higher values than the column chromatographic procedure (G26), attributable to the possibility that some lysine and valine adducts with glucose did not alter the mobility of glycosylated albumin. Since the valines (39) and lysines (58) make up one-sixth of the 585 amino acid residues in human albumin, the degree of glycosylation (8%) is similar to that in adult human hemoglobin, with 28 valine and lysine residues (one-fifth) in the 145 amino acid β chain, and exhibiting 5% Hb A_{1c}, plus other minor hemoglobin glycosylations.

The biokinetics of albumin glycosylation, studied with radioactive glucose (B6), indicated that the incorporation of glucose into the acid-labile Schiff base adducts of HSA (aldimine and N-substituted aldosylamine) was complete in 1 to 2 hours and that some 2% HSA contained glucose bound in this form under physiologic conditions (5.5 m*M* glucose, pH 7.4, 37°C). From the kinetic constants involved in the formation and dissociation of the cyclic glycosylamine (aldosylamine) and its Amadori rearrangement product, N-substituted 1-amino-1-deoxyketopyranose, an estimate of 29% glycosylation by the radiochemical procedure was found (B6). This is in close agreement with the value of 28% based on the furosine assay (S11). The *in vivo* glycosylation at 8% reflects the considerable binding capacity of HSA for endogenous metabolites (e.g., bilirubin) and exogenous substances (e.g., drugs).

2.2.2. *In Vivo Biosynthesis*

The biosynthesis of Hb A_{1c} in humans was followed after an injection of [^{59}Fe]transferrin into a human volunteer (B41). The specific activity of Hb A_{1c} increased slowly and at day 60 started to exceed that in Hb A_0. It was concluded that Hb A_{1c} is formed continuously at a slow rate over the 120-day life span of normal human red cells. Young red cells, separated by density gradient, have lower concentrations of Hb A_{1c} than do old cells (F7).

The biokinetics of the formation of Hb A_{1c} was studied in patients with acute changes in blood glucose levels. Rapid increases in Hb A_{1c} levels were observed by column chromatographic assays. The enigma was resolved when the thiobarbituric acid estimation for glycosylated hemoglobin did not reflect such increases or changes. Short-column chromatographic procedures were not able to differentiate between the aldimine intermediate and the stable ketoamine product. Transfer of red blood cells, incubated with high glucose concentrations, to a glucose-free or low-glucose medium or active insulin treatment of diabetics with high blood glucose resulted in rapid decrease of the aldimine fraction with no change of the ketoamine moiety.

In summary, the formation of Hb A_{1c} is a two-stage process, the initial reaction being rapid to form the aldimine adduct, a labile form, which can either proceed by Amadori rearrangement to form the covalently bound and almost irreversible ketoamine as a result of continued time–concentration pressure of glucose or proceed to reverse, liberating free glucose (or phosphorylated glucoses), should the pressure concentration of blood and tissue glucose decrease. The kinetic constants are given in Fig. 2. Many of the reports on rapid or fast increases or decreases in Hb A_{1c} in diabetic patients failed to appreciate the biokinetic implications in the formation of the ketoamine, Hb A_{1c}; in general, their conclusions are erroneous, and such references are not included in this review. Normally, about 10% of Hb A_{1c} is in pre-Hb A_{1c} form. In human hyperglycemia, the rate of Hb A_{1c} increase and the final Hb A_{1c} value attained depended upon the integrated time duration–glucose concentration of hyperglycemia and the red cell life span.

2.3. Functional Aspects

2.3.1. *Glycosylated (Glycated, Glyco-) Hemoglobin*

The covalent binding of β-Val 1 to glucose in Hb A_{1c} blocks the binding of 2,3-diphosphoglycerate (DPG) at this site, thus altering the oxygen dissociation curve (shift to the left) and increasing the oxygen affinity of Hb A_{1c} (D13). Hemoglobin A_{1d3}, with α-Val 1 bound to glucose, has β-Val 1 free to

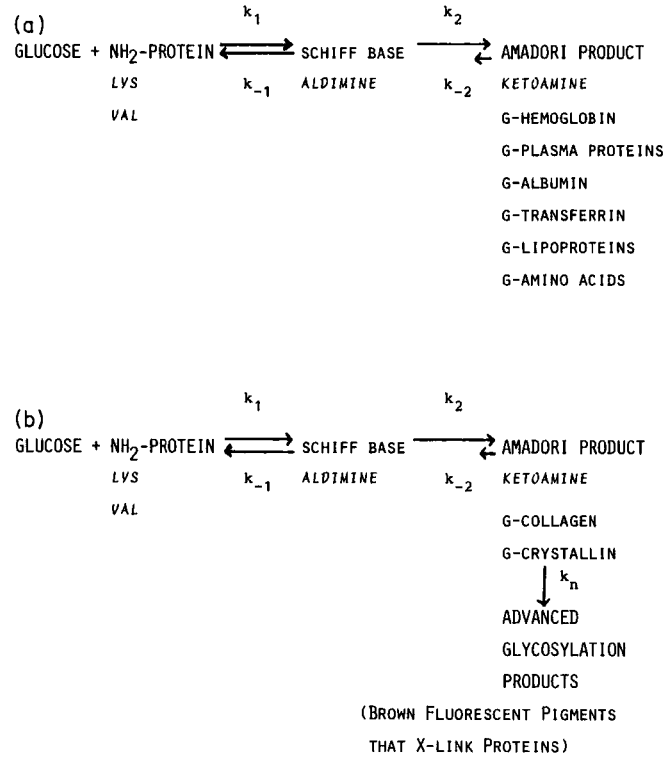

FIG. 2. Formation of ketoamines and advanced glycosylation final products in proteins: (a) proteins with short and intermediate life spans; (b) long-lived structural proteins. Advanced glycosylation final products are mainly confined to long-lived structural proteins. The kinetic constants k_1 and k_{-1} for pure hemoglobin A_{1c} and the glycosylated hemoglobin species A_2, F, and S, prepared from human red blood cells, were similar at $0.1 \times 10^{-3}\, M^{-1}\cdot\text{second}^{-1}$. The k_2 rate constants for the aldimine to ketoamine conversion were $\frac{1}{8}$–$\frac{1}{8}$ the k_1 value for the hemoglobin species and $\frac{1}{2}$ for albumin. The rate constant k_{-2} averaged 7.5% of the forward reaction for the dissociation of Hb A_{1c} and some 12% for the conversion of glycosylated (ketoamine) albumin to its labile aldimine form (B13a).

bind DPG, and thus an oxygen dissociation curve indistinguishable from Hb A_0.

2.3.2. *Glycosylated Plasma Proteins*

Plasma albumin has binding sites for various natural metabolic substances (e.g., glucose, fatty acids, bilirubin) as well as xenobiotics and drugs (e.g., digitoxin, Warfarin). While chromatographic and solubility differences from pure human serum albumin occur in some glycosylated albumins, there does

not appear to be any alteration in functional properties (D6). Incubation of fresh serum with [^{14}C]glucose and electrophoresis of the trichloracetate precipitate resulted in highest radioactivities (and therefore protein glycosylation) in the albumin and α_1-globulin fraction, with 55–58% incorporation (and formation) in the other globulin fractions (D6).

Fibrinogen, particularly its lysine-rich γ fragment, undergoes extensive glycosylation (B13); assessed by thrombin time and fibrin aggregation, glycosylated fibrinogen exhibited reduced clottability (B13). Further, glycosylation of fibrinogen and fibrin decreases the susceptibility of fibrin to plasmin degradation by 10 to 90%, depending on the conditions of incubation with glucose or glucose 6-phosphate (B30).

Some 2% of lysine residues in low-density lipoproteins (LDL) of plasma were glycosylated in euglycemia and up to 5% in the outpatient diabetic (S9); with modification of some 2–5% lysine residues, the catabolism of LDL was decreased by 5 to 25% (S40). In poorly controlled diabetics, the decrease in LDL catabolism in diabetics could produce an 8–27% reduction in LDL cholesterol (A4).

Other plasma proteins exhibit glycosylation, but there are currently few established reports of functional effects. Of interest is glycosylated transferrin; with its short half-life of 8 days (K4), glycosylated transferrin assays can be used to follow short-term variations in glycemia (K3).

2.3.3. *Red Cell Membrane Proteins*

Glycosylation of the red cell cytoskeleton [e.g., membrane proteins (M25) and in particular spectrin (M13)] leads to decreased deformability and a concomitant decrease in erythrocyte life span. This has been monitored by the centrifugation packing coefficient, filtration, and negative-pressure micropipette techniques with red cell suspensions incubated *in vitro* with 25 m*M* glucose or from *in vivo* preparations from normal and diabetic individuals (B13). The relevance of these observations will be discussed in Section 5.5.1.

2.3.4. *Long-Lived Proteins*

The nonenzymatic glycosylation of lens crystallins (little or no turnover during the individual's life span) and membrane proteins (elastin, collagens, and basic myelin protein) with very slow turnovers is not only increased in states of persistent hyperglycemia, but may in time proceed to the formation of browning products of the Maillard reaction (M28, M30, S14). Nonenzymatic glycosylation of this group of proteins may herald particular aging processes (e.g., cataract formation, arteriosclerosis, glomerular basement membrane thickening).

3. Methodological Aspects of Assays

3.1. Specimen Collection and Storage

Any method of blood sampling (finger prick, venepuncture, venous line) is satisfactory. Anticoagulated specimens (heparin, EDTA, acid citrate) are more convenient, since separation of red cells and plasma permits diagnostic assays of glycosylated hemoglobin and glycosylated albumin to be performed on one blood sample.

Samples may be taken at any time, except after a meal when hyperlipidemia and chylomicronemia (D15) interfere with sample preparation and analyses. If an oral glucose tolerance test (OGTT) for diabetic screening or diagnosis is also to be performed, blood from the fasting (resting) blood sample is preferable, since this will require less washing of red cells to remove plasma glucose. Its presence would continue the glycosylation of hemoglobin as a time–concentration-related event.

As for most procedures in clinical chemistry, prompt analysis is preferable, but often not feasible. Whole blood samples at room temperature or 4°C should be processed within 6 and 24 hours, respectively. For storage for any length of time beyond this, preparation of a dilute hemolysate or more vigorous procedures involving dialysis to produce stroma-free hemolysates is mandatory to provide correct values in subsequent assays of glycosylated hemoglobins (G2, H3, S7). Storage artifacts may produce either increases or decreases in glycosylprotein values. The former arises from the presence of lactescence or stroma or free glucose in hyperglycemic specimens; in addition, this may occur from the formation of hemoglobin breakdown products (H17) or progressive formation of a complex between hemoglobin and oxidized glutathione, Hb A_{1d} (H16), which, for example, cochromatographs with Hb A_{1a+b} in short-column cation exchange resin chromatography (S7). The latter arises from aldimine dissociation when hyperglycemic specimens are stored in glucose-poor or glucose-free media. Methods estimating Hb A_1 and not the ketoamine Hb A_{1c} component are particularly subject to errors in this regard. For long-term storage at 4°C (preferably −20°C to −80°C), hemolysate dilution and/or dialysis is essential; the glucose of the biologic fluid must be removed, and a sulfhydryl reagent (e.g., cysteine) added to the diluted hemolysate.

3.2. Sample Preparation

Although many different procedures have been described for preparation prior to glycosylated hemoglobin assay, a standard method applicable to

most techniques would be to centrifuge red cells to remove plasma, with washing the packed erythrocytes one or two times with isotonic saline to remove glucose from the medium. The red cells are then hemolysed with hypotonic saline, or more vigorous extraction procedures including dialysis are adopted to produce stroma-free hemolysates.

Total plasma proteins have been processed directly for their glycosylated fraction from plasma after trichloracetic acid precipitation (J6, K6, M12, Y6). For plasma (or serum) albumin, a plethora of methods have been described for the preparation of highly purified human serum albumin; the author has scaled down the chromatographic method of Curling *et al.* (C20), with estimation of the glycosylated fraction by column chromatography or the spectrophotometric thiobarbituric acid method (G26). Similar methodologic considerations apply to the study of glycosylation in fibrinogen, lipoproteins, and the long-lived tissue proteins, such as collagen, crystallins, and basic myelin protein.

3.2.1. *Removal of Free Glucose in Plasma*

A major cause for erroneous glycosylated protein analyses is the presence of plasma glucose in bloods after sampling. Many of the early studies were subject to this source of error (e.g., G2). For assays of Hb A_1, Hb A_{1c}, and glycosylated erythrocyte membrane proteins (i.e., spectrin), plasma should be removed as soon as possible and the red cells suspended in glucose-free isotonic saline for washing one or two times or incubated overnight to discharge the aldimine moiety of glycosylation.

3.2.2. *Removal of Labile Glycosylated Hemoglobin*

In those methods that do not distinguish the labile aldimine adduct from the stable ketoamine component, the former may be removed by incubating red cells in glucose-free isotonic saline or buffered media for 12 hours (D2). However, specific acid catalysis of the aldimine adduct is rapid (30 minutes) and complete at pH 5–6 and is achieved by incubating red cells or hemolysate with concentrated (0.2–0.6 *M*) buffers (e.g., borate, acetate) in the pH 5.0–6.5 range. This was preferable and more effective than to discharge the aldimine complex by poisonous chemicals, namely, at pH 5 for 30 minutes with 30 m*M* semicarbazide and 12 m*M* aniline (N3) or sodium acetate-buffered saline (B15). Adaptations of these principles in commercial methods have been reported (N4).

If preparatory prerequisites are followed, samples for glycosylated hemoglobin and plasma protein assays may be stored, depending on refrigeration temperatures, for months and years without loss of activity.

3.3. Methods of Measurement

Assay procedures for glycosylated proteins incorporate varied methodologies (A1) and have been tailored to accommodate certain specific requirements. The identification and quantitation of the ketoamine link in a variety of proteins have rested mainly on the spectrophotometric thiobarbituric acid assay. The standpoint is emphasized that short laboratory time assays for ketoamine-adducted proteins hold preeminence over rapid reporting of Hb A_1 values as representing glycohemoglobin, unless strict attention to the control of sources of variation is adhered to. Exclusion of free glucose and the labile aldimine adduct is mandatory in all glycosylprotein assays.

For routine estimations, where rapidity of measurement with a reasonable degree of accuracy is called for, the short-column cation-exchange resin chromatographic technique has been most extensively used. Nevertheless, after its introduction in 1977, many modifications were required to eliminate sources of interference, and only recently described or introduced commercial methods should be used. Current developments, particularly the introduction of *m*-aminophenylboronic acid as an affinity chromatographic medium, have improved the specificity and accuracy for Hb A_1 and Hb A_{1c} by short-column analysis. For large diabetic clinics some form of automation is preferable, for example, automated high-pressure liquid chromatography, spectrophotometry using the Auto-Analyzer, agar gel electrophoresis with scanning densitometry.

Essential requirements in assay procedures include (1) for sample storage, the removal of free glucose from the medium containing glycosylated protein, and dilution and dialysis (or ultrafiltration) for hemolysate or protein preparation; (2) specific assay for the stable ketoamine adduct or prior removal of the labile aldimine adduct; and (3) provision of a standard and quality control.

3.3.1. *Column Chromatography*

The reference procedure for glycosylated hemoglobin was the original long-column cation-exchange resin method (T8), adapted from standardized procedures for separating minor and variant hemoglobins from Hb A_0. The chromatographic run took 24 hours and Hb A_{1c} separated clearly from other fast-eluting hemoglobins. Logistic characteristics of long-column methodology rendered it unsuited to routine clinical use, and a universal trend has been the development of short-column procedures and their modification.

A short-column (5 cm in a 20-ml syringe) variation of the Trivelli (T8) procedure was first described in 1977 (K21) and required $2\frac{1}{2}$ hours; a similar

method took 20 minutes (W4). The technique eluted "fast-moving hemoglobins" as a composite fraction and, as indicated by the authors, measured Hb A_1 and other fast hemoglobins such as hemoglobin F and certain hemoglobin variants. A modified microcolumn method described at this time permitted the separation of Hb A_{1c} from other Hb A_1 fractions (J5). A number of commercial minicolumn methods, utilizing disposable columns and resin, were developed from these findings (C2, M10, M11). All these procedures sacrificed resolution and accuracy for rapidity and simplicity in resin preparation and buffer requirements; in particular, authors and workers did not take cognizance of the physical and chemical problems involved in the use of cation-exchange resins in minicolumns (short) and microcolumns (narrow). Sources of variation and critical factors in short-column chromatography soon became apparent (R15, S7, S27). They include the composition of the first eluting buffer and its optimum pH (pH 6.7, S7; pH 6.78, S27), sample load (1.3–2.0 mg Hb/ml resin, S7), and the rate of elution of the buffer. The use of two elution buffers did not discriminate between Hb A_1 fractions; however, by employing a three-buffer system, it was possible to separate Hb A_{1c} from A_0 and other rapid hemoglobins [e.g., A_{1a}, A_{1b} (B43, M6)]; the former method is automated.

Temperature control of the short resin column was of particular importance. At 22–24°C, results corresponded closely to values found on macrocolumns. For every 1°C rise in temperature, there is an increase of Hb A_1 concentration by a 0.5% (D16) or 1% (R10) point value. Most commercial methods supply temperature correction charts (R10) or factors (H6). While some have found a linear relation for assay temperatures between 16 and 30°C and the increase of Hb A_1 value (D16, R10), others have reported it as only linear between 16°C and 22°C (H6). Alternatives are to use a temperature-controlled room or a Perspex water bath to take 20 to 40 minicolumns, with water at 23°C circulated from a constant-temperature water bath. The smaller the elution volume, the more critical is the influence of temperature and the necessity for strict control (S42). The results for *all* short-column methods should be corrected to a 23°C value and so expressed. Hammons *et al.* (H4) have presented a valuable evaluation of three commercial minicolumn kits. Recently, an automatic low-pressure liquid chromatographic system has been described (B18), and Diamandis *et al.* (D11) have reported favorably on an automated Hb A_{1c} analyzer, whose separation principle is a combination of reversed-phase partition and cation-exchange chromatography.

A modification of the resin column mode is centrifugation, at 150–200 *g* for 1 minute, of resin with overlying hemolysate from a plastic eluent container threaded onto a centrifuge tube (C2, D4). Sixteen tests can be com-

pleted in 15 to 30 minutes. The plastic connection and resin are reused. Subject to control of the same factors as for the resin column, similar results are obtained more rapidly.

Batch chromatography represents resin separation of hemoglobins in its simplest form (R11). The resin is equilibrated with a phosphate buffer, pH 6.7, as a slurry and placed on a rotatory mixer with the prepared hemolysate for 30 minutes. Hb A_1, which does not attach to the resin, is separated from it by a serum separator (more rapid than centrifugation) and estimated spectrophotometrically at 415 nm. Several commercial kits are available, with mixing times of 5-, 10-, 15-, and 25-minute rotation, and using centrifugation to separate the resin fines.

These rapid and simple techniques (short column, centrifugation, batch separation), with reusable supplies and equipment, are cost-effective and provide reasonable accuracy, provided that sources of variation are rigidly controlled. In final analysis, accuracy is to be preferred over rapidity and low cost. Glycosylated hemoglobin assays, in contrast to frequent or daily blood glucose determinations, are only required once every 4–12 weeks for diabetic control surveillance.

3.3.2. *High-Performance Liquid Chromatography*

High-performance liquid chromatographic (HPLC) systems, using cation-exchange resins (e.g., Bio-Rex 70) or other column packings, have great sensitivity for the isolation of glycosylated hemoglobins and other glycosylproteins and exhibit excellent reproducibility. Techniques require small samples, are rapid, and can be automated. While initially more costly for equipment, they offer precision of a high order, with intra- and interassay coefficients of variation of 1–3%. Cole *et al.* (C15) were among the first investigators to assemble such a system for the determination of glycosylated hemoglobins and, subsequently, to describe an automated system (D29). Another method for automated HPLC assay of glycosylated hemoglobins (G25) was described shortly thereafter, and the addition of ethanol to the phosphate buffer in a step-gradient system enabled the time for column equilibration between samples to be reduced (S8). Currently, complete systems for "high-pressure" liquid chromatography applicable to glycosylated protein analysis are available from Beckman Instruments, Inc. (2500 Harbor Boulevard, Fullerton, CA 92634); Gilson Medical Electronics (3000 West Beltline, Middleton, WI 53562), Hewlett-Packard (1050 Page Mill Road, Palo Alto, CA 94304), LKB Instruments (9319 Gaither Road, Gaithersburg, MD 20877), Perkin-Elmer Instruments (Main Avenue, Norwalk, CT 06856), Pharmacia (800 Centennial Avenue, Piscataway, NJ 08854; FPLC, fast-protein polypeptide liquid chromatography, Shimadzu Instruments (7102 Riverwood Drive, Columbia, MD 21046), and Waters (34 Maple Street,

Milford, MA 01757). The report of Ellis *et al.* (E4) is an example. Using the cation exchanger Synchropak CM 300 (SynChrom, Inc., Linden, Ind.) and Perkin-Elmer HPLC equipment, superior separation of glycosylated hemoglobin and hemoglobin variants was obtained (H18). While Hb A_{1c} could be eluted as a single zone and accurately quantitated, as was also Hb F_1, fetal hemoglobin could interfere with Hb A_{1c} estimates when present in elevated amounts. Using this procedure (W12), the presence of Hb F_1, Hb S_1, and Hb C_1 could be demonstrated in cases of homozygous Hb SS (sickle cell anemia) and Hb CC. By the use of high-performance liquid chromatographic separation and quantitation of glycosylated hemoglobin A_2, Bisse *et al.* (B15a) found that normal and variant hemoglobins are glycosylated at the same rate *in vivo*, and they propose assay of glycosylated Hb A_2 as an alternative index of intermediate glycemic control. Essentially, concentrations of glycosylated hemoglobin components are influenced primarily by diabetes mellitus and hemolytic anemias.

3.3.3. *Affinity Column Chromatography*

From the finding that boryl-substituted celluloses and other chromatographic supports were capable of forming specific complexes with sugars (W2), it was established that boronate had a high affinity for 1,2-*cis*-diols, as present in the stable ketoamine glycosylprotein complex, but not for the labile aldimine fraction. Dihydroxyboryl-substituted celluloses, or polyacrylamide, or *m*-aminophenyl-boronic acid immobilized on agarose beads bound a variety of ketoamine glycosylated amino acids, peptides, and proteins. Thus glycosylated valine and lysine was identified and quantitated in the urine of diabetic patients (B29), glycosylated hemoglobin in human and mammalian red cells (K13, Y4), and glycosylated albumin and proteins in plasma or serum (G19, Y1). An affinity chromatographic method, utilizing *m*-aminophenylboronic acid immobilized on 6% cross-linked agarose beads, forms the basis for a short-column procedure (A1, H1, Z2) that is a simple, rapid, accurate, and precise alternative to the ion-exchange column with "substantial freedom" from the errors and interferences of that procedure. *Inter alia*, the affinity chromatography method exhibits (1) a small temperature factor, namely, 0.1–0.2% glycosylated hemoglobin for each 1°C increase between 18 and 27°C, about one-tenth that for ion-exchange procedures, although one report (F12) introduces modifications that diminish this error; (2) a pH optimum of 8; variation of pH between 7.75 and 8.15 gives a net change of 0.5% glycosylated hemoglobin; (3) failure to remove the aldimine component giving an error about one-tenth that for ion-exchange chromatography; (4) no effect of hemoglobins F, S, and C, nor up to 20% methemoglobin; (5) no effect for lipemia, icterus, hemolysis, or type of anticoagulant used; (6) no effect from other hemoglobin adducts, for exam-

ple, carbamylation in uremia (B34), acetylation as occurs with fetal hemoglobin (F_{1c}) or on taking high doses of aspirin (N5), or the acetaldehyde adduct responsible for false high values of Hb A_1 in alcoholics (S44). Taken together with further recent reports (F2, J3, M22, T1) confirming the absence or slight effect of interfering factors, affinity chromatography, with its reasonable cost, must be considered *the* short-column procedure of choice. Several commercial kits are now available, (e.g., Pierce, Rockford, Ill.; Isolab, Akron, Ohio).

Goldstein *et al.* (G12) have criticized the nonuniformity of Glyco-Gel (Pierce, Rockford, IL 61105); however, the reviewer has not found this to be the case, and the specificity of the affinity method has been substantiated (W9).

3.3.4. *Electrophoresis*

The electrophoretic separation of adult hemoglobin from other hemoglobin species on cellulose acetate membranes and subsequently on agar and agarose gels has been extensively conducted in many routine and clinical research laboratories. Hemoglobin A_{1c} is more negatively charged than Hb A_0, but such that its isoelectric point differs by only 0.01 pH units (R7). Hence assay for Hb A_{1c} or other glycosylated proteins cannot be achieved successfully with ordinary electrophoretic techniques. The use of a nontransparent plastic support for the cellulose acetate membrane and addition of dextran sulfate to the buffers results in binding of the sulfate groups to nonglycosylated hemoglobins and acceleration of their mobility—"mobile affinity electrophoresis." After the electrophoretic run of 40 minutes, the hemoglobin bands are stained for 10 minutes; the excess stain is washed out for 5 minutes and the strip cleared with heating for 10 minutes prior to scanning densitometry. This adds appreciably to the time required for the method. However, using a commercial kit (A9), with optimal conditions determined as 33 mmol/liter for buffer, pH 6.4 and temperature of 18°C, it was found that buffers of 25 and 50 mmol/liter, pH conditions of 6.2 and 6.6, and operating temperatures of 4°C and 22°C had little effect on assay results, nor did the presence of Hb C and Hb F interfere to any degree. The active ligand is in the mobile phase so that only a simple support medium is required. The reported precision (coefficient of variation of 4.9% and 3.9%) for replicate analyses of specimens containing 7.5% and 15% glycosylated hemoglobin was satisfactory.

Affinity electrophoresis on agar, agarose, and polyacrylamide gels, with the active ligand immobilized in the gel matrix, for the identification and quantitation of glycosylated proteins, was reported in 1977 (B20, H14). In view of the advantages recorded for affinity chromatography in recent years, it is surprising that the possibilities for affinity electrophoresis have not been further explored.

In agar gel electrophoresis, the fixed negative charge of the matrix results in differential interaction with hemoglobins of different charge (W13). Thus, Hb A_0 is more positively charged than Hb A_{1c}, so that the latter migrates more rapidly to the cathode by electroendosmosis. The procedure, available as a commercial kit, gave results that compared favorably with reference short-column and HPLC methods (M17, R5). The assay was little affected by changes in buffer pH from 5.8 to 6.5 and temperatures at 19, 24, or 37°C. Aleyassine *et al.* (A6) found that hyperlipidemia and temperature variations from 4 to 30°C did not influence the results and that glycosylated Hb A_1, Hb S_1, and Hb C_1 could be detected. However, accuracy and precision were not reported; others, employing a similar technique, reported precision coefficients of variation of 9% and 4.6–8.0%, respectively (D2, H7).

The application of differences in isoelectric points for the separation of hemoglobins was first employed in polyacrylamide gels some 15 years ago (D25, L5, U1). Subsequently, developments indicate that, while equipment and gels are more costly than for other electrochromatographic techniques, isoelectric focusing is unique in its exceptional resolving power. It provides a more definitive separation of glycosylated hemoglobins than either chromatography or size- and charge-dependent electrophoresis. Generally, column chromatographic methods do not separate Hb F from Hb A_{1c}, but isoelectric focusing does so clearly (S26). Similarly, glycohemoglobin S (F6) and glycohemoglobin A_2 (T2) are detectable in hemolysates from sicklemic, normal, and diabetic individuals. In two cases of sickle cell anemia, Hb S_{1c} and Hb F_{1c} were detected in a pH 6–8 carrier column (Bernstein, 1977, unpublished).

Flat bed electrophoresis with thin-layer (0.5-mm) polyacrylamide slabs and the use of a pH gradient of one unit (pH 6.5–7.5) produced separation superior to that obtainable with commercially available ampholines or pharmalytes of pH 6–8 and pH 7–9 (B4, B7, S16). The expansion of a linear pH gradient of 0.1 pH units over the whole separation distance (with resolution of p*I* differences of 0.001 pH units) has been made possible by covalently linking acryloyl monomer buffers to the polyacrylamide gel matrix (R7). The use of two separators incorporated into the gel provides a suitable method for routine clinical laboratory use (C19). For hemoglobin, the buffer has the composition $CH_2 = CH - CO - NH - R$, with R a tertiary amino group to give a p*K* of 7. The ketoamine component is readily separable from the aldimine by isoelectric focusing, thus providing two parameters of diabetic control.

3.3.5. *Immunoassay*

This highly specific and sensitive method has been insufficiently explored and exploited. A specific radioimmunoassay for Hb A_{1c} has been described (J1). Sheep antiserum was developed against purified human Hb A_{1c}. After

absorption, the antibodies were virtually Hb A_{1c} specific, with minimal reaction to Hb A_0, and 5% and 10% cross-reactivity to Hb A_{1a} and Hb A_{1b}, respectively.

Chou *et al.* (C10) used a rabbit antiserum against Hb A_{1c} to estimate Hb A_1 separated by column chromatography and to quantitate the glycosylated hemoglobin by laser nephelometry. More recently, a preliminary report appeared (W15) describing an immunoassay for nonenzymatically glycosylated plasma lipoproteins. In view of the considerable specificity both of enzyme and radioimmunoassays, it is probable that this methodology will be extended to other nonenzymatically glycosylated proteins.

3.3.6. *Photometric Methods*

Reactions between reducing sugars and amino acids (G18, H12, H13, R6) precede the browning (Maillard) reaction that may occur in food products, especially milks. Such adducts, subjected to acid pyrolysis are hydrolyzed to 5-hydroxymethylfurfural, HMF (K1, K2). The latter is reacted with 2-thiobarbituric acid (TBA) for photometric analysis. Flückiger and Winterhalter (F11, W14) described a method for glycosylated hemoglobin that has been extensively used both for glycosylated hemoglobin and other glycosylated protein assays and also for the identification of ketoamine adducts in a wide variety of proteins. The original method called for a 1-hour pyrolysis at 100°C with 0.1 *N* oxalic acid and a 30-minute period of color development at 40°C.

Various modifications have been investigated in order to enhance the capabilities of the TBA method. In the acid pyrolysis step, the oxalic acid concentration has been increased to 0.3–0.5 *N* (F4, F9, S37) and heating to 100°C for 2 hours (R12), 5 hours (S37), and even longer. This requirement made the method both laborious and cumbersome. The author found (1978, unpublished) that at the altitude of Johannesburg (1585 m), an added hour was required to obtain the identical HMF formation as at Cape Town (at sea level); the use of an autoclave reduced this to 1 hour. Similarly, Paisey *et al.* (P3) reported that at the high altitude of Mexico City, where water boils at 93.5°C, the acid pyrolysis step was not complete even after 6–7 hours in a boiling water bath; using a pressure cooker, the reaction was complete in 2 hours. Others (K14, N11, P6) found that 20 or 60 minutes in a pressure cooker gave the same results as 5 hours in a boiling water bath. The formed HMF is increasingly destroyed by heat, and 81–83% recovery (S37) was reported for the 5-hour hydrolysis step; the loss after 1 hour at 120°C was similar (P6).

Thereafter, the samples are cooled (an ice bath is preferable) and the proteins precipitated with 40% trichloracetic acid (most reports) and centrifuged or filtered. For color development, incubation has been at 40–56°C for 10–30 minutes (F4, N11, S37). Automatic processing of the protein

precipitation and color development steps by the Auto-Analyzer or similar automatic instruments (B43, K14, M19, R12) combined with high temperature for the initial acid pyrolysis has permitted the analysis of 100 to 200 samples per day with excellent precision.

The yellow color product generated from HMF and TBA was originally read at 443 nm (K2); tests with repurified and recrystallized TBA and precision spectrophotometers indicated a maximum absorbance at 445 nm (B10) and were used by Postmes *et al.* (P14). Automatic instruments were monitored at 440 nm. The color developed from 1-valyl-1-deoxyfructose (viz., the β-*N*-valyl terminus of hemoglobin, i.e., Hb A_{1c}) is greater than that for the 1-lysyl-1-deoxyfructose (S24); alternatively, this depends on the position of the valyl and lysyl residues in the configuration of hemoglobin and other proteins.

While there are few reports regarding the availability and use of standards for the other methods, chemical methods are suited to the inclusion of standards. Fructose, 1-deoxy-1-morpholino-D-fructose (J4), HMF (S37), lyophilized hemoglobin (D9, N11), and purified Hb A_{1c} have all been used. It must be noted that HMF as standard does not monitor the initial acid pyrolysis step, and its heat lability has already been indicated. The rate of HMF production from fructose differs from that for the deoxy-aminosugars, but the use of 1-deoxy-1-morpholino-D-fructose gives a good approximation. The preparation and use of lyophilized hemoglobin, obtained from normal and diabetic bloods so as to contain Hb A_{1c} (ketoamine only), is advocated.

The TBA method has been used not only to assay glycosylated hemoglobin, but also almost exclusively for the following glycosylated proteins: plasma albumin (D22, E1, N10, M12), plasma proteins and albumin (K6), capillary whole-blood protein collected on filter paper (L14), erythrocyte spectrin (M13), skin collagen (P2), lens basement membrane (M5), and crystallins (M28). In assays on hair and epidermal keratin, cyclohexanone extraction of the color developed with TBA and reading the absorbance of the cyclohexanone layer at 433 nm has provided increased sensitivity and accuracy (T4). A possible objection to the procedure is the use of oxalic and thiobarbituric acids, which are potentially toxic chemicals.

With regard to precision, the within- and between-assay coefficients of variation were 2.8% and 2.6%, respectively, at 7% glycosylated hemoglobin, and 3.2% and 4.0%, respectively, at 13.6% glycosylated hemoglobin (N12); an overall coefficient of variation of 5% and 4%, respectively, was reported for low and high ranges of glycosylated hemoglobin (N11). For an extended trial of high-temperature (autoclave) acid pyrolysis and automated processing of color development, intra- and interassay coefficients of variation of 1.6% and 2.6%, respectively, were recorded.

Other photometric methods have been described. The phenol–sulfuric acid method (N8, R3) calls for the use of dangerous chemicals, namely, 80%

phenol and concentrated sulfuric acid, but was claimed to be more sensitive and reliable than the TBA method (N8). In a modified method for glycosylhemoglobin (R3), globin is isolated, and the phenol–sulfuric acid color reaction for bound hexose determined at 480 nm before and after hydrochloric acid–oxalic acid hydrolysis. The before-hydrolysis value may represent mainly glucose bound to valine (presumably HbA_{1c}), while the after-hydrolysis value may represent furfural derivatives released from glucose bound to other valines and lysine. The specificity of the described method requires further study.

The periodate oxidation of sugars on α- and ϵ-amino groups of proteins can be determined by fluorometric assay of the formaldehyde formed (G4). This estimates both labile aldimine and stable ketoamine adducts; it thus suffers from the same disadvantage as short-column ion-exchange chromatography in that the aldimine moiety must first be discharged. However, in conjunction with $NaBH_4$ reduction, which will open the sugar ring to form a second formaldehyde, the method permits the determination of nonenzymatic glycosylation in proteins that are also enzymatically glycosylated.

A method, applicable only to glycosylated hemoglobin, depends on the addition of inositol hexaphosphate (phytic acid), which binds and saturates the 2,3-diphosphoglycerate (DPG) sites on hemoglobin that are not bound by glucose. Glycosylated hemoglobin does not bind inositol hexaphosphate. The estimation of the extent of glycosylation has been performed as follows: (1) Binding of inositol hexaphosphate produces a spectral change, recorded at 560 and 633 nm, the extent of which is inversely related to the concentration of glycosylated hemoglobin (W1); or (2) the addition of haptoglobin, which binds glycosylated hemoglobin, with fluorometric determination of the glycosylated hemoglobin/haptoglobin complex by its peroxidase activity (D8). Both methods can be automated.

A method for the estimation of serum glycosylprotein, based on the ability of ketoamines (fructosamines) to reduce nitroblue tetrazolium at pH 10.8, was reported in 1982 (J4). More recently, an automated assay (75 samples per hour) has been described, with excellent precision (coefficient of variation 2%) and low cost (D1).

Compared with glycosylated hemoglobin and glycosylated plasma protein assays, plasma fructosamine was found to be the best discriminator between four groups of diabetic and nondiabetic patients (R13). However, the reviewer believes that the assay of a more specific glycosylated protein is warranted (viz., the estimation of albumin fructosamine) (W1a). This has two advantages, namely, the determination of glycosylated albumin with a well-defined biological half-life of some 20 days (reflecting a 2–4-week time-integrated change in blood glucose) and with albumin representing some 60% of total plasma proteins. Second, the remainder are an assortment of

carrier, acute phase, coagulation, and other proteins with a very wide range of concentrations, functions, and life spans.

Hayashi and Makino (H6b) have described a simple and rapid fluorometric assay of glycosylated albumin with dansylated phenyl boronic acid [*N*-(5-dimethyl amino-1-naphthalene sulfonyl)-3-aminobenzene boronic acid]. The emission spectrum of this reagent changes on binding to *cis*-diols present on glycosylated albumin, permitting quantitation using excitation and emission maxima of 330 and 490 nm, respectively. The reaction is almost entirely with glycosylated albumin, so that the serum sample can be directly analyzed.

3.4. Aspects of Sample Control

Normal and variant hemoglobins exhibit posttranslational modifications, with reactive molecules other than glucose, to form adducts. Such nonenzymatic additions render hemoglobin more negatively charged than Hb A_0 and have chromatographic and electrophoretic properties similar to Hb A_1. Methods specific for glycosylprotein are therefore required for their exclusion, for example, affinity (not cation-exchange) chromatography (A1, M22), TBA (N11), and isoelectric focusing (C19). These adducts include acetylation, principally resulting from the ingestion of aspirin (B24, N5); modification by acetaldehyde adduction and its metabolic product, 5-deoxyxylulose-1-phosphate in alcoholics (H11, S44); and penicilloylation of hemoglobin (F9), a reactant produced by most penicillin drugs. Hemoglobin carbamylation, resulting from condensation of urea-derived cyanate with valine and lysine residues, is responsible for the increased Hb A_1 value in uremia (B34, F10), but only in part, since acidosis (D10), anemia, and blood transfusions in chronic renal failure also play a part (B3). The use of vacutainers with iodoacetate for blood glucose estimations may produce an artifact due to iodoacetate adduct formation (S17).

Many variant hemoglobins migrate rapidly in chromatographic and electrophoretic media and do not separate from Hb A_1. A more specific identification, Hb A_{1c} or ketoamine, should be employed. This also applies to Hb F_1, which is present in children less than 4 years old, increases in the pregnant female near birth (E5), and is increased in hereditary persistence of fetal hemoglobin (B10) and in various hematologic diseases. On the other hand, in geographic or ethnic groups (blacks, Asiatic Indians, Mediterranean people) among whom there are high incidences of Hb S and Hb C, Hb A_1 assessment will underestimate overall hemoglobin glycosylation considerably, and a ketoamine assay is called for (B10).

In chromatographic methods, the total hemoglobin of hemolysates and the glycosylated hemoglobin in eluates and fractions has been almost entirely

determined by absorbance at 415 nm (with readings also ranging from 412 to 417 nm). This wavelength peak is assumed to be the only hemoglobin species. The internationally standardized hemiglobincyanide (cyanmethemoglobin) method for estimating hemoglobin in human and other bloods has now received general acceptance as a reference method (V1) and is to be preferred as a control on the accuracy and reproducibility of the hemoglobin estimation. Most investigators using photometric methods have used this procedure for total hemoglobin. Chlorohemin may also be used as a primary reference standard for hemoglobin assay (W19).

3.5. Comparison of Methods

Several investigative groups have compared four to six different methods for glycosylated hemoglobin (B22, M34, N13, P10), while others have evaluated their major or new technique against one or more comparison methods (A1, A6, A9, D8, F4, H7, J4, K13, L14a, M17, M18, N11, P8, T6, W1). A detailed analysis of these reports is not presented; it is noteworthy that much emphasis has been placed on rapid methodology. In contrast to the blood glucose or urea in a comatose or uremic patient, a report on glycosylated hemoglobin, albumin, and other factors is not urgent. A glycosylated hemoglobin assay is not required more often than once every 4–8 weeks and glycosylated albumin every 3–4 weeks to monitor diet/therapy control in the diabetic. Accuracy and precision of assay for the specific ketoamine adduct, with quality control and establishment of reference values, should not be subordinated to a "stat" result. Currently, the following assessment appears to be warranted. The photometric thiobarbituric acid assay for ketoamine is the method of the choice both for routine laboratory and specialized studies. Unlike most of the other methods, it has been applied to the estimation of many glycosylated proteins in addition to hemoglobin and serum albumin.

Short-column affinity chromatography with phenylboronate has been found to have remarkably few interferences and problems and is superior to cation-exchange chromatography. It provides a rapid and precise method, and large numbers can be processed, with automatic hemoglobin analysis used for the eluates. While requiring expensive equipment, HPLC is of considerable accuracy and sensitiveness and can easily be automated to process a large number of samples. Mobile affinity electrophoresis is the method of choice for this charge-dependent separation technique. For specialized studies, particularly where hemoglobin variants other than Hb A_0 may be involved, isoelectric focusing, with acryloyl buffers producing immobilized pH gradients incorporated into the flat bed polyacrylamide gel, is unsurpassed: it can separate Hb A_0 from Hb A_{1c} by 5–6 mm with clear demarcation.

4. Glycosylated Protein Values

4.1. Normal Human Proteins

A representative list of normal values for human glycosylated proteins is presented in Tables 2 and 3. Some degree of divergence for the mean values for glycosylated hemoglobin, using the same procedure and additional to individual laboratory variables, may reflect age, sex, diet, and other factors in the composition of apparently healthy "normal" subjects examined. The variation in the normal range of red cell parameters (e.g., life span, DPG content, inorganic phosphate uptake) is also involved. While most attention has been devoted to glycosylated hemoglobins A_{1c} and F_{1c} because of their diagnostic relevance, an appreciable record of values has now been established for other human glycosylated proteins, peptides, and amino acids (Table 3). Nonenzymatically lysine-bound glucose has also been determined in nondiabetic and diabetic samples of tendon, coronary and aortic arteries, femoral nerve, glomerular basement membrane, and lung parenchyma from human autopsies (V8), as well as skin, hair, fingernails, and bone. Skeletal and cardiac muscle values were below detection limit (0.06 nmol glucose/mg wet tissue) for the sensitive furosine HPLC method (S11). Examination of normal urines indicated the presence of glycosylated amino acids (mean, 2.8 μmol/24hours/kg body weight), of which lysine (67%–86%) was present as the major adduct (B29).

4.2. Glycemic States

Alterations in glycosylated protein values occur principally in diabetes, glucose intolerance, other conditions of hyperglycemia, hypoglycemia of some duration, and disorders of the particular protein involved. In diabetics, the values for most glycosylated proteins are two to three times normal concentrations, and in the majority of reports, the range of results is often clearly separable from the normal (see Section 5). For glycosylated hemoglobin, with a normal range of 4 to 8% by most methods, the values of diabetes may extend to 20–30% in extreme cases. Graf *et al.* (G21) concluded that hemoglobin glycosylation was an irreversible and saturable process, attaining a maximum of some 23%; however, Mortensen *et al.* (M33), with an *in vivo* and *in vitro* biokinetic model, have established that hemoglobin–ketoamine formation is slowly and slightly reversible and that after a change in blood glucose level, a steady-state Hb A_{1c} level is reached in 3 to 4 weeks.

4.3. Experimental Animals

Comparative values for glycosylated hemoglobin in man and several experimental (mammalian) species are detailed in Table 4. Examination of the

TABLE 2
NONENZYMATIC GLYCOSYLATED HEMOGLOBINS IN NORMAL SUBJECTS[a]

Protein	Number of subjects	Method[b]	Glycosylation (%)[c]	CV (%)[d]	References
Hb A_1	22	CIESCC	7.5 ± 1.5 SD		G26
	48	CIESCC	4.98 ± 0.49 SD		S7
	85	CIESCC	7.2; 6.0–8.8	4.8, inter	S27
	18	CIESCC	6.8 ± 0.6 SD	2.5	W1
	84	Batch	6.3 ± 1.9 (2 SD)	8.0, intra	R11
	9	Agar gel electr.	6.1 ± 0.5 SD, SEM 0.1	9.0	H7
	50	Agar gel electr.	6.6 ± 0.5 SD; 5.6–7.5	5.2	M17
Hb A_{1c}	10	HPLC	5.06 ± 0.32 SD, SEM 0.1; 4.6–5.6		C15
	20	HPLC	6.36 ± 0.55 SD; 5.3–7.5		K13
	92	IEF, gel pH 6–8 or 7–9	4.66 ± 1.05 SD; 3–7		S16
		IEF, gel pH 6–8	4.9; ref. interval, 3.9–6.4	12.6, inter; 8.5, intra	S26
GHb[e]	107	Affin. chr.	4.96 ± 0.65 SD; 4.0–6.7	1.6, inter	F2
	124	Affin. chr.	6.36 ± 0.55 SD; 5.3–7.5		K13
	44	Affin. chr.	7.1 ± 0.52 SD; 5.5–8.2	5.7, inter; 6.9, intra	M22
	62	Affin. chr.	7.31 ± 0.92 SD; 5.25–9.70	2.0, intra	H1
Labile GHb	12	Affin. chr.	−0.34 ± 0.44 SD; +0.48 to −1.04		H1

GHb	19	TBA	4.76 ± 0.80 SD; 3.8–6.3		F4
	65	TBA	5.51 ± 0.62 SD; 4.3–6.7 (2 SD interval)		S37
	30	TBA, automated	1.62, SEM 0.05 nmol HMF/mg protein	4.8	M19
	22	TBA, automated	7.9 ± 1.1 SD; 5.4–9.9	2.0–4.8 intra; <11, inter	B43
	18	Phytic acid	7.7 ± 1.3 SD	3.8	W1
	28	Phenol–H_2SO_4	0.286 ± 0.051 SD globin-bound hexose, mole/mole Hb		R3
Hb F_1	48	IEF	10		B4
	14	IEF	7.1; 4.8–8.8		O6
	17	CIESCC	18.7; 11.0–21.0		O6

[a] Selection of reports where authors took particular care in method and result analysis.

[b] CIESCC, cation ion exchange short column chromatography; batch, cation ion exchange resin mixed with sample and subsequent centrifugation or filtration or decantation; agar gel electr., agar gel electrophoresis (mobile affinity electrophoresis); HPLC, high-pressure (performance) liquid chromatography; IEF, isoelectric focusing in polyacrylamide and other media; Affin. chr., *m*-aminophenylboronic acid affinity column chromatography; TBA, thiobarbituric acid assay at 443 nm after acid pyrolysis.

[c] Mean ± standard deviation; standard error of mean and range.

[d] CV, coefficient of variation; inter, interassay, between runs; intra, intraassay, replicates on single or more specimens.

[e] GHb, glycosylhemoglobin.

TABLE 3
NONENZYMATIC GLYCOSYLATED PROTEINS IN NORMAL SUBJECTS[a]

Proteins	Number of subjects	Method	Glycosylation (%)	CV (%)	References
Plasma proteins	58	Affin. chr.	6.29 ± 1.87 SD; 2.9–10.3	6.2, inter; 4.0, intra	G19
Serum proteins	12	Fructosamine	1.75 (mean) mmol fructosamine/liter		J4
	82	TCA ppt.; then TBA	0.32 ± 0.01 SEM nmol HMF/mg protein		K7
	20	TBA	0.81 nmol glucose/mg protein; 0.61–1.31		M12
	50	Affin. chr.	9.95; 8.3–11.6	<6.5	Y1
	12 rats	TBA	0.75 ± 0.12 nmol HMF/mg protein		D5
Albumin	8	TBA	86 ± 18 SD pmol HMF/mg protein		D22
	10	Albumin purified; then TBA	64 ± 4.6 SD pmol HMF/nmol albumin; 56–72		D23
	25	CIESCC	7.0 ± 1.9		G26
	25	TBA	8.3 ± 2.2		G26
	100	Affin. chr.	3.72 ± 0.85	4.4; 5.4	J3a

	50	Affin. chr.	8.6; 6.8–10.3	<6.5	Y1
	12 rats	TBA	1.12 ± 0.14 nmol HMF/mg protein		D5
	7 rats	TBA	8		D5
Urinary amino acids	9	Affin. chr.	2.8 μmol G-amino acids/kg body wt		B29
Spectrin	7	TBA	0.059 ± 0.19 A443 nm/mg spectrin		M13
Collagen (skin)	33	Acid autoclave hydrolysis and TBA	0.072 ± 0.12 μmol fructosamine/ 100 mg wet wt.		P2
Lens crystallins	—	Isotopic	0.028 ± 0.011 nmol glucitol–lysine/nmol protein		G7
Lens crystallins (cortex)	11	TBA	1.72 ± 0.65 nmol HMF/mg protein		L7
Lens crystallins (nucleus)	11	TBA	1.17 ± 0.40 nmol HMF/mg protein		L7
Lens capsule (basement membrane)	6	TBA	29.1 ± 4.9 nmol/mg protein; 23–37		M5

[a] Annotations as in Table 2. HMF, 5-hydroxymethylfurfural; TCA, trichloracetic acid.

TABLE 4
GLYCOSYLATED HEMOGLOBINS AND RED CELL PARAMETERS IN SEVERAL ANIMAL SPECIES[a]

Species	Cell glucose (m*M*)	Life span (days)	Glucose exposure[b]	HbA_{1c} (%)	References
Human	3.2 ± 0.8	120	385	5.0	H9
Human				4.1 ± 0.28	A8
Baboon	3.4 ± 0.4	45	150	3.6	H9
Baboon		30–60		2.4 ± 0.4	A8
Monkey	2.4 ± 0.3	95	225	3.4	H9
Monkey		100[c]		2.8	S30
Dog	1.5 ± 0.6	100	150	3.2	H9
Dog		110[c]		2.8 ± 0.35	W20
Dog				5.4	Y4
Rabbit	1.2 ± 0.4	60	70	1.2	H9
Rabbit		60		3.9	C3
Pig	0.2 ± 0.4	90	20	0	H9
Rat		55–65[c]		1.2	D5
Rat				6.7	Y4
Rat				2.3–2.5	F16
Mice		45[c]		1.6–2.3	F16
Mice		50		1.74 ± 0.06	K16

[a] Adapted from Higgins *et al.* (H9), with additions.
[b] Red cell glucose × red cell life span.
[c] From Berlin, N. I., and Berk, P. D. (1975). *In* "The Red Blood Cell" (D. M. Surgenor, Ed.), Vol. II, pp. 957–1019. Academic Press, New York.

differences in amino acid composition of these hemoglobins, as well as the factors of deoxygenation, pH, variations in DPG, and inorganic phosphate content, indicated that these did not affect *in vivo* glycosylation to any appreciable degree and did not explain the divergence in values. Higgins *et al.* (H9) drew attention to the correlation in six species between glucose exposure (expressed as a multiple of red cell life span in days multiplied by cell glucose in millimoles per liter) and their glycosylated hemoglobin concentration. However, there is some uncertainty as to animal glycosylated hemoglobin values (Table 4), and significantly different values are recorded for the dog (W20, Y4) and other animals (A8, C3, D5, S30).

5. Diagnostic Relevance of Assays in Diabetes

Nonenzymatic glycosylation involves the amino acids valine and lysine almost entirely, and presumably glycosylation of peptides and proteins containing these residues in a reactive form is universal. Most attention has

been directed toward glycosylated proteins that are readily accessible for sampling, to provide (1) a diagnostic record and (2) insights into metabolic processes (e.g., complications in diabetes, aging, and specific clinical disorders). In man, this has involved studies in glycosylated proteins with a short biological half-life (viz., young erythrocytes; transferrin, $t^{1/2}$-8 days; human serum albumin, $t^{1/2}$-20 days), a medium biological life (red cell membrane protein and hemoglobin, 120-day life span), and a long biological life (collagens, elastin, lens crystallins, and myelin protein).

5.1. Glycohemoglobin

Hemoglobin A_{1c} or glycosylated (ketoamine) hemoglobin values as a percentage of total circulating hemoglobin reflect the integrated time–concentration levels of plasma glucose (more specifically, the free glucose of erythrocytes), conditioned by an almost continuous formation during the normal 120-day life span for human red cells. *In vitro* biokinetic studies on the formation and breakdown of the ketoamine adduct (M33, M35) indicate that the altered steady state of Hb A_{1c} concentration took 3 to 4 weeks after a change in blood glucose to a new level. In chemically induced diabetes in experimental animals with red cell life span and glucose concentration and metabolism similar to that in man, Hb A_{1c} increased from 2.81 ± 0.35% in normal dogs to 6.52 ± 0.76% after 26 weeks of hyperglycemia (W20). In the clinical situation, while 10 normal subjects aged 21–39 years and 10 normal subjects aged 44–57 years had Hb A_1 concentrations of 6.8 ± 0.56% and 6.5 ± 0.99%, respectively, type I and II newly diagnosed diabetics had values of 16.24 ± 3.23% and 13.39 ± 2.70%, respectively, compared with 11.92 ± 1.56% and 12.71 ± 1.62%, respectively, for types I and II on treatment (C22). Similarly, 15 elderly diabetics, on oral hypoglycemic drugs, had Hb A_1 concentration of 12.6 ± 0.8%; 3 weeks after discontinuing the drug therapy, the Hb A_1 had plateaued at 15.2 ± 0.8% and continued at this level until week 22. Therapy was reinstituted at week 20, and the Hb A_1 levels were 14.1 ± 0.9% and 12.3 ± 0.8% at weeks 24 and 28, respectively (B19).

Thus no significant decrease in Hb A_{1c} level is seen in the newly diagnosed or out-of-control diabetic until after some 4 to 5 weeks of strict metabolic control (D14). The Hb A_{1c} levels reflect the red cell glucose oscillations integrated over the previous 5–8 weeks and represent the diet–drug response of the patient for this period. For this reason, assays to monitor progress and compliance are not required more often than once every 4–6 weeks.

The aim in treatment for type II (non–insulin-dependent diabetic) patients is to bring the Hb A_{1c} level to normal or close to the upper limit of normal. With type I insulin-dependent diabetics, and particularly in the

early stages of therapy, such a strategy will increase the number of hypoglycemic episodes and is to be avoided. Testing for Hb A_{1c} monitors compliance; it provides a check on the falsification of the home monitoring of blood glucose levels and of urine glucose records. Further, a patient may present at the monthly clinic visit with a reasonable blood glucose and absent glycosuria simply by following a 3–4-day strict low-carbohydrate–low-calorie diet; however, the Hb A_{1c} result would indicate the true position. An accurate test (ketoamine specific) is not confuted by an episode of labile glycemia nor subject to patient manipulation. Values of Hb A_{1c} have advantages over urine tests or fasting plasma glucose, which present a discrete moment only in the metabolic life history of the diabetic. The results for Hb A_{1c} may be relied on to provide a correct indicator of long-term diet–drug therapy and a reliable monitor of compliance.

5.2. Labile Glycohemoglobin

By 1975–1977 it had been definitively established that nonenzymatic glycosylation of hemoglobin involved formation of an initial rapid and reversible aldimine adduct with glucose, followed by a slow and nearly irreversible formation of ketoamine, Hb A_{1c} that persisted for the life span of the red cell (B40, B41, K15). The use of rapid methods (short-column cation-exchange chromatography and electrophoresis) to quantitate Hb A_{1c} was held to yield results equivalent to the long-column Trivelli technique (T8). Thus acute changes in blood glucose levels were equated with changes in Hb A_{1c}, casting doubt on Hb A_{1c} values reflecting the glycemic record of the patient for the previous 1–2 months. Many reports of 1977–1981 attested to this (e.g., G17, S49, W7); in most instances, the increases were almost entirely due to pre-Hb A_{1c} (labile aldimine adduct). Specific assays for Hb A_{1c} (ketoamine) and, in particular, the thiobarbituric acid photometric method do not indicate any changes in Hb A_{1c} with temporary and short-term alterations in blood glucose (C17, G14, M32, N2). Normally, up to 10% of the total glycosylated hemoglobin is labile (B15, I1, N2, N3); in unstable diabetics, it may represent up to 40% of the total glycohemoglobin (N4) and is an indicator of those insulin-dependent diabetics (usually the young labile subject) who have wide fluctuations in blood glucose levels. To monitor this instability in juvenile or insulin-dependent type I diabetes, labile (aldimine) glycohemoglobin can be determined by several methods, such as estimation of total glycohemoglobin by short-column cation-exchange chromatography and ketoamine products by thiobarbituric acid assay to give labile (aldimine) or pre-Hb A_{1c} content by difference; or the estimation of Hb A_{1c} (or Hb A_1) before and after discharge of the labile component (q.v. Section 3.2.2) to give total and ketoamine glycosylated hemoglobin with aldimine concentra-

tion by difference. Assessment of the labile moiety every 3 or 7 days will diminish the requirement of venepuncture or capillary sampling for blood glucose estimations.

5.3. Glycosylation in Hemoglobin Variants

Diabetes concurrent with variant hemoglobins occurs in many ethnic and geographic groups. While there are now over 400 hemoglobin variants described (I2), most are rare and only one-third, of which the most common are hemoglobins S and C, cause clinical symptoms.

Some 5–20% of American and African blacks, Indians of the Indian subcontinent and of Papua, New Guinea, Mauritius, and eastern and southern Africa, and peoples of Mediterranean origin are heterozygous for hemoglobin S or C. In β variants of hemoglobin (S, C, etc.), the variant can comprise up to 45% of the total circulating hemoglobin. Photometric methods will correctly reflect the glycosylated hemoglobin status of diabetics with hemoglobinopathies, since they determine the ketoamine content in all the hemoglobin present. Charge-dependent (chromatography, electrophoresis) techniques will underestimate total hemoglobin glycosylation since they will only record Hb A_{1c} or Hb A_1 (A5, B10, O2, S31), and not the component from S_{1c} or C_{1c}. By means of mobile affinity electrophoresis, affinity chromatography, or isoelectric focusing using immobilized pH gradients, it is possible to separate and quantitate each glycosylated hemoglobin fraction, A_{1c}, S_{1c}, C_{1c}, F_{1c}, and so on (in sickle cell anemia, homozygous Hb SS, only sickle and fetal hemoglobin and their glycosylated moieties are present). In areas where there is any degree of hemoglobinopathy, the laboratory method employed should reflect the correct percentage glycosylation of the total circulationg hemoglobin for any assessment of diabetic metabolic control.

5.4. Glycosylated Plasma Proteins

5.4.1. *Total Protein and Albumin*

Because the presence of free glucose in plasma or serum will continue the glycosylation process (K5), it is not feasible to store such samples unless the glucose is first removed by dialysis. Preferably, the total plasma protein must be precipitated and stored as such or with further purification; alternatively, fractions may be prepared by suitable fractionation procedures and stored in solid form or reconstituted in glucose-free media.

While the methods for estimating glycosylation in total plasma or serum proteins are technically simple (K6, K7, M1, M12, Y6), many variable factors can cause problems in interpretation. These include the different half-lives

for the protein fractions, different number of reactive valine and lysine residues in the complex of plasma proteins, and competitive binding of particular proteins to other molecules. Two reports selected at random reflect this situation: McFarland *et al.* (M12) found 20 control subjects to have protein glycosylation equivalent to 0.63–1.31 nmol hydroxymethylfurfural/mg protein, clearly separable from 29 diabetics with 1.54–3.97 nmol hydroxymethylfurfural/mg protein. On the other hand, Kennedy *et al.* (K7), using similar conditions for the TBA assay, reported 82 controls (mean 0.32, range 0.1–0.65 nmol hydroxymethylfurfural/mg protein) and 107 diabetics (mean 0.74, range 0.2–1.5 nmol hydroxymethylfurfural/mg protein).

A variation of this, namely, the measurement of glycosylated whole-blood protein obtained from capillary blood collected on filter paper, provides an easily performed and rapid technique (L14), but interpretation involves many variables associated with the complex assemblage of proteins.

For individual plasma proteins, most attention has been devoted to glycosylated albumin, with a half-life of 20 days, as a short-term diagnostic indicator of metabolic control in the diabetic. While glycosylation of horse serum albumin had already been known (M21), it was not until 1979 that studies indicated increased glycosylation of human serum albumin in diabetic patients (D22, D23, G26, M19). A typical finding is that of Guthrow *et al.* (G26), who give normal values of 7.0 ± 1.7% by carboxymethylcellulose chromatographic separation and 8.3 ± 2.2% by thiobarbituric acid assay, compared with 12.8–29.7% for diabetic subjects by the two methods. These reports all record that albumin becomes more highly glycosylated than hemoglobin; taking into account the shorter half-life of albumin compared with hemoglobin, it is highly reactive toward nonenzymatic glycosylation. Nearly 50% of the nonenzymatic glycosylation of human serum albumin is at Lys 525 (G6), and this alters the conformation and function of the glycosylated albumin (S23). Glycosylation also occurs at Lys 199, a known site for albumin acetylation (G6). Ingestion of aspirin in high doses (e.g., in rheumatoid arthritis therapy) will cause a false increase in glycosylated albumin (B24) when measured by column chromatography or electrophoretic methods. Isoelectric focusing and photometric assays will distinguish glycosylated albumin from the acetylated form. A similar situation has been reported for glycosylated hemoglobin (B24, N5) in man and for aspirin and sodium salicylate in respect of glycosylated collagen (Y5) in experimental diabetic rats.

Many methods for the preparation of pure serum albumin are available, and some can be scaled down from techniques used by blood transfusion services to process protein components from outdated blood. Assay can be by affinity chromatography (G10a), HPLC, isoelectric focusing, mobile affinity electrophoresis, and thiobarbituric acid photometry (N10). The last method employed acid (pH 1) hydrolysis for 8 hours at 115°C to liberate the

HMF, and a 50-minute incubation with 12.5 mmol/liter (final concentration) thiobarbituric acid for color development. Acid pyrolysis could be reduced to 2 hours by using a pressure cooker or autoclave. Some 50 specimens can be processed (albumin purification, autoclave pyrolysis, and automated color development and recording) every 2 days. Glycosylated serum albumin values should be monitored every 3 weeks.

Instead of isolating albumin initially from serum in a pure or relatively pure form, a more accurate and rapid method is to separate glycosylated (ketoamine only) albumin from nonglycosylated albumin on an affinity agarose–boronate column and then to assay the separated glycosylated albumin for its ketoamine content (W9) or its albumin content as a percentage of total plasma albumin (J3a).

5.4.2. *Transferrin and Other Trace Plasma Proteins*

Transferrin has an 8-day half-life, and glycosylated transferrin levels have been advocated as a short-term indicator of glycemic control (K4). The glycoprotein fibronectin has an extensive capacity for binding cell matrix and basement membrane components, and this is diminished by nonenzymatic glycosylation (C12). Simplified methodology for glycosylated transferrin and other trace protein elements [e.g., fibronectin (C12)] will be required for any extensive diagnostic use of such components to be made.

5.4.3. *Lipoproteins*

The relation between Hb A_{1c} levels and other metabolic parameters (A4) in the diabetic state has been assessed. Gabbay and co-workers (G1, S34) found that plasma cholesterol and LDL, known risk factors for atherosclerosis, correlated positively with the degree of hyperglycemia and glycosylation (Hb A_{1c} greater than 14%) in 77 insulin-dependent diabetics over a 5–9-month period. However, in 40 insulin-requiring diabetics who had a higher mean age (E2), no correlation was found between blood glucose and Hb A_1 compared with cholesterol and HDL. On the other hand, Strobl *et al.* (S47) found a close relationship between glycosylated serum protein (an intermediate indicator of metabolic control) and serum levels of LDL cholesterol.

Both low- and high-density lipoproteins (LDL and HDL) contain lysine residues that can be extensively glycosylated. Similarly, plasma apolipoproteins AI, AII, B, CI, and E are glycosylated in hyperglycemic diabetes (C21). Modification of some 40% of lysines in LDL by reductive glycosylation will completely block receptor-mediated LDL catabolism in cell culture (W3), in experimental animals (G15, W17), and in man (K8). In man 2% of LDL lysine residues glycosylate normally; in diabetics, some 2–5% of LDL lysines are glycosylated, and LDL catabolism is decreased some 5–25% (S9). Such slowing of LDL catabolism could increase plasma LDL levels and so

increase cholesterol deposition in tissues. In contrast to LDL, catabolism of HDL is reported to be accelerated by glycosylation (W16), but only significantly after 15% of lysine residues react. These are lower limits for effects on receptor binding and lipoprotein catabolism and, combined with the short half-life of apolipoprotein, render this of slight consequence in human diabetes.

5.4.4. *Coagulation Proteins*

In preliminary experiments, fibrinogen exhibited nonenzymatic glycosylation *in vitro* (B9, M16); this was confirmed, together with the finding that excess glycosylated fibrinogen is present in diabetics (B26, C1, L17, M16). Fibrinogen undergoes extensive glycosylation, particularly its lysine-rich γ fragment. Glycosylated fibrin formed will reflect that of its fibrinogen precursor. Activated factor XIIIa has transglutaminase activity and stabilizes the fibrin clot by forming intermolecular cross-links between glutamine and lysine residues of adjacent fibrin monomers. Glycosylation, occurring at lysine residues, will block cross-linking and thus destabilize the fibrin clot. Further, fibrin deposits are hydrolyzed specifically at lysine bonds, and increased glycosylation of fibrinogen (fibrin) will diminish the capacity of plasmin to degrade fibrin (B30).

Specific lysine residues of antithrombin III bind heparin to act as a major inhibitor of serine–protease plasma coagulation factors. Nonenzymatic glycosylation of antithrombin III, by preferentially binding to lysines and decreasing the affinity of antithrombin III for heparin, will decrease its thrombin inhibitory activity (B31, C7). *In vivo* inhibition of antithrombin III occurs in type I diabetics (D27, S35). Decreased antithrombin III and fibrinogen activity in severe diabetics can be reversed by heparin infusion.

The excess vessel wall fibrin accumulation and atherosclerosis seen as a long-term complication of diabetes may well result from the glycosylation-induced inhibition in fibrinogen (fibrin)–plasmin degradative function and heparin-catalyzed antithrombin III activity.

5.5. Glycosylation of Membrane Proteins

5.5.1. *Red Blood Cells*

Indications of nonenzymatic glycosylation of red cell membrane proteins was first reported in 1976 (B2). Analysis of the various protein components of the erythrocyte membrane indicated that glycosylation of red cell ghosts in 18 diabetics was twice that in normal individuals and correlated with levels of Hb A_{1c} (M25). Comparison of individual membrane protein bands on sodium dodecyl sulfate–polyacrylamide electrophoresis did not indicate any

significant selective glycosylation. Glycosylated erythrocyte membrane proteins were significantly increased ($p < 0.001$) in 12 type I diabetics (11.3 ± 0.7 nmol HMF/mg protein) compared with 10 controls (4.9 ± 0.3 nmol HMF/mg protein) and correlated positively with other metabolic parameters, for example, raised glycosylated serum proteins, Hb A_{1c}, and fasting plasma glucose (L4).

The major protein of the inner membrane surface of erythrocytes, spectrin, showed increased glycosylation in diabetics (M13). On a protein weight basis, spectrin glycosylation was about twice that for hemoglobin, although spectrin contains 6.8% lysine compared with 7.8% for α-hemoglobin and 7.6% for β-hemoglobin.

Spectrin forms an inner membrane stretchable protein network with elastic energy responsible for returning deformed red blood cells to their original shape. Its increased glycosylation, by inhibiting membrane protein transglutamidation (mechanism similar to that reported for fibrinogen) may account for the increased number of rigid (poorly deformable) red cells found in the mature and old fraction of erythrocytes in diabetics. Alterations in the physicochemical properties of proteins in erythrocytic membranes and decreased red cell deformability are associated with hyperglycemia in diabetic persons (C16, H6a, M14, S10).

5.5.2. *Endothelial Cell and Other Membranes*

Disease of the microcirculation involving the glomerulus, retina, and other microvessels is a hallmark of complications in long-standing diabetes mellitus. Current studies in man and experimental animals indicate that successful control of plasma glucose levels will prevent or ameliorate microangiopathy. The role of glycosylated albumin (and fibrin) and endothelial membrane proteins in this context is under investigation. Williams *et al.* (W10), in studies on the capillary endothelium of the microvessels in rat epididymal fat, found that glycosylated albumin is avidly taken up by endothelial micropinocytic vesicles, whose protein components in turn become glycosylated. Rapid vesicle-mediated extravasation of glycosylated albumin occurs at concentrations met with in diabetic sera; persistent microvascular leakage of glycosylated albumin may be significant in the pathogenesis of diabetic microangiopathy. While hyperglycosylated LDL is internalized and catabolized in guinea pig tissues and cultured human fibroblasts, the amount is less than for LDL (K8, S5, S41, W3). In view of the short half-lives of lipoproteins, this may be relevant to the grossly elevated plasma LDL values in human genetic disorders of LDL metabolism, but probably of lesser significance in the diabetic.

Clinical microvascular complications are seen late in the course of diabetes, but attempts to relate the degree of microangiopathy to the prevail-

ing glycemic state have not been conclusive. Both Raskin *et al.* (R4) and Sosenko *et al.* (S33) have examined the capillary basement membrane thickness in skeletal muscle biopsies from type I diabetics with long-standing glycemia and have shown a positive correlation of membrane thickness to glycosylated hemoglobin levels. Intervention to control blood glucose levels reduced the thickness of the capillary basement membrane; Sosenko *et al.* (S33) found that this only held for postpubertal subjects.

Preliminary studies indicate nonenzymatic glycosylation of human platelet membranes (S2); whether there is any connection with the abnormalities in platelet function in diabetes mellitus is under investigation (M37).

5.5.3. *Glomerular Basement Membrane*

The concept that precise control of diabetic hyperglycemia has a part in reducing the microvascular complications in the diabetic comes from several lines of investigation in animals (M8, M15) and man (G10, L16, S12, S15, V2). Nephropathy is the most serious complication of type I (insulin-dependent) diabetes, and renal failure eventually occurs in some 40%. Within 7 to 10 years of the onset of persistent albuminuria, about 50% of patients will have died.

McVerry *et al.* (M15) found that after 12 weekly injections of glycosylated plasma proteins into seven normal mice (a rather small experimental sample), five of the seven had glomerular basement membrane (GBM) thickening on electron microscopy. Cohen *et al.* (C14) reported similar findings in rats. However, there is a contrary finding that neither glycosylated nor normal albumin from rats and humans binds to the renal basement membrane of diabetic and control rats (J2, N7). Nevertheless, *in vitro* studies demonstrate that increased nonenzymatic glycosylation of basement membranes can be readily achieved (C13); and isolation from autopsy tissues of pure glomerular basement membranes (K16, M8) from six long-standing maturity onset diabetics and 10 normal subjects showed that not only was the albumin and immunoglobulin G (IgG) content of such membranes higher in the diabetic group, but also the nonenzymatic glycosylation of the whole glomerular basement membrane was significantly increased (S12).

In 18 diabetic children (40 healthy controls) and 20 diabetic children with no proteinuria or evidence of retinal vascular complications on fundoscopy (10 healthy controls), the excretion of GBM antigens was examined by immunoelectrophoresis using a rabbit antihuman GBM serum (L15, S15). Eleven of the 18 and 10 of the 20 diabetic children, respectively, exhibited α-1 mobility, while the remaining diabetics and *all* the healthy children presented with α-2 mobility. On *in vitro* incubation with glucose, GBM antigens of healthy children now possessed α-1 mobility. Examination of GBM, isolated from kidneys obtained at surgery or within a few hours of

death at necropsy, indicated the presence of ketoamine-linked glucose (S12). Further, diabetics with Hb A_{1c} of 10% or more had significantly greater immunoelectrophoretically altered GBM antigen in urine than subjects with Hb A_{1c} less than 10%. Plasma glucose levels and increased protein glycosylation, leading to glomerular basement membrane changes, may represent one facet of a multifactorial pathogenesis in the renal microangiopathy of the diabetic.

As the amount of urinary albumin in diabetics increased from microalbuminuria in 22 patients to an average of 1.23 g/day in 7 subjects (G10), so the proportion that was glycosylated decreased, despite the elevation of glycosylated serum albumin compared with normal subjects. The explanation may reside in the finding that, in GBM preparations, digestion by nonspecific proteases is considerably less for nonenzymatic glycosylated samples as compared to normal (L16). This situation probably also occurs *in vivo*.

In two multicenter studies (K19, S43), insulin-dependent diabetics with retinopathy showed consistent and significant declines of microalbuminuria (less than 1 g/24 hours) on continuous subcutaneous insulin infusion, but not on conventional injections, over a period of 8 months. The results of the Viberti–Keen group (V2, V3, V4) extend and confirm the belief that vigorous treatment and control of blood glucose levels will delay the progression (and possibly the onset) of nephropathy. No definitive statement is, however, possible at the present time.

5.6. Glycosylation in Eye Proteins

Subsequent to, and consequent on, glycosylation reactions in long-lived proteins (e.g., lens crystallins, collagens, elastin, myelin protein), the long life of these structural proteins leads to the formation of post-Amadori products. Prominent features are the occurrence of brown fluorescent pigments and participation in protein cross-linkages. Although Amadori ketoamine formation attains an equilibrium constant (being almost, but not quite irreversible), post-Amadori substances are irreversible once formed, and their concentration increases during the life of the protein glycosylated in proportion to the equilibrium constant of the Amadori product. While primarily an aging phenomenon, it is relevant here since diabetes is a life-long disease and the majority of diabetics are a mature and elderly opulation.

5.6.1. *Lens Crystallins, Basement Capsule, and Cataract*

Studies in experimental animals and man, instituted promptly after the production or diagnosis of the diabetic state, indicate that the development of cataract and retinopathy can be definitely inhibited by good glucose con-

trol. Sustained hyperglycemia is essentially cataractogenic as a result of at least two mechanisms: (1) excess formation of sorbitol from glucose under the influence of lens aldose reductase (this metabolic path may be blocked by the enzyme inhibitor sorbinil) and (2) nonenzymatic glycosylation of the lens proteins and α-, β-, γ-, and δ-crystallins.

Since the lens does not shed cells or lose any protein, the lens crystallins represent the oldest protein in any animal. The lens grows throughout life from the periphery, and cells at the center (nucleus) of the lens are as old as the animal or human itself. Although structural and biochemical modifications in crystallins occur with aging and cataractogenesis, no other protein persists for so long. The applicability to man of the results and conclusions on experimental animals, with regard to *in vitro* incubation of normal, senile, and cataractous lenses with glucose and in lenses obtained for chemical-induced diabetes, is indirect because of the considerably longer human life span, although relevant qualitative conclusions can be deduced (C8, C9, L12, M28, S45). In summary, animal lens crystallins undergo nonenzymatic glycosylation, accentuated in the diabetic (chemical-induced) state; there is an age-related increase in nonenzymatic glycosylation in calf and mature bovine lenses. Nonenzymatic glycosylation leads to partial unfolding of the crystallin protein molecule and facilitates its sulfhydryl group oxidation. While clear solutions were obtained from normal lenses, solutions from cataractous lenses were cloudy due to the formation of crystallin protein aggregates of high molecular weight.

In studies on human lenses, nonenzymatic glycosylation in normal and senile cataractous lenses was found to be similar (A11, G7, P4); in diabetic cataractous lenses there was a twofold (G7) and 35% increase. The investigators concluded that nonenzymatic glycosylation did not appear to play a major role in the formation of insoluble lens protein or protein disulfides or cataract formation. Lee *et al.* (L7) demonstrated that glycosylation of human lens cortical proteins, but not lens nuclear proteins, was significantly higher in diabetics with senile cataract. This difference on aging of the crystallin element in the lens will be discussed later. The same group (M5) found that nonenzymatic glycosylation *in vivo* of the human lens capsule (basement membrane, type IV collagen) was significantly increased in diabetic cataracts (average subject age, 67 years) compared to nondiabetics with senile cataracts (average age, 71 years).

Pirie (P11) was the first to investigate the yellow–brown pigment in nuclear cataracts. Examination of 660 cataractous lenses (B37, D12, P11) revealed 25% to be uniformly pale yellow and a further 29% with a hazel to deep brown nucleus. She isolated a fraction of insoluble crystallin protein held together by covalent cross-links that were *not* disulfides. A brown cross-linked material was isolated that appeared to contain some nonprotein mate-

rial; it had an ultraviolet absorption peak at 325–355 nm and exhibited fluorescence at wavelengths over 400 nm or near 450 nm. This compound has been identified as one post-Amadori glycosylation product (B28, B33, M29, P13). One such fluorescent chromophore, identified as 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole, has an ultraviolet absorbance maximum at 278 nm with a prominent shoulder at 330–335 nm; the tailing of this shoulder into visible wavelengths is responsible for its yellow–brown color. It exhibits a fluorescence maximum of 440 nm. This compound incorporates two peptide amino nitrogen atoms derived from lysine and two glucose residues, indicating that its peptide-bound precursors are concerned in the cross-linking of structural proteins by glucose *in vivo*. Assay of this and similar compounds derived from Maillard intermediates would monitor yellow-brown post-Amadori substances, which form in aging and in diabetes, and their role in complications (H6a).

It is suggested that in diabetic cataractous lenses, the ketoamine content of the lens cortical crystallins will be higher than for the older nuclear crystallins, while the post-Amadori glycosylation products, while they may be present in the cortex, will be elevated in the nucleus. The deeper the yellow–brown of the nucleus and the higher the concentration of heterocyclic imidazole and similar compounds, the greater the time-averaged exposure of *all* long-lived structural proteins to high concentrations of glucose over the years will be reflected.

5.6.2. *Retinal Microvascular Basement Membrane*

Davis *et al.* (D3) examined 69 female and 69 male diabetics, of whom 33% had retinopathy; those with retinopathy had a mean glycosylated hemoglobin of 12.5% compared with 11.3% in those without this complication. Despite the fact that others have not found this association (G16, K19, M36), the former workers (D3) suggest that such a level of hyperglycemia is probably associated with the complications of the disease, since their subjects did not have regular access to specialist facilities and complications were recognized for the first time. The crucial point is that good metabolic control from the outset of discovery of the diabetic state delays the onset and ameliorates the progression of retinopathy. This contention is supported from various sources (D18, H15, N1). A combination of Hb A_1 and fasting blood glucose is considered useful for long-term control of diabetes and retardation of retinopathy.

A study on the *in vitro* nonenzymatic glycosylation of bovine retinal microvessel basement membranes is of particular interest (L10). Type IV (and probably type III) collagen was purified from the intact retinal microvessels or isolated retinal microvascular basement membranes, and the rate constants for the glycosylation process, first examined for hemoglobin glycosyla-

tion, were determined, Ketoamine (Amadori rearrangement) production was shown to be the rate-limiting step, suggesting that *in vivo* recurrent or sustained high blood glucose levels will produce substantial ketoamine production in the basement membranes of the retinal microvasculature. Consequences have been examined in bovine retinal capillary pericyte cultures (L11). Increasing glucose concentration increased the synthesis of soluble collagen at the expense of insoluble collagen. This was shown to be due to the inhibition of the normal lysyl-derived cross-linked collagen formation, due to increased nonenzymatic glycosylation of the epsilon-amino group of lysyl residues of newly synthesized collagen. The later Maillard reaction, involving polymerization and dehydration of the Amadori enolized compound, results in reduced collagen solubility (K17, M30, Y5). These alterations in basement membrane structure of retinal capillaries may play a part in the function–structure abnormalities seen in diabetic retinopathy.

5.7. Glycosylated Collagen

Knowledge of ketoamine concentrations and postketoamine modification products is becoming of increasing clinical importance in relation to the assessment and control of complications in diabetics, which are mainly referable to function–structure alterations in long-lived structural proteins. Routine collagen analyses using human fingernail, skin, or hair samples are readily performed (O5, P1, P2). In a furosine assay, fructose–lysine extracted from fingernail protein showed a twofold increase in diabetics compared with normals (O5). In autopsy skin samples, the mean glycosylation for 33 subjects was 0.072 ± 0.012 μmol fructosamine/100 mg skin wet weight, compared with 0.127 ± 0.034 μmol fructosamine/100 mg skin wet weight in 10 diabetics (P2, P15). Lyons and Kennedy (L18) reported increased nonenzymatic glycosylation of skin collagen in patients with type I diabetes mellitus, and Buckingham *et al.* (B36) found a 13-fold increase in ketoamine-linked hexose bound to skin protein in children with poorly controlled insulin-dependent diabetes mellitus and exhibiting a sclerodermalike syndrome and waxy skin. An annual skin biopsy for long-term follow-up is suggested in type I diabetics or those with nephropathy, frequent infections, or episodic complications; 200 mg of skin is adequate for duplicate assays, expressed in terms of wet weight of tissue, or 500 mg for duplicates expressed in terms of dermal collagen based on hydroxyproline estimation (B8, B13). Two technicians can determine the glycosylated collagen in 50 skin biopsies in less than 2 days.

Investigative studies on the nonenzymatic glycosylation of collagen have elucidated several aspects of its effect in altering the structural and functional properties of collagens. In rats rendered diabetic, examination of tail

tendon or thoracic aorta collagen (A10, L1, R9, Y5) demonstrated an increased collagen glycosylation with altered properties. Glucose acts to form bonds between the ϵ-amino of lysyl or hydroxylysyl residues in collagens, and the effect of elevated glucose concentrations in diabetics is to inhibit intermolecular cross-links and covalent bridges between lysine and non–glucose-derived Schiff bases during the formation of collagen or its maturation. The result is inhibition of collagen fibril formation, altered solubility of collagen (F17), decreased susceptibility to proteolysis, and increased resistance to collagenase action. However, Lyons and Kennedy (L19) suggest that this last effect is due instead to the formation of advanced glycosylation final products. Once the collagen fibers are cross-linked, nonenzymatic glycosylation is diminished. In human dura mater, diaphragmatic tendon, and abdominal or thoracic skin samples obtained at autopsy (M30, S13, S14), findings were in accord with the concept that there was an age-related increase in glycosylated collagen in normal subjects, but that type I and young diabetics exhibited considerable excesses of glycosylated collagen related to ongoing collagen synthesis and maturation.

Albumin and immunoglobulin G were found to bind to nonenzymatic glycosylated collagen at a rate fourfold that of unmodified collagen (B28). Could this structurally modified collagen, by aggregating immunoglobulins and albumin and retaining their capacity to form immune complexes *in situ* with free antigen and antibody, cause chronic tissue damage like that associated with diabetes mellitus? Similarly, during the process of nonenzymatic glycosylation of collagen, LDL molecules are covalently bound (B32), and such a mechanism could contribute to accelerated atherosclerosis in the diabetic.

The influence of a lifetime of aging and long-sustained hyperglycemia in the diabetic state is responsible for post-Amadori formation of glycosylated brown collagen. LaBella (L1) described changes in human Achilles tendon with age in terms of an increased resistance of its collagen to solubilization, with progressive greater fluorescence at 405 nm and accumulation of yellow pigment absorbing at 340 nm. Some 20 years later, an autopsy study of insoluble dura mater revealed increased yellow (absorbance at 350 nm) and fluorescent (emission at 440 nm) material with age, accentuated by the diabetic state (M30). Collagen samples (rat tail tendon fibers incubated at 37°C for 19 days in 100 m*M* glucose) showed identical absorbance and fluorescence spectra and nonenzymatic browning. Although not proven as yet, the substance is probably similar to that associated with the brown nuclear cataract (crystallins) and isolated from browned albumin and polylysine (B33, P13).

The age-corrected fluorescence of collagen browning was greater in insulin-dependent diabetics with increased degrees of retinopathy and joint

and arterial stiffness (M31a). The authors suggested that there was an overall correlation between the severity of diabetic complications and many years of cumulative glycemia.

5.8. Glycosylated Nervous System Protein

About 25% of diabetics have a symptomatic polyneuropathy, and most present with electrophysical evidence of abnormal peripheral nerve function. Some of the metabolic abnormalities (G24) can be attributed to glycosylation reactions (ketoamine formation and post-Amadori products) in central and peripheral nervous system myelin components. Neuropathic complications related to diabetes mellitus are reversible with improved metabolic control (W6), while the degree of decrement in motor nerve conduction velocity is a measure of hyperglycemic levels in untreated diabetes (G20). In experimental diabetic rats and dogs, there was a greater than twofold increase in nonenzymatic glycosylation of the primary axonal and myelin proteins of sciatic and femoral nerves compared to controls (V5, V6). Glycosylated lysine and its hydrolyzed rearrangement product were the major glycosylation elements identified, together with an increased accumulation of peripheral nerve myelin by macrophages in diabetic rats (V7). Nonenzymatic glycosylation of α and β rat brain tubulins occurred *in vitro* and *in vivo* under conditions of untreated diabetic hyperglycemia (W11) and led to the formation of amorphous tubulin aggregates. Such changes could compromise neuronal functions dependent on microtubule formation. The methods employed fall within the ambit of clinical neurochemical laboratories.

5.9. Animal Studies

Mechanisms of nonenzymatic glycosylation in normal, senile, spontaneously diabetic, and chemically induced (alloxan and/or streptozotocin) diabetic animals have been intensively investigated (selected references, C3, D5, F16, H9, K16, S25, V6, W20, Y5). However, since the life span of proteins in experimental animals differs very appreciably from that of those in the human, their applicability should only be regarded in general terms. Where pertinent, such experimental studies have been discussed under the relevant sections.

5.10. Utility

Approximately 1 in 40 persons in the United States suffer from some form of diabetes (P9), and incidences higher and lower than this occur worldwide. Both sophisticated and relatively simple accurate methods are now available

for glycosylated protein determination. The usefulness of such assays is predicted on three phases in the natural history of the diabetic, namely, diagnosis, metabolic control, and development of complications (in particular, retinopathy, nephropathy, and neuropathy). Reviews on the measurement and clinical use of glycosylated hemoglobin measurements (G5, G12, M9, P7, S19) and the utility of glycosylated protein assays in general (B1, B38, D28, K11, M23, R13) have appeared. In recent assessments of the use of glycosylated hemoglobin in monitoring diabetic control, there are indications that by the use of more specific analytic techniques the normal range of values for glycosylated hemoglobin are lower than those given previously (G8).

5.10.1. *Screening for Diabetes*

A combination of glycosylated hemoglobin (accurate Hb A_{1c} or stable ketoamine) and fasting blood glucose assays is an accurate predictor for the diagnosis of the diabetic state (C22, D17, F8, F13, J7). This endogenous assessment was applicable to diagnosis in both type I and II patients, and the numerous technical and scoring problems of the artificial oral glucose tolerance test (OGTT) were avoided. There are at least eight procedures for a "standard" OGTT to establish the diagnosis of diabetes mellitus (M7). An alternative is that if the OGTT result is equivocal, a glycosylated hemoglobin assay at the time of the repeat OGTT is highly desirable.

There are some reports in which Hb A_{1c} or A_1 values were regarded as inadequate criteria for diagnosis (D17, L9, S3), or where its value was equivocal (D30). These discrepant views may partly be due to differences in methodology or equating results of Hb A_1 or aldimine plus ketoamine with stable ketoamine values. Final assessment must await the subsequent history of patients who have been grouped according to Hb A_{1c} or OGTT evaluation or some other combination of tests.

5.10.2. *Control of Glycemia*

Most diabetologists and diabetic care personnel would accept the premise that good metabolic control in diabetics decreases the incidence of the manifold complications of this worldwide disorder. Urinary and preferably blood glucose levels must be used to regulate short-term insulin or oral hypoglycemic drug dosage, diet, and adjuvant therapies. Several possibilities are available to monitor the adequacy of control by assay of glycosylated proteins and postglycosylation rearrangement products.

5.10.2.1. *Short-Term Monitors.* These include labile pre-Hb A_{1c} (several days), glycosylated serum transferrin, half-life of 8 days (K3, K4) and glycosylated serum albumin, half-life of 20 days (D23, J6). These estimations

reflect glycemic status for 1 to 2 weeks and 2 to 4 weeks prior to their measurement.

By centrifugal or density fractionation of a red cell sample, the top 10% of the red cell column will represent young erythrocytes with a 0–10-day life in circulation and will thus reflect the immediate past exposure to blood glucose concentrations (B11).

The preferred short-term parameter is plasma glycosylated albumin. The method has technical advantages and is preferable in cases of diabetic pregnancy, hemolytic disease, and high Hb F (M9) and in monitoring control after a hyperglycemic episode (D19).

5.10.2.2. *Intermediate Monitor.* Glycosylated (ketoamine) hemoglobin assays provide an overview of control for the previous 5 to 8 weeks. Such assays are independent of patient compliance; an assay every 2 to 3 months will also reduce the number of blood samplings required for blood glucose estimation.

Various aspects of the clinical utility of glycosylated hemoglobin have been examined (B39, C17, G13, J7, N6). Particular emphasis was laid on serial monitoring in type I diabetes, and its use in other aspects of the labile diabetic (infections, burns, trauma and surgical operations, pregnancy) has been advocated (D14). By home collection of blood on filter paper (L14b) or by a capillary blood collection system (L4a), it becomes feasible for the clinician to have glycosylated hemoglobin results available at the time of the clinic visit or appointment.

5.10.2.3. *Long-Term Assessment.* Glycosylated collagen of skin or nail, taken every 12 months, will monitor cumulative control and the development of post-Amadori glycosylation products. These, particularly the formation of yellow–brown pigments, are indicative of progressive cell aging and may be used to probe the possibilities of accelerated aging in diabetics.

5.10.3. *Relationship to Diabetic Complications*

Overall, the life span of a person, once diagnosed and treated as a diabetic, is one-third less than that of their normal fellows. While there are other candidates as causes of the major long-term complications in diabetics (neuropathy, nephropathy, retinopathy, arteriosclerosis), there is good evidence that sustained hyperglycemia, especially with reference to non–insulin-sensitive tissues (lens, red cells, etc.), is a prime factor in initiating and aggravating these disorders.

6. Various Clinical and Other Aspects

6.1. Other Glycemic Conditions

6.1.1. *Hyperglycemia*

Hyperglycemia in excess of a brief interlude occurs in a few other conditions only. Reports of an elevated Hb A_{1c} or A_1 have been made in glucose intolerance and prediabetic states; stress hyperglycemia in coronary artery disease; hormonal disturbances such as growth hormone excess (G9), excess glucocorticoids, Cushing's syndrome; obesity with or without diabetes mellitus (T5); and prolonged usage of thiazide diuretics (B17). In these situations, it would be more instructive to perform glycosylated albumin assays.

6.1.2. *Hypoglycemia*

While there are many causes of short-term hypoglycemia, there are few that are long term with significantly low plasma glucose. These conditions would include neonatal hypoglycemia, insulinoma (nesidioblastosis), adrenocortical and pituitary failure, malnutrition and liver necrosis (cirrhosis), glycogen storage disease type 1, and galactosemia.

Values of Hb A_{1c} or A_1 out of harmony with plasma glucose levels may be a clue to prolonged, unrecognized nocturnal hypoglycemia and the necessity to reduce insulin dosage (A14, D24, G3); for example, the patient of Gale *et al.* (G3) had blood glucose levels of 8.4 to 13.1 (mean 10.9) mmol/liter at 2 to 3 PM with an Hb A_1 of 6.3%, when a value of 11% to 13% would have been expected. Finally, serial samples taken at 3, 6, and 9 AM indicated that for 6 hours or more per day, the blood glucose was 2 mmol/liter or lower.

In the diagnosis of insulinoma, Hb A_1 assay has been claimed to be a more accurate index of metabolic disorder than the overnight fasting blood glucose (S6); in 10 cases, Hb A_1 was lower in untreated patients than in controls (5.5 $\pm$ 0.2% against 6.9 $\pm$ 0.1%, $p < 0.001$). In a further case (F15) with Hb A_1 of 4.6% (7.1 $\pm$ 0.7% in 17 normals) and blood glucose of 2.4 and 0.9 mmol/liter after a fast of 12 and 20 hours, respectively, and two cases (S29) with Hb A_1 of 3.7 and 3.8% (normals, 4–6%), a similar conclusion was reached. However, in 13 patients preoperatively with surgically confirmed insulinomas (N9), Hb A_1 values ranged from 5.2 to 6.6% (mean 5.9 $\pm$ 0.5% SEM) compared with 30 nondiabetics (4.5–8.9%, mean 6.5 $\pm$ 1.2% SEM). Neither the blood glucose nor the Hb A_1 levels were consistently low in these subjects, and the finding of an inappropriate hyperinsulinemia was considered diagnostically more definitive.

In glycogen storage disease (GSD type I, van Gierke's disease), a low Hb A_{1c} level has been recorded. In galactosemia, the inherited inability to

metabolize galactose leads to high blood galactose with reciprocal low blood glucose and the formation of galactosylated serum albumin (U3). In a retrospective survey, hemolysates and plasma stored at −25°C from five galactosemic patients were found to have galactosylated hemoglobin and plasma albumin three times that for normal stored controls (author, unpublished).

6.2. Various Other Conditions

6.2.1. *Red Cell Disorders*

In hemolytic anemias (immune, hereditary spherocytosis), levels of glycosylated hemoglobin (3.9 ± 0.1% SD) were significantly lower ($p < 0.0005$) than in patients and normal controls with 7.0 ± 0.7% SD and 6.7 ± 0.7% SD, respectively (F14). The actual levels of glycosylated hemoglobin in nondiabetic patients with hemolytic anemias are indicative of the degree of hemolysis during the preceding weeks (P5). The estimation of glycosylated hemoglobin could serve as a rapid screening test and make labeled red cell survival studies unnecessary in many cases.

In miscellaneous conditions such as chronic renal failure, renal transplants, pernicious anemia, rheumatoid arthritis, and hemolytic anemia, with reduced erythrocyte survival (^{51}Cr half-life less than 15 days), glycosylated hemoglobin values were 4.0–6.8% in comparison with the normal of 7.3% (F14). In a diabetic with a drug-induced hemolysis caused by dapsone, glycosylated hemoglobin was 4.2% and 3.7% a month later—the upper limit of normal for the microcolumn chromatographic method in use being 8.5% (K9). This highlights the age-related changes of Hb A_{1c} that occur during the life span of the red cell (F7); because of the slow process of ketoamine formation, young red cells of short life span have accumulated a small content of glycosylated hemoglobin, which increases with their maturation and aging.

In glycosylated hemoglobin, since the sugar displaces the 2,3-diphosphoglycerate, there is an increased affinity for O_2, and it has been proposed that this leads to tissue hypoxia and a relative polycythemia in the diabetic (D13). Nevertheless, at the *in vivo* concentrations of glycosylated hemoglobin generally present in diabetes, this would not appear to play a significant role (B14, S1).

In iron deficiency anemias, patients often have raised glycosylated hemoglobin values, even rising into the diabetic range, which subside on treatment (B25). Different explanations have been given to account for this phenomenon (M27, S28).

Various hemoglobin variants have charge characteristics similar to Hb A_{1c} or A_1 and thus will separate chromatographically or electrophoretically as

"fast-moving hemoglobins" in the same position (S31). Other variants that cochromatograph with Hb A_1 are hemoglobin Raleigh (D26), Wayne (G13), Hope (K10), and hemoglobins G and H (K18, K20). The opposite error was found with hemoglobins clearly separable from hemoglobin A (e.g., Hb F, S and C). Estimation of Hb A_{1c} or A_1 underestimated *total* hemoglobin glycosylation (A5, B10). Only total hemoglobin ketoamine or separation techniques identifying F_1, S_1, C_1, and so on would be satisfactory; for example, in sickle cell anemia, only Hb S and F and their glycosylated moieties S_1 and F_1 occur.

6.2.2. *Miscellaneous Disorders*

6.2.2.1. *Cystic Fibrosis.* Glycosylated hemoglobin in 34 cystic fibrosis patients (age $1\frac{1}{2}$–20 years) was significantly higher ($7.97 \pm 1.16\%$) compared with 150 normal children ($6.8 \pm 0.8\%$). This correlated with their decreased insulin response and impaired glucose tolerance (B16), although diabetes mellitus is reported to be rare in cystic fibrotics (B16).

6.2.2.2. *Steroids.* Subjects on ethinylestradiol–norethisterone contraceptive pills had significantly lower Hb A_1 levels than women using barrier or antiestrogen techniques (O1). This is in harmony with observations that synthetic estrogens lower plasma glucose, possibly via an effect on the portal insulin : glucagon ratio.

Oxlund *et al.* (O8) found that corticosteroid treatment in rats for 14 or 60 days increased the strength and stability of lumbar skin collagen, but that 120-day treatment decreased skin thickness and collagen content and increased amounts of glucose were attached to the ϵ-amino group of lysine residues in collagen.

6.2.2.3. *Hypoalbuminemia.* In chronic malnutrition and kwashiorkor, serum albumin levels are markedly decreased to 1.5–2.5 g/dl. Glycosylated albumin expressed as a percentage of total serum albumin or per milligram albumin was decreased in parallel with the subjects' hypoglycemia. Glycosylated hemoglobin and urinary glycosyl–lysine were at the lower limit of normal for age-matched controls (author, unpublished).

6.2.2.4. *Salicylate Administration.* Hemoglobin F is acetylated and glycosylated endogenously, and glycosylated Hb F_1 is best detected by isoelectric focusing. With continuous high aspirin intake in children and adults, acetylation competes with glycosylation for free-amino valine and lysine in proteins; and albumin (M20), collagen type I (L1), and collagen type IV (Y5) are preferentially acetylated. Assays, unless specific for the ketoamine linkage, will not reflect time-averaged blood glucose concentra-

tions. Structure–function changes occur both in acetylated and glycosylated collagens.

6.2.2.5. *Immune Complexes.* Binding of both albumin and immunoglobulin G (IgG) to glycosylated collagen averaged four times that of unmodified collagen (B28). Both albumin and IgG bound to nonenzymatically glycosylated collagen retained their capacity to form immune complexes *in situ* when exposed to free antibody and antigen. Further, antibodies specific for glycosylated LDL have been detected by immunoassay in diabetics, producing an increase in the clearance of glycosylated LDL (W18). Nonenzymatic glycosylation of plasma proteins and long-lived structural proteins may render them immunogenic and result in the production of autoantibodies that react specifically with glycosylated lysine groups. In this way, the antibodies will recognize not only the particular immunogen, but also possibly other cross-reacting glycosylated plasma and tissue proteins. Chronic tissue damage in long-term diabetes may arise in this way.

6.2.2.6. *DNA.* Glucose and glucose 6-phosphate react *in vitro* with DNA to produce browning reactions, with significant structural and biological alterations in the DNA (B35). Since nucleic acids are long-lived molecules in the resting cell, accumulation of nonenzymatic browning products could be related to the decreased genetic variability characteristic of the aged organism. Glycosylation of nucleic acids has been little studied and may have considerable functional implications.

6.2.2.7. *Protein Hormones.* Glycosylated insulin has been prepared (D21) and its biologic function shown to be reduced by 20%. However, the life of protein hormones *in vivo* is short, and so that negligible *in vivo* glycosylation occurs. The use of glycosylated insulin complexed to concanavalin A in a closed-loop insulin delivery system has been described (B27); it presented stability advantages over conventional insulins in these situations.

6.2.2.8. *Enzymes.* Several enzymes, for example, β-*N*-acetyl-D-glucosaminidase, EC 3.2.1.30 (D20), ribonuclease A, EC 3.1.27.5 (E1), and the sulfhydryl protease, cathepsin B, EC 3.4.22.1 (C18), when incubated with glucose *in vitro*, lose considerable or all enzyme activity. The enzymes undergo nonenzymatic glycosylation resulting from the formation of reversible covalent intermediates (aldimine and ketoamine) at active-site ε-amino lysines. However, the short half-life of the majority of enzymes *in vivo* indicates that glycosylation of cell enzymes is limited and mainly confined to the formation of reversible aldimine adducts.

6.3. Renal Failure

Several authors have reported an increase in Hb A_1 in both diabetics and nondiabetics with chronic renal failure (D7, G22, O3). However, it was observed that such increases were not in harmony with the metabolic status of the patient. The increased Hb A_1 values in uremia were mainly not due to glycosylation of hemoglobin, but reflect carbamylation of the hemoglobin at the same site by urea-derived cyanate (S21). When the specific thiobarbituric acid assay (F10) or the specific affinity chromatographic measurement (D7, O4, S21) of glycosylated hemoglobins in uremic patients was employed, the increase in Hb A_{1c} was slight. It was attributed to impaired glucose tolerance and shortened red cell life span (B34, F10, L2, L3) or acidosis (D10, O4).

In diabetes with the development of microalbuminuria and subsequent renal failure, there is an increase in urinary glycosylated amino acids (B29) as well as an increase in nonenzymatic glycosylation of urinary proteins (G23) and specifically glycosyl albumin (G10).

Zawada *et al.* (Z1) have reported that in 24 renal transplants and particularly in 2 diabetics, as well as in 34 subjects undergoing hemodialysis including 4 diabetics, the Hb A_1 by an affinity chromatographic column method (which presumably excluded nonglucose adducts with hemoglobin) was significantly elevated compared with normal controls. These observations conflict with those who found decreased glycosylated hemoglobin in renal transplants or chronic renal failure (F14) and correlated this with a decrease in red cell survival.

In continuous ambulatory peritoneal dialysis, the use of a peritoneal solution with 4.25% glucose caused plasma glucose levels to reach above 5.5 mmol/liter for 4 hours during the dialysis session and produced a marked insulin response (A12). It is probable that Hb A_{1c} values would be somewhat elevated in such subjects, but this would not occur with the use of 1.5% glucose solutions.

6.4. Pregnancy

The natural history of a diabetic woman who becomes pregnant is one of considerable morbidity and mortality for mother, fetus, and neonate. In normal pregnancy, glycosylated hemoglobin changes are minor (W22). In pregnant diabetics, there are a number of studies in which initial elevated values of glycosylated hemoglobin decreased appreciably by the third trimester and were often below age–sex-matched diabetics (B23, J8, K12, L8, M24, M26, O7, S20, W8, Y2, Y3). Generally, better metabolic control was achieved in the pregnant than nonpregnant female diabetic; this may be

attributed to the pregnant woman's greater motivation and compliance. Further, in those fetuses and neonates with malformations, the initial maternal Hb A_{1c} or A_1 recorded was considerably elevated (K12, L8, M26, M31c, Y2, Y3), while no malformations occurred in those with lesser maternal elevations initially and with reductions during progress of pregnancy. The incidence of macrosomia and metabolic abnormalities, such as hyperbilirubinemia and hypoglycemia, was greater where maternal glycosylated hemoglobin values were raised. Some authors (F1, S32, W21) have examined Hb F_{1c} levels in cord blood and concluded that fetuses of diabetic mothers are hyperglycemic in utero. However, Olesen *et al.* (O6), in a meticulous analysis of their own results, concluded that while the Hb F_{1c} and birth weight of those born of diabetic mothers are increased above normal, metabolic control in pregnancy limits the increase to a satisfactory level (low or statistically insignificant).

Gestational diabetes occurs in 1–2% of pregnant women. Artal *et al.* (A13) examined 82 nondiabetic pregnancies and found no case of gestational diabetes; they also concluded that glycosylated hemoglobin assays were unsatisfactory as a screening procedure. Shah and co-workers (S22) selected 90 subjects at 28–30 weeks with defined risks for diabetes; Hb A_1 alone or in combination with an OGTT (50 g glucose) was not found to be a sensitive test for detection of diabetes. However, in obese gestational diabetics, significant Hb A_{1c} elevation (8.77 ± 1.03%) was found compared with obese nondiabetic pregnants (S20). Further, in a very extensive study on 180 patients with a history or clinical features of prediabetes (B5), 21 of 33 patients with gestational diabetes had elevated glycosylated hemoglobin levels, and 50% of these delivered macrosomic infants. Pollak and associates (P12) examined glycosylated hemoglobin levels in mothers of large-for-gestational-age infants and found this a worthwhile postpartum test for undetected diabetes.

The concentrations of glycosylated albumin or glycosylated plasma proteins decreased by 50% or more after 4 weeks of effective metabolic control in pregnant diabetics; by contrast, the rate of decrease in glycosylated hemoglobin values in the same patients was significantly less and gave no indication of glycemic improvement until the 12th week. Thus measurement of glycosylated plasma (serum) proteins is an important adjunct to serial blood glucose monitoring in clinical circumstances such as diabetic pregnancy, where early confirmation of good diabetic control is important (L7a, M31b).

7. Significance in Aging Processes

The formation of post-Amadori glycosylation rearrangement products and yellow-brown pigments is a reflection of normal blood glucose concentra-

tions over a lifetime, "three score and ten" or more years in structural long-lived proteins, and is accelerated in the diabetic by high glucose levels over a number of years. The glycosyllysines (ketoamine) formed prevent the lysyl cross-linking between collagen or crystallin or elastin fibrils, interfere with the structure and function of these structural proteins, and render the glycosylated products more susceptible to protease degradation. Glucose interference with collagen cross-linking and enchanced collagenolytic degradation may be responsible for the prolongation of wound healing in diabetics and elderly people (L13, S25).

While glycosylated collagen and crystallin are increased in elderly subjects and diabetics of long standing, attention should be directed to the detection of subsequent compounds formed, namely, brown pigments (absorbance at 335 nm and fluorescence at 440 nm) and rearrangement products (B13a, B33, L1, M29, M30, P11). An aromatic heterocyclic compound, derived from two lysyl and two glucose groups, 2-(2-furoyl)-4(5)-(2-furanyl)-1*H*-imidazole (C7a, P13) and ϵ-*N*-carboxymethyllysine (A4a), have been identified as degradation products of glycosylated (glycated) protein. Definitive assays for these and related compounds are promising. The applicability of the Maillard browning reaction (M2, M3) to long-lived biological proteins is now firmly established, and investigations aimed at practical assays are required.

8. Summary

Nonenzymatic glycosylation takes place in all proteins with a free-reacting lysine or valine in the presence of glucose. The formation of glycosylated plasma albumin, hemoglobin (Hb A_{1c}), and skin collagen provides a diagnostic index of short- to long-term time–concentration of glucose *in vivo*. A wide range of assay methods are available, with affinity chromatographic, isoelectric focusing, and spectrophotometric methods providing the best accuracy and versatility. Glycosylated hemoglobin assays indicate glucose pressure over the previous 2 to 3 months and are of diagnostic value in general diabetic control, while glycosylated plasma albumin determinations are preferable in acute episodes in the life of a diabetic (e.g., pregnancy, infection, stress, trauma, surgery), since they provide an overview of changing blood glucose values of the previous 2 to 4 weeks. Glycosylated collagen estimations reflect tissue aging and are relevant in healing processes. Glycosylation alters the biologic activity of proteins, and these may relate to the manifold complications concomitant on the lifelong elevation of blood and tissue glucose in the diabetic (C6a). Assays for glycosylated hemoglobin have been routinely performed in clinical chemistry laboratories for a dec-

ade, and convenient determination for other nonenzymatically glycosylated proteins is proceeding apace.

ACKNOWLEDGMENTS

Interest in the subject was initiated in 1974 while the author was head of the Metabolic Research and Human Biochemical Genetics Unit, South African Institute for Medical Research (SAIMR), Johannesburg. Literature to June 1986 has been consulted. I am indebted to Ronald Georges, head of Biochemical Methods and Equipment Unit, SAIMR, for many fruitful discussions and material assistance in obtaining reference material.

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BIOCHEMISTRY OF THE NERVOUS SYSTEM

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1. Introduction

The discipline of neurochemistry has burgeoned so swiftly in the past decade that the very length of this article limits the detail with which all areas of the field can be covered. Indeed, monographs have been written on virtually each item that is discussed only briefly in this article. Nevertheless, insofar as this chapter represents an introduction to the primary biochemical mechanisms that contribute to the functioning of the peripheral and central nervous systems, those basic neurochemical processes that are thought to contribute to the normal physiology of the nerve cell and its ability to communicate with other nerve cells can be described reasonably well.

Fundamental to our understanding of neuronal physiology is the concept of the neuron as an excitable cell, that is, a cell that is capable of maintaining an electrical charge across its cell membrane and that, under well-defined circumstances, can alter that potential charge so that an electrical impulse can be carried along the length of the neuron. Many of the properties that make the neuron excitable and capable of electrical impulse propagation can be explained through an understanding of the basic morphology of the neuron and of the biochemical nature of the neuronal cell membrane and the myelin sheath that envelops a large portion of the nerve cell. Cell excitation not only alters the internal chemistry of a particular neuron but also permits one neuron to communicate with another by means of chemical, and rarely electrical, transmission. The chemical transmission occurs at a specifically defined neuroanatomic site known as the synapse, and a great deal of research has been directed toward understanding the myriad processes that contribute to what has been deemed synaptic transmission. This particular

area of neurochemistry deals with the release of neurotransmitters, their binding to specific recognition sites (receptors) on neurons, and those factors that contribute to the inactivation of the transmitter, such as neuronal reuptake and enzymatic catabolism. Efforts have also been focused on understanding mechanisms by which the synthesis of neurotransmitters can be controlled and ways by which neurons can respond intracellularly to the binding of neurotransmitters to cell membranes. One such means of response is through the generation of second messengers such as cyclic nucleotides and prostaglandins. Last, important to our present theoretical constructs about the biochemistry of neurons is an understanding of the intermediary metabolism of carbohydrates, amino acids, lipids, and nucleic acids, which, as in all cells, organizes the chemical machinery of the cell to support those particular functions for which the cell has undergone differentiation.

It is worthwhile to emphasize at this point that there is a large gap between the descriptive knowledge covered in this article and a fundamental understanding of the molecular processes that lie behind the neurochemical events that have been studied to date. For instance, although elevations in the second messenger cyclic adenosine monophosphate have been described in many brain regions after stimulation of neurons by a variety of neurotransmitters, the precise consequences of such increases in cyclic AMP are unknown. Although cyclic AMP can lead to the phosphorylation of numerous cellular proteins, the role of the majority of these proteins in cell function is not understood, and, at best, only hypothetical possibilities can be construed. Another example of the emphasis on our descriptive knowledge of the brain is the observation that a large number of neuropharmacologic agents have been shown to bind specifically to recognition sites on neuronal cell membranes. Whether such binding reflects competition with endogenous substances or is primarily artifactual represents a point of contention among neuroscientists. Certainly the meaning of such binding is often highly speculative.

It is hoped that this article will give the reader the essential background necessary to understand the basic biochemical events thought to contribute to normal neuronal functioning. Where appropriate, review articles on particular subjects will be suggested for the reader who wishes to pursue a certain area in more detail. Because the literature in this rapidly expanding field is immense, no attempt is made to annotate each concept or research finding in a thorough manner. Such specific references will be found in the suggested review articles. However, all the concepts and findings presented in this chapter are generally accepted by the scientific community, and wherever a controversy exists, a point is made to emphasize the disagreement to the reader.

2. Morphology of Neurons

2.1. Basic Anatomy

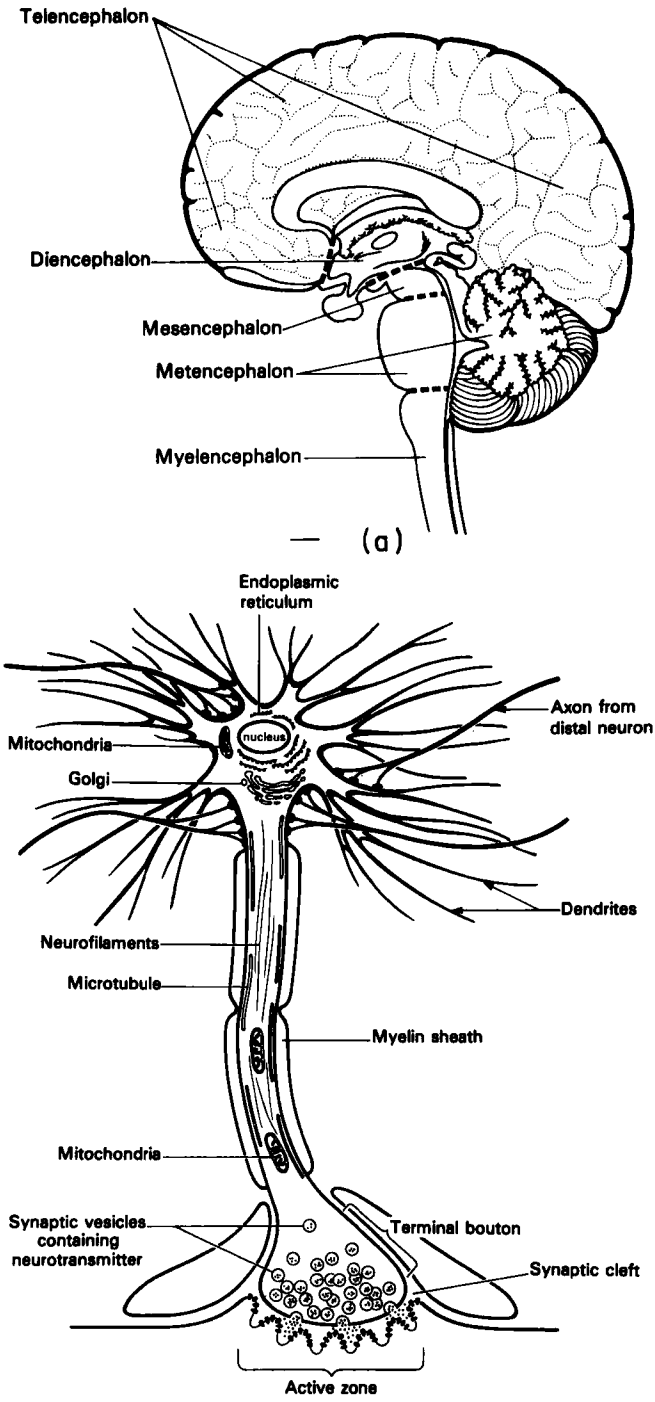
2.1.1. *General Structure of the Peripheral and Central Nervous Systems*

Prior to a description of the cell types present in the nervous system, it is helpful to delineate in rudimentary fashion the principal regions and divisions of the peripheral and central nervous systems, both of which are comprised of parenchymal cells (neurons) and their closely associated supportive neuroglial cells. The entire central nervous system, which includes the brain and the spinal cord, is bathed with cerebral spinal fluid, a plasma ultrafiltrate elaborated by the ependymal cells that cover the choroid plexus, which is the lining of the cerebroventricular regions. Cerebrospinal fluid normally contains 5–15 mg protein/100 ml in the ventricles, although values can double in areas around the spinal cord. Cerebrospinal fluid occupies both the subarachnoid space and the four brain ventricles (the two lateral, third and fourth ventricles) and spinal cord. The cerebrospinal fluid has a number of important functions, such as maintaining the balance of electrolytes and pH, regulating ventricular pressure (normally 70–180 mm H_2O), protecting the head and spinal cord from mechanical trauma, and acting as the primary mode for the removal of end products of neuronal metabolism. Cerebrospinal fluid is often sampled through ventricular or lumbar puncture in experimental animals and clinically in patients for the determination of metabolite levels of important neurotransmitters and as a diagnostic aid for cerebral injury and infection.

The mature adult brain can be divided into five primary regions that begin to develop in humans during the sixth week of gestation. These are the myelencephalon (medulla oblongata), the metencephalon (pons and cerebellum), the mesencephalon (midbrain), the diencephalon (thalamus and hypothalamus), and the telencephalon (cerebral cortical hemispheres and associated structures) [see Fig. 1(a)].

The mature adult peripheral nervous system is composed of the sensory and motor systems emanating from the spinal cord; the autonomic nervous system, which is composed of both the sympathetic and parasympathetic systems; and the enteric nervous system, which, due to its unique structural, chemical, and functional features, is presently considered to be separate from the autonomic nervous system even though it demonstrates profound interactions with the sympathetic and parasympathetic systems.

The vast majority of the biochemical properties that will be discussed in this article apply to both the peripheral and central nervous systems. It is beyond the scope of this chapter to discuss particular differences in the


Telencephalon
Diencephalon
Mesencephalon
Metencephalon
Myelencephalon
(a)
Endoplasmic reticulum
Axon from distal neuron
Mitochondria
nucleus
Golgi
Dendrites
Neurofilaments
Microtubule
Myelin sheath
Mitochondria
Synaptic vesicles containing neurotransmitter
Terminal bouton
Synaptic cleft
Active zone
(b)

biochemistry of the two systems, and for the purposes of the present discussion they are not important.

2.1.2. *Cell Types of the Peripheral and Central Nervous Systems*

With the exception of the endothelial and smooth muscle cells associated with the blood vessels that extend throughout the nervous system, there are two primary cell types that are present in the nervous system: the parenchymal neurons and their associated neuroglial cells. Curing embryonic development, the ectodermal germ layer gives rise to the neural tube, which directly divides into the neurons of the central nervous system and its supporting neuroglia. The neural tube can further develop into the neural crest, which results in the elaboration of the neurons of the peripheral nervous system and its supporting neuroglia. Only the microglia evolve from a different embryonic tissue, the mesoderm.

2.1.2.1. *Neurons.* Neurons are unique among cell types in mammalian organisms for their heterogeneity in size and shape and for their characteristic diversity of secretory compounds [see Fig. 1(b)]. For instance, neurons can release the quaternary amine acetylcholine; a number of monoamines such as the catecholaminmes, serotonin, and histamine; amino acids; and more complex peptides and proteins. After birth, neurons can no longer divide, a property that is in marked contrast to most other cell types. Central to our present understanding of neuronal functioning is the so-called neuron theory of Ramón y Cajal, which states that neurons form a contiguous, but not a continuous, network of intercommunicating cells. Prior to the work of Ramón y Cajal, there was a general belief that neurons existed as a protoplasmic continuous mass separated only by syncytia. However, the stellate-shaped cells originally described by Purkinje in 1839 are now believed to be separate entities, each one consisting of a cell body (soma or perikaryon), long, efferent axons, and short, afferent dendrites. The neuron theory has been fully confirmed by histological and cytological studies employing light and electron microscopy, neurophysiological methods utilizing the latency of nerve-to-nerve impulse propagation, and neuropharmacological and biochemical techniques. The individual neuronal cells, which have polymorphous qualities, are known to communicate with each other at the synaptic junction, a term first coined by Sherrington in 1897.

FIG. 1. (a) Cross section of mature human brain with major embryological regions outlined. (b) Diagrammatic representation of a single neuron with major organelles depicted. Cleft noted on myelin sheath represents node of Ranvier. Details of neuronal morphology are presented in text.

There are general structural features peculiar to the neuron that make it ideal for carrying electrical impulses. These include the dendrites that receive incoming signals from other neurons, the perikaryon through which the impulse is processed, the axon hillock through which impulses from numerous dendrites are integrated, and the axon through which a newly integrated impulse is carried to the nerve terminal, or terminal bouton, for communication to still other neurons.

The perikaryon is composed of a nucleus and a variety of subcellular organelles, most of which contain membranous cisternae. The nucleus, as in all cells, is the repository of the genetic material and in neurons is invariably spherical and has a different shape than nuclei associated with neuroglia. The nuclear envelope extends to the smooth endoplasmic reticulum that comprises the Golgi apparatus, which is present in the perikaryon and the dendrites. The Golgi apparatus is thought to be involved in the synthesis and packaging of secretory substances, and many researchers believe that the packaging of certain peptidergic neurotransmitters into vesicles may occur at the Golgi prior to transportation down the axon to the synaptic nerve terminal, where neurosecretion occurs. The Golgi may also be involved in the synthesis of lysosomes, those organelles that are involved in the proteolytic degradation of numerous proteins. Last, the Golgi apparatus may be an important site for the synthesis of glycoproteins and glycosphingolipids that eventually will be translocated to the cell membrane and nerve terminal.

Important to the transport of packaged vesicles and glycoconjugates synthesized at the Golgi are the elongated structures found throughout the neuron known as neurofibrils, which are composed of either microtubules or neurofilaments. Microtubules (diameter 20–30 nm) have been implicated in the movement of chemicals and organelles within the cytoplasm. The movement often occurs from the perikaryon down the axon (axoplasmic flow), but can also occur in the reverse direction (retrograde axoplasmic flow). Microtubular movement can be effectively inhibited by colchicine and the *Vinca* alkaloids. Neurofilaments (diameter 10 nm) may play a role in maintaining the architectonic integrity of the perikaryon and neuronal processes, but their function is clearly controversial.

Other organelles include the rough endoplasmic reticulum, the subsurface cisternae, and the mitochondria. As in other cell types, the rough endoplasmic reticulum is the primary site of protein synthesis. In neurons, this granular organelle studded with ribosomes is also known as the *Nissl substance*, due to its chromophilic nature. It is highly developed in neurons and reflects the active protein synthesis that is constantly occurring in these cells. Few of the proteins synthesized at the Nissl bodies are secreted and the majority of them remain within the cell or are incorporated into the cell membrane. After ribosomal translation, many of the proteins are transported

from the Nissl bodies to the Golgi, where the addition of carbohydrate units can modify the protein after ribosomal translation. In a number of neurons, a system of membrane-bound granules can be found on the inner layer of the cell membrane, but the function of the so-called subsurface cisternae is unknown. Some have speculated that these organelles may aid in the assembly of the cell membrane or be involved in the active uptake of chemicals from the extracellular space. Last, mitochondria can be found throughout the perikaryon, axonal, and dendritic processes and in nerve terminals. They are the centers of oxidative phosphorylation in neurons.

The short, afferent processes that give the neuron its stellate appearance on light microscopic examination are the dendrites. They appear to be the recipients of a great deal of neuronal traffic, and it is generally believed that their primary function is to carry electrical impulses into the perikaryon. However, there is considerable evidence, especially in invertebrates, that they can also communicate to other neurons via dendrodendritic connections. The long efferent processes that emanate from the axon hillock of the perikaryon are axons and are the primary route through which neuronal impulses pass from one cell to another. At the end of the axon is the synaptic nerve terminal, invariably highly enriched in neurotransmitters and that, upon receiving the electrical impulse, releases transmitter into the synaptic cleft. Most nerve cells have only one axon (unipolar), but it is not unheard of to discover bi- or multipolar neurons. Electron microscopic studies indicate that synaptic terminals of axons can abut other axons (axoaxonal), dendrites (axodendritic), or cell bodies (axosomatic).

2.1.2.2. *Neurologia.* The supportive cells of the nervous system are known as neuroglia. Although their function is thought to be primarily architectonic, recent research also suggests that these cells may be involved in the inactivation of certain neurotransmitters, may modulate the responses of certain neurons, and may also act as general scavengers for waste products of neuronal metabolism. Another important function of these cells is to synthesize the myelin sheath that covers the processes of many neurons. Neuroglia are not known to make any synaptic connections and, unlike neurons, can divide throughout the life span of the organism.

Macroglial cells are of ectodermal origin and include the astrocytes, ependymal cells, oligodendrocytes, and Schwann cells. Astrocytes appear to be primarily supportive in function and under microscopic examination can be seen to envelope axons and dendrites and buttress against the adventitial layer of blood vessels. In the gray matter of brain, which is composed primarily of perikarya, they have a clear protoplasmic appearance, whereas in the white matter, which is composed primarily of neuronal processes, they appear fibrous. Ependymal cells line the ventricles of the brain, are ciliated,

and actively secrete the cerebrospinal fluid. Oligodendrocytes elaborate myelin around one or more axons or dendrites in the central nervous system; their counterpart in the peripheral nervous system is the Schwann cell or satellite cells of peripheral ganglia. Microglial cells are of mesodermal origin and enter the central nervous system at the same time as blood vessels. There are usually few microglial cells that can be detected microscopically, except after neuronal injury, when they appear to migrate to the site of inury to phagocytize debris from neuronal damage.

Excellent reviews covering the gross and microscopic anatomy of the peripheral and central nervous systems can be found in Willis and Grossman (1977), Barr (1974), and Raine (1981). Detailed descriptions of the subcellular organization of neurons are discussed by Novikoff and Holtzman (1970) and Peters *et al.* (1970); the biology of neuroglia is reviewed by Windle (1958) and Schoffeniels *et al.* (1977). For those interested in neuroanatomic methodologies, the review by Cowan (1975) provides a good starting point.

2.2. Subcellular Fractionation

Numerous techniques are available to the researcher who wishes to study the chemical behavior of subcellular fractions of neurons or glia. It is obviously beyond the scope of this chapter to detail the various methodologies that may be employed for these purposes. One excellent review of the techniques available has been written by Appel and Day (1976).

Generally, whole brain or distinct brain regions are homogenized or sonicated into a sucrose solution or any number of buffers to disrupt neurons and cells. The choice of buffer and the osmolality of the homogenizing solution is important for the preparation of certain subcellular fractions or for the assay of particular enzymatic reactions or receptor binding studies. For instance, the technique of Gray and Whittaker (1962) is still among the most commonly employed preparative techniques for the study of synaptosomal processes. This technique is emphasized because, although it is by no means the sole method by which brain tissue can be used to prepare synaptosomes (nerve terminals), many investigators have used this standardized technique to study mechanisms that control the release of neurotransmitters at the nerve terminal. Most subcellular fractionation techniques employ the concept of differential centrifugation to prepare various organelles or subcellular fragments. For instance, a brain homogenate can be centrifuged at approximately 1000 *g* for 10 minutes to prepare a pellet containing cell debris and nuclear material. The resulting supernatant fraction can then be centrifuged at 10,000 *g* for 30 minutes to yield a pellet enriched in brain mitochondria, myelin, and synaptosomes. These particles can be purified further by dis-

continuous sucrose density centrifugation. Microsomal fractions are prepared by further centrifugation at 100,000 *g* for 60 minutes; these fractions contain the Golgi apparatus and smooth and rough endoplasmic reticulum. The 100,000 *g* supernatant is generally termed the cytosolic fraction.

Techniques are also available for the separation of various cell types in the central nervous system. Among the most widely used methods to separate neuroglial cells from neurons are the techniques described by Sellinger and Azcurra (1974), but other techniques using acetone–glycerol separation of cells or trypsinization of cell suspensions are also available (see Rose, 1968, for a discussion). Methods for the isolation of myelin, postsynaptic membranes, lysosomes, and the vesicles present within nerve terminals that contain neurotransmitters are also available. Invariably, these preparative methods employ some form of density-gradient centrifugation.

An important aspect of the preparation and isolation of subcellular particles from brain regions is the criteria by which purity is assessed. Electron microscopy of the various subcellular fractions can provide among the best pieces of evidence for the presence in the preparation of the organelles or subcellular fragments of interest. However, a number of biochemical markers (usually enzymes) that have been established to be present in certain fractions can also be assayed to demonstrate the enrichment of the organelle of interest. For instance, acetylcholinesterase is a common marker for synaptosomes; dopamine-β-hydroxylase is a marker for catecholamine storage vesicles within the synaptosome; and cytochrome *c* oxidase is a marker for mitochondria. Most of the enzymatic markers can be assayed routinely.

3. Biochemistry of the Cell Membrane and the Myelin Sheath

3.1. The Cell Membrane

3.1.1. *Structure and Biosynthesis*

As in most other vertebrate cells, the neuronal cell membrane (often referred to as the plasmalemma or axolemma in neurons) is composed almost entirely of proteins and lipids. Estimates of the percentage of the membrane composed of proteins and lipids vary, but approximations indicate that the axolemma is 40% protein, 40% lipid, and 20% water and carbohydrate. Membranous water is associated with the internal portions of the membrane as a rigid hydrogen-bonding structure. Lipids can be further subdivided into cholesterol, phospholipids, and glycolipids. The molecular forces that bind the membrane together are primarily ionic, hydrogen, and van der Waals bonds; few covalent bonds appear to be present.

Most contemporary biochemists agree with the Singer–Nicholson fluid

mosaic model of the membrane, which stresses the lipid bilayer nature of the axolemma in which is embedded a mosaic of asymmetric proteins. In this model, the membrane is thought to be composed of two layers of lipids that expose hydrophilic regions at the surface of the membrane and hydrophobic regions toward the interior of the membrane. The interior of the membrane is thought to be similar to a fluid organic phase. X-Ray diffraction studies and electron microscopy with freeze-fracture experiments support this concept. The fluid mosaic model is also thermodynamically the most sound, maximizing both hydrophobic and hydrophilic interactions.

Membrane-associated proteins can be either *peripheral* or *integral*, their classification being contingent on their ease of solubilization from the membrane with detergents. Integral proteins, by definition, are resistant to treatment by detergents. Most membranous proteins appear to be globular, α-helix proteins that expose their hydrophobic regions to the inner "organic phase" of the membrane and their outer hydrophilic regions to the extra- or intracellular space. Microviscosity studies suggest that certain globular units of proteins can "float" within the lipid matrix of the cell membrane. As the cholesterol : phospholipid ratio of the membrane increases, proteins that have been experimentally tagged with fluorescent probes are displaced and move with a greater speed within the lipid bilayer. Fluorescent photobleaching studies further indicate that there are two classes of membranous proteins, mobile and stationary. The latter class appears to be bound through the membrane to the intracellular network of neurofibrils. Diffusion coefficients for mobile membranous proteins are on the order of 10^{-10} to 10^{-11} cm^2/second; lipids appear to move much more rapidly, with diffusion coefficients on the order of 10^{-8} cm^2/second. Many of the membrane-associated proteins span the lipid bilayer in an asymmetric manner, with complex carbohydrate chains often folding into the extracellular space. Membrane fluidity can be controlled by at least two events: The alteration of fatty acid composition within the plasmalemma and by the methylation of phospholipids such as phosphatidylethanolamine by membrane methyltransferases. Methylation appears to be well correlated with decreases in membrane fluidity. The molecular organization of the plasmalemma is reviewed by Singer (1974), Bretscher and Raff (1975), and Rothman and Lenard (1977).

The question of membrane biosynthesis and its regulation is controversial. It is known that virtually all of the proteins that eventually are incorporated into the cell membrane are synthesized at the rough endoplasmic reticulum. These proteins and the complex lipids destined for the cell membrane are often further modified at the Golgi apparatus, where postribosomal glycosylation of proteins is known to occur and where many complex membrane-associated lipids are processed. An understanding of the mechanisms that

control the translocation of these substances to the plasmalemma is only rudimentary. Even less is known about the processes that control the assembly of lipids and protein to form the cell membrane. A key question asks whether the membranous suprastructure is under strict genetic control or whether components are assembled randomly. One hypothesis has been proposed to explain the mode of membrane biosynthesis. Known as the *sequential transformation* hypothesis, it propounds a physical continuum starting at the nuclear membrane, passing through the rough endoplasmic reticulum, and eventually arriving at the plasmalemma through the Golgi apparatus and the smooth endoplasmic reticulum. The functional and physical continuity among the subcellular organelles suggested by this hypothesis is thought to occur by a subtle sequential transformation of the endoplasmic reticulum to plasmalemma. Some experimental evidence exists for this hypothesis (see Grove *et al.*, 1968, for further details). However, since the axon contains a sparse, if present, endoplasmic reticular network, the above hypothesis does not explain how membrane-associated proteins and lipids are assembled in the axon and the specialized synaptic structures, which contain a complex array of lipids (phospholipids, neutral glycosphingolipids, and gangliosides) and proteins. There is considerable evidence that a large number of axolemmal and synaptic lipids and proteins can be synthesized at the rough endoplasmic reticulum of the neuronal perikaryon, modified (if necessary) postribosomally at the Golgi, and translocated to the axon and synaptic nerve terminal via axoplasmic flow mediated by microtubules. It is clear from protein turnover studies that in most neurons the initial rate of incorporation of radiolabeled amino acids into protein is highest in the rough endoplasmic reticulum of the perikaryon and that the mean rate of incorporation into mitochondrial, axolemmal, and synaptic protein is considerably slower. These findings are consistent with a translocation of proteins from the perikaryon to the varied processes of the neuron. Further information on the assembly of the cell membrane can be found in the review by Lodish and Rothman (1979).

3.1.2. *Impulse Initiation and Propagation in the Neuron*

3.1.2.1. *The Resting Membrane Potential.* The neuronal cell membrane possesses the ability to separate and store bioelectric charges. The electrical force that separates the positive and negative charges that span the neuronal plasmalemma is known as an electrochemical potential. The property of maintaining an electrochemical potential makes the entire neuron an "excitable" cell, that is, one that can maintain an electrochemical gradient at rest and that can rapidly respond to appropriate stimuli by manifesting rapid fluctuations in the electrical charge across the membrane. Among the primary, specialized functions of neurons is the ability to carry information in the

form of electrical charges from the neuronal perikaryon down the length of the axon to the nerve terminal, where the information associated with the electrical charge is converted to chemical energy in the form of neurotransmitter release.

Electrical properties of neurons are measured by a variety of techniques. A review of these methodologies is beyond the purview of this chapter. However, it is important to note that changes in the electrochemical potential, ionic diffusion or current, and membrane conductance or permeability can be determined experimentally by intracellular and extracellular recording techniques that can be performed *in vitro* and *in vivo*. Hubbard *et al.* (1969) describe in detail a variety of intracellular techniques, such as voltage clamping, and extracellular techniques, such as sucrose-gap recording.

It is by virtue of the fact that the neuronal plasmalemma can separate charges and establish electrochemical potentials that neurons are capable of becoming "excited." By *excitability* is meant the property of the neuron to initiate and propagate an electrical impulse that is at disequilibrium with the electrochemical potential associated with the resting neuron. A key question in neurochemistry to which a definitive answer has proved elusive to date is whether the initiation of an electrical impulse in an excitable cell commences with a biochemical event (such as the hydrolysis of ATP) or a physical event (such as a conformational change in a plasmalemmal protein) or whether the two events are inseparable. The process of initiation occurs within milliseconds, and as a result, an accurate separation of biochemical and biophysical processes has been difficult.

The electrochemical potential of a neuron at rest is known as a resting membrane potential. The resting membrane potential is a prerequisite for the excitation of the neuron and for the propagation of the neuronal impulse. The resting membrane potential, which is ordinarily measured in millivolts, is the result of three factors: an interfacial potential, a series of diffusion potentials, and potentials generated by electrogenic active transport systems. Conventionally, it is measured with respect to the inside of the cell membrane and under resting conditions is therefore negative. Extensive details about each of these three potentials can be found in Hubbard *et al.* (1969), Aidley (1971), and Thomas (1972).

The Donnan interfacial potential results from the unequal distribution of electrolytes across a colloidal-type membrane that contains polyvalent charges, such as the fluid mosaic membrane model discussed previously. In biologic membranes, the unequal distribution of ions is caused by the negative surface charge at pH values about 7.0 on that portion of the membrane facing the extracellular fluid space. These negative charges are associated with phosphate and carboxyl groups. As described previously, charged (polar) portions of the membrane align themselves with the hydrophilic environ-

ments of the extra- and intracellular spaces, while the lipophilic portions of the membrane concentrate themselves toward the interior of the membrane. To balance the fixed negative charges on the outside of the membrane, extracellular cations will associate with the plasmalemma. The extent of binding is determined by the net amount of negative charges and by the dissociation constants of the particular cations for the fixed membrane-associated anionic species. On the neuronal plasmalemma, therefore, the Donnan interfacial potential contributes to the total resting membrane potential by allowing for the accumulation of positive charge to the exterior of the cell membrane. In terms of electrical theory, this accumulation of positive charge may be considered to be proportional to the capacitance of the biomembrane.

Diffusion potentials for the primary biological ions (potassium, sodium, and chloride) represent the primary source of the resting membrane potential. The diffusion potential for a given ionic species can be calculated from the modified Nernst equation developed by Hodgkin and Huxley. The equation is ordinarily written as

$$E = \frac{RT}{ZF} \ln \frac{[\text{cation}]_o}{[\text{cation}]_i} \quad \text{or} \quad E = \frac{RT}{ZF} \ln \frac{[\text{anion}]_i}{[\text{anion}]_o},$$

where E is the membrane potential for the given ionic species at equilibrium, R the gas constant at 25°C, T the temperature in Kelvin, Z the valence of the ion, and F the Faraday constant. The subscripts o and i designate extra- and intracellular concentrations, respectively. A diffusion potential is the result of selective permeability of the cell membrane to a particular ion, resulting in the formation of an electrochemical gradient. The energy (or force) required to maintain the gradient across the membrane under the given permeability conditions of the membrane is the diffusion potential. Permeability of the membrane for an ionic species refers to unspecified factors or properties of the membrane that regulate the net diffusion of the ion from one membrane interface to the other. Underlying molecular concepts for the permeability properties of the membrane will be discussed later in this section. For now, permeability may be thought of as a mathematical constant derived from Ohm's law, where the electrochemical force or energy required to maintain a given concentration gradient across a membrane for a given ion is proportional to the membrane's permeability (or conductance) for that ion. Obviously, the greater the permeability of the membrane for a given ion, the greater the contribution of that ion to the resting membrane potential under the conditions of a given chemical gradient.

For instance, at rest the neuronal plasmalemma has a relative per-

meability constant of 1.0 for K^+, 0.04 for Na^+, and 0.45 for Cl^-. As the extra- to intracellular concentration* gradients for K^+, Na^+, and Cl^- are not very different, it can be readily seen that the diffusion potential for K^+ has the greatest contribution of all three ions to the resting potential. Indeed, when the values for the permeability constants and the intra- and extracellular concentrations of K^+, Na^+, and Cl^- are applied to the modified Nernst equation, a resting membrane potential due to diffusion potentials alone is calculated to be -70 mV, a value not dramatically different than the actual resting membrane potentials recorded in most neurons. It must be remembered that one reason that the calculated value does not agree with the experimentally determined value is that the Nernst value does not take into consideration interfacial and electrogenic pump potentials.

Electrogenic active transport systems also contribute to the resting membrane potential. The Na^+–K^+ active transport system is thought to be electrogenic since it leads to the net efflux of three Na^+ ions for the influx of every two K^+ ions. Hence, electronegativity is established within the cell. The contribution of the electrogenic sodium pump to the total resting potential was first discovered in experiments in which extracellular K^+ concentrations were altered. If one were to predict the effect of increasing extracellular K^+ concentrations on the resting potential of the cell membrane from the modified Nernst equation, depolarization (a drop in intracellular electronegativity) would be expected. However, increasing extracellular K^+ also stimulates the sodium–potassium active transport system (discussed later in this article). Stimulation of the transport system results in the hyperpolarization of the cell due to the net extrusion of positive charge (in the form of Na^+) from the cell and counteracts the change in potential that would be predicted from the alteration in chemical gradients alone.

Hence, at rest the neuron is in a state of temporary electrochemical equilibrium in which the resting membrane potential maintains a chemical gradient of ions. The chemical gradient is present due to the diffusion equilibrium potentials for each ion, but also as a result of the interfacial potentials and the electrogenic separation of charges caused by the Na^+–K^+ active transport system. Excitability of neurons depends on disequilibrium.

3.1.2.2. *The Action Potential.* The electrical equilibrium of the resting neuron is rapidly changed upon excitation. Opening and closing of ionic pores appear to be passive processes that do not require the direct input of metabolically derived energy. This important conclusion is based on experi-

* The extracellular and intracellular concentrations for K^+ in mammals are approximately 3 and 155 m*M*, respectively; for Na^+ approximately 145 and 12 m*M*, respectively; and for Cl^- approximately 120 and 4 m*M*, respectively.

ments that alter the ionic environment of the axon. Large axons, such as those derived from the squid, can be easily depleted of cytoplasm that is then replaced by artificial ionic solutions that lack sugars and other organic metabolites. So long as appropriate gradients for ions are maintained, normal action potentials can be recorded; this provides strong evidence that the small molecules of metabolism, such as adenosine triphosphate, are not required for the opening and closing of the numerous selective ion channels involved in the propagation of an impulse.

Action potentials are ordinarily recorded *in situ* or *in vitro*, and the potential is generated by stimulating electrodes that generate a direct current pulse. The actual potential is recorded distal to the stimulating electrodes by a set of recording electrodes. Since the stimulating pulse is increased, the initial signal detected by the recording electrodes is only the passive cable currents (electrical field) initiated by the direct current pulse. Eventually, the intensity of the stimulus is such that a characteristic deflection of the membrane potential is observed; this deflection is known as the action potential and is a unique property of neuronal tissue. Further enhancement of the stimulus pulse does not change the characteristic form of the action potential; in other words, once a given stimulating current is achieved, the action potential is propagated and its characteristic form is not altered by increasing the stimulating current further. This phenomenon is known as the "all-or-nothing" law. The stimulating current that is required to initiate the action potential is known as the threshold stimulus intensity.

As shown in Fig. 2, a characteristic action potential has four clearly defined phases: the rising phase (in which the membrane potential with respect to the intracellular space becomes more positive), the peak, the falling phase (in which the membrane potential returns to its original negativity), and the "underswing" phase (in which the membrane potential is actually more negative than under resting conditions and which eventually returns to normal). The duration of an action potential at a given site on the axon is only a few milliseconds. Its velocity of propagation down an axon depends on the size and type of axon and from which organism it is derived. Even more important, it depends on whether the neuron is myelinated or unmyelinated, a concept that will be discussed shortly.

Simply put, the action potential is caused by a state of disequilibrium between ideal electrical potentials for two ions, sodium and potassium. The equilibrium potentials for Na^+ and K^+ can be thought of as the electrical force required to maintain the given ionic gradients across the cell membrane for each ion. For Na^+, the equilibrium potential is approximately 50 mV (with respect to the inside of the membrane); for K^+, it is approximately -75 mV. (These values apply to the giant squid axon on which the early investigations on action potentials were conducted. Of course, these values

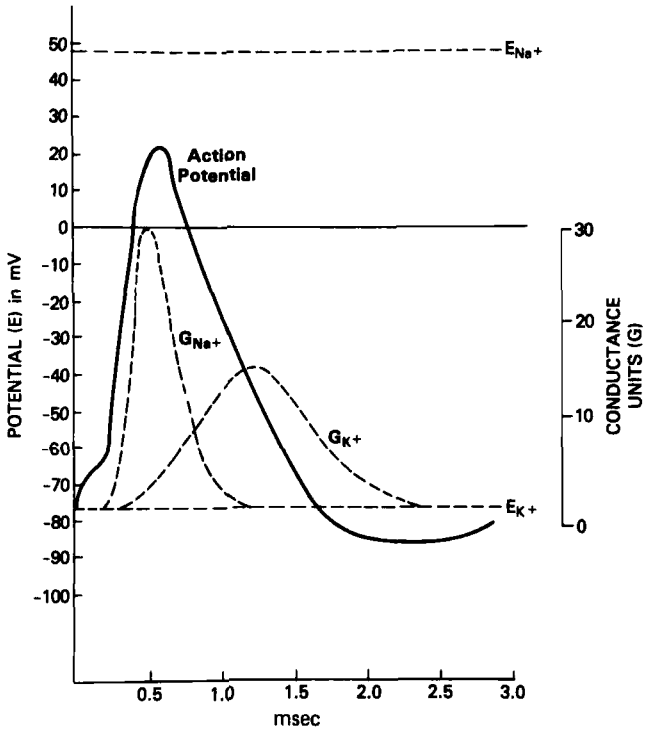


FIG. 2. Diagrammatic representation of oscilloscope tracing of typical action potential. Conductance (G) changes for particular ions are shown as a function of time. Equilibrium potentials for Na^+ and K^+ ($E_{Na}+$ and E_K+) are also depicted.

are different, depending on the ionic gradient and membrane permeability values present in a given species).

Once the threshold stimulus intensity is achieved, an action potential is created at the site of stimulation and then it propagates down the length of the axon. The rising phase occurs as a result of an increased membrane permeability toward Na^+, which causes the membrane potential to move from its resting potential, which is determined primarily by permeability for K^+, toward the equilibrium potential for Na^+. During this period the permeability toward K^+ remains the same as at rest, but exerts less influence on the total membrane potential because of the large increase in permeability to Na^+. However, within a few milliseconds the permeability toward K^+ begins to rise, an event that counteracts the increasing permeability toward Na^+. As a result, the membrane potential begins to return toward resting conditions. The permeability to Na^+ again returns to resting

values (very low relative to values for K^+) and the falling phase occurs. The underswing phase is a result of the excessive permeability to K^+ that has not yet returned to resting values. Accompanied by various anions such as Cl^- to which the membrane is also permeable, Na^+ and K^+ actually cross the membrane during the development of the action potential. However, the small amounts of Na^+ and K^+ that cross the membrane during the action potential are a result of, and not the cause of, the action potential. The action potential is a result of a change in the permeability (or conductance) of the membrane to a given ionic species. In and of itself, the change in permeability, without accompanying ionic flow, is sufficient to cause a change in potential. The Na^+ and K^+ ions cross the membrane, however, because the equilibrium potentials for each ion are never achieved and because the permeability (i.e., physical property of the membrane) to each ion is constantly changing. Accumulations of a given ion within the cell are then corrected by active transport systems, such as the Na^+–K^+ exchange pump, which will be discussed shortly.

Voltage-clamping techniques, which hold the electrical potential across the neuronal membrane constant, while still allowing for the transmural flux of ions, have provided detailed analyses of the rates at which various ions cross the membrane and the kinetics of ion channel activation and inactivation. Specific information about a given anion or cation can be obtained by altering the ionic composition of the bathing medium (Hodgkin and Huxley, 1952; also see Aidley, 1971).

By using voltage-clamping techniques, it was found that virtually all of the electrical current associated with the action potential was derived from Na^+ and K^+ channels. Furthermore, it also became clear that the energy for gating "passive" channels was obtained from the work performed by the electric fields with which the axolemma was stimulated. It was surmized that the electric fields acted upon axolemmal macromolecules of which the ion channel was presumably built. A simplified scheme of the development of an action potential could then be described as follows: Depolarization of the axon could be achieved by introducing current to the cell membrane, which then would immediately respond by rapidly changing the configuration of macromolecules associated with the Na^+ channel. Hence, the permeability to Na^+ would increase, and the Na^+ ions coupled to Cl^- would begin entering the cell at an ever more rapid pace because the new electrochemical equilibrium for Na^+ had not yet been achieved. However, before the new Na^+ electrochemical equilibrium could be achieved, the K^+ channel would also open. That event would counteract the approach to Na^+ equilibrium and the transmembrane potential would again return to resting conditions. Local circuit currents, also known as *cable* or *electrogenic* currents, continue to carry the action potential down the length of the axon.

These so-called cable currents should not be confused with the ionic currents established during an action potential; they are not the result of distinct properties of the membrane. An equation that accurately describes the events that occur during an action potential would have to include both the ionic currents across the cell membrane with the cable currents, which result from a passive spread of electricity along the intra- and extracellular media. The membrane in this case acts as a resistor.

All neuronal cells appear to behave as described. They maintain a low conductance to K^+ with little permeability to Na^+ under resting conditions. However, upon depolarization, the membrane Na^+ channels open up and a transient high conductance to Na^+ is seen. The high Na^+ conductance is partially reversed by a dramatic increase in the membrane's conductance to K^+ and then all conductances return to basal levels. The characteristic shape of the action potential is the result of the fact that the membrane cannot achieve appropriate new conductances to balance the sodium electrochemical gradient and the inward flux of Na^+ within the time frame required. Therefore, the action potential can be thought of as a transition state between a membrane potential based primarily on the K^+ electrochemical gradient to a membrane potential based primarily on the Na^+ electrochemical gradient. At no time is the absolute equilibrium potential ever achieved, and the potential at any given second is the result of the sum of the various ion conductances and the direction of their chemical gradient.

Conduction velocity of an action potential can be increased dramatically by the presence of myelin, a fatty sheath that surrounds the axon and that is interrupted into gaps every millimeter or so at the nodes of Ranvier. Myelin is elaborated by Schwann cells in the peripheral nervous system and by oligodendrocytes in the central nervous system (the biochemistry of myelin will be discussed later in the article). The presence of myelin will dramatically alter the mode and velocity of conduction of the action potential in the axon. As in unmyelinated nerves, the action potential is still transmitted from one section of the axon to another by the presence of local circuit currents. However, the fatty sheath of myelin has poor conduction properties and therefore acts as an insulator. Hence, the local circuit currents "jump" from one gap to another at the nodes of Ranvier and the rate of conduction is enhanced as local circuit currents travel faster than the action potential itself. This process of discontinuous conduction is known as saltatory conduction. Numerous diseases involving myelin deficiency have been described clinically. As one might predict, demyelinating diseases have profound effects on neuronal conduction and on the well-being of the patient. A few of these conditions will be described briefly in the upcoming section on myelin biochemistry.

Molecular models for impulse initiation and propagation have been proposed by many investigators, but in general, no unifying theory of the molecular basis of impulse propagation has emerged. It is almost universally accepted that the ionic channels or pores that must underlie membrane permeability are macromolecules, most probably single proteins or protein complexes that form aqueous pores within the lipid bilayer of the membrane to allow passage of a given ionic species. A general model of such a channel can be constructed from the large number of diverse experiments that have been reported in the literature over the past 20-odd years (see Armstrong, 1975, and Ulbricht, 1977, for detailed reviews). The outer portion of the channel, which faces the extracellular environment, most probably has a selectivity filter of unknown composition that can recognize specific cations or anions. Within the channel itself some form of sensor factor must be present that recognizes the electric field surrounding the ionic pore and that can rapidly induce a conformational change in the macromolecule, including that portion that is responsible for the "gating" of ions.

Such concepts have been based largely on studies employing pharmacological agents to describe the structure of ionic channels indirectly. A number of agents that interact with various portions of the Na^+ channel have been identified. Tetrodotoxin, isolated from the puffer fish, and saxitoxin, isolated from the paralytic shellfish, apparently both bind to the outer selectivity filter of the ionic channel with dissociation constants on the order of 1×10^{-9} *M*. Radiolabeled tetrodotoxin has been used in the biochemical isolation of the Na^+ channel from the cell membrane and has also been employed in experiments designed to estimate the number of Na^+ channels on the axolemma. Local anesthetics, such as lidocaine and procaine, also block the Na^+ channel, apparently by binding to the central portion of the ionic pore. The hydrophobic portion of the local anesthetic molecule is thought to bind to portions of the macromolecular structure that are involved in gating mechanisms. Batrachotoxin, a lipophilic steroid, on the other hand, is thought to allow the Na^+ channel to remain open for prolonged periods of time by somehow interacting with the macromolecular structure of the pore from a position within the lipid bilayer. Compounds that are known to interact specifically with the K^+ channel are rarer; probably the most widely used are tetraethylammonium salts. Verapamil and D-600 are two compounds known to block the Ca^{2+} channel, although the mechanism by which they exert their activity is unknown. Numerous divalent cations, such as Mn^{2+}, Cd^{2+}, Co^{2+}, La^{3+}, and Ni^{2+} can effectively compete with Ca^{2+} for binding to extracellular sites (such as at sialic acid residues connected to glycoproteins and gangliosides) and for passage through passive Ca^{2+} channels and active Ca^{2+} transport systems.

The Hodgkin–Huxley description of neuronal impulse propagation does not attempt to address the underlying molecular mechanisms that alter ionic permeability and hence the membrane potential. Since the purification of ionic channels has been relatively slow to date, it is safe to conclude that at this time the molecular and biochemical basis of impulse propagation is unknown. However, a few points may be made with a reasonable degree of certainty. It appears from the pharmacological studies described in the previous paragraph that more than one ionic channel (or permeability factor, to be more precise) is involved in the transmural flux of ions. From turnover experiments for Na^+ and K^+ flux across the membrane, most investigators in the field feel confident that ionic channels that allow for the passive flux of ions are most likely responsible for Na^+ and K^+ movements. Turnover values for these ions are orders of magnitude higher than those for any known active ion transport system. Furthermore, membrane potentials can be more easily accounted for by calculations that assume a passive flow of ions. Certain antibiotics, such as monazomycin, gramicidin A, and alamethicin, which form "pores" within bio- or artificial membranes spontaneously, also provide a useful model for studying structure–function relationships in passive diffusion pore systems. Results obtained by using these antibiotics lend further support to the concept of passive ionic channels regulating the permeability of the membrane to Na^+ and K^+. The use of proteolytic enzymes such as pronase and certain phospholipases has resulted in a permanent opening of Na^+ channels, presumably by cleaving off portions of proteins and phospholipids within the cell that are necessary for the closing of the Na^+ gating system. Traditional kinetic studies with voltage clamping have helped define the different rates of the various steps involved in the opening and closing of the ionic channels; however, the information provided by these experiments is primarily mathematical and leaves open questions revolving around the biochemical nature of these events. Last, two new electrophysiological methods have been developed that allow for ultrasensitive recording of electrical events in the membrane. One is known as the "patch clamp" technique, which permits unit recording of ionic currents from a single ionic channel as it opens and closes within a small portion of the membrane (Conti and Neher, 1980). Another useful technique is known as the "gating current" technique, which allows for the measurement of movements caused by charged components of the macromolecular structure of the channel after the inhibition of all ionic current across the membrane (Armstrong, 1975). A great leap in our knowledge concerning impulse propagation awaits the purification of the numerous ionic channels in functional states and reconstitution of these pores in artificial micellar membranes for an accurate correlation of structural and functional properties

(Cahalan, 1980). Further details on impulse propagation in neurons can be found in the excellent books by Hodgkin (1964) and Junge (1981).

3.1.3. *Active Ion Transport Systems*

In order to maintain the necessary concentration of nutrients, metabolites, and ions within a cell so that normal metabolic activity can continue, it is often necessary for transport systems to participate in the transmural flux of the solute in question. Although this section will deal primarily with transport systems that are located in the cell membrane, it is important to remember that many of these transport systems exist in the membrane of cellular organelles such as the mitochondria and the endoplasmic reticulum and that they often appear to be structurally and functionally similar to the transport systems on the cell membrane.

Often the rate of diffusion of a solute (i.e., an ion or a metabolite) can be totally accounted for by the electrochemical gradient for the solute. This simple diffusion is termed *passive diffusion*, since the forces that act upon solutes to cause them to cross a membrane are solely electrical and chemical. Obviously, an electrical gradient is only important for charged solutes such as ions or organic molecules that maintain a charge at the extra- and intracellular pH. Passive diffusion does not require metabolic energy derived from cellular processes. The rate of passive diffusion is described by Fick's law: $dn/dt = -PA(C_1 - C_2)$, where n is the number of molecules, t the unit time, P a constant derived from the diffusion coefficient and membrane thickness, A the area traversed, and $C_1 - C_2$ the concentration difference of solute in compartments 1 and 2 (i.e., extra- and intracellular compartments). In the case of biological membranes, the solute can either traverse the membrane through pores (such as the Na^+ or K^+ channels described previously) or diffuse through the lipid bilayer itself (such as uncharged species of an organic molecule). In the case of an acidic or basic organic compound, a certain percentage of the molecule will remain uncharged at physiological pH. Depending on the lipid–water partition coefficient of the solute, its rate of flux through the lipid bilayer can be predicted. When diffusion of a solute cannot be accounted for solely on the basis of Fick's law, a transport system most probably will account for the additional movement of solute molecules.

There are two primary transport systems in neurons: facilitated and active. Facilitated transport systems do not require metabolic energy from the cell. These systems are believed to be carrier mediated, since the presence of carriers can account for the kinetic findings observed in facilitated transport systems, such as saturability, competition by other substrates, and specificity toward certain families of molecules. Facilitated transport systems can

include the movement of only one solute molecule by the carrier per transport event (such as with glucose or glycerol) or the exchange of one solute as it moves into the cell with another solute as in exits the cell (such as the Na^+-Ca^{2+} exchange system). It is not understood at this time whether the carrier actually moves from one end of the membrane to the other or whether it simply rotates or changes its conformation while remaining stationary in the membrane mosaic.

Active transport systems require metabolic energy derived from the cell for their activity. They too are saturable, are specific toward given solutes, and can often be inhibited by competitive solutes. They are carrier mediated. One example of an important active transport system is the Na^+-K^+ exchange pump, which transports Na^+ out of the neuron in exchange for K^+ into the neuron. The pump requires the presence of Mg^{2+} and adenosine triphosphate (see later discussion), the source of metabolic energy. The Na^+-K^+ pump is especially active in the axolemma immediately after the action potential, and its presence is essential for the maintenance of both the Na^+ and K^+ concentration gradients across the axolemma. Indeed, during the action potential, a small amount of Na^+ leaks into, and a small amount of K^+ leaks out of, the neuronal cytoplasm. Although relatively insignificant after one or two action potentials, an enormous alteration of the intracellular Na^+ and K^+ concentrations would ensue if not constantly corrected by the Na^+-K^+ pump. The change in intracellular concentrations of these two cations alone would grossly affect electrical and enzymatic activity of the neuron, as well as cytoplasmic volume (due to the change in ionic and osmotic content).

For the purposes of this article, the most important ion transport systems are those involving Na^+, K^+, and Ca^{2+}. Transport systems for neurotransmitters will be discussed subsequently. Most probably Cl^- is distributed across the axolemma passively, and because the concentration of Mg^{2+} appears to be very similar in both extra- and intracellular compartments, the active transport of this cation has not received close scrutiny. Hence, the Na^+-K^+ exchange system and systems involved in the transport of Ca^{2+} will receive primary attention. Excellent reviews on the transport of Na^+ and K^+ will be found in Skou and Norby (1979); an excellent source on calcium transport and its intracellular roles can be found in Scarpa and Carafoli (1978).

3.1.3.1. *The* Na^+-K^+ *ATPase Pump.* The Na^+-K^+ adenosine triphosphatase (ATPase) transport system is the primary active transport process that maintains the normal chemical gradients for Na^+ and K^+ in cells of both vertebrates and invertebrates. Although the concern of this article is with neuronal tissue, it should be stressed that the Na^+-K^+ ATPase pump

is located in a number of tissues, such as all electrically excitable cells (skeletal, cardiac, and smooth muscle; neurons; and electric organ of the eel) and in cells that need to maintain a specific electrochemical gradient for Na^+ and K^+ (such as neuroglia, erythrocytes, the choroid plexus, and kidney parenchymal cells).

In subcellular distribution studies, Na^+-K^+ ATPase has consistently been shown to be present almost exclusively in particulate fractions. The 100,000 *g* supernatant fraction has very low Na^+-K^+ ATPase activity. When particulate fractions from brain are further purified, Na^+-K^+ ATPase activity appears to be associated with the microsomal fraction and with enriched synaptosomal fractions derived from crude mitochondrial fractions subjected to discontinuous sucrose density centrifugation. Enriched mitochondria and synaptic vesicles are virtually devoid of Na^+-K^+ ATPase activity, but the synaptic plasmalemma is enriched in enzyme activity. These findings, confirmed by many laboratories, are consistent with the role of Na^+-K^+ ATPase in Na^+ and K^+ transport across the cell membrane.

Although investigators have been successful in localizing Na^+-K^+ ATPase to discrete regions of tissues such as the kidney (i.e., in the thick ascending loop), similar cellular distribution studies in the more complex central nervous system are still forthcoming. The relative amounts of Na^+-K^+ ATPase in neuroglia and neurons are still unsolved and reports vary among laboratories. Popular techniques for histochemical visualization of Na^+-K^+ ATPase include [^{3}H]ouabain binding to enzyme and immunocytochemical methods involving binding of mono- or polyclonal antibodies raised against the purified enzyme.

Under ordinary circumstances in mammalian brain, the extracellular Na^+ and K^+ concentrations are 145 and 3 m*M*, respectively; the intracellular concentrations 12 and 155 m*M*. However, because the resting membrane potential is a composite of a variety of factors (see earlier section), neither the Na^+ nor the K^+ gradient is at equilibrium with the electrical potential spanning the membrane. Hence, even at rest there is a small inward drift of Na^+ and a small outward drift of K^+. The Na^+-K^+ ATPase pump therefore maintains this gradient by utilizing energy derived from oxidative phosphorylation of the neuron.

The energy requirements of the neuron needed to maintain the Na^+ and K^+ gradients are pronounced. The maximal rate of ATP hydrolysis under conditions of maximal cation exchange has been estimated to be between 15 and 30% of the total hydrolysis of ATP within the neuron. In comparison, the combination of protein and lipid turnover and biosynthesis of neurotransmitters appears to require only 10% of the total ATP hydrolysis of the nerve. (The remaining hydrolysis occurs during active transport of other substances and for a variety of enzymatic reactions.)

There are four primary factors that determine the amount of energy (i.e., ATP hydrolysis) required in a neuron to extrude Na^+ and take up K^+. One factor is the degree of electrical activity within the neuron at a given time; it is estimated that the activity of the pump increases approximately 20-fold during the appearance of an action potential. Furthermore, the frequency at which action potentials appear also affects the pump activity. Whether a neuron is myelinated or not represents another critical factor in ATP hydrolysis, since Na^+-K^+ ATPase appears to be present primarily at the portion of the axolemma that corresponds to the node of Ranvier. Hence, it is ordinarily thought that myelinated neurons expend less energy than unmyelinated neurons on Na^+-K^+ exchange. Last, the radius of the neuron itself seems to be crucial in determining ATP hydrolysis. Apparently, an impulse generates a greater functional decrease in the Na^+ concentration gradient in smaller axons, a process that ultimately requires a greater expenditure of energy than in larger axons to reestablish the concentration gradient. The energy of ATP hydrolysis (12 kcal/mol ATP) is transduced to pump activity (4 kcal/mol cation). Hence, each mole of ATP yields sufficient energy to lead to the exchange of 3 mol of cations, as calculated from the Gibbs free energy equations. The molar ratio of ATP hydrolysis to K^+ and Na^+ transfer appears to be 1 : 2 : 3.

There are a number of properties of the Na^+-K^+ pump system that allow investigators to couple the pump to the ATPase reaction. Both the Na^+-K^+ exchange and the ATPase activity are membrane bound. Adenosine triphosphate and Mg^{2+} are required for Na^+-K^+ exchange. The rate of ATP hydrolysis is proportional to the rate of cation flux and is also proportional to the amount of Na^+ and K^+ present in the surrounding fluids. Both the membranous pump and the purified ATPase show high affinities for Na^+ and K^+ concentrations present in both intra- and extracellular studies. However, under physiological conditions the rate-limiting cation is invariably intracellular Na^+. In erythrocyte ghosts, the ATPase has a high affinity for Na^+ (K_D = 0.2 m*M*) and a low affinity for K^+ (K_D = 10 m*M*) intracellularly; extracellulary, the affinities are reversed with a K_D of 30 m*M* for Na^+ and a K_D of 0.5 m*M* for K^+. On either side of the membrane, the ATPase binds 3 mol Na^+/mol and 2 mol K^+/mol. Last, the cardiac glycosides such as ouabain and digitonin are sensitive and specific inhibitors of both Na^+-K^+ exchange and ATP hydrolysis. No other specific transport system or enzyme is known to be inhibited by cardiac glycosides at this time.

A peculiarity of the Na^+-K^+ ATPase transport system is the observation that multiple operational modes for the pump exist *in vitro*. Whether the following observations have any bearing on physiological situations is unknown, and yet from a biochemical view they demonstrate the flexibility of the enzyme transport system and the key fact that it is the concentration of

cations, ATP, and its metabolites that dictate how the pump will work. Five basic operational modes of Na^+-K^+ ATPase have been demonstrated: (1) normal Na^+-K^+ exchange in which Na^+ leaves and K^+ enters the cell in the presence of intracellular ATP and Mg^{2+}; (2) reversed Na^+-K^+ exchange in which Na^+ moves into, and K^+ moves out of, the cell when extracellular Na^+ is above normal, intracellular K^+ is above normal, and the intracellular ADP:ATP ratio is higher than normal; (3) uncoupled Na^+ extrusion in which both Na^+ and K^+ are absent from the extracellular space and intracellular concentrations of K^+ and Na^+ are normal (three Na^+ are extruded per ATP hydrolyzed); (4) Na^+-Na^+ exchange in which the extracellular compartment is devoid of K^+ and extracellular Na^+ exchanges with intracellular Na^+ without any net change in ion concentrations (ATP is not hydrolyzed in this exchange, but is required along with ADP and Mg^{2+}); and (5) K^+-K^+ exchange in which Na^+ is depleted from the extracellular space and extracellular K^+ exchanges with intracellular K^+ in the presence of ATP and inorganic orthophosphate (again ATP is not hydrolyzed). It is clear from the variety of exchanges that are possible that the pump does not always require metabolic energy for its activity. The reason ATP is not hydrolyzed in all these reactions is unknown.

The basic enzymatic ATPase reaction has been studied with fragmented membranes or solubilized, purified enzyme. Such systems are different than those described earlier because the enzyme is exposed to all components of the reaction simultaneously: Na^+, K^+, ATP, and Mg^{2+}. Careful studies suggest that Na^+ binds to ATPase prior to phosphorylation of the enzyme, a process that is a prerequisite for K^+ binding. Subsequent to the latter event, orthophosphate uncouples from ATPase and then Na^+ and K^+ are unbound in that order. There appear to be both Mg^{2+} and Mg^{2+}–ATP binding sites on the enzyme, each having low- and high-affinity sites. It is speculated that the low-affinity sites are regulatory, rather than mandatory, for ATP hydrolysis; ATPase is thought to have four separate conformations: one in which Na^+ is bound to the dephosphorylated enzyme; a second in which Na^+ is bound to a phosphorylated form of the enzyme in the absence of Mg^{2+}; a third in which Na^+ is bound to a phosphorylated form of the enzyme in the presence of Mg^{2+}; and a final form in which both Na^+ and K^+ are bound to the dephosphorylated enzyme. These conformational states have been supported by many kinetic experiments with the purified enzyme.

Two inhibitors of the exchange mechanism and ATP hydrolysis are ouabain and vanadate. Ouabain binds only to that portion of ATPase that would be exposed to the extracellular surface *in situ*. It appears to stimulate the incorporation of orthophosphate into the same site that is phosphorylated by ATP under normal conditions, but ouabain inhibits all pump and hydrolytic

activity of ATPase. Studies with ouabain have demonstrated that phosphorylation only occurs on the intracellular portion of the enzyme. [^{3}H]Ouabain has been used as a marker to localize the enzyme and in 1979 the first report of an endogenous ouabainlike substance in brain was reported in the literature. Another inhibitor of ATPase is vanadate, a congener of phosphate that crosses the cell membrane and inhibits activity and exchange from the intracellular surface, a process that can be potentiated dramatically in the presence of extracellular K^+.

Na^+–K^+ ATPase has been purified from dog and sheep kidney, eel electric organ, and the shark rectal gland. The brain enzyme has only been partially purified. Because Na^+–K^+ ATPase is an integral membrane protein, satisfactory purification schemes have been few. Techniques often begin with treating particulate fractions high in activity with sodium iodide to remove membranous impurities followed by detergent solubilization of the membrane-bound enzyme. Subsequent steps involve routine column chromatography and gradient centrifugation. Purified enzyme preparations are usually at least 90% pure. The enzyme appears to be composed of two subunits, one approximately 85,000–120,000 Da, the other, a glycoprotein, approximately 40,000–60,000 Da.

Work with the purified enzyme has led to the development of polyclonal rabbit antibodies to the holoenzyme and its subunits. It has been found that Na^+–K^+ ATPase has multiple antigenic sites, and antibodies will often inhibit only one portion of the complex flip–flop reaction mechanism. For instance, certain antibodies raised against the holoenzyme might inhibit Na^+-stimulated phosphorylation but have no effect on K^+-stimulated dephosphorylation of the enzyme. Studies have also indicated that a good deal of cross-organ and cross-species immunologic heterology exists, suggesting, at the very least, the presence of isozymes of Na^+–K^+ ATPase in one species.

3.1.3.2. *Calcium Transport Systems.* Because intracellular Ca^{2+} has an enormous range of effects in both excitable and quiescent cells, transport systems that affect the level of cytoplasmic Ca^{2+} have been receiving increasing attention. Intracellular Ca^{2+} concentrations are on the order of 1×10^{-7} to 1×10^{-8} *M*; if the Donnan equilibrium were solely responsible for the gradient of intra- to extracellular Ca^{2+} concentrations, the level within the cell would be many orders of magnitude higher. Hence, the presence of transport systems is very important for the maintenance of the observed cytoplasmic Ca^{2+} concentrations.

Free cytoplasmic Ca^{2+} concentrations are controlled at three levels: (1) the plasmalemma, (2) the mitochondrial membranes, and (3) the endoplasmic reticular membrane. The flux of Ca^{2+} across each of these mem-

branes is either the result of passive diffusion, facilitated transport, active transport, or a combination of these processes. A good review of each type of flux can be found in Bronner and Peterlik (1981).

Passive diffusion accounts for the vast majority of Ca^{2+} flux across the cell membrane into the cytoplasmic space. However, there are two primary pumps located on the plasmalemma that appear to extrude Ca^{2+} from the cytoplasm into the extracellular space. The first appears to require energy in the form of ATP. Both the ATP-coupled pumping of Ca^{2+} and the Ca^{2+}-dependent ATPase activity are stimulated several fold by the presence of calmodulin, an important Ca^{2+}-binding protein, which will be discussed later in this article. The Ca^{2+}–ATPase pump appears to be present in many tissues, including brain, but has not yet been purified. It is presumed that *in vivo* the pump activity is regulated by the influx of Ca^{2+} and subsequent binding of Ca^{2+} to calmodulin during the action potential. As pump activity is increased, free cytoplasmic Ca^{2+} is returned to below the micromolar levels that occur during neuronal excitation. The second pump in the plasmalemma is not known to require metabolic energy and, unlike the Ca^{2+}–ATPase pump, requires the presence of extracellular Na^+. It is presumed that the coupled exchange of Na^+ for Ca^{2+} is a form of facilitated transport, but little is known about the molecular properties of this exchange system, which has not been isolated. The facilitated process can be inhibited by replacement of Na^+ in the extracellular space with choline or lithium. It should be stressed that at this time the overall importance of these two Ca^{2+} extrusion pumps in the plasmalemma for the regulation of free intracellular Ca^{2+} is only speculative.

Mitochondria are probably involved with the regulation of free Ca^{2+} levels in the cytoplasm by affecting both uptake and release. Calcium ion uptake from the cytoplasm appears to require Mg^{2+}–ATP or the oxidation of a respiratory substrate such as succinate. The actual driving force for the transfer may be the proton gradient that exists across the inner mitochondrial membrane. The K_m for Ca^{2+} uptake is between 20 and 40 μM. Nevertheless, Ca^{2+} uptake into the mitochondria is energy dependent and can be blocked by Ruthenium Red. Calcium ion release into the cytoplasm is insensitive to Ruthenium Red and is presumed to involve different processes. Evidence for passive diffusion has been reported. Of interest is a Na^+–Ca^{2+} exchange system that may not be unlike the one present in the plasmalemma. Increases in cytoplasmic Na^+ have been coupled to an increased release of Ca^{2+} from mitochondria. Half-maximal release has been shown to occur at 10 mM Na^+, a concentration that presumably can occur *in vivo* during neuronal excitation. However, such Na^+–Ca^{2+}-coupled exchange in mitochondria appears to be tissue specific, and a large number of tissues do not display Na^+ modulation of mitochondrial Ca^{2+} release.

Whether such a system occurs in neurons is not clear. Nevertheless, the presence of a Na^+–Ca^{2+} exchange system in the mitochondria of certain tissues such as cardiac and skeletal muscle (but interestingly not in smooth muscle) has led certain investigators to postulate the partial regulation of free cytoplasmic Ca^{2+} concentrations by the Ca^{2+} uptake and release mechanisms of the mitochondria.

An even more sensitive system for Ca^{2+} uptake from cytoplasm exists in microsomal preparations that are enriched in endoplasmic reticulum. The K_m for Ca^{2+} uptake in microsomes has been reported to be approximately 1 μM, which makes the uptake system 20 to 40 times more sensitive than the mitochondrial systems. Initial studies indicate that the endoplasmic reticular uptake systems require the hydrolysis of ATP. To date, these transport systems have not been purified and little is known about their physiological role in Ca^{2+} regulation.

It should be clear that in comparison to the present knowledge on Na^+–K^+ ATPase, the current understanding of Ca^{2+} transport is sparse. The relative importance of plasmalemmal, mitochondrial, and reticular systems in the overall regulation and control of free cytoplasmic Ca^{2+} remains to be elucidated and is obviously a fruitful area of research for the future.

3.2. Myelin

Myelin is a heterogeneous substance with a high lipid-to-protein ratio that is elaborated by the Schwann cells of the peripheral nervous system and the oligodendrocytes of the central nervous system. In the brain, it is useful to separate regions morphologically into white matter, composed of myelinated axons, glial cells, and capillaries, and gray matter, composed of the neuronal perikaryon, its dendritic processes, and various amounts of the components found in white matter. The white matter drives its characteristic appearance from the high lipid content in myelin, which is a modified plasmalemma that wraps itself in a sheath around the axon. Fifty percent of white matter is myelin.

Techniques for isolating myelin from the peripheral and central nervous systems exist and usually include homogenization of tissue in isotonic sucrose solutions followed by discontinuous density centrifugation. Because of its high lipid content, myelin has an intrinsically low specific gravity and forms large vesicles in aqueous solutions. The large vesicular size and low density are the two primary properties of myelin that are utilized for its isolation.

It has been difficult to establish criteria for the purity of myelin because only recently have workers developed an *a posteriori* description of essential constituents of the substance. There are a few biochemical markers that are

thought to be intrinsic (and unique) to myelin, many markers that are believed to be absent in myelin, and a large number of markers that are distributed both in myelin and the neuronal axolemma. One obvious criterion for myelin purity is its ultrastructural appearance under electron microscopy, which will be discussed shortly. Another is the appearance of galactosylceramide (cerebroside), 2′, 3′-cyclic nucleotide 3′-phosphohydrolase, the pH 7.2 cholesterol ester hydrolase, the 24,000-Da proteolipid and myelin basic protein, all of which will be described when the biochemical composition of myelin is discussed. A third criterion for determining the purity of myelin is to assay for contamination by markers for mitochondria, microsomes, the plasmalemma, and cytosol. Often highly purified myelin can trap axonal and synaptic components within its large vesicles, but osmotic shock of the myelin vesicles in distilled water will usually separate myelin membranes from the smaller neuronal contaminants. A vast majority of the myelin components (i.e., lipids and many proteins) can then be extracted in chloroform–methanol (2 : 1).

Myelin is approximately 75% lipid and 25% protein. Carbohydrate residues are associated with both the lipid and the protein components of myelin. High proportions of cholesterol, phospholipid, and glycolipid are found in the lipid fractions. Phospholipids include ethanolamine phosphatides, phosphatidylserine, and phosphatidylinositol; glycolipids include both neutral (cerebroside, sulfatide, galactosyldiglyceride) and polar (gangliosides, especially GM_1 and GM_4) lipids. A classification and discussion of the metabolism of brain lipids is beyond the scope of this article; readers are referred to Lajtha (1969), Davison (1968), Awasthi and Srivastava (1980), and Suzuki (1981).

The proteins present in brain myelin can conveniently be organized according to their molecular weight as determined by the reducing, denaturing conditions of polyacrylamide gel electrophoresis. These proteins appear to be either specific for myelin (such as 110,000-Da proteolipid protein, DM-20, and the basic proteins) or nonspecific (a vast array of enzymatic activities). The highest-molecular-weight protein so far characterized that appears to be specific for myelin is a 110,000-Da glycoprotein. During ontogenic development, its molecular weight decreases, but its function remains unknown. The Wolfgram proteins (50,000–60,000 Da) make up about 15% of the total myelin protein. The next major protein appears to be 2′-3′-cyclic nucleotide 3′phosphohydrolase (subunit of 44,000 Da), and myelin contains over 60% of the total brain activity of this enzyme, which appears to be involved in the formation of myelin. The 24,000-Da proteolipid protein, which is very hydrophobic (60% nonpolar amino acids), has attracted considerable attention to date primarily because of its unique physicochemical properties. Basic protein, DM-20, and prelarge and presmall basic proteins

have all been studied as unique components of myelin, but as with almost all of the myelin proteins, their role in myelin metabolism is unclear.

Myelin proteins in the peripheral nervous system are different than those found in brain. They include P_0 (about 50% total peripheral myelin protein), protein Y and protein X, all glycoproteins, and two basic proteins, P_1 and P_2. Protein P_2 appears to be the antigen in experimental allergic neuritis, the peripheral counterpart of experimental allergic encephalomyelitis, a condition in which central myelin basic protein is thought to be an antigen. See Morell *et al.* (1981), Martenson (1980), and Agrawal and Hartman (1980) for further details on myelin basic protein and its presumed physiologic and pathologic roles in myelin function.

Myelination of nerves appears to correlate well with the appearance of neuronal function and proceeds in the peripheral nervous system first, followed by the spinal cord, and finally the brain. Within the brain itself, myelination of axons occurs at different rates, and the intracortical neurons of the cerebrum appear to acquire a myelin sheath last. However, certain neurons in the periphery and in the brain never appear to develop a myelin coating. All Schwann cells or oligodendrocytes are capable of synthesizing the constituents that can develop into a myelin sheath; however, it appears that there are distinct chemical signals, heretofore undefined, that are communicated to the Schwann cell or oligodendrocyte and determine whether the axon will become myelinated. The Schwann cell is responsible for the myelination of one segment of one peripheral nerve; the oligodendrocyte, on the other hand, has been reported to be responsible for the myelination of single segments of up to 42 axons.

The structure of myelin has been elucidated from studies utilizing three separate, physical techniques: polarization of light, X-ray diffraction, and electron microscopy. The earliest ultrastructural studies on myelin employed the birefringence of polarized light. These studies strongly suggested that myelin was composed of contiguous layers, with lipids oriented radially to the axonal axis and the protein oriented tangentially to the axon. These conclusions were confirmed by experiments employing X-ray diffraction, which demonstrated a bilayer of lipids cushioned by proteins. Each bilayer of lipid and protein was calculated to be approximately 80–90 Å thick, so that two fused unit bilayer membranes were thought to be 160–180 Å thick. In contrast, the most recent electron microscopic studies have indicated a unit bilayer of 120 Å. The discrepancy is presumably due to the considerable shrinking that occurs upon preparation of the tissue for microscopy. Magnification of the myelin sheath at ×350,000 clearly reveals stained lines thought to be composed of proteins, followed by clear areas thought to be filled with fluid hydrophilic lipids; between each densely stained protein line are two regions, which are fused layers of proteins. The distance from the

center of one large protein band to the center of the next large protein band (i.e., protein–lipid–protein–lipid–protein) represents a unit bilayer. Certain axons may be covered by as many as 100-unit bilayers.

As explained earlier in this chapter, both Schwann cells and oligodendrocytes elaborate sufficient myelin to cover a portion of the axon. Each segment of the myelin sheath is interrupted at the nodes of Ranvier. These periodic interruptions may appear every 1 mm or so down the length of the axon. The node itself may only be a few micrometers wide. The local cable circuits may be thought to "jump" from one node to another (hence, the term "saltatory" conduction), which greatly enhances conduction velocity. It should be emphasized that the local cable currents that precede the action potential move discontinuously with time, whereas the action potential itself moves at a constant velocity. The primary function of myelin, then, is, by virtue of its high resistance and low capacitance, to increase conduction velocity of large neurons. Second, some investigators feel that myelin also prevents neuronal "cross-talk," or ephaptic conduction. An important corollary to these functions of myelin is that its presence allows for less utilization of energy because only the nodes of Ranvier are excited and reequilibration of ionic gradients is only required at discrete locations. Furthermore, the presence of myelin allows for rapid conduction without the necessity of extremely large neurons.

The importance of myelin to the proper functioning of the nervous system raises the question of biologic control of myelin turnover. Myelin synthesis is most rapid during that period that corresponds to rapid cellular proliferation in the brain. This period is the earliest stage of myelination in animals or man. The mammalian adult demonstrates slow turnover (e.g., slow rates of synthesis and degradation) of myelin once steady-state levels of the myelin sheath have been established. It has been calculated that during the periods of rapid myelination in animals (days 10–20 in the rat, for instance), each oligodendrocyte that is actively involved in the synthesis of the components of myelin is making about three times its own weight in myelin. The overall rate of synthesis of myelin is dependent on three factors: (1) the rate of synthesis of each lipid and protein component, (2) the rate of degradation of each component, and (3) the rate at which each component is assembled into the myelin membrane.

Estimates of the rate of myelin synthesis can be obtained *in vitro* from the measurement of cerebroside synthesis, which increases fourfold from day 10 to day 20 in the rat and then slowly decreases along with the overall rate of myelin synthesis. *In vivo* studies employing radiolabeled precursors of specific myelin components, such as sulfate into sulfatide, yield results similar to those obtained *in vitro*. *In situ* studies with tissue slices confirm *in vitro* and *in vivo* experiments. These studies also indicate that the appearance of

myelin occurs first in the periphery, then in the spinal cord and brainstem, and finally in higher structures.

The sequence by which myelin components are assembled in the myelin sheath is presently a subject of active research. Such studies will not only clarify key kinetic steps in myelin assembly, but also might offer interesting insights into the synthesis and assembly of biomembranes. Certain proteins appear to be synthesized at, or close to, the site of myelin assembly (i.e., basic protein and Wolfgram protein), whereas others appear to be synthesized at a site remote from the assembly locale and are transported by specific lipoproteins (i.e., 24,000-Da proteolipid protein). It is also known that the assembly of lipid components occurs independently of the assembly of protein components. and researchers have therefore concluded that the organization and assembly of myelin does not occur as a complete unit. The following sequence of myelin assembly has been postulated. Certain high-molecular-weight proteins and the Wolfgram proteins are brought together in a membrane low in lipids. To this initial structure are added additional Wolfgram proteins and myelin basic protein. After the subsequent addition of the 24,000-Da proteolipid protein, considered by many to be "rate limiting," the remaining lipids are added to the myelin sheath. Of course, the postulated sequence is based on numerous experiments that are beyond the coverage of this article (see Benjamins and Morell, 1978).

Recent studies confirm the initial observations of a decade ago that the turnover of myelin in adults is extremely slow. Myelin as a whole can be considered relatively stable, but various components are metabolized at different rates. The turnover rates (as measured by metabolic half-lives) of phosphotidylcholine, phosphotidylethanolamine, and phosphotidylserine are at least one-half as fast in myelin as in microsomes. Other lipids most probably turn over more slowly. Most lipids have turnover times in myelin on the order of weeks or months. Proteins have similar turnover times. In summary, the original proposal that myelin has a long-term metabolic stability appears accurate. However, various components turn over at different rates, and each component undergoes two phases, one of slow and one of fast degradation. Further information on the biochemistry, ultrastructure, and metabolism of myelin can be found in Bunge (1968), Davison and Peters (1970), Morell (1977), and Norton (1975).

A final note should be made concerning diseases involving demyelination of axons. A large number of neuronal disorders exist that have profound repercussions for the afflicted individual. Disorders of demyelination can be primary (destruction of myelin with sparing of neurons) or secondary (damage to myelin after primary neuronal lesion). Demyelinating diseases are divided into four basic etiologies: (1) genetically determined metabolic disorders, (2) acquired inflammatory disorders, (3) toxic and nutritional disorders, and (4) disorders of secondary demyelination.

The vast majority of genetically determined degenerative disorders involving myelin occur in humans prior to age 10. They all appear to be due to inborn errors of metabolism and involve the loss of one or more enzyme activities. They involve loss of myelin from axons and central white matter; diffuse lesions (often symmetrical) in the brain; and few, if any, inflammatory sequelae. These diseases include Krabbe's globoid leukodystrophy, Canavan's disease (spongy degeneration of white matter), Alexander's disease, Refsum's disease, and adrenoleukodystrophy (Schilder's disease). Acquired inflammatory disorders include Guillain–Barré syndrome, an acute disease of the peripheral nerves, and multiple sclerosis, a disease with an autoimmune basis that may be related to earlier viral exposure. Numerous chemicals have been implicated as demyelinating agents, including lead, carbon monoxide, triethyltin, and hexachlorophene. Copper deficiencies, especially in neonates, may result in decreased myelinogenesis. Two conditions worth noting that result in secondary demyelinatin include Wallerian degeneration, which results from loss of axonal integrity following trauma to neuron, and subacute sclerosing panencephalitis, in which demyelination occurs subsequent to viral destruction of neurons. Although any clinical neurology textbook would be a more than adequate source for those readers interested in pursuing this subject, Morell *et al.* (1981) also provide an excellent starting point and outline of these dibilitating disorders.

4. The Synapse and Its Function

4.1. General Principles of Synaptic Transmission

The synapse refers to the discrete anatomical unit that encompasses an axonal or dendritic nerve terminal with a second cell, which can be either another neuron or a different cell type such as a muscle or gland. Separating the pre- and postsynaptic cell membranes is the extracellular fluid of the synaptic space. The principal mode by which a neuron is thought to communicate with another neuron—or with a target cell type such as striated, smooth, or cardiac muscle—is chemical transmission. The process of synaptic transmisstion involves the biosynthesis and storage of the chemical neurotransmitter, its release from the presynaptic terminal into the synaptic cleft, its interaction with specific recognition sites known as receptors on the postsynaptic neuronal or target cell membrane, and finally the inactivation of the transmitter by extracellular catabolism or by reuptake into the nerve cell. However, some evidence for electrical transmission between neurons of the central nervous system exists and must always be considered in electrophysiological studies of the central nervous system. General reviews of chemical and electrical transmission can be found in Shepherd (1974) and Weight (1971).

The first neurotransmitter to be discovered was acetylcholine, discovered by Loewi in the early 1920s. These studies were conducted with isolated preparations of heart and indicated that a substance inhibitory to the chronotrophy of the heart was released by inhibitory nerves. Since then, numerous chemicals have been proposed as putative neurotransmitters, and reasonable estimates suggest that there are at least 50 candidates for the role of neurotransmitters. Like acetylcholine, many of the other neurotransmitters that were discovered early were found in peripheral tissues such as the heart or the vertebrate neuromuscular junction. At such sites, there is the release of a neurotransmitter at discrete sites where the pre- and postsynaptic membranes are in intimate contact. However, as research progressed over the past two decades, it became apparent that there were many situations where a nerve terminal was not closely associated with a postsynaptic membrane containing a unique receptor for the transmitter in question, but in fact was so situated that the released chemical could either diffuse into the circulation (i.e., neurohypophysis or adrenal medulla) or into the extracellular space to act on a distant receptor. Chemicals so released are usually not considered neurotransmitters so much as neurohormones or neuromodulators.

In all cases it appears as though calcium entry into the nerve terminal is an absolute requirement for the release of a neurotransmitter or neuromodulator. In many cases the stored neurotransmitter, enclosed within a storage granule or vesicle, fuses with the plasma membrane and through the process of exocytosis releases its contents into the extracellular space, be it the synaptic cleft or the circulation. The entry of Ca^{2+} into the nerve terminal is triggered by the appearance of the action potential at the nerve ending. It is also important to bear in mind that the subsequent release of neurotransmitter following Ca^{2+} entry into the nerve terminal results in a quantal release of chemical transmitter, an amount that is dependent on the vesicular storage size and the synthetic machinery of the nerve for the transmitter in question.

Originally, all neurons were thought to follow *Dale's principle*, a set of propositions originally construed by Eccles (1957) and named after Sir Henry Dale, who originally suggested the terms *cholinergic* and *adrenergic* for neurons that release acetylcholine and norepinephrine (noradrenaline), respectively. Dale's principle expounds that a single neuron can contain and release only one neurotransmitter and that the neuron can act in only an inhibitory or excitatory manner, but that it cannot exert different activities at different sites. Recently, however, evidence suggesting the presence of at least two neurotransmitters in a single neuron has been reported; adrenal chromaffin cells, for instance, have been shown to contain the endogenous opioid peptide met-enkephalin. Furthermore, neurons in certain invertebrates such as *Aplysia*, the marine mollusk, have been shown to have both

excitatory and inhibitory effects on different adjoining neurons. Hence, the model systems for synaptic transmission have become considerably more complicated since the time only a quarter-century ago when Eccles first proposed Dale's principle. Yet, in many instances, the framework outlined by Eccles is still applicable to a large number of nerves.

4.1.1. *Putative Neurotransmitters and the Criteria for Their Identification*

As mentioned earlier, there are at least 50 putative transmitters that could potentially play a role in synaptic transmission or in neurochemical processes. These include acetylcholine, the aromatic monoamines (catecholamines and indole amines), a variety of primary and polyamines, certain amino acids, certain purine nucleosides and nucleotides, and a large number of peptides. The biosynthesis and regulation of the turnover of these putative transmitters will be discussed in another section of this article. However, it is worth noting here that the synthesis of these sundry transmitters is complex and variable, and in most cases the biologic control of the synthesis and degradation of these compounds is poorly understood at best.

The aromatic monoamines include the catecholamines such as dopamine, norepinephrine, and epinephrine as well as the indole amine 5-hydroxytryptamine (serotonin). These monoamines have been conclusively demonstrated to be neurotransmitters in both peripheral and central tissues. Furthermore, they have been implicated in a number of neuropsychiatric conditions and hence have gained wide research attention. Among the diseases in which they are thought to play a role are Parkinson's disease, Huntington's disease, Wilson's disease (all of which involve the basal ganglia); anxiety syndromes; manic–depressive psychosis; schizophrenia and related psychoses; and hypertension. Indeed, a variety of pharmacological manipulations are presently available for the clinician that in some way interfere with monoamine metabolism, release, reuptake, or receptor binding. This interesting area is beyond the scope the present chapter, but can be explored further in Sourkes (1981a,b) and in Berger and Barchas (1981). The development of fluorescent histochemical techniques has also aided the understanding of the neuroanatomical localization of neurons containing the aromatic monoamines.

It may be of interest to review briefly certain of the major disease states just outlined. Possible abnormalities in the metabolism of central neurotransmitters, particularly biogenic amines, have been implicated in a number of pathological states. Perhaps the most well-recognized example is Parkinson's disease. In this disease, for unknown reasons, there is a degeneration of a significant number of neurons that utilize dopamine as a neurotransmitter. The cell bodies of these neurons normally reside in the substan-

tia nigra, and they project to the caudate nucleus, an area of the brain that is important for motor coordination. An understanding of the biosynthetic mechanism for dopamine made possible the development of significant advances in drug therapy. L-Dopa, the precursor of dopamine, can be administered to patients with Parkinson's disease. This compound crosses the blood barrier and is converted to dopamine, which in turn stimulates the dopamine receptors. Both manic–depressive psychosis and schizophrenia have also been postulated to involve biogenic amines. In the former disease, inhibitors of norepinephrine or serotonin uptake are often found to be effective antidepressants. These drugs would tend to prolong the amount of time these neurotransmitters are present in the synapse. Thus, it has been suggested that possibly the fundamental defect in this disease relates to a subnormal production or release of norepinephrine or serotonin in the central nervous system. Schizophrenia, on the other hand, has been suggested to arise from a relative overproduction of dopamine, as compared to norepinephrine, within certain neurons. These hypotheses are at best only tentative, and considerably more work is needed to define such pathological mechanisms.

Huntington's disease, a choreiform motor disorder, apparently has a complex etiology involving many different neurotransmitters, and an understanding of the pathological basis of the disease is only now emerging. Finally, defects in a number of neurotransmitter systems such as catecholaminergic, serotonergic, and cholinergic pathways have been studied in relationship to abnormalities in blood pressure control. Indeed, although the exact neural pathways that regulate blood pressure are still under speculation, it is becoming increasingly clear that the central nervous system is extremely important in the overall control of blood pressure and may contribute to the etiology of hypertension.

Other amines that potentially are involved in neurotransmission are phenylethanolamine, histamine, octopamine, and certain polyamines such as putrecine, spermine, and spermidine. Many amino acids might also be transmitters in the central nervous system or the periphery, such as γ-aminobutyric acid (GABA), taurine, glycine, glutamate, aspartate, and alanine. Obviously, any study of amino acids as transmitters is obfuscated by their ubiquitous presence in all cells and in many cell processes. Glycine and GABA are believed to be inhibitory neurotransmitters, in other words, capable of inhibiting the formation of, and frequency of action potentials in, the axolemma of the neurons to which they bind. Glutamate and aspartate, on the other hand, appear to be excitatory. Of special interest is the recent work that suggests a coupling of a subtype of GABA receptor with a recognition site for the widely employed benzodiazepine tranquilizer diazepam. Although the scientific case for synaptic transmission by the purines, ade-

nosine, and inosine/hypoxanthine is considerably weaker than for the amines and amino acids, a preponderance of purinergic receptors have been found in neuronal tissues, suggesting a potential role in neuronal regulation.

In the past few years it is the small peptides that have received enormous attention for their possible role as neurotransmitters. These peptides include substance P, thyrotropin-releasing hormone, somatostatin, arginine–vasopressin, neurotensin, cholecystokinin, vasoactive intestinal peptide, angiotensin II, bradykinin, and the enkephalins and endorphins. This list is by no means complete, but is representative of the large number of peptides that have recently achieved candidacy as putative neurotransmitters. It is obvious that many of the peptides listed have well-documented hormonal actions in peripheral tissues. Furthermore, considerable homology of amino acid sequences exists among certain groups of peptides, such as the enkephalins, endorphins, and adrenal corticotropin hormone. A number of steps are useful for the analysis of putative neuropeptidergic transmitters, which include development of a sensitive bioassay and purification techniques; chemical and physical characterization of the peptide, including the amino acid sequence; and production of antibodies to the peptide, which should ultimately yield sensitive radioimmunoassays for quantitation and immunocytochemical techniques for cellular localization studies.

For all the preceding classes of putative neurotransmitters, a number of criteria must generally be met for the particular compound to be considered a known and established neurotransmitter. Furthermore, none of the following criteria in and of itself is considered adequate to establish that a substance is a transmitter, and the more of the criteria that can be met, the more certain an investigator might be that the substance of interest is in fact a transmitter: (1) The neurotransmitter must be present in the nerve terminals from whence it is released. Ideally, its presence in the nerve terminal should be demonstrated by both chemical (i.e., synaptosomal localization) and immunological or histofluorescent techniques. Because synthesis may occur only in the nerve terminal, it is not necessary to demonstrate the presence of the transmitter in the perikaryon, dendrites, or proximal portions of the axon. If specific chemical or surgical denervation of selected neurons containing the putative transmitter is possible, such treatment should result in the depletion of transmitter pools. (2) The transmitter is quantally released upon nerve stimulation by either electrical impulse or high extracellular K^+. However, many substances, including cations, proteins, and small-molecular-weight molecules involved in energy transport may be coreleased with the transmitter. (3) Application of the putative neurotransmitter to postsynaptic membranes of neurons or other effector cells yields the same response as electrical or K^+-depolarized stimulation of the presynaptic nerve. These effects should occur in a concentration range that approximates

estimates of released endogenous transmitter concentration in the synaptic cleft. (4) Biochemical evidence for a postsynaptic recognition site for the transmitter should be obtained. (5) A direct inhibition of the dose–response curve of the putative neurotransmitter and its evolved action potentials should be observed with increasing concentrations of specific receptor antagonists of the transmitter. (6) A local mechanism for the inactivation of the putative transmitter should be present in the discrete area of the synaptic cleft. This mechanism may include a soluble extracellular enzyme, a reuptake system for the transmitter into the neuron or supportive glial cells, or a combination of reuptake and intracellular catabolism. Dilution and diffusion of the substance in the extracellular space are not ordinarily considered an effective means of local inactivation. (7) Supersensitivity of postsynaptic cell to the neurotransmitter can be demonstrated after chronic denervation. (8) The biosynthetic machinery for the transmitter should be present in the nerve terminal.

4.1.2. *Transmitter Release and Mechanisms of Inactivation*

4.1.2.1. *Release.* It is presently believed that a neurotransmitter is released during the exocytotic process from the presynaptic membrane in discrete quanta, or finite amounts; these quanta are released spontaneously in nerves at rest at the rate of approximately one or two per second. Excited nerves increase the quantal release of transmitter by about fivefold. It has been estimated that at the neuromuscular junction each quantum contains about 10^5 molecules (0.166×10^{-18} mol) of the transmitter acetylcholine. It is also established that neurotransmitters such as acetylcholine can passively diffuse across the cell membrane by mechanisms unrelated to exocytosis. The number of moles of transmitter per quantal release for neurotransmitters in the central nervous system has not been accurately quantified.

The *vesicle hypothesis* of synaptic transmission states that one synaptic vesicle, for instance, containing acetylcholine, contains one quantum of transmitter (i.e., 10^5 molecules of acetylcholine). A variety of supportive evidence exists for the vesicle hypothesis. First of all, it has been clearly demonstrated that isolated synaptic vesicles from a variety of sources contain neurotransmitters. For example, cholinergic vesicles from the torpedo electric organ and vesicles isolated from certain areas of mammalian brain have been shown to contain abundant quantities of acetylcholine; adrenal chromaffin granules have also been shown to contain both norepinephrine and epinephrine. Second, examination of synapses following prolonged neuronal stimulation supports the vesicle hypothesis through two separate findings: (1) the presynaptic membrane surface examined under the electron microscopy clearly increases in surface area, an observation consistent with the fusion of vesicles with the plasmalemma; (2) at adrenergic nerve terminals,

the protein dopamine-β-hydroxylase is released concomitantly with the catecholamine transmitter, a finding that can only be readily explained by the exocytotic release of vesicular contents. Third, freeze-fracture electron micrographs of preparations such as the neuromuscular junction clearly indicate that synaptic vesicles in the process of exocytosis can be observed and that the number of vesicle–membrane fusions increases with the extent of presynaptic neuronal stimulation.

The regions of the presynaptic membrane where fusion of vesicles and the plasmalemma occur are limited to what has been termed the "active zones." Closely associated with these active zones are what electron microscopists believe may be clusters of calcium ionophores necessarv for the entry of Ca^{2+} for the initiation of exocytotic release. Other morphological entities at the active zone have also been identified, but their physiological role in transmitter release has not been elucidated. It should be noted that numerous freeze-fracture micrographs taken of active synapses reveal many more vesicle fusions than would be predicted for one or two release events. These observations are not consistent with the one vesicle–one quantum hypothesis that was briefly discussed earlier. To date, no explanation for the discrepancy between the number of vesicles and the number of quanta released has been proposed except to suggest that the vesicle, in fact, only releases a fraction of the quantum, which has been termed a microquantum.

The synaptic vesicle is thought to be recycled by the nerve terminal after opening to release its contents. The precise nature of the recycling, however, is unknown. Some investigators have suggested that a particulate substance is added to the active zone where the vesicle has fused with the plasmalemma and that this portion of the membrane is then internalized and collected a few seconds later (termed endocytosis of coated vesicles). Others have suggested a simple process of direct invagination. The debate lingers on and other events may yet be discovered that will more clearly define the process of recycling.

In general, the process of vesicular release can be summarized as follows: During or after the biosynthesis of the neurotransmitter, the substance is packaged into synaptic vesicles at the nerve terminals. Here the transmitter is stored until the nerve terminal is depolarized by the appearance of an action potential, at which time Ca^{2+} enters the cell and permits the exocytotic process that involves the apparent fusion of vesicular membranes with the plasmalemma. Such fusion allows for the release of the transmitter that is packaged within the vesicle. Regulatory mechanisms that are not presently clear then lead to the recycling of the vesicle within the nerve ending. In-depth reviews of release processes can be found in Cooke *et al.* (1973), Krnjevic (1974), Katz (1969), Rubin (1970), Zimmerman (1979), and Kelly *et al.* (1979).

A number of pharmacological agents are known to affect the release of

specific neurotransmitters and warrant brief mention at this point. Interference with the synthesis of acetylcholine or any of the catecholamines can be elicited by hemicholinium or α-methyl-*p*-tyrosine, respectively, and subsequently block release of the transmitters by reducing the intracellular and vesicular pool size. In the case of adrenergic (i.e., catecholaminergic) vesicles, the active transport of transmitter into the vesicle can be blocked by covalent binding of rauwolfia alkaloids such as reserpine. The effects of reserpine are characterized first by a transient increase in catecholamine release, followed by a prolonged depletion of the neurotransmitter. The effects are so long, in fact, that radiolabeled reserpine has been used as a marker for the turnover of adrenergic storage granules in peripheral tissues such as the adrenal medulla. Both carbachol and tetraethylammonium have been shown to promote a similar transient release of acetylcholine in cholinergic nerve terminals, but their effects do not appear to be followed by the prolonged depletion associated with reserpine in adrenergic nerves. Guanethidine, by mechanisms that are still unclear, can also displace catecholamines from adrenergic terminals in a manner not dissimilar to that of reserpine. Tyramine, ephedrine, and amphetamine can elicit a brief release of transmitter from adrenergic neurons, but presumably their actions are not on vesicular catecholamines, but on a free, cytoplasmic mobile pool. Last, there appear to be compounds that are capable of inhibiting the release of selective transmitters. For instance, botulinus toxin has been shown to inhibit the release of acetylcholine from cholinergic nerve terminals, presumably by blocking selectively that portion of the axon immediately proximal to the terminal bouton. Iproniazid and bretylium, on the other hand, have been shown to block the release of catecholamines, although their mechanism of action is even less clear than for compounds such as botulinum toxin. As can be readily seen, the drugs interfere exclusively with cholinergic or adrenergic systems and have been widely employed in studies on the regulation of chemical transmission in the autonomic nervous system. Pharmacological agents affecting other aminergic or peptidergic systems are also available, but the length of this article precludes a discussion of their pharmacodynamics. It would be safe to state, however, that at this time those compounds affecting cholinergic or adrenergic release have been the most widely studied. A more detailed discussion of these compounds, with accompanying references, can be found in Mayer (1980).

4.1.2.2. *Mechanisms of Inactivation.* It was noted previously that one of the criteria for the identification of a neurotransmitter is the presence of a local means by which a transmitter can be inactivated. If this process were not present, it stands to reason that a transmitter would be exposed to a postsynaptic recognition site for a prolonged period of time, an event that

might eventually lead to the malfunctioning of the postsynaptic response apparatus. For instance, in tissues replete with catecholamines it has been demonstrated repeatedly that prolonged release of transmitter by compounds such as amphetamine, or the blockade of their reuptake for inactivation by compounds such as imipramine, results in supra-activation of postsynaptic receptors and their subsequent desensitization. Hence, the process of inactivation of the chemical transmitter is extremely important. To date, two primary modes of inactivation have been identified: (1) inactivation by an extracellular enzyme and (2) inactivation by reuptake of the transmitter into presynaptic, postsynaptic, and glial compartments.

The only clear case of a transmitter that is known to be inactivated by an extracellular enzyme is acetylcholine. The enzyme responsible for the breakdown of acetylcholine is acetylcholinesterase, which occurs in neurons and in the surrounding extracellular space, in a variety of tissues and at the neuromuscular junction. Within milliseconds after release and binding of acetylcholine to its postjunctional receptor, acetylcholinesterase is hydrolyzing the transmitter to choline and acetic acid. A similar enzyme, butyrocholinesterase, which is less specific for acetylcholine, can also catabolize the neurotransmitter, however. Numerous compounds, most notably the bisquaternary compounds such as edrophonium, physostigmine, and neostigmine and the organophosphorous compounds such as diisopropyl fluorophosphate, are extremely potent inhibitors of acetylcholinesterase and are used for pharmacological, insecticidal, and military purposes. It is also quite possible that other putative transmitters such as peptides are catabolized by extracellular enzymes or membrane-bound enzymes anatomically situated to face into the extracellular space. Some evidence exists that the enkephalins may be inactivated following release by endo- and exopeptidases that are either present in the interstitial fluid or are bound to the extracellular surface of the cell membrane.

In contrast to the relatively simple mode of inactivation described for acteylcholine and perhaps some neuropeptides, the reuptake systems that have been the subject of intensive study, especially for the aromatic monoamines, are considerably more complex. These uptake systems demonstrate a high affinity for the transmitter that they are meant to accumulate. They exist in addition to transport systems that possess a lower affinity for the substrate in question. These low-affinity transport systems may exist for the accumulation of substrate for general metabolic requirements of the cell. Of interest is the fact that all studies on the high-affinity reuptake systems for monoaminergic transmitters have demonstrated an absolute requirement of the system for Na^+. The system does not appear to require adenosine triphosphate, however, and so can be readily distinguished from the $Na^+ - K^+$ ATPase exchange system described earlier in this article. The high-

affinity reuptake systems require concentrations of 0.1 to 0.2 *M* Na^+ for maximal activity, concentrations that are ordinarily present in the extracellular fluid. At these concentrations of cation, transmitter binding to the transport system is favorable, and following the traversal by the transport system of the cell membrane, transmitter is readily released into the intracellular space, where Na^+ concentrations are considerably lower.

The transport systems for the monoamines have been divided into two uptake types: one of high affinity (uptake 1), which is thought to be associated exclusively with nerve terminals, and one of lower affinity (uptake 2), which is thought to be associated with glial and postsynaptic sites. Lineweaver–Burk analysis of uptake kinetics can readily distinguish between these two types of uptake. Although the uptake systems are not thought to require ATP, as described in the preceding paragraph, it is generally believed that the reuptake systems are closely linked with a Na^+–K^+ ATPase pump. This conclusion is based on the observation that ouabain and other metabolic inhibitors slow transmitter reuptake and that low extracellular Na^+ or high extracellular K^+ can block reuptake. Furthermore, the uptake system may also be a form of facilitated transport because it is both temperature and glucose dependent and uptake is saturable and follows Michaelis–Menten kinetics.

The high affinity of the reuptake systems for monoamines, as measured by the Michaelis constant, is on the order of 0.1–0.4 μM; the low affinity is about 10-fold higher. Specific uptake inhibitors for each monoamine have apparently been developed. Chlorimipramine and Lilly 110/40 are presumed to be specific for serotonergic uptake systems. Benztropine and amphetamine (an action separate from its ability to enhance catecholamine release) are thought to block specifically dopamine uptake and desmethylimipramine and cocaine are believed to inhibit norepinephrine uptake.

The transmitter that is taken up into the neuron and liberated into the intracellular fluid can then either be recycled into vesicular storage granules or metabolized by intracellular enzymes. The two enzymes that are of major importance for the catabolism of aromatic monoamines are monoamine oxidase, of which there may be two or more isozymes, and catechol-*O*-methyltransferase. In neurons, monoamine oxidase is associated with mitochondria, whereas catechol-*O*-methyltransferase is associated with the soluble cytoplasmic fraction. Both monoamine oxidase and catechol-*O*-methyltransferase can act on monoamines sequentially to form a variety of metabolic products. These have been identified by radioisotopic and chromatographic experiments. The major metabolites of the aromatic monoamines have been identified: 3-methoxy-4-hydroxyphenylethylene glycol and 3-methoxy-4-hydroxymandelic acid (vanylmandelic acid) for norepinephrine and epinephrine; 3,4-dihydroxyphenylacetic acid and 3-meth-

oxy-4-hydroxyphenylacetic acid (homovannilic acid) for dopamine; and 5-hydroxy-indole acetic acid for serotonin.

On the basis of specific substrates and inhibitors, two forms of monoamine oxidase have been postulated and termed types A and B. Type A is thought to be specific for norepinephrine and serotonin as substrates and inhibited by clorgyline and Lilly 51641. In contrast, type B is thought to be specific for phenylethylamine and benzylamine as substrates and is preferentially inhibited by pargyline and deprenyl. Both types of enzymes are found in neurons, and it is important to stress that substrate and inhibitor specificity is only relative and that cross-reactivity will be observed at high enough concentrations.

It is beneficial to conclude this section on inactivation by noting that little is known about the *in vivo* degradation of most other neurotransmitters. Although the literature on GABA especially and to the lesser extent on other amino acids is beginning to demonstrate an understanding of those processes that are responsible for the degradation of the putative neurotransmitters, it is also safe to say that little has actually been reported on the regulation of neuropeptide catabolism. One reason for this dearth of information is the methodological difficulties in assigning specific functions to the large number of peptidases that have been reported to date. Perhaps investigators will find that the substrate specificity to which they are accustomed when studying enzymes such as acetylcholinesterase and monoamine oxidase will not find a parallel in the peptidases responsible for the catabolism of the large number of putative neuropeptidergic transmitters. Further information on mechanisms of inactivation can be found in Iversen (1970), Costa and Sandler (1972), and Mayer (1980).

4.1.3. *Postsynaptic Responses*

Postsynaptic responses to neurotransmitters are invariably initiated by the binding of the transmitter to a specific recognition site, or receptor. This finding is true both for interneuronal communications and the transmission of signals from neurons to effector cells. Perhaps the only known exception to this observation is the presumed communication in the central nervous system between electrotonic synapses, a topic beyond the scope of this chapter [see Weight (1971) and Schmitt *et al.* (1976) for further details].

A brief comparison between electrical and chemical synapses, however, is instructive. Electrical transmission, by definition, requires no chemical substance for the transmission of an electrical signal from one neuron to another; rather, electrotonic impulses are thought to move from one cell to another via syncytoid connections. The rate of transmission is orders of magnitude faster in electrical, as versus chemical, synapses. Although inhibition of chemical transmission can occur in a variety of ways (i.e., inhibition of

release or synthesis of transmitter), inhibition of electrical transmission probably does not take place. Amplification of a signal also most likely takes place only at a chemical synapse. Furthermore, to prevent "cross-talk" between two or more neurons, rectifier circuits, which allow current to pass in only one direction, are probably required. In contrast to chemical synapses where the frequency of transmission is limited by transmitter availability, electrical synapses can sustain a more rapid frequency of impulse propagation. Last, the energetic efficiency of an electrical synapse is much higher than a chemical synapse, which must expend energy on transmitter synthesis, storage, release, and degradation.

The postsynaptic response to a chemical messenger appears to occur at postsynaptic active zones, which can be recognized morphologically at sites where nerve terminals make contact with other neurons or effector cells such as striated muscle. They consist of a pronounced density of intramembranous particles as viewed under electron microscopy. These particles are at least 100-fold more enriched in active zones when compared to the remainder of the membrane. At the cholinergic nerve–muscle junction, evidence exists to suggest that these intramembranous particles are in fact ion channel–receptor complexes. Portions of the particles, thought to be the receptors, turn over with a time course of days, but the overall integrity of the active zones remains intact. In the cerebral cortex of the central nervous system, dendritic spines of neurons have been shown to be concentrated with active zones. These active zones appear to be intimately associated with portions of the neuronal cytoskeleton, since the cytoplasmic portion of the active zone displays a prominent band of fuzzy material, which, in turn, makes contact with microfilaments.

In the preceding two examples (i.e., neuromuscular junction and cerebral cortical dendritic spines), active zones are present in their most organized form. However, there are cases where active zones are considerably more diffuse and are not associated solely with portions of the neuron or effector cell adjacent to the terminal of the presynaptic neuron. For instance, active zones of peripheral end organs of the sympathetic nervous system appear to be spread out over larger sections of the cell membrane. The morphological aspects of the active zone are discussed in more detail by Heuser and coworkers (1976) and Heuser and Reese (1977).

The best-understood mechanism by which transmitters affect changes in a postsynaptic cell is though receptor binding. Such interactions can result in either an electrical or a biochemical change in the postsynaptic cell. In the case of electrical changes induced by receptor binding, neurotransmitters can alter the excitability of the postsynaptic cell by either depolarizing or hyperpolarizing that portion of the cell surrounding the receptor. Such a change in potential is the result of an alteration in the transmembrane per-

meability of the cell to one or more ions. The effect on the potential will depend largely on the extent of permeability of the membrane to a given ion, as was discussed earlier in this chapter. These permeability changes occur rapidly and ordinarily do not require metabolically derived cellular energy. Receptor binding can also induce biochemical changes within the cell, often through second messengers such as the cyclic nucleotides, calcium–calmodulin, or prostaglandins. These effects will be discussed later in the article in the section on second messengers.

It has often been observed that a given transmitter may either inhibit or excite different neurons in vertebrate brain and in the marine mollusk *Aplysia*. The variability in response is thought to be due to differences in the receptor complex in different cells. The receptor complex is composed of a transmitter recognition site and an ionophore, a membrane structure, most probably a protein, that lends selective permeability of the membrane to a given anion or cation. In *Aplysia*, for instance, a serotonergic receptor can be associated with a Cl^-, K^+, Na^+, or Ca^{2+} ionophore, depending on the cell type on which the receptor complex is located. In vertebrates, acetylcholine can cause excitation at the neuromuscular junction (receptor plus excitatory Na^+ ionophore), inhibition at some neurons in the brain (receptor plus inhibitory Cl^- ionophore), or any number of slow depolarizations or hyperpolarizations depending on the nature of the receptor complex. Hence, no substance (or its receptor) is exclusively excitatory or inhibitory. The response of a neuron to a given transmitter will depend, if one is to be seen at all, on the ionophore to which the receptor is coupled. Furthermore, in some cases a receptor will not be coupled to an ionophore at all but to another macromolecule such as an enzyme. In this latter case, receptor occupancy will result in a biochemical, rather than an immediate bioelectrical, alteration of the neuron. One such example, which has been widely studied, is the coupling of the β-adrenergic receptor or the dopaminergic receptor to the membrane-bound enzyme adenylate cyclase, which is responsible for the synthesis of the second messenger cyclic adenosine 5′-monophosphate from ATP.

Because so much of the postsynaptic response to a neurotransmitter depends upon the interaction of the substance with a specific recognition site, it would be useful at this point to attempt to describe criteria by which a receptor can be assessed. Much research over the past two decades has been directed at characterizing receptors for a large number of putative neurotransmitters, and in light of the abundance of reports concerning recognition sites for both endogenous and exogenous compounds, it is useful to analyze the significance of specific recognition sites. For a binding or recognition site to be considered a specific receptor for an endogenous ligand, a number of criteria must be met, as will be described shortly. The receptor itself is

considered to be a macromolecule, most probably proteinaceous in nature, that specifically recognizes and binds a hormone, neurotransmitter, or endogenous compound. The ligand–receptor interaction must then yield a quantifiable response. Therefore, although binding may be measured, this parameter and its characterization do not represent a complete study of the receptor, nor do they provide definite proof for a specific recognition site that possesses physiological relevance. One must always remain alert to the question of the localization of the recognition site. Is the site pre- or postsynaptic? Is the binding to a storage granule, an uptake transport system, or an enzyme, or is the binding to a classical postsynaptic membrane-bound receptor?

The following characteristics of a recognition site must generally be met before it is considered a receptor. (1) The receptor displays strict structural and steric interactions with its ligand. (2) The specific binding is saturable, and therefore has a limited number of binding sites. This finding is in marked contrast to nonspecific binding, which is usually not saturable. (3) Binding exhibits a tissue specificity and is not present in all tissues at the same specific activity (moles ligand bound/milligram protein). (4) The receptor displays a high affinity, as measured by the dissociation constant K_d for its ligand; K_d's are usually in the low micromolar or nanomolar range. (5) Binding of the ligand to the receptor is reversible. (6) Binding is temperature, pH, and often ion dependent and should be linear within a certain range with respect to tissue concentration. (7) Binding of the ligand to the receptor results in some physiological or biochemical perturbation, such as an opening of ionophores and a change in membrane potential or activation of an enzyme, such as adenylate cyclase. Binding is ordinarily measured with radiolabeled ligands and in the case of peptide ligands it is worth keeping in mind that iodination of peptides can affect biological activity. Detailed reviews of receptor theory, identification, and methodology can be found in Yamamura *et al.* (1978) and the Society for Neuroscience Handbook (1980).

Receptor binding that meets the preceding criteria has been reported for virtually every putative and accepted neurotransmitter known. It has also been reported for exogenous compounds with no known endogenous ligand cogeners. For instance, specific and saturable binding with high affinity to membrane preparations of brain has been reported for phencyclidine and tetrahydrocannabinol; there are no known endogenous ligands that may share sufficient structural and steric requirements with these drugs to be considered potential competitors for the reported recognition sites. Hence, data should be interpreted with caution, especially when one considers the pronounced lipophilicity of both compounds.

Although it would be impossible to survey the literature on all receptors for neurotransmitters in this chapter, a few key examples will be discussed

briefly. These will include the acetylcholine receptors, which can be subdivided into nicotinic and muscarinic receptors; the pre- and postsynaptic catecholamine receptors; serotonin and GABA receptors; and opioid peptide receptors.

4.1.3.1. *The Acetylcholine Receptors.* The receptors for acetylcholine can be divided into nicotinic and muscarinic subtypes. The former is associated with the rapid and excitatory depolarizations that are traditionally seen after application of nicotine to neurons containing these receptors. However, chronic depolarization of these cells by nicotine or acetylcholine eventually results in blockade of the response. The latter subtype is associated with the slow and either excitatory or inhibitory responses that can be evoked with the mushroom alkaloid muscarine. Both receptor types are integral membrane proteins and retain most of their binding capacity following solubilization. Using techniques that label the acetylcholine receptor with alkylating agents that are structurally related to acetylcholine or with the venon neurotoxins α-bungarotoxin and α-toxin, the nicotinic receptor has been purified from the electric organ of *Electrophorus electricus;* progress has been made with the muscarinic receptor from vertebrate sources.

The nicotinic receptor is a glycoprotein with a native molecular weight of 250,000. It is composed of a number of subunits, the smallest of which is 40,000 Da and has been shown to possess protein kinase activity. At least one strongly anionic site is present, and it is believed to be involved in the binding of the electropositive group that is invariably found in nicotinic agonists and antagonists. At least one disulfide bond is thought to be present due to the reversible inhibition of agonist-induced responses by dithiothreitol. Classical antagonists include hexamethonium and tetraethylammonium for ganglionic nicotinic receptors and decamethonium and tubocurarine for nicotinic receptors at the neuromuscular junction. Efforts at reconstitution suggest that receptor subunits inserted into artificial membranes and subsequently exposed to acetylcholine can increase the membrane's cation conductance.

The muscarinic receptor has been difficult to purify in reasonable yield for a number of reasons, not the least of which is its resistance to solubilization with detergents and the lack of tissues with high receptor concentrations. Nevertheless, recent reports indicate that the muscarinic receptor has a molecular weight of 80,000 and is a monomer. For further information on nicotinic and muscarinic receptors, see Rang (1974), Briley and Changeux (1977), and Potter (1976).

4.1.3.2. *Catecholamine Receptors.* Catecholamine receptors are divided into presynaptic autoreceptors and classical postsynaptic receptors. Auto-

receptors respond to neurotransmitter molecules released by the same neuron on which the autoreceptors are located. In contrast, the traditionally conceived postsynaptic receptor responds to transmitter molecules released from an adjacent neuron. In other words, at the nerve terminal, autoreceptors respond to transmitter released into and present in the synaptic cleft.

Autoreceptors appear to regulate transmitter synthesis and/or release. The mechanisms by which these receptors exert their activity in the nerve terminal is unknown, although processes involving protein phosphorylation, calmodulin, and protein carboxymethylation have been proposed. Released transmitter is believed to "feed back" to the terminal from which it was released and inhibit further release by binding to the autoreceptor. Although autoreceptors have been identified to dopamine, norepinephrine, serotonin, and GABA, the most detailed information to date concerns the norepinephrine autoreceptor, which shares properties with the α-receptor.

The postsynaptic dopamine receptor can be divided into two types, D-1 and D-2 receptors. The D-1 receptor is linked to adenylate cyclase (for the formation of cyclic AMP), is blocked by bromocryptine, is only weakly antagonized by butyrophenone neuroleptics, is not present in the pituitary, and can be depleted in the corpus striatum following kainic acid lesions. The D-2 receptor is not linked to adenylate cyclase, is stimulated by bromocryptine, is strongly antagonized by butyrophenones, is present in the pituitary, and is not depleted in the corpus striatum following kainic acid, suggesting a presence largely on axons and nerve terminals.

The receptors to norepinephrine can be divided into α and β receptors. Each of these, in turn, can be further divided into α_1/α_2 and β_1/β_2. These classifications are based primarily on pharmacological studies. Prazosin and WB-4101 are selective antagonists for α_1 receptors, whereas clonidine is a selective agonist and yohimbine a selective antagonist of α_2 receptors. Norepinephrine, but not epinephrine, interact with the receptors at physiological concentrations. The α receptors are not linked to adenylate cyclase. Both β_1 and β_2 receptors, however, are linked to adenylate cyclase. Epinephrine and norepinephrine are equipotent at β_1 receptors, but epinephrine is a far more potent agonist at β_2 receptors. Practalol is a selective β_1 antagonist; terbutaline and salbutamol are selective β_2 agonists.

Although the physiological events occurring subsequent to α- or β-receptor stimulation are beyond the scope of this article, a brief outline of the major events following pharmacological stimulation or blockade of these receptors may be of some use to the reader. Activation of α receptors is associated with a peripheral excitatory action on certain types of smooth muscle, such as in blood vessels supplying skin and mucous membranes; a peripheral inhibitory action on other types of smooth muscle, such as in the gut; and excitatory actions generalized in the central nervous system, such as

increased wakefulness and decreased appetite. Activation of β receptors is associated with a peripheral inhibitory action on smooth muscle in the bronchial tree and in blood vessels specifically supplying skeletal muscle, a cardiac excitatory action leading to an increase in heart rate and contractility, and metabolic effects resulting in increased hepatic and muscle glycogenolysis and a liberation of free fatty acids from adipose tissue. Blockade of α or β receptors by numerous pharmacological agents can effectively reverse these actions of agonists. Stimulation or blockade of α or β receptors is of value where pathological abnormalities exist that require the reversal of the condition at the level of the receptor.

Catecholamine receptors appear to be responsive to physiological and pharmacological manipulations. Destruction of catecholamine-containing neurons by either denervation or 6-hydroxy-dopamine results in the development of supersensitivity of receptors on which catecholamine-containing nerve terminals impinged. These effects have been observed in a number of different brain regions. Further readings on catecholamine receptors can be found in Creese *et al.* (1978), Kebabian and Calne (1979), Langer (1974), and Starke (1977).

4.1.3.3. *Serotonin Receptors.* Of the nonpeptidergic receptors, the cholinergic and catecholaminergic receptors described earlier have received the most scrutiny. Nevertheless, because of the important role of serotonin in such neuronal functions as sleep, anterior hypophyseal regulation, pain appreciation, blood pressure and temperature control, and appetite, a word or two about its receptors is appropriate. Microiontophoretic studies indicate the presence of presynaptic serotonin autoreceptors on serotonin-containing neurons and postsynaptic receptors on nonserotonergic neurons. The serotonin autoreceptors apparently decrease the rate of spontaneous firing. The predominant effect of serotonin on postsynaptic neurons containing the appropriate receptors is an inhibition of neuronal firing, although some excitatory receptors in the central nervous system have been reported.

Serotonin agonists appear to be more potent presynaptically rather than postsynaptically. These analogs include lysergic acid diethylamide and dimethyltryptamine. Serotonin antagonists such as methysergide and cyproheptadine have little effect on central inhibitory receptors, but are quite effective on the few excitatory receptors that have been identified. No clear explanation for this apparent dichotomy has been put forth.

Direct binding studies with radioligands have employed 5-hydroxytryptamine (serotonin) and *d*-lysergic acid diethylamide, whose binding is stereospecific and favors the D isomer. Antagonists can displace lysergic acid from its binding sites more effectively than they can serotonin. These and other studies led to the formulation of a hypothesis proposing two (serotonin

1 and serotonin 2) receptors for serotonin. Whether the two receptors suggest different conformations of the same macromolecule or do, in fact, represent different binding sites remains to be elucidated. Haigler and Aghajanian (1977) and Bennett and Snyder (1978) provide two reviews on serotonin receptors for interested readers.

4.1.3.4. *GABA Receptors.* In recent years, considerable efforts have been undertaken to define the role, biochemical characteristics, and regulation of GABA receptors. Although two forms of high-affinity binding of GABA are discernable, the Na^+ dependency of the presynaptic binding (which reflects uptake sites) allows it to be distinguished from the postsynaptic site (which apprently does not require Na^+ and represents a classical receptor). The latter Na^+-independent, high-affinity binding site for GABA also recognizes the specific GABA analogs muscimol and *N*-methylbicuculline. Binding studies also suggest the presence of more than one type of Na^+-independent GABA receptor. Some of these receptors are coupled to a benzodiazepine recognition site, and this observation has received considerable attention.

Benzodiazepines are a widely used group of tranquilizers whose efficacy in the treatment of anxiety and convulsive states has been well documented but poorly understood, and GABA had been proposed as a participant in the mechanism of action of benzodiazepines, but until recently their interrelationship had been unclear. It is now believed that benzodiazepines can increase the maximal binding of GABA at its high-affinity sites and that this response is further potentiated by chelation of Ca^{2+}. Furthermore, GABA can enhance the affinity of benzodiazepines for their binding site. The presumed sites of GABA and benzodiazepine binding, which are thought to be separate, are hypothesized to be connected by the peptide GABAmodulin, which has recently been isolated and whose amino acid sequence has been reported. The peptide GABAmodulin is also suspected to be involved in the coupling of the benzodiazepine recognition site to the chloride ionophore. It may also decrease GABA binding by masking a portion of GABA receptors, and detergent treatment of brain tissue, a process that may remove GABAmodulin, greatly enhances GABA binding. A number of reviews of GABA binding and its relationship to benzodiazepine actions are available, among them De Feudis (1975), De Feudis (1978), and Costa and Guidotti (1979).

4.1.3.5. *Opioid Peptide Receptors.* The nature and biologic regulation of opioid receptors have received enormous attention since 1973, when their presence in vertebrate brain homogenates was reported independently by three separate laboratories. In recent years it has become clear from phar-

macological evidence that there are at least three subtypes of the opioid receptor, which were named after the prototype compound that bound preferentially at a given site: μ for morphine, κ for ketocyclazocine, and σ for SKF 10047. To date, none of these receptors has been purified to any great extent, presumably due to both their intrinsic chemical instability and their sensitivity to both ionic and nonionic detergents.

The very presence of endogenous opioid receptors in the brains of all vertebrate species studies from fish to man originally prompted the question of the reason for highly specific receptors for alkaloids produced by opium poppies. Questions of this nature resulted in investigations that demonstrated the presence of two classes of endogenous peptide ligands for the opioid receptors described earlier: methionine- and leucine-enkephalin. Furthermore, it was discovered that the sequence of methionine-enkephalin was present in the pituitary hormone β-lipotropin, and that three separate peptide sequences within β-lipotropin corresponded to an endogenously circulating second class of opioidlike compounds termed α-, β-, and γ-endorphin.

Binding sites for opiate alkaloids and endogenous opioid peptide ligands are found on vertebrate smooth muscle cells and in the central nervous system. There is considerable heterogeneity in the extent of binding among various regions of the brain and spinal cord. Regions that are high in subtypes of opioid recognition sites include the limbic system and all areas that have been implicated in pain transmission or perception to date. These latter regions include the periaqueductal and periventricular gray regions, the substantia gelationosa of the dorsal spinal cord, the medial thalamus, and the nucleus raphe magnus. The precise cellular localization of the receptors, however, is still a matter of controversy. Although considerable evidence has been published suggesting that the majority of, if not all, opioid receptors are presynaptic (autoradiographic evidence coupled with studies on receptor number following dorsal rhizotomy or effects of opioids on K^+-evoked release of other neurotransmitters), there is also strong evidence that a fair proportion of central opioid receptors are postsynaptic. It may well be that as with catecholamine receptors, the opioid receptors are both pre- and postsynaptic.

The chemical nature of the opioid receptor has been studied extensively, but investigations are limited by the fact that the receptor has not been purified to a homogenous state. Specific antagonists such as naloxone and naltrexone are available to interfere with agonist (i.e., morphine or enkephalin) binding and thereby aid in defining the biochemical properties of the receptor. Binding of both agonists and antagonists is stereospecific, saturable and of high affinity, with a pH optimum on the physiological range. Phospholipids may play a role in ligand binding by maintaining the receptor

in a conformation that is capable of recognizing agonists. At least two conformations of the receptor occur, one that favors agonists (inhibited by Na^+ or to a lesser extent by Li^+ but by no other cation) and a second that favors antagonists (stimulated by Na^+). Sodium is thought to be an "allosteric effector" that promotes an elevated affinity of the receptor for antagonists and a lower affinity for agonists. The receptor can be inactivated by sulfhydryl alkylating reagents such as iodoacetamide or *N*-ethylmaleimide, which suggests the presence of essential sulfhydryl groups in receptor conformation. The effects of iodoacetamide and *N*-ethylmaleimide can be attenuated by the presence of agonists, antagonists, or Na^+. Further chemical clarification of these receptors awaits purification. Interested readers are guided to Simon and Hiller (1978), Beaumont and Hughes (1979), and Simon and Hiller (1981) for further information.

4.2. Synaptic Integration and Neurotransmitter Turnover

Synaptic integration involves processes that allow a neuron or whole sets of neurons to respond dynamically to a large amount of input. It is composed of at least two stages. The first stage involves a single neuron, whose response to the varied inputs it receives at dendritic and axonal postsynaptic zones is predicated upon the summation of excitatory and inhibitory synaptic signals. The second stage involves the communication of one signal summated at the axon hillock of a single neuron to numerous other neurons. Responses can be complex. For instance, excitation of inhibitory neurons can shut down a neuronal network. Inhibition of inhibitory neurons can result in disinhibition or excitation of a system. The effect of the second system on a third system becomes even more complex.

A single neurotransmitter can therefore affect the response to, or the release of, a second or third neurotransmitter presynaptically either by an interaction with an autoreceptor or by enhancing the conductance to chloride anion. A transmitter can also alter the response of a second transmitter at a postsynaptic site by affecting the rate of transmitter inactivation (changing rate of reuptake and/or catabolism) or by potentiating or inhibiting the response of the postsynaptic receptor to its ligand either through competitive effects, allosteric effects, or nonselective changes in membrane responsiveness. The complexities and theories of synaptic integration have been discussed by Bloom (1979a).

Synaptic integration often affects the rate of synthesis and degradation of a neurotransmitter or its rate of renewal or turnover. The *turnover rate* of a specific transmitter is the rate of renewal of that transmitter under steady-

state conditions. The measurement of turnover has a few advantages over physiological monitoring of neuronal activity, including the fact that one can follow neuronal activity of populations of neurons and that one can ascertain the type of neuron that is being studied by the transmitter that is being quantified. However, for the turnover rate to correlate with neuronal activity, two criteria must be met: (1) any observed alterations in turnover after physiological, pharmacological, or biochemical manipulation must occur only in specific areas of the brain and not be generalized, and (2) the change in turnover rate must not alter the pool size of the neurotransmitter, but simply its rate of synthesis and corresponding rate of degradation.

Two primary methods of determining turnover rates of neurotransmitters are available to the investigator: the perturbation method (nonisotopic) and the steady-state method (isotopic). In the former technique, an enzymatic step in the synthesis or degradation of a transmitter is blocked and either the substrate or the product of the enzyme is followed as a function of time. For instance, if one wished to measure the turnover rate of dopamine, an inhibitor of its rate-limiting synthetic enzyme tyrosine hydroxylase (see the next section) could be administered (i.e., α-methyl-*p*-tyrosine), and the loss of dopamine could be followed as a function of time. In many cases, the loss of transmitter follows first-order kinetic behavior, so that the natural logarithm of the transmitter ($\ln T_t$) at a given time point is equal to the natural logarithm of the transmitter ($\ln T_0$) at an initial time point minus the product of time t and the rate constant of degradation k: $\ln T_t = \ln T_0 - kt$. At steady state, the rate of synthesis is equal to the rate of degradation, and so the product of the rate constant k, which can be determined experimentally, and the steady-state transmitter concentration is equal to the turnover rate: turnover rate = $k[\text{transmitter}]_{ss}$. An alternative technique is to pulse label the transmitter pool with radiolabeled precursor and follow the change in specific activity as a function of time. Both the perturbation method and the isotopic steady-state method should yield similar results. A mathematical and experimental treatment of turnover measurements for various transmitters can be found in Marchbanks (1977), Neff *et al.* (1969), Costa and Neff (1970).

The measurement of neurotransmitter turnover is popular because it provides the investigator with potential modes of regulation of the dynamic equilibrium that controls transmitter pools. It is also an important measurement in light of the observation that in many cases transmitter tissue levels are barely disturbed after various pharmacological interventions or following neuronal depolarization and transmitter release. The question then presents itself as to how tissue pool sizes of transmitter remain relatively stable when the neuron is at disequilibrium. Turnover studies are an attempt to answer this question.

5. Synthesis of Neurotransmitter Substances

The concept of synaptic transmission postulates the release and subsequent recognition of specific chemical substances by adjacent cellular elements. In the central and peripheral nervous systems, neurons appear to form communication networks where the specificity of information transfer resides in the specific neurotransmitters and the appropriate receptors. Although large number of compounds have been suggested to be neurotransmitters, there seem to be no more than one or two expressed in any one cell type. Specificity appears to lie in the genetic expression of the enzymes necessary for the biosynthesis of each of the substances.

A major feature of neurotransmitter synthesis is the observation that the neurotransmitters themselves are all nitrogenous substances and in most cases are ultimately derived from dietary protein (Fig. 3). A singular exception to this concept is acetylcholine. Choline can be derived from serine, although a significant portion is derived from dietary lecithin. While dietary protein is not directly converted into neurotransmitters, the amino acids that are derived from these proteins can be used unchanged (glycine, glutamate, or aspartatic acid), metabolized to specific derivatives (catecholamines, etc.),

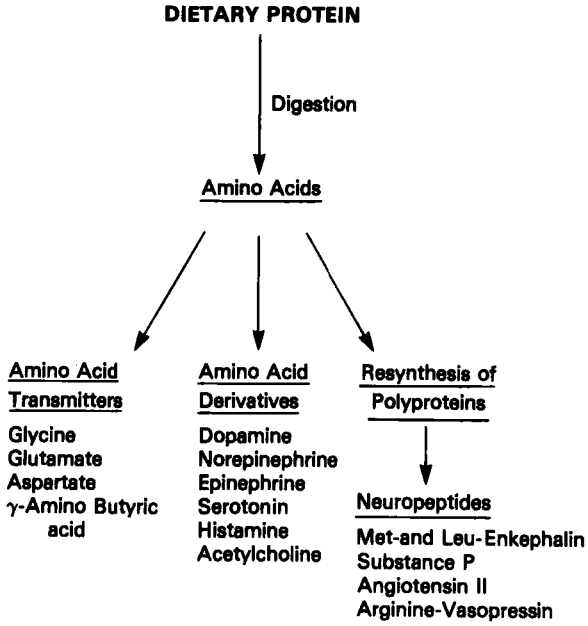


FIG. 3. Relationship of neurotransmitters to dietary protein.

or reassembled into proteins that are then cleaved into specific peptide fragments in certain cells. In the ensuing discussion, the biosynthetic mechanism for each of the major neurotransmitter groups will be considered in the most rudimentary fashion. The synthesis of neurotransmitters has probably received the most scientific attention of all the areas of research in neurochemistry. For a more detailed but general discussion of the material covered in this section, the reader is referred to the numerous chapters on the various neurotransmitters found in Siegel *et al.* (1981) or Cooper *et al.* (1974).

5.1. Catecholamines

The catecholamines (dopamine, norepinephrine, and epinephrine) are three related compounds all derived from either tyrosine or phenylalanine. All three are neurotransmitters in the central and peripheral nervous systems. The biosynthetic pathway for these catecholamines is shown in Fig. 4. The catecholamine-utilizing neurons are among the most thoroughly studied, perhaps in a large part because analytical tools have long been available for detecting these compounds. It appears that each catecholaminergic neuron is specific for its particular neurotransmitter and that the specificity is determined by the complement of biosynthetic enzymes that are genetically expressed. For instance, dopaminergic neurons have only tyrosine hydroxylase and aromatic L-amino acid decarboxylase, whereas noradrenergic cells have dopamine β-hydroxylase in addition to the above two enzymes. Cells responsible for the synthesis of epinephrine have all four enzymes. In each of these types of neurons, however, the tyrosine hydroxylation step appears to be to a rate-limiting step in neurotransmitter synthesis.

Tyrosine hydroxylase is an iron-containing mixed function oxidase that utilizes tetrahydrobiopterin, oxygen, and L-tyrosine as cosubstrates. Molecular oxygen is transferred from O_2 to the aromatic ring of tyrosine. In recent years the molecular properties and the mechanisms that regulate the activity of this enzyme have been studied in depth by several laboratories (Kuhn and Lovenberg, 1983).

In brief, it has long been known that the activity of this enzyme is powerfully inhibited by catecholamine end products (dopamine, norepinephrine, and epinephrine). This inhibition is expressed as a competition of the catecholamine with tetrahydrobiopterin. During the past decade, however, ideas on this regulation have been further refined.

Tyrosine hydroxylase is a substrate for protein kinases (see following section on second messengers), and once phosphorylated, the enzyme is less sensitive to end-product inhibition and thereby appears to have a greater affinity for hydroxylase cofactor. One study suggests that under physiologic

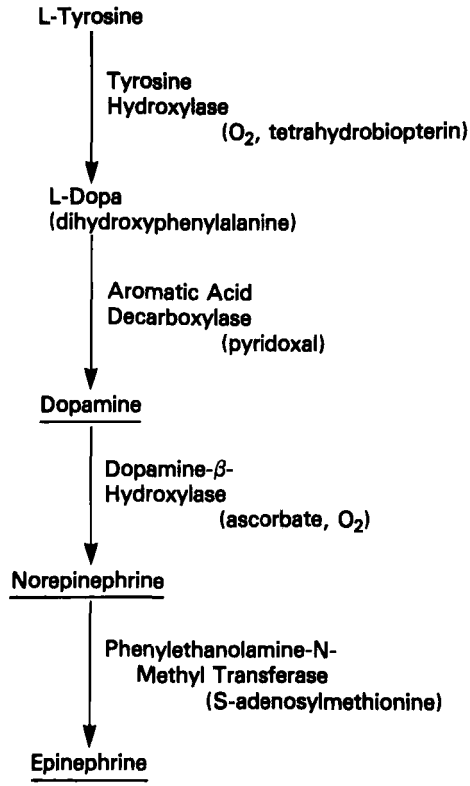


FIG. 4. Biosynthetic pathway for the three major catecholamine neurotransmitters: dopamine, norepinephrine, and epinephrine.

conditions only the phosphorylated form is active. The question remains, however, as to how the cell recognizes the need for more transmitter and activates the appropriate protein kinase, which is responsible for the phosphorylation of the hydroxylase. An increased proportion of tyrosine hydroxylase in an activated and presumably phosphorylated state correlates well with enhanced synthesis of catecholamines and an increase in dopaminergic neuronal activity. The nature of the endogenous kinase is not resolved. *In vitro* either cAMP-dependent or cAMP-independent or Ca-dependent kinase can cause an activation of the hydroxylase. Thus the intracellular signal for this activation remains to be determined.

While it is known that increased neuronal activity and increased catecholamine synthesis occur in concert, it is not known whether pharmacologically inhibiting or stimulating synthesis results in significant decreases or increases in functional activity. The logic for much research

resides in attempting to clarify this potential relationship. Clearly one can limit the rate of catecholamine synthesis by substrate analogs such as α-methyl-*p*-tyrosine. However, it is not clear whether the physiologic effects that ensue are the result of reduced transmitter synthesis or metabolites of the exogenous inhibitor. Conversely, it may be possible to stimulate synthesis by increasing the availability of the precursor. Conventional wisdom has long held that the tissue concentration of tyrosine is close to kinetic saturation and that catecholamine synthesis is little affected by tyrosine intake. Recent studies from a number of laboratories have shown small increases in the turnover of norepinephrine as a result of tyrosine administration. It has also been shown that when the dopamine system is activated pharmacologically that the rate of dopamine turnover is proportional to the circulating or tissue concentration of tyrosine. Although a large proportion of endogenous tyrosine hydroxylase is subsaturated with regard to tetrahydrobiopterin, enhancing catecholamine synthesis by administration of the hydroxylase cofactor has been largely unsuccessful because this compound appears to penetrate cells so poorly.

Noradrenergic and adrenergic neurons also contain the enzyme dopamine β-hydroxylase. Like tyrosine hydroxylase, this enzyme is a mixed function oxygenase. The electron donor in this case is ascorbic acid rather than tetrahydrobiopterin, and dopamine is the primary substrate. The enzyme has been well characterized and is a tetramer of 75,000-Da subunits, which are copper-containing glycoproteins. No major regulatory systems are known for this enzyme. It appears that there are sufficient enzyme molecules present in cells in which this enzyme is expressed to completely convert all the dopamine that is formed into norepinephrine.

The subcellular localization of the enzyme is interesting. In contrast to tyrosine hydroxylase, which is localized to the cytoplasmic compartment, dopamine hydroxylase is largely contained within the aminergic storage vesicles. Thus, it appears that dopamine, which is synthesized in the cytosol, must be taken up into these vesicles in order to be converted to norepinephrine. Approximately 50% of the enzyme is associated with the membranous portion of the vesicles, whereas the other 50% is in a soluble form within the vesicle. During synaptic transmission noradrenergic neurons release both norepinephrine and its biosynthetic enzyme by an exocytotic mechanism. While most of the released norepinephrine is taken back up by the terminal, the enzyme is believed to diffuse of the synaptic cleft into the extracellular fluid and eventually into the serum. There are relatively large amounts of dopamine β-hydroxylase in human serum that are believed to arise from release from sympathetic neurons.

The epinephrine-forming enzyme appears to be present only in neurons that synthesize epinephrine and in adrenal medullary cells. Phenylethanola-

mine-*N*-methyltransferase utilizes norepinephrine and *S*-adenosylmethionine as cosubstrates with epinephrine and *S*-adenosylhomocysteine being the reaction products. Like dopamine β-hydroxylase, there appears to be no short-term major regulatory mechanism controlling the state of catalytic activity. Like most methyltransferases, this enzyme is very sensitive to end-product inhibition by *S*-adenosylhomocysteine.

The factors controlling genetic expression of the biosynthetic enzymes within catecholaminergic neuron and that result in their specificity are unknown. Of particular interest, however, are some very recent studies that show that each of the biosynthetic enzymes contains considerable amino acid homology and that the genes for each of these proteins may have been derived from a common ancestral gene. One of the major questions facing neuroscientists relates to how the expression of genetic information for catecholaminergic synthetic enzymes is controlled.

5.2. Serotonin

The serotonergic neuronal system is one of the most extensive in the central nervous system. The cell bodies of this system are clustered in approximately 10 groups known as Raphe nuclei in the midbrain and lower brain stem. Nevertheless, the axons and terminals permeate every major brain region and the neurons functionally subserve a variety of physiologic functions, including food and water intake, sexual activity, sleep thermoregulation, regressiveness, and blood pressure control.

The ultimate precursor of serotonin, the neurotransmitter of these cells, is tryptophan. Although some food substances contain significant amounts of serotonin, the majority of neuronal serotonin is synthesized endogenously by a biosynthetic pathway that resembles that for catecholamines. In fact, the second step of this pathway is catalyzed by the same enzyme that catalyzes the second step of the catecholamine pathway. Another similarity is that the rate of serotonin synthesis is controlled by the initial enzyme in the pathway, tryptophan hydroxylase.

Although tryptophan hydroxylase has many properties that are similar to tyrosine hydroxylase, it is a distinct enzyme that is only present in serotonergic cells in the central nervous system. It should be noted that serotonin also appears to be synthesized in cells of the intestinal mucosa, although the enzymes for this pathway have not been characterized. Tryptophan hydroxylase is also a mixed function oxygenase that is present in the cytosol of serotonergic neurons. The enzyme requires tetrahydrobiopterin as an electron donor and utilizes molecular oxygen and tryptophan as cosubstrates. Compared to tyrosine hydroxylase, much less is known about the molecular properties of tryptophan hydroxylase, largely because of difficulties scientists have encountered in attempting to purify this enzyme.

Another similarity to the catecholamine system is the fact that the initial enzyme, tryptophan hydroxylase, is subject to protein phosphorylation, which appears to increase its affinity for tetrahydrobiopterin. Of interest is the finding that, in this case, the protein kinase appears to be a specific calcium–calmodulin-dependent enzyme. While it is not known whether this regulation is physiologically significant, it has long been recognized that conditions that lead to increased activity of serotonin neurons and increased Ca^{2+} fluxes also lead to increased synthetic rates for serotonin.

A second and perhaps more important regulatory mechanism appears to revolve around endogenous concentrations of the substrate tryptophan. Kinetic analysis of the partially purified tryptophan hydroxylase indicates that the K_m value for tryptophan was close to, or slightly greater than, the average endogenous concentration of this amino acid. Thus either increasing or decreasing the endogenous concentration of tryptophan should result in either increases or decreases in the rate of serotonin synthesis. This phenomenon has been clearly demonstrated in both experimental animals and man. Since tryptophan is an essential amino acid, it appears that the amount of tryptophan consumed in the diet can have a significant impact on the rate of serotonin synthesis and consequently on the regulation of several physiologic systems.

Other pharmacological approaches to controlling serotonin synthesis involve both inhibitors of tryptophan hydroxylase and administration of tetrahydrobiopterin to stimulate synthesis. With regard to the latter approach, similar problems of delivery of the cofactor to the appropriate cells as described for tyrosine hydroxylase exist, although several lines of experimentation suggest that serotonin synthesis can be augmented if intracellular levels of tetrahydrobiopterin are increased. With regard to inhibitors of tryptophan hydroxylase, one of the most interesting metabolic inhibitors to be described is parachlorophenylalanine. This compound is a relatively weak competitive inhibitor of the purified enzyme but causes an irreversible inactivation of tryptophan hydroxylase in neuronal cells *in vivo*. While this compound has been a most useful tool in the study of the serotonin system in experimental animals, it has not been useful in diseases involving serotonin overproduction because of its toxicity.

5.3. Other Biogenic Amines as Putative Neurotransmitter

A number of amines derived from aromatic amino acid are present in brain in trace quantities. Since aromatic L-amino acid decarboxylase shows a broad substrate specificity, it is not surprising that compounds such as tyramine, tryptamine, phenylethylamine, and histamine are present in brain. These amines are derived from the simple decarboxylation of the corresponding

amino acid. Although some researchers have suggested that these biogenic amines are neurotransmitter substances in specific neurons, this hypothesis has not been firmly established in most cases. Nevertheless, this area should be the object of continuing research in the future.

5.4. Glutamate and Aspartate

There is now considerable evidence that the two amino acids glutamate and aspartate can serve as neurotransmitter substances. However, detailed mapping of these neurons is difficult because these compounds are both derived from protein and are synthesized by intermediary metabolic mechanisms in all cells. This fact means that there are no unique biosynthetic enzymes that can be used as specific markers for these neurons. There are several lines of evidence, however, that suggest that these amino acids, particularly glutamate, are important neurotransmitter substances. Many neuronal cells are excited electrically when exposed to these acidic amino acids, and therefore they are known as excitatory transmitters. Second, brain contains relatively high concentrations of these amino acids. There is an uneven distribution of these acidic amino acids in brain that suggests that they may be concentrated in certain groups of nerve cells. Finally, glutamate or aspartate can be shown to be released following electrical stimulation.

5.5. γ-Aminobutyric Acid (GABA) and Glycine

The two amino acids GABA and glycine are thought to be inhibitory neurotransmitter substances and are contained in high concentrations within specific neurons. GABA is synthesized from glutamic acid by the action of a glutamic acid decarboxylase that is found largely within neural tissue. This is a pyridoxal-requiring enzyme that utilizes the nonessential amino acid glutamic acid as a substrate. Evidence that GABA is a neurotransmitter of specific neurons subserving specific physiologic roles is now widely accepted.

Likewise, the role of glycine as an inhibitory neurotransmitter, particulary in the spinal cord, is well established. This compound, which is the most simple amino acid known, can likewise be derived from the breakdown of proteins or from glyoxylate via a specific transaminase. It appears that nerve terminals in the spinal cord have specific high-affinity uptake systems for glycine and that these neurons contain unique vesicles for storage of glycine. The fraction of neurotransmitter stores of glycine derived from intracellular metabolism as compared to the circulation is unknown. The reader is referred to Hockman and Bieger (1976) for a more detailed discussion of the excitatory and inhibitory neurotransmitters.

5.6. Acetylcholine

Neuronal systems using acetylcholine as a neurotransmitter have been among those more widely studied. Acetylcholine was the first neurotransmitter isolated by scientists. The precursor of this neurotransmitter is choline, which is derived either from dietary lecithin or from intracellular metabolism. Choline can be formed from ethanolamine, the decarboxylated product of serine, via a complex series of reactions. The relative normal contributions of endogenous or exogenous choline to the precursor pool is not established. It is known that choline loading can substantially enhance the level of choline within the brain.

The enzyme catalyzing the formation of acetylcholine has recently been extensively characterized. Choline acetyltransferase catalyzes a reaction between choline and acetyl-coenzyme A to yield the neurotransmitter. The enzyme is localized in cholinergic neurons. Kinetic characterization of choline acetyltransferase suggests that the K_m for choline is substantially higher than prevailing concentrations of the precursor. Thus, administration of choline should markedly increase the rate of acetylcholine synthesis and turnover. Although there is some evidence that levels of acetylcholine and functional activity of cholinergic neurons increase following choline administration, there remain many questions concerning the precise relationship between acetylcholine and neuronal activity. One of the major problems is that there are no unique metabolites of acetylcholine that can be used as markers for neurotransmitter turnover.

In contrast to the biosynthetic systems for catecholamines and serotonin discussed earlier, there appear to be no posttranslational modifications such as protein phosphorylation or proteolytic activation that regulate the catalytic state of choline acetyltransferase. A more detailed discussion of acetylcholine synthesis may be found in Blusztajn and Wurtman (1983).

5.7. Peptides

The number of known specific peptides that serve neuromodulator neurotransmitter or neurohumoral roles is expanding at a rapid rate, and a discussion of all the individual peptides is beyond the scope of this article. The detailed mechanisms controlling the synthesis of these peptides are not well understood, although general concepts concerning peptide synthesis are now emerging. The reader is referred to a recent volume (Krieger *et al.*, 1983).

It is clear that most, if not all, neuropeptides are derived from larger precursor proteins, which are translations of messenger RNA transcripts of specific genes. In many cases it appears that the precursor protein contains

the amino acid sequences for more than one biologically active peptide or multiple copies of a particular peptide. Because of these features, the proteins have been called polyproteins. The synthesis of peptides often begins with the hydrolysis of proteins by protease enzymes followed by sequential processing of peptides by specific peptidases.

Perhaps the best example of a widely studied polyprotein is proopiomelanocortin. This protein, found in certain cells of the pituitary, contains amino acid sequences for α, β, and γ melanocyte stimulating hormone, adrenal-corticotrophic hormone, β and γ lipotropin, corticotropin-like intermediate lobe peptide, and β-endorphin. Another example is proenkephalin, which is a protein molecule containing four sequences of Met-enkephalin and one each of Leu-enkephalin, Met-enkephalin Arg-Phe_1, and Met-enkephalin Arg-Gly-Leu. The biologically active peptides are generated by the action of specific proteases and peptidases. In many cases the biologically active sequence is encompassed by pairs of basic amino acids in the polyprotein, and it is thought that trypsinlike enzymes are responsible for the initial cleavage. The resulting peptide, which contains a terminal basic amino acid, is then "finished" by the action of a carboxypeptidase B-type enzyme.

The renin–angiotensin system originally described in renal tissue has now been shown to be present in brain. In this case, the proteolytic enzyme renin cleaves a precursor molecule angiotensinogen between two leucine residues to yield angiotensin I. This decapeptide is converted to angiotensin II by removal of His-Leu by a dipeptidyl carboxypeptidase call the angiotensin converting enzyme. It is the octapeptide that is the biologically active peptide in the periphery and possibly a neurotransmitter in brain.

Another important putative neurotransmitter is substance P. There are relatively well defined neuronal systems in the central nervous system that contain this 11-amino acid peptide. A group of Japanese scientists using recombinant DNA technology have been able to deduce the primary amino acid sequence of the precursor molecule. In this molecule they found the substance P sequence enclosed by pairs of basic residues. Of particular interest is the fact that the sequence for another biologically active amphibian peptide, kassinin, is also present in the precursor molecule. This sequence also is enclosed by pairs of basic residues. Thus while kassinin has yet to be found in brain, it can be predicted to be a naturally occurring neuropeptide. The use of cell biology techniques will clearly be of utmost importance as the mechanisms of neurotransmitter synthesis are unraveled.

6. Second Messengers

The consequences of transmitter interaction with specific membrane-bound recognition sites have been stressed throughout this chapter. In par-

ticular, direct alterations in the structure of the plasmalemma and subsequent alterations in ionic conductance have been noted. However, an equally important mechanism exists for changing ionic conductance; it is also the mode by which most cells, including neurons, react to membrane stimuli through the initiation of intracellular biochemical changes. This molecular mechanism by which cells respond to extracellular signals such as hormones or neurotransmitters to produce diverse metabolic and physiologic responses is through the production of second messengers. Second messengers are chemicals synthesized intracellularly subsequent to the interaction of an extracellular signal such as a hormone or transmitter with its recognition site. (The first messenger, of course, is the hormone or neurotransmitter that initiated the intracellular cascade.) The second messengers that will be discussed in this section are the cyclic nucleotides, calmodulin, and the prostaglandins.

6.1. Cyclic Nucleotides

The response of target neurons to a number of neurotransmitters, such as catecholamines, acetylcholine, histamine, serotonin, and peptides, appears to depend upon the formation of the cyclic nucleotides cyclic adenosine 3′,5′-monophosphate (cAMP) or guanosine 3′,5′-monophosphate (cGMP). The biochemical pathway for cAMP formation and the subsequent effects of the second messenger are depicted in Fig. 5. Both cyclic nucleotides are considered potent second messengers, although the body of evidence supporting this hypothesis is considerably stronger for cAMP. These cyclic nucleotides have been implicated in the secondary changes in membrane permeability to ions that are noted after transmitter–receptor interactions. They have also been implicated in the intracellular movement of synaptic vesicles and other organelles; carbohydrate, lipid, and glycoconjugate metabolism; neurotransmitter synthesis and release; and the structural development of neurons. It should be remembered that the cyclic nucleotides are only a link in what is apparently a very long chain of enzymatic processes. It would be safe to state, however, that the primary, if not sole, intracellular binding proteins for the cyclic nucleotides are specific protein kinases. The intracellular actions of cyclic nucleotides appear to be solely due to a change in the level of kinase activity and subsequent levels of specific phosphoproteins.

A number of criteria must be met to conclude that a cyclic nucleotide is involved in a postsynaptic event. First of all, electrical stimulation of the presynaptic neuron or microiontophoresis of the putative neurotransmitter at the synapse should increase the level of cyclic nucleotide. This increase should be blocked by receptor antagonists or low Ca^{2+}, which would prevent neurotransmitter release after electrical stimulation. Second, the pres-

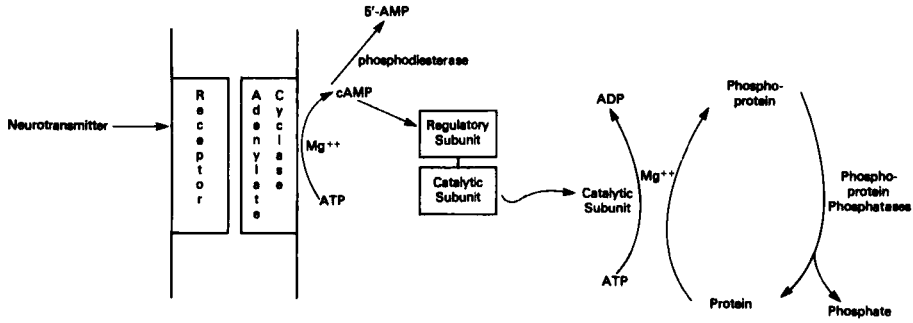


FIG. 5. Schematic representation of cAMP synthesis and degradation and effects on protein kinase regulatory and catalytic subunits.

ence of an adenylate or guanylate cyclase that can be stimulated by the appropriate neurotransmitter should be present in the postsynaptic membrane. The activation should be blocked by appropriate receptor antagonists. Third, application by iontophoresis of derivatives of cyclic nucleotides to which the neuronal membrane is permeable (i.e., dibutyryl and 8-bromo derivatives of cAMP or cGMP) should mimic the effects of electrical or chemical stimulation. Last, phosphodiesterase inhibitors should potentiate and prolong the effects of the cyclic nucleotides. It is often impossible to measure tissue levels of a cyclic nucleotide in the absence of a phosphodiesterase inhibitor because the turnover of the second messenger is so rapid that the cyclic nucleotide is synthesized and catabolized within seconds.

6.1.1. *Cyclic AMP*

Those neurotransmitters that elevate the intracellular level of cAMP are known to do so by stimulating a membrane-bound enzyme that is coupled to the neurotransmitter receptor. This enzyme, which some investigators believe is actually a subunit of a larger receptor–enzyme complex, is known as adenylate cyclase. Adenylate cyclase catalyzes the formation of cAMP from ATP. The enzyme has a K_m for ATP of 0.3–1.5 m*M* and requires the presence of a divalent cation, preferably Mg^{2+}. Calcium, by binding to another second messenger known as calmodulin, can exert potent regulatory actions on the enzyme, as can guanosine triphosphate, which is thought to regulate adenylate cyclase responsiveness to neurotransmitter receptor interactions by binding to a guanine nucleotide regulatory protein. It appears as though this latter protein is essential for the proper alignment and coupling of the receptor to adenylate cyclase. Adenylate cyclase is found in the highest concentration in synaptic regions of the brain. Norepinephine, dopamine,

serotonin, histamine, and a number of neuropeptides have been shown to stimulate adenylate cyclase activity in membrane preparations. Specific subtypes of neurotransmitter receptors appear to stimulate adenylate cyclase, such as β-adrenergic, D_1-dopaminergic, and H_2-histaminergic receptors, while others such as muscarinic, opiate, and α-adrenergic receptors have been shown to inhibit the enzyme.

Cyclic AMP is catabolized to 5′-adenosine monophosphate by the enzyme cyclic nucleotide phosphodiesterase, which terminates any further cAMP-initiated reactions. This enzyme also requires Mg^{2+} for activity. Calcium, again in consort with calmodulin, can stimulate phosphodiesterase activity. Phosphodiesterase appears to exist in multiple forms, each with specificity toward different substrates. Calcium and calmodulin activate only one form of the enzyme. The enzyme is potently inhibited by methyl xanthines, such as caffeine, theophylline, and theobromine. It is believed that at least part of the pharmacological effects of such compounds can be explained through their inhibition of phosphodiesterase and the consequent reduction in the catabolism of cAMP.

Once synthesized, cAMP exerts its activity by binding to another enzyme, cAMP-dependent protein kinase. Protein kinase allows the transfer of phosphate groups from ATP to specific protein substrates to occur. Cyclic AMP can stimulate the kinase 10–20-fold under ideal conditions. The K_m of the kinase for cAMP is approximately 0.3–0.5 μM, a concentration that is usually exceeded following adenylate cyclase stimulation. Cyclic-AMP-dependent protein kinase has been identified to exist as two isozymes (types I and II), each of which is composed of two different subunits: a *regulatory subunit* (to which cAMP binds) and a *catalytic subunit* (which allows for the transfer of phosphate from ATP to protein substrates). Four moles of cAMP bind to the inactive holoenzyme (a tetramer), which then uncouples to form a dimer regulatory subunit (with 4 mol of cAMP) and a now active dimer catalytic subunit. The two isozymes differ only in their regulatory subunits. The holoenzyme has a molecular weight of 170,000, while the native catalytic subunit and the native regulatory subunit (both dimers) have molecular weights of approximately 82,000 and 86,000, respectively. A specific inhibitor of the catalytic subunit, known as the *Walsh inhibitor*, has been purified to homogeneity from skeletal muscle. It has no effect on non-cAMP kinases and can therefore be used as a marker for this specific kinase.

Phosphoprotein phosphatases are responsible for the cleavage of phosphate groups from their protein acceptors. Some reports suggest that these enzymes can be inhibited by zinc. The actions of cAMP, then, can be seen to be terminated by two mechanisms: the breakdown of cAMP by phosphodiesterase and the removal of phosphate groups from phosphoproteins by phosphatases.

Phosphate groups are generally attached to acceptor proteins at serine residues, although under many conditions threonine can also act as an amino acid phosphate acceptor. The phosphate–serine/threonine bond is covalent. It is the identity of the protein substrates that are subjected to cAMP-dependent phosphorylation that determines the ultimate effect of an elevation in cAMP levels within a neuron. Phosphorylation of proteins by cAMP-dependent protein kinase displays high specificity. For instance, in the synaptic plasmalemma, when membrane-bound proteins are resolved using slab gel electrophoresis following *in vitro* or *in vivo* phosphorylation, it has been determined that only a few proteins (out of dozens) specifically demonstrated an increase in their phosphate content in the presence of cAMP. These studies not only suggest substrate specificity, but also vicinal localizations of the kinase and its substrates. Besides proteins in the plasmalemma, proteins in ribosomes, the Golgi apparatus, myelin, neurotubules, and synaptic vesicles have also been shown to display enhanced phosphate incorporation in the presence of cAMP. It is worth noting that in most cases the subcellular distribution of cAMP-dependent protein kinase, its protein substrates, and the phosphoprotein phosphatases have all been shown to be similar.

As might be noted in the preceding description, the specificity of neurotransmitter effects that are mediated by cAMP is determined at three basic levels. First of all, the nature of the neurotransmitter receptor will determine whether membrane-bound adenylate cyclase will be stimulated, inhibited, or left unaffected. Second, the nature, concentration, and intrinsic activity of the protein kinase and the phosphoprotein phosphatase will determine the pattern and degree of protein phosphorylation. Last, the identity and availability of the protein substrates will determine the ultimate biochemical consequences of cAMP elevations.

Cyclic AMP has been implicated in synaptic transmission due to its actions on a number of important synaptic and neuronal events, such as membrane permeability, synaptic membrane phosphorylation, neurotransmitter synthesis, and cell growth and differentiation. As pointed out earlier, neurotransmitter–receptor interactions can result in direct physical perturbations of the membrane with consequent alterations in membrane permeability to specific ions. This effect is particularly the case when the ionophore is located near the receptor. However, if the ionic channel is distant from the receptor, mechanisms such as phosphorylation can result in an alteration of channel permeability. Cyclic AMP is known to lead to a hyperpolarization of neurons in a number of brain regions such as the cerebral cortex, the caudate nucleus, the peripheral paravertebral sympathetic ganglia, the cerebellar cortex, and the hippocampus. Although it has been hypothesized that this hyperpolarization is the consequence of the phosphorylation of specific neuronal membrane proteins, the relatively short duration of hyperpolarization

has made it impossible to correlate changes in membrane permeability to the extent of membrane protein phosphorylation. The correlation has been made, however, in tissue where hyperpolarizations due to cAMP are considerably longer, such as heart, bladder epithelium, and erythrocytes.

Certain proteins that appear to be present only in the synaptic plasmalemma have been shown to be phosphorylated specifically by cAMP-dependent kinase. Termed proteins Ia and Ib, these proteins are rapidly phosphorylated (in less than 5 sec) by electrical stimulation of presynaptic fibers and by application of the neurotransmitter believed to be released by the presynaptic neuron. Depolarizing agents, such as potassium and veratridine, when applied to the postsynaptic fibers, cause an enormous increase in the phosphorylation of proteins Ia and Ib. Dibutyryl cAMP and 8-bromo cAMP both mimic the actions of the neurotransmitters, as do phosphodiesterase inhibitors. The function of these proteins is at present unknown.

Cyclic AMP can affect the synthesis of catecholamines by two separate modes of action on the rate-limiting catecholamine-synthesizing enzyme tyrosine hydroxylase. Cyclic AMP-dependent kinase can phosphorylate tyrosine hydroxylase, which in turn results in activation of the enzyme. Furthermore, in peripheral tissues such as the adrenal medulla, cAMP can result in the *de novo* synthesis of tyrosine hydroxylase by causing a derepression of gene expression due to the translocation of the catalytic subunit of the kinase to the chromaffin cell nucleus.

Other actions in which cAMP has been implicated in the brain but that are outside the scope of this chapter are neurotransmitter release, neuronal cell growth and differentiation, control of cerebral microvasculature resistance, long-term memory, vision, and the phosphorylation of microtubules.

6.1.2. *Cyclic GMP*

In general, a similar system to that described for cAMP exists for the formation and degradation of cGMP. However, notable exceptions are present. Less is known generally about cGMP than about cAMP, although it seems reasonably certain at this time that cGMP may mediate the actions of acetylcholine at muscarinic receptors and of histamine at H_1 receptors. With the exception of cerebellum, where cGMP levels are approximately equal to those for cAMP, all brain regions possessing cGMP have levels that are one-tenth those of cAMP. The enzyme that converts GTP to cGMP, guanylate cyclase, is, unlike adenylate cyclase, associated to a large degree with soluble fractions of brain. It is unclear, however, whether the enzyme is loosely integrated with the membrane matrix *in vivo* and is easily dissociated upon homogenization. Specific cGMP phosphodiesterases also exist, although a certain degree of cross-reactivity of cAMP and cGMP phosphodiesterases exists with their respective substrates. A specific cGMP-dependent protein

kinase has also been found to exist with its own unique protein substrates. (For instance, proteins Ia and Ib do not incorporate phosphate in the sole presence of cGMP kinase.) The cGMP kinase holoenzyme has a molecular weight of 150,000. In contrast to cAMP kinase, cGMP kinase is composed of two apparently identical subunits with molecular weights of approximately 75,000. Furthermore, it appears as though cGMP binding and catalytic activity reside on the same subunit polypeptide chain. The holoenzyme is therefore not uncoupled into subunits by the binding of cGMP.

Cyclic GMP levels can be elevated in cerebellum by nerve stimulation, acetylcholine, depolarizing agents, and phosphodiesterase inhibitors. In many cases, these elevations can be antagonized by GABA. The presence of cGMP in sympathetic ganglia has also been the subject of intense interest. There it appears to antagonize many of the actions of cAMP. In general, however, the role of cGMP in neuronal function has received considerably less attention than cAMP. The full extent of the involvement of either cAMP or cGMP in neuronal function is presently unknown, and the nature and degree of involvement should be an area of study in the future. An important finding will be to what extent phosphorylation of proteins and synaptic or neuronal actions in fact correlate.

Numerous reviews of cyclic nucleotide, protein phosphorylation, and neuronal function are available. These include Greengard (1976, 1978, 1979), Williams and Rodnight (1977), Nimmo and Cohen (1977), Bloom (1979b), Wilson (1980), Cohen (1982), and Nestler and Greengard (1983).

6.2. Calmodulin

Calmodulin is an acidic protein of 148 amino acids (16,700 Da) that appears to be one of the primary binding sites for free intracellular calcium. Interestingly, it contains approximately a 50% homology with the sequence of the muscle contractile protein troponin C. Its presence within cell membranes and in cytoplasm lends credence to the concept that Ca^{2+} can act as a second messenger in a manner not unlike cAMP. Ironically, calmodulin was first discovered not as an intracellular receptor for Ca^{2+}, but as an activator of a particular form of cyclic nucleotide phosphodiesterase. Since its discovery in 1970, however, many laboratories have directed their efforts toward describing the physiological role of calmodulin. The protein seems to be involved in the regulation of the intrinsic activity of a number of enzymes, in many cellular processes that are associated with cell motility and the structure of the cytoskeleton, and in the release of hormones and neurotransmitters from various cells. It seems that calmodulin is highly conserved throughout life forms and has been described in virtually all cell types studied.

Much of the current literature has been aimed at establishing that many of the known Ca^{2+}-dependent processes in muscle and nerve cells require calmodulin as an intermediate. Although such studies are relatively straightforward when purified enzyme preparations are employed, it is much more difficult to devise physiological experiments with intact tissue preparations that can conclusively demonstrate a calmodulin requirement for a particular Ca^{2+}-mediated process. Phenothiazines have been shown to bind to calmodulin and thereby decrease its affinity for Ca^{2+} and have thus been utilized as pharmacological tools to study the calmodulin dependence of a given cellular event. However, it is extremely difficult at this time to establish whether the antagonizing actions of these drugs are due solely to their interaction with calmodulin or whether other specific or nonspecific actions of the drugs could account for their effects. (For instance, it is well known that phenothiazines interact with dopaminergic and α-adrenergic receptors, possess general local anesthetic properties, and have antihistaminergic activity.)

Calmodulin can bind 4 mol of Ca^{2+} per mole protein. When binding commences, calmodulin becomes increasingly more hydrophobic and can only express activity in the bound form. The dissociation constant of calmodulin for Ca^{2+} is 2–3 μM. When bound to Ca^{2+}, calmodulin then has a variety of actions, of which only a few can be discussed in the interest of brevity. These effects include activation of adenylate cyclase and phosphodiesterase and enhanced turnover of cyclic nucleotides and the stimulation of noncyclic nucleotide-dependent protein phosphorylations. However, prior to discussing calmodulin's actions on cyclic nucleotide turnover and on protein phosphorylation, it is important to point out some of the other proposed actions of the protein, including its stimulation of neurotransmitter release (which may well be related to calmodulin-dependent phosphorylation), its enhancement of vesicle–plasmalemmal membrane interactions, and its activation of numerous other neuronal and nonneuronal enzymes (i.e., myosin light chain kinase, phosphorylase kinase, glycogen synthetase kinase, Ca^{2+}/Mg^{2+}-ATPase and tryptophan 5′-monooxygenase). It is also worth mentioning its profound effects on microtubule assembly and disassembly, on Ca^{2+} transport, and intestinal ion secretion. Reviews of calmodulin's actions on those processes, which, although of considerable interest, exceed the capacities of this article, can be found in Means and Dedman (1980), De Lorenzo (1980, 1981), Carafoli (1981), and Means *et al.* (1982). For those interested in the pharmacology of calmodulin antagonists or physicochemical studies on calmodulin structure, Vincenzi (1981) and Krebs (1981) are suggested. Reviews that include substantial sections on the adenylate cyclase–phosphodiesterase systems and calmodulin or on calmodulin-dependent protein phosphorylations can be found in Cheung (1981), Brostrom and Wolf (1981), and Stoclet (1981).

Calmodulin has been reported to stimulate membrane-bound adenylate cyclase in the presence of Ca^{2+}. Some reports suggest that Ca^{2+} is required for the stimulation of dopamine-dependent adenylate cyclase and that the sensitivity of adenylate cyclase to a neurotransmitter is regulated by the presence of membrane-bound calmodulin, which binds the Ca^{2+} required for dopamine stimulation of the enzyme. Furthermore, these reports present data that support the concept that cAMP-dependent phosphorylation of the plasma membrane leads to a release of membrane-bound calmodulin, thereby providing a feedback loop for the control of adenylate cyclase activity. The appearance of calmodulin in soluble fractions of the cell would also result in the activation of phosphodiesterase, which would further attenuate cAMP activity.

It has been stated earlier that only certain forms of phosphodiesterase can be activated by calmodulin. In particular, the "high-K_m" phosphodiesterase, which has a greater V_{max} and higher K_m for cAMP than for cGMP, seems to be particularly sensitive to calmodulin activation. The effect of calmodulin is to increase its V_{max} for the formation of 5'-AMP from cAMP. It has been hypothesized that the high-K_m phosphodiesterase, which is found to soluble compartments of the neuron and which has been purified to homogeneity, is present to catabolize cAMP that is some distance from the cell membrane and helps prevent increases in cAMP concentration away from the membrane. The "low-K_m" phosphodiesterase, which is membrane bound and presumably has immediate access to cAMP generated locally by adenylate cyclase, is also sensitive to calmodulin, but apparently has equal affinity for both cAMP and cGMP. It is worth noting at this point that the effects of calmodulin on cGMP turnover, both at the level of guanylate cyclase and cGMP phosphodiesterase, have been reported to be similar to those for cAMP, although, as is usually the case for cGMP systems, the evidence is not as well documented. Nevertheless, the role of calmodulin in regulating the turnover of cyclic nucleotides has been established to be important, although many aspects of calmodulin's regulation are still unclear. Considerable research is required to define precisely the interrelationships of these two second messengers.

In brain, a large number of particulate soluble proteins are phosphorylated in the presence of Mg^{2+}–ATP and Ca^{2+}–calmodulin. These phosphorylations can be blocked by phenothiazines and do not occur in the presence of cAMP or cGMP. Furthermore, they are unaltered by the Walsh inhibitor, which is specific for the cAMP catalytic subunit. Hence, investigators have concluded that there is present in both particulate and soluble fractions of brain a Ca^{2+}–calmodulin-dependent protein kinase that is insensitive to cyclic nucleotides and that contains its own set of protein substrates. A few reports have appeared in the literature claiming purification of a calcium–calmodulin-dependent kinase from mammalian brain. The en-

zyme is reported to have a number of subunits with a native molecular weight of 650,000. There appears to be general agreement that among its favored substrates are the microtubule-associated protein, myelin basic protein, and tubulin. The net effect of calmodulin-stimulated phosphorylation of these proteins is at present unknown.

An interesting corollary to Ca^{2+}–calmodulin actions can be noted here. Coupled to an increase in intracellular Ca^{2+} and subsequent binding to calmodulin, an enhanced breakdown of phosphatidylinositol in the neuronal plasmalemma has been observed by investigators. Although the increased phosphatidylinositol breakdown has been seen in a number of tissue preparations, its fundamental effect on membrane properties is presently unknown. However, a number of receptor types, including muscarinic, α-adrenergic, histaminergic, serotonergic, and peptidergic, have been demonstrated to accelerate phosphatidylinositol breakdown. Of further interest is the presence in the central nervous system of a noncyclic nucleotide-dependent, non–calmodulin-dependent protein kinase called C-kinase. This kinase is activated by phospholipids, including diacylglycerol, a breakdown product of phosphatidylinositol. Hence, neurotransmitter activation of Ca^{2+} influx might not only stimulate calmodulin-dependent kinase, but also activate C-kinase by the release from the membrane of metabolites in the phospholipid cascade. The reader is referred to Downes (1982) for further details on this potentially interesting relationship.

6.3. Prostaglandins

A large number of physiological and pathological stimuli can lead to the enhanced synthesis of a third group of second messengers, known as the prostaglandins. The prostaglandins derive their name from their original discovery in seminal plasma over 50 years ago. Since that time the purification and elucidation of their structure have indicated that they can be separated into three general classes: the primary prostaglandins comprised of the E series (due to solubility in ether) and the F series (due to their solubility in phosphate buffer), the prostacylin series, and the thromboxane series. It is known that the prostaglandins of both series are a family of unsaturated, oxygenated, 20-carbon cyclopentane carboxylic acids with a vast array of potent physiological actions. Studies on prostaglandins have been complicated by the fact that they do not accumulate intracellularly, but after synthesis and exerting activity, they are rapidly degraded to inactive metabolites. Nevertheless, in brain both classical neurotransmitters and neuropeptides have been shown to increase prostaglandin formation, which is thought to contribute to stimulus–secretion coupling and the regulation of synaptic transmission.

The synthesis of prostaglandins is a complex event due to the numerous

levels of control that can exert influence on their formation. The first step in their biosynthesis is the release of the precursor, arachidonic acid, from complex membrane lipids. Deacylase enzymes such as phospholipase A_2, diglyceridelipase, and cholesterol esterase are thought to be responsible for de-esterifying arachidonic acid from more complex lipid molecules. Following the release of arachidonic acid, a peroxyeicosatetraenoic acid intermediate is formed that is then rapidly converted to a cyclopentane derivative (PGG_2) by one or more enzymes termed fatty acid cyclooxygenases, which require heme as a cofactor. Intimately coupled to the cyclooxygenase is another heme-requiring enzyme, a peroxidase that reduces the PGG_2 substrate to the PG endoperoxide. Purification of these enzymes has been achieved only recently.

It is now a well-known fact that nonsteroidal anti-inflammatory agents such as acetylsalicylic acid, indomethacin, and ibuprofen are potent, irreversible inhibitors of fatty acid cyclooxygenase. Furthermore, significant evidence has appeared in the literature that indicates that steroidal anti-inflammatory agents may prevent the release of arachidonic acid from the more complex lipids to which it is normally attached.

The PG endoperoxide can be further processed by three metabolic routes: (1) the primary prostaglandin pathway, (2) the prostacyclin pathway, and (3) the thromboxane pathway. The two major prostaglandins that are synthesized in the primary pathway are $PGF_{2\alpha}$ and PGE_2. The major prostacyclin is PGI_2, and thromboxane A_2 is the principal thromboxane formed. As mentioned earlier, all the prostaglandins turn over rapidly and their metabolic products can be found in urine and feces.

Synthesis of prostaglandins (PGE_2 and $PGF_{2\alpha}$) and their release into cerebral ventricular fluid have been reported to occur in mammalian central nervous system tissue. Levels of prostaglandins tend to range from 0.1 to 2.5 μg/g tissue. Their presence has been reported in cerebral cortex, cerebellum, and hippocampus, and levels can be enhanced in the presence of norepinephrine and other catecholamines. Thromboxane synthesis has also been reported to occur. The trace amounts of prostacyclin are probably the result of contamination of brain preparations with vascular elements.

The rate-limiting step in prostaglandin synthesis appears to be the release of arachidonic acid from membranes. This process is affected by calcium fluxes across the membrane and by the rearrangement of phospholipid ratios in the membrane that were discussed earlier. It is therefore interesting that a single membrane interaction with a neurotransmitter can influence three different second messenger systems, all of which appear to interrelate. Adenylate or guanylate cyclase activation, calcium fluxes and calmodulin activation, phospholipid turnover, and arachidonic acid release can all occur within a few seconds of a receptor activation. Both cyclase and phosphodiesterase

activity as well as phospholipid turnover and arachidonic acid release can be influenced by calcium–calmodulin.

Prostaglandins have been associated with neurotransmitter release and synaptic regulation at specific nerve synapses. They have also been reported to influence cyclic nucleotide turnover and the release of anterior pituitary hormones. They may also be involved with fever and temperature regulation. Their participation in many other central nervous system functions as second messengers has also been documented, but in deference to brevity only the topics listed above will be briefly discussed.

Stimulation of specific neurons at autonomic ganglia of sympathetic or parasympathetic origin results in the postsynaptic increase in prostaglandin synthesis and release. This effect can be duplicated by application of the specific neurotransmitter in lieu of stimulation, and the enhanced synthesis can be blocked by the appropriate receptor antagonists. One hypothesis put forward suggests that released prostaglandins of the E series feed back on presynaptic sites to prevent the further release of neurotransmitter and thereby form a negative feedback loop. Although this hypothesis was first formulated from evidence collected in the peripheral organs such as heart, oviduct, and vas deferens, more recent evidence indicates that PGE_2 can also inhibit release of neurotransmitter from central neurons.

It has also been documented that prostaglandins of the E series stimulate adenylate cyclase in various brain regions. Prostaglandins can act synergistically with certain neurotransmitters to activate the cyclase. However, morphine and opioid peptides appear to attenuate the prostaglandin-induced increases in cAMP formation. Interestingly, prostaglandins of the F series are reported to stimulate guanylate cyclase activity.

Coupled to the actions of prostaglandins on cyclic nucleotide levels is the finding that microinjections of prostaglandins can stimulate the release of hypophyseal hormones such as follicle-stimulating hormone, luteinizing hormone, and adrenocorticotropic hormone, presumably by elevating the levels of these hormones' respective releasing factors (i.e., FSH-releasing hormone, LH-releasing hormone, corticotropin-releasing hormone) in the hypothalamus. Changes in the production of cAMP in the hypothalamus have been linked to the increased synthesis of pituitary releasing hormones. Furthermore, inhibitors of prostaglandin synthesis, such as acetylsalicylic acid or indomethacin, have been shown to decrease the secretion of hypophyseal hormones.

Last, an additional hypothalamic action of prostaglandins may be the regulation of body temperature. Prostaglandins may be especially important in the etiology of fever. E-series prostaglandins, injected intraventricularly, cause a large increase in core temperature. This effect can be blocked by cyclooxygenase inhibitors, and it is well known in man that acetylsalicylic

acid is an effective antipyretic. It has also been reported that prostaglandin levels are elevated in cerebrospinal fluid in patients with elevated body temperatures. Of present interest is whether prostaglandins enhance the synthesis of endogenous pyrogens in hypothalamic areas.

The area of prostaglandin biochemistry and physiology has attracted considerable attention over the past two decades, especially in the pharmaceutical industries, where various antagonists of the synthesis or actions of these lipids are being tested yearly. A true appreciation of their complex actions is, unfortunately, beyond the scope of the present chapter, but the reader is referred to any of the following reviews: Bergstrom *et al.* (1968), Coceani (1974), Brody and Kadowitz (1974), Samuelsson *et al.* (1978), Lands (1979), and Wolfe and Coceani (1979).

Acknowledgment

The authors are indebted to Ms. Theresa Wilson for her patience and diligence in the preparation and typing of this manuscript.

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CLINICAL AND METABOLIC ASPECTS OF SULFOHYDROLASES IN MAN

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1. Introduction

Sulfohydrolases catalyze the hydrolysis of sulfate esters ($R{\cdot}O{\cdot}SO_3^-$) according to the following reaction, where R·OH can be an alcohol, a phenol, a carbohydrate, or a steroid:

$$R{\cdot}O{\cdot}SO_3 + H_2O \rightarrow R{\cdot}OH + H^+ + SO_4^{2-}$$

The best known of these enzymes are the aryl-sulfate sulfohydrolases (EC 3.1.6.1), which catalyze the hydrolysis of a variety of arylsulfates and have been detected in most animal tissues, bacteria (Dodgson and Rose, 1975; Roy, 1976), and plants (Farooqui *et al.*, 1977).

For many years arylsulfate sulfohydrolases did not attract attention because it was difficult to understand their physiological function. Mammals produce and excrete arylsulfates as a part of their detoxification mechanisms against phenolic compounds. Once formed, the subsequent hydrolysis of arylsuflates by sulfohydrolases seems to be pointless and would only reexpose the animal to the toxicity (Dodgson and Rose, 1975). Further it was suggested (Roy, 1960) that these enzymes may not be active *in vivo* because they would be inhibited by normal concentrations of phosphate and chloride ions. However, the studies of James Austin and his associates (1963, 1965) in the mid-1960s indicated the involvement of these enzymes in several human genetic disorders. Our present knowledge of the enzymatic properties and physiological role of sulfohydrolases has been acquired only in the past 20 years. The aim of the present review article is to provide a cumulative overview of the properties and role of sulfohydrolases to a wider audience and to stimulate further studies on the enzymology and treatment of human genetic disorders caused by deficiencies of the sulfohydrolases.

2. Classification

Aryl-sulfate sulfohydrolases have been classified on the basis of their cellular location, substrate specificities, physicochemical properties, and the effect of certain reagents on the enzymatic reaction constants (Dodgson and Rose, 1975). Two major types of aryl-sulfate sulfohydrolases occur in vertebrates. Type I enzyme, the microsomal aryl-sulfate sulfohydrolase C, is insoluble and thus seldom detectable in body fluids. This enzyme shows a high activity toward such synthetic sulfate esters as *p*-nitrophenyl, acetylphenyl, and phenyl sulfates. Aryl-sulfate sulfohydrolase C is inhibited by cyanide but not by phosphate or sulfate ions. Type II enzymes, aryl-sulfate sulfohydrolases A and B, have been detected in most vertebrates (Roy, 1958; Farooqui and Bachhawat, 1971) and invertebrates (Mraz and Jatzkewitz, 1974). They are much more active toward *p*-nitrocatechol sulfate than toward *p*-nitrophenyl sulfate and are strongly inhibited by phosphate and sulfate ions. Besides these enzymes, human tissues also contain several other sulfohydrolases that hydrolyze a variety of carbohydrate sulfate esters. In the following discussion we will first consider the physicochemical and kinetic properties of all sulfohydrolases and then discuss their association with various human genetic disorders.

3. Physicochemical and Kinetic Properties of Sulfohydrolases

3.1. Arylsulfohydrolase A

Homogeneous preparations of this enzyme have been prepared from several human tissues (Breslow and Sloan, 1972; Shapira and Nadler, 1975a; Stevens *et al.*, 1975; Draper *et al.*, 1976; Farooqui, 1976a; Luijten *et al.*, 1978; James and Austin, 1979; Laidler *et al.*, 1985). The purified human arylsulfohydrolase A is a lysosomal acidic glycoprotein with an isoelectric point at pH 4.6 (Table 1). The human placental arylsulfohydrolase A exists as a monomer or tetramer, depending on the protein concentration, pH value, and ionic strength of the medium. The monomer and tetramer of human arylsulfohydrolase A have molecular weights of 102,000 and 400,000, respectively (Farooqui, 1976a). The carbohydrate composition of human arylsulfohydrolase A is not known, but the human placental enzyme contains 15% neutral carbohydrate and 0.6% *N*-acetylneuraminic acid (A. A. Farooqui, unpublished). The ox liver, rabbit testicular, and sheep brain arylsulfohydrolases A contain 10, 20, and 25% carbohydrate (Graham and Roy, 1973; Balasubramanian and Bachhawat, 1975; Farooqui and Srivastava, 1979).

TABLE 1
PHYSICOCHEMICAL PROPERTIES OF HUMAN ARYLSULFOHYDROLASES

Property	Arylsulfohydrolase A	Arylsulfohydrolase B	Arylsulfohydrolase C
Nature	Glycoprotein	Glycoprotein	Glycoprotein
Subcellular location	Lysosome	Lysosome	Microsome
Isoelectric point	4.6	8.0	6.5
Molecular weight	102,000	48,000	166,000
$A^{1\%}_{280}$	6.6	17.0	—
Effects of sulfate ions	Competitive inhibition	Noncompetitive inhibition	No effect
Sedimentation coefficient	6.5	4.5	5.4
Diffusion coefficient	4.9×10^{-7}	6.6×10^{-7}	—

The glycoprotein nature of human arylsulfohydrolase A is also reflected in its rather strong binding to concanavalin A Sepharose, a property that has allowed the efficient purification of this enzyme from various sources (Bishayee *et al.*, 1973; Balasubramanian and Bachhawat, 1975; Helwig *et al.*, 1977; Farooqui and Srivastava, 1979; Sarafian *et al.*, 1982; Laidler *et al.*, 1985). The amino acid composition of human urine and liver arylsulfohydrolase A is known (Breslow and Sloan, 1972; Draper *et al.*, 1976; Laidler *et al.*, 1985). The enzyme contains a rather high content of hydrophobic amino acid residues (leucine, isoleucine, tyrosine, phenylalanine) and proline, which are probably responsible for the relative instability at 0°C or below and the greater stability at elevated temperatures (Graham and Roy, 1973; Waheed and Van Etten, 1980a). According to Waheed and Van Etten (1980a), the relatively low content of α-helix and the higher amounts of β-sheet and unordered structure in rabbit liver arylsulfohydrolase A can be attributed to the high content of proline. A relatively high content of glutamic and aspartic acids is reflected in the low isoelectric point of this enzyme. Studies from several laboratories have indicated that arylsulfohydrolase A has essential histidine (Jerfy and Roy, 1974; Lee and Van Etten, 1975) and arginine residues (James, 1979) in its active site.

The removal of neuraminyl residues from pure arylsulfohydrolase A by bacterial neuraminidase does not alter the specific activity, kinetic properties, and general stability but does change the elution profile through a DEAE-cellulose column (Fig. 1) and the electrophoretic mobility on a polyacrylamide gel (Graham and Roy, 1973; Farooqui and Srivastava, 1979). Das and Bishayee (1980) claimed that the treatment of partially purified sheep brain arylsulfohydrolase A with bacterial neuraminidase did not cause a

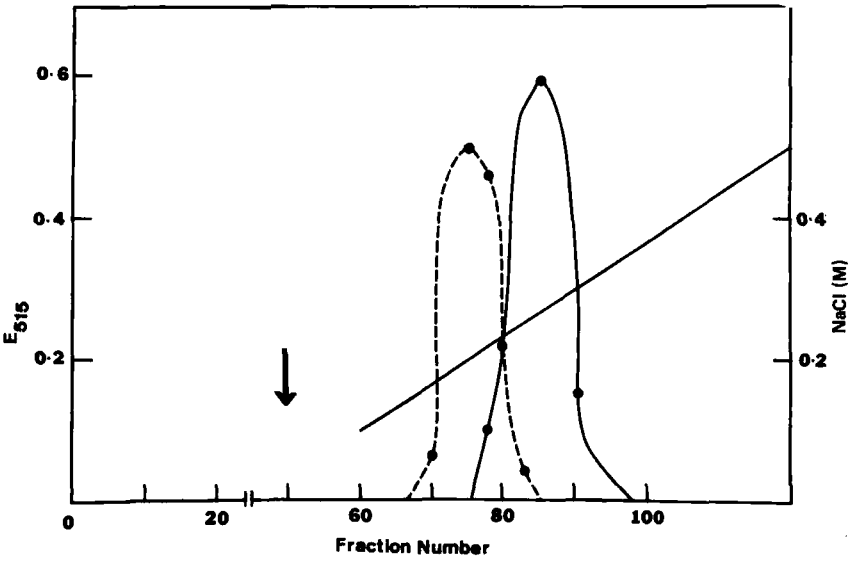

FIG. 1. Elution profile of arylsulfohydrolase A through a DEAE-cellulose collumn (1.5 × 40 cm). Native arylsulfohydrolase A (——) and neuraminidase-treated arylsulfohydrolase A (- - - -).

significant decrease in electrophoretic mobility of this enzyme. But the treatment of sheep brain arylsulfohydrolase A with alkaline phosphatase significantly reduced the electrophoretic mobility. Sarafian *et al.* (1984) have treated arylsulfohydrolase A with neuraminidase, alkaline phosphatase, and other glycohydrolases (α-*N*-acetylglucosaminidase, endoglycosidase D, and endoglycosidase H). Only neuraminidase treatment changes the electrophoretic mobility of arylsulfohydrolase A, indicating the involvement of neuraminyl residues in the heterogeneity of this enzyme. Treatments with other enzymes had no effect on arylsulfohydrolase A, implying that neuraminidase-resistant charge microheterogeneity is not due to a structure associated with the carbohydrate moiety of this enzyme.

The kinetics of arylsulfohydrolase A are quite complex, because the reaction velocity shows an abnormal relationship with the enzyme concentration and time of incubation (Roy, 1953; Baum and Dodgson, 1958) (Fig. 2). The anomalous reaction kinetics of arylsulfohydrolase A are manifested as a time-dependent decrease in hydrolytic rate during incubation with *p*-nitrocatechol sulfate, followed by a partial recovery of the initial rate. Baum and Dodgson (1958) have proposed that "during the interaction of enzyme with substrate a new site capable of binding with substrate as well as the reaction product (nitrocatechol and sulfate) and certain other inhibitory compounds (phosphate

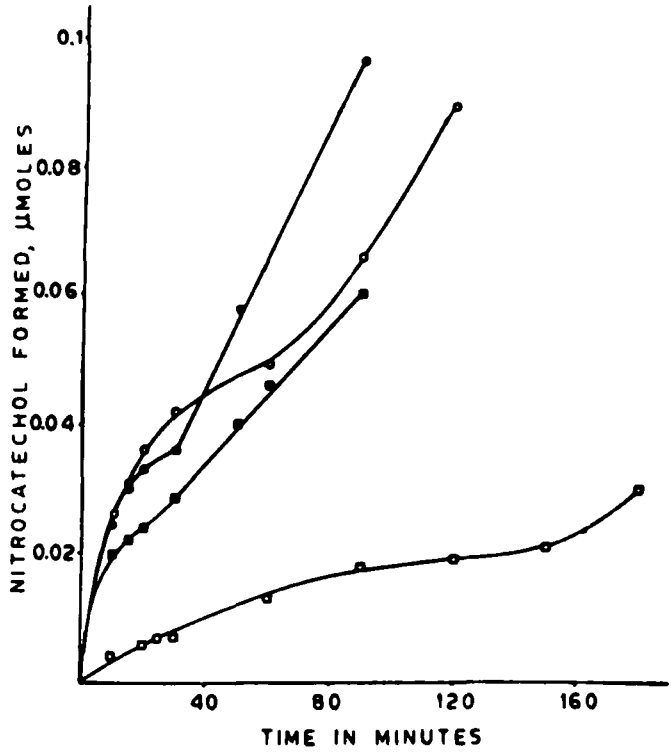

FIG. 2. Anomalous time-activity curves of arylsulfohydrolases A of various animal species. Rat brain, ●; monkey brain, ○; sheep brain, ■; and chicken brain, □. [Modified from Farooqui and Bachhawat (1971).]

and pyrophosphate) is slowly exposed in the enzyme. When the substrate is bound to the new site of the modified enzyme the latter is virtually inactive. On the other hand when the reaction products or other inhibitory compound are bound to the new site, the modified enzyme is active, although the active center may still be inhibited if these compounds are present in excess." This hypothesis was recently confirmed by several investigators (Nicholls and Roy, 1971; Waheed and Van Etten, 1980b; Prosser and Roy, 1980), who actually isolated the so-called inactive modified enzyme from the incubation mixture. According to Waheed and Van Etten (1980b), inactivation of arylsulfohydrolase A during hydrolysis of ^{35}S-labeled substrate at pH values near the pH optimum (pH 5–6) is accompanied by the incorporation of radioactivity into the protein molecule. The stoichiometry of the incorporation of radioactivity corresponds to 2 g atom of sulfur per mole of enzyme monomer or 1 g atom per equivalent peptide chain. No comment was made

about the nature of the bound sulfate in the modified enzyme. Prosser and Roy (1980) suggested that sulfate is esterified with hydroxy amino acids (tyrosine, serine, or threonine) as a sulfate ester ($R{\cdot}O{\cdot}SO_3^-$) or attached to ring nitrogen of histidine as a sulfamate ($R{\cdot}NH{\cdot}SO_3$) in modified enzymes. Here it should be recalled that arylsulfohydrolase A has an essential histidyl residue in its active site that can be modified by diazotization and carboxymethylation (Jerfy and Roy, 1974; Lee and Van Etten, 1975). Waheed and Van Etten (1986) indicated that rabbit liver arylsulfohydrolase A contains two free sulfhydryl groups. The chemical modification of these groups does not alter the polymerization property of arylsulfohydrolase A. Roy (1985a,b) proposed a new explanation for the abnormal kinetics of arylsulfohydrolase A. According to him, the abnormal kinetics of this enzyme can be explained by hysteretic effects arising through interaction of the enzyme with its substrate and one of the reaction products, sulfate, which behaves as an activator of the modified enzyme.

The kinetic parameters of arylsulfohydrolase A with *p*-nitrocatechol sulfate are shown in Table 2. Optimal activity is at pH 5.0 with a K_m value of 0.4 m*M*. This enzyme is strongly inhibited by sulfate, sulfite, and phosphate ions with K_i values of 0.052, 0.026, and 0.034 m*M*, respectively (Table 3). Thus sulfite is the most potent inhibitor, followed by phosphate and sulfate. Such a powerful inhibition by sulfite suggests that this ion may be a transition-state analog intermediate of the arylsulfohydrolase A catalyzed reaction. This enzyme is also strongly inhibited by ascorbic acid 2-phosphate (Carlson *et al.*, 1976). The inhibition by ascorbic acid 2-phosphate is competitive with a K_i value of 0.3 μ*M*. This metabolite binds very tightly to the enzyme molecule and may serve as controlling metabolite for arylsulfohydrolases.

TABLE 2
KINETIC PARAMETERS OF HUMAN ARYLSULFOHYDROLASE A WITH DIFFERENT SUBSTRATES[a]

Substrate	pH optimum	K_m value (m*M*)	V_{max} (μmol/min/mg protein)
p-Nitrocatechol sulfate	5.5	0.40	160
Ascorbic acid 2-sulfate	4.8	2.8	85
Tyrosine *O*-sulfate	5.5	35.0	6.6
Cyclic AMP	4.3	11.6	0.07
Sulfogalactosylceramide	4.5	0.15	6.6
Sulfogalactosylalkylacylglycerol	4.5	0.18	5.0
Sulfogalactosylsphingosine	4.5	0.20	3.0
Sulfolactosylceramide	4.5	0.18	4.0

[a] Modifed from Farooqui (1976a) and Farooqui (1981).

TABLE 3
KINETIC PROPERTIES OF FREE AND CON A IMMOBILIZED ARYLSULFOHYDROLASES A AND B[a]

Kinetic parameter	Arylsulfohydrolase A		Arylsulfohydrolase B	
	Free	Immobilized	Free	Immobilized
pH optimum	5.5	5.25	5.4	5.0
K_m value (m*M*)	0.77	2.20	2.5	7.5
V_{max} (μmol/min/mg protein)	95.0	93.0	85.0	89.0
K_i (m*M*) for sulfite	0.026	0.027	—	—
K_i (m*M*) for phosphate	0.034	0.066	—	—
K_i (m*M*) for sulfate	0.052	0.137	1.55	5.0

[a] Modified from Farooqui and Srivastava (1981).

In addition to aryl-sulfates, human arylsulfohydrolase A hydrolyzes sulfate groups from several sulfate esters, including cerebroside 3-sulfate (Mehl and Jatzkewitz, 1968; Porter *et al.*, 1972; Farooqui and Bachhawat, 1972, 1973; Jerfy and Roy, 1973; Stevens *et al.*, 1975), seminolipid (Fluharty *et al.*, 1974; Farooqui, 1976a), psychosine sulfate (Eto *et al.*, 1974a,b; Farooqui, 1976a), ascorbic acid 2-sulfate (Roy, 1975; Fluharty *et al.*, 1976; Carlson *et al.*, 1977; Farooqui and Srivastava, 1979), and tyrosine *O*-sulfate (Fluharty *et al.*, 1979a). The kinetic properties of human placental arylsulfohydrolase A with different sulfate esters are shown in Table 2. An interesting aspect of the hydrolysis of sulfolipids by arylsulfohydrolase A is the requirement for the heat-stable factor that activates the hydrolysis of sulfolipids several fold without having any effect on *p*-nitrocatechol sulfatase activity (Mehl and Jatzkewitz, 1965, 1968). This heat-stable protein has been renamed activator protein and has been purified to homogeneity from human liver. The purified activator is a glycoprotein with a molecular weight of 21,500 and an isoelectric point at pH 4.3 (Fischer and Jatzkewitz, 1975; Mitsuyama *et al.*, 1985).

The mechanism of action of this activator has not been established, but it is known (Fischer and Jatzkewitz, 1978; Mitsuyama *et al.*, 1985) that the activator forms an equimolar complex with cerebroside 3-sulfate prior to hydrolysis by arylsulfohydrolase A. Similar heat-stable activator proteins are also known for other acid hydrolases (Li and Li, 1983; Inui and Wenger, 1983; Wenger and Inui, 1984; Conzelmann *et al.*, 1982; Christomanou and Kleinschmidt, 1985; Burg *et al.*, 1985). It is becoming increasingly evident that many lysosomal hydrolases have specific activators, although some of these activators may be common to more than one enzyme (Li and Li, 1983, 1984; Li *et al.*, 1985). The desulfation of cerebroside 3-sulfate also occurs in the absence of activator protein. In these experiments, Tween-20 or sodium

taurodeoxycholate is used for solubilizing the sulfolipids (Farooqui and Bachhawat, 1972, 1973; Porter *et al.*, 1972; Jerfy and Roy, 1973). According to Jatzkewitz (1978), the activator protein acts as a detergent and solubilizes the sulfolipids *in vitro* and *in vivo*. Studies also indicate that arylsulfatase A hydrolyzes cyclic AMP to 5'-AMP (Uchida *et al.*, 1981; Farooqui and Lujan, unpublished). The activity of arylsulfohydrolase A with cyclic AMP is quite low when compared to the activity with other physiological substrates (Table 2).

As with *p*-nitrocatechol sulfate, arylsulfohydrolase A becomes inactivated (forms a modified enzyme) when acting on sulfolipids and ascorbic acid 2-sulfate (Roy, 1976), but no reactivation by sulfate or phosphate has been reported. It remains to be seen whether this substrate-induced inactivation has any physiological significance in sulfate metabolism.

Arylsulfohydrolase A forms an enzymatically active insoluble complex with a plant lectin, concanavalin A (Con A), at a high salt concentration, where electrostatic interactions are minimal (Bishayee *et al.*, 1973; Bishayee and Bachhawat, 1974; Farooqui and Srivastava, 1981). This insoluble complex can be isolated by centrifugation. Con A-immobilized enzyme has greater thermostability (Ahmad *et al.*, 1973; Farooqui and Srivastava, 1981) and displays a broad pH optimum between pH 3.5 and 4.5. Immobilization on Con A also induces changes in the affinity of substrate with arylsulfohydrolase A. The kinetic properties of free and immobilized arylsulfohydrolase A are shown in Table 3.

Waheed *et al.* (1982a) studied the biosynthesis of arylsulfohydrolase A in human fibroblasts and reported that this enzyme is synthesized as a precursor protein with a mean apparent molecular mass of 62,000. Intracellularly this precursor protein is converted into a 60,500 M_r polypeptide within a period of 1 to 7 days. The 60,500 M_r product in polyacrylamide gels corresponds to one of the two polypeptides present in human placenta arylsulfohydrolase A. In human fibroblasts, arylsulfohydrolase A has an unusually long half-life of 2 months (Waheed *et al.*, 1982b). Confirmation of this work has been made by Bach and Neufeld (1983).

3.2. Arylsulfohydrolase B

Human arylsulfohydrolase B is a lysosomal glycoprotein with a slightly basic isoelectric point of 8.0 (Shapira and Nadler, 1975a; McGovern *et al.*, 1982). It can be clearly distinguished from arylsulfohydrolase A by its lower molecular weight and higher isoelectric point (Table 2). This enzyme has been obtained in a homogeneous state from human liver, placenta, and eosinophils (Shapira and Nadler, 1975b; Gniot-Szulzycka, 1972; Agogbua and Wynn, 1976; McGovern *et al.*, 1982; Weller and Austen, 1983), ox brain

(Bleszynski and Roy, 1973), and ox liver (Farooqui and Roy, 1976). Human liver arylsulfohydrolase B has a molecular weight of 48,000 and exists in multiple forms at least in ox tissues (Farooqui and Roy, 1976; Bleszynski and Roy, 1973), human placenta (Gniot-Szulzycka, 1972), human brain, and human liver (Harzer *et al.*, 1973). These multiple forms (arylsulfohydrolases B_1, B_2, and B_3) can be separated from each other by ion exchange chromatographic and electrophoretic methods (Bleszynski *et al.*, 1969; Gniot-Szulzycka, 1972). The origin and significance of the occurrence of these multiple forms is not clear, but the treatment of arylsulfohydrolase B_2 and B_3 forms with bacterial neuraminidase results in the formation of arylsulfohydrolase B_1 (Fig. 3). Arylsulfohydrolases B_1, B_2, and B_3 can be separated into α and β forms by pH gradient CM-Sephadex chromatography (Farooqui and Roy, 1976; Farooqui, 1976b). The relation between the α and β forms of

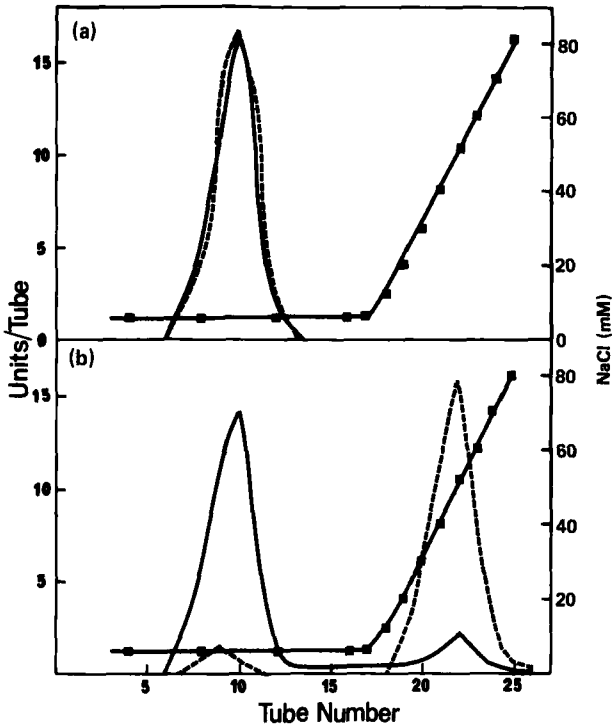

FIG. 3. Elution profiles of arylsulfohydrolases B_1 and B_2 through DEAE-cellulose column. (a) After treatment with neuraminidase and (b) before treatment with neuraminidase. Arylsulfohydrolase B_1 (——), arylsulfohydrolase B_2 (- - - -), and sodium chloride (—■—). [Modified from Farooqui (1976b).]

arylsulfohydrolase B is not known. However, it has been suggested that the carbohydrate composition may be responsible for this heterogeneity (Farooqui and Roy, 1976). The carbohydrate composition of arylsulfohydrolase B is not known. However, the hydrolysate of this enzyme contains glucosamine (2–3 residues/mol) (Farooqui and Roy, 1976). Furthermore, arylsulfohydrolase B is strongly absorbed on a concanavalin A-Sepharose column and can be eluted with methyl α-D-mannoside. These observations strongly support the view that arylsulfohydrolase B is a glycoprotein. Human liver arylsulfohydrolase B is strongly inhibited by iodoacetate (Agogbua and Wynn, 1976) and by a variety of phosphate esters derived from amino acids, amines, and simple sugars (Rao and Christe, 1984). This enzyme is also inactivated by freezing, but the lost enzymic activity can be restored completely by rewarming at 37°C for 30 minutes. Long storage of arylsulfohydrolase B at −20°C results in irreversible inactivation (Agogbua and Wynn, 1976). SDS-gel electrophoresis has indicated that human eosinophil sulfohydrolase B is composed of four subunits of 15,000 M_r (monomer). The tetrameric form seems to be the most active species (Weller and Austen, 1983).

Lakshmi and Balasubramanian (1980) showed the presence of a new multiple form of arylsulfohydrolase B in human and monkey brain. Arylsulfohydrolase B_m can be separated by DEAE-cellulose chromatography (Mathew and Balasubramanian, 1984). The B_m form totally binds to Sephadex G-200 and was not eluted with 1.0 *M* NaCl, 0.5 *M* glucose, 0.5 *M* glucose plus 0.5 *M* NaCl, 0.5 *M* KSCN, 1 *M* urea, or 1% Triton X-100. The treatment of arylsulfohydrolase B_m with *Escherichia coli* alkaline phosphatase results in the formation of a less acidic form, presumably due to dephosphorylation. The dephosphorylated form does not bind to DEAE-cellulose. Inorganic phosphate and serine phosphate but not mannose 6-phosphate can inhibit this dephosphorylation. The kinetic properties of the phosphorylated and dephosphorylated arylsulfohydrolase B_m are quite similar. The possibility that arylsulfohydrolase B is a dephosphorylated form of B_m has been ruled out by the significant differences between substrate concentration and activity curves of these enzymes.

The amino acid composition of human arylsulfohydrolase B is known (McGovern *et al.*, 1982). The enzyme contains a low amount of methionine and cysteine. The amino acid compositions of human and cat liver arylsulfohydrolases B differ remarkably from those from ox brain (Bleszynski and Roy, 1975) and liver (Farooqui and Roy, 1976). The latter two contain rather large amounts of proline, leucine, glycine, and tyrosine. Relative to arylsulfohydrolase A, the arylsulfohydrolases B from ox brain and liver contain a higher proportion of basic amino acids, accounting for their higher

isoelectric point. Arylsulfohydrolase B also has large amounts of tyrosine compared to arylsulfohydrolase A, which is probably responsible for the higher extinction coefficient of the former. Similarly to arylsulfohydrolase A, ox liver arylsulfohydrolase B also has an essential histidyl residue in its active site (Farooqui, 1976c). The sedimentation and diffusion coefficients of arylsulfohydrolase B are 4.5 S and 6.6 $\times$ 10^{-7} cm^2/second, respectively (Table 1).

The kinetic properties of these multiple forms are quite similar (Farooqui, 1976b). With *p*-nitrocatechol sulfate as substrate, arylsulfohydrolase B has a K_m value at least three times higher than that of arylsulfohydrolase A. Sulfate ions produce a noncompetitive inhibition of this enzyme with a K_i value of 1.2 m*M*. Weller *et al.* (1986) reported that purified preparation of eosinophil arylsulfohydrolase B is strongly inhibited by nanomolar concentrations of leukotrienes C_4, D_4, and E_4 but not by leukotriene B_4 and isomeric 5,12-dihydroxyeicosatetraenoic acid. This indicates a requirement of a thiopeptide at C-6. The authors (Weller *et al.*, 1986) suggest that sulfidopeptide leukotrienes and their sulfoxide derivatives may be involved in regulation of arylsulfohydrolase B activity. Besides *p*-nitrocatechol sulfate, arylsulfohydrolase B also hydrolyzes UDP-*N*-acetylgalactosamine 4-sulfate (Fluharty *et al.*, 1975; Farooqui and Roy, 1976; Farooqui, 1976d; Fluharty *et al.*, 1982; McGovern *et al.*, 1982) (Table 4). The rate of hydrolysis of UDP-*N*-acetylgalactosamine 4-sulfate is 200 times lower than the rate for *p*-nitrocatechol sulfate (McGovern et al., 1982). For ox liver and rabbit kidney arylsulfohydrolases B this factor is 1300 and 900 times less than the synthetic substrate (Farooqui and Roy, 1976, Helwig *et al.*, 1977). In addition to UDP-*N*-acetylgalactosamine 4-sulfate, homogeneous preparations of arylsulfohydrolase B can also hydrolyze glucosamine 4,6-disulfate but not glucosamine 6-sulfate (Farooqui, 1976a). Gorham and Cantz (1978) have indicated that purified human kidney arylsulfohydrolase B acts as an exosulfohydrolase against tetrasaccharide prepared from chondroitin 4-sulfate.

The biosynthesis of arylsulfohydrolase B has been studied in hamster and human skin fibroblasts (Warburton and Wynn, 1976; Steckel *et al.*, 1983). Pulse-chase labeling and uptake studies have indicated that arylsulfohydrolase B is synthesized and secreted as a 64,000 M_r precursor. This precursor is processed within 24 hours via short-lived intermediates to two different forms. Form I (chains of 47,000 and 11,500 M_r) is labeled first and is twice as stable as form II (chains of 40,000 and 31,000 M_r) (Steckel *et al.*, 1983). Arylsulfohydrolase B activity is associated with the 64,000 M_r precursor and with form I but not with form II. The precursor (64,000 M_r) and the 40,000 M_r chain of form II contain oligosaccharide units that are resistant to endo-β-*N*-acetylglucosaminidase H. This suggests that, like other lysosomal

TABLE 4
RATE OF HYDROLYSIS OF VARIOUS HEXOSAMINE SULFATES BY ARYLSULFOHYDROLASE B[a]

Substrate	Sulfate formed (nmol/min/mg protein)
UDP-*N*-acetylgalactosamine 4-sulfate	95
Carbobenzoxyglucosamine 4,6-disulfate	26
Carbobenzoxygalactosamine 6-sulfate	0
Glucosamine 4,6-disulfate	35
Galactosamine 6-sulfate	0
p-Nitrocatechol sulfate	130,000[b]

[a] Modified from Farooqui (1976c).
[b] nmol 4-nitrocatechol formed/min/mg protein.

hydrolases, arylsulfohydrolase B is synthesized as a large precursor and subsequently processed to the native enzyme by limited proteolysis (Hasilik, 1980).

3.3. ARYLSULFOHYDROLASE C

This enzyme was discovered in rat liver microsomes by Dodgson *et al.* (1954) and then described in microsomal fraction of human tissues (Dodgson *et al.*, 1956; French and Warren, 1967; Perumal and Robins, 1973). Steroid sulfohydrolases that resemble arylsulfohydrolase C in many properties (Dolly *et al.*, 1972; Balasubramanian, 1976; Chang *et al.*, 1985) are also found in microsomal fractions. It has been suggested that two separate enzymes are responsible for the hydrolysis of *p*-nitrophenyl sulfate and estrone sulfate in human placenta (French and Warren, 1967). On the basis of pH optima, thermal stability, inhibition characteristics, and comigration on isoelectric focusing, Dolly *et al.* (1972) and Iwamori *et al.* (1976) concluded that the hydrolysis of *p*-acetylphenyl sulfate, *p*-nitrophenyl sulfate, and estrone sulfate by microsomal and solubilized microsomal preparations can only be attributed to arylsulfohydrolase C, but this enzyme was different from dehydroepiandrosterone sulfohydrolase, which also occurs in microsomal fractions. Mathew and Balasubramanian (1982) reexamined this controversy by treating their enzyme preparation with Russel's viper phospholipase A. This completely inactivates estrone sulfate sulfohydrolase without affecting arylsulfohydrolase C. The latter is partially inactivated by *Staphylococcus aureus* (Cowan strain) phospholipase C. This observation suggests that the lipid environments around the catalytic site of these enzymes are different. Further hydrophobic chromatography on phenyl-Sepharose results in the separation of arylsulfohydrolase C and estrone sulfohydrolase activities. This

suggests that arylsulfohydrolase C and estrone sulfate sulfohydrolase are different enzymes. Further studies are needed to identify the topological distribution of arylsulfohydrolase C and estrone sulfate sulfohydrolase in microsomal membranes. Monospecific antibodies against human placental steroid sulfatase have been prepared (Van Der Loos *et al.*, 1984). These antibodies can now be used for studying differences in the properties and localization of arylsulfatase C and steroid sulfatases.

Arylsulfohydrolase C has been purified to homogeneity from human placental and rat liver microsomes (Moriyasu *et al.*, 1982; Burns, 1983; Noel *et al.*, 1983) by using multiple-column chromatographic procedures. The purified human enzyme is a glycoprotein with Stoke's radius and sedimentation coefficient values of 56 Å and 4.85 S, respectively. In the purified state this enzyme still has traces of Triton X-100 and has a molecular weight of 166,000. SDS-gel electrophoresis indicated that the subunits have a molecular weight of 62,000. The rat liver enzyme, which was purified by hydrophobic chromatography, is a tetramer with a molecular weight of 280,000. Monomeric forms of this enzyme have a molecular weight of 72,000 (Moriyasu *et al.*, 1982). Human placental arylsulfohydrolase C exhibits microheterogeneity showing two main peaks of activity with apparent isoelectric points of 6.5 and 6.8, respectively (Noel *et al.*, 1983; Gniot-Szulzycka and Komozyneski, 1972). The liver microsomal enzyme has an isoelectric point of pH 8.1. Arylsulfohydrolase C is rich in mannose and *N*-acetylglucosamine. Moriyasu and Ito (1982) studied the topology of arylsulfohydrolase C in microsomes and found that the catalytic site of this enzyme is exposed to the cytoplasm, whereas its carbohydrate chains are exposed on the luminal side of the endoplasmic reticulum. Because rat liver arylsulfohydrolase C consists of four identical subunits, it is proposed that each subunit has a transmembranous disposition. Chaotropic agents have been used for solubilizing arylsulfohydrolase C. A low-molecular-weight peptide that enhances the activity of arylsulfohydrolase C in the presence of antichaotropic agents is present in various tissues (Lakshmi and Balasubramanian, 1979). Human placental arylsulfohydrolase C loses its activity on treatment with α-mannosidase and *N*-acetylglucosaminidase, indicating the involvement of a carbohydrate moiety in the catalytic site (Dibbelt and Kuss, 1984). Apart from its insolubility, arylsulfohydrolase C differs strikingly from arylsulfohydrolases A and B_1 (Table 2), the most obvious difference being the insensitivity to sulfate ions.

3.4. Iduronate Sulfate Sulfohydrolase

This enzyme removes the sulfate group from L-iduronate 2-sulfate residues found in heparin, heparan sulfate, and dermatan sulfate. It has been

TABLE 5
PHYSICOCHEMICAL PROPERTIES OF CARBOHYDRATE SULFOHYDROLASES AND NUCLEOTIDE SULFATE SULFOHYDROLASE

Enzyme	Nature	Subcellular location	Isoelectric point	Molecular weight
Iduronate 2-sulfate sulfohydrolase	Glycoprotein	Lysosome	3.4	110,000
N-Acetylglucosamine 6-sulfate sulfohydrolase	Glycoprotein	Lysosome	5.4	97,000
N-Acetylgalactosamine 6-sulfate sulfohydrolase	Glycoprotein	Lysosome	—	90,000
Heparan sulfate *N*-sulfohydrolase	Glycoprotein	Lysosome	4.7	110,000
PAPS sulfohydrolase	Protein	Cytosol	—	—
APS sulfohydrolase I	Protein	Cytosol	—	—
APS sulfohydrolase II	Protein	Lysosome	8	56,000

purified from human liver (Yutaka *et al.*, 1982) and plasma (Wasteson and Neufeld, 1982). The purified iduronate sulfate sulfohydrolase is an acidic glycoprotein with an isoelectric point about 3.4. The enzyme has a molecular weight of 110,000 as determined by Sephadex G-200 gel filtration (Table 5). Analytical gel electrophoresis in a nondenaturating system gives a molecular weight of 90,000 (Wasteson and Neufeld, 1982). Purified iduronate sulfate sulfohydrolase has optimal activity at pH 4.0 and has a K_m value of 10 to 20 μM with α-1-iodopyranosyluronic acid 2-sulfate. The enzyme is inhibited by a variety of salts, but the effect appears to be largely due to the anions. Sodium chloride, which enhances the activity of many acid hydrolases, is inhibitory at concentration above 10 m*M*. Phosphate, sulfate, and citrate ions at 1 m*M* produce 50% inhibition of enzymic activity. Zinc, cadmium, ferrous, and mercuric chlorides cause 50% inhibition at 50 m*M* (Yutaka *et al.*, 1982). *p*-Nitrocatechol sulfate, a substrate for arylsulfohydrolases A and B, produces a competitive inhibition of iduronate sulfate sulfohydrolase with a K_i value of 3 m*M*, which is close to the K_m value of arylsulfate sulfohydrolases. This suggests that iduronate sulfate sulfohydrolase is related to arylsulfohydrolases to the extent that it can bind *p*-nitrocatechol sulfate at the active site, but it is unable to hydrolyze it (Yutaka *et al.*, 1982). The amino acid composition, carbohydrate composition, and physicochemical properties of this enzyme are not known.

3.5. *N*-ACETYLGLUCOSAMINE 6-SULFATE SULFOHYDROLASE

This enzyme hydrolyzes the sulfate group from *N*-acetylglucosamine 6-sulfate, which is found in heparin, heparan, and keratan sulfate (Bhavanan-

dan and Meyer, 1966). It has been purified from human urine (Basner *et al.*, 1979a) and has also been reported in human liver, kidney, brain, placenta, and skin fibroblasts. *N*-Acetylglucosamine 6-sulfate sulfohydrolase is a glycoprotein of molecular weight 97,000. It has an isoelectric point between 5.4 and 8.3. The purified enzyme displays optimal activity at pH 5.5 and is also active toward *N*-acetylglucose 6-sulfate and glucose 6-sulfate. The apparent K_m values for tetrasaccharide, *N*-acetylglucosamine 6-sulfate, and glucose 6-sulfate are 0.15, 1.5, and 7.7 m*M*, respectively (Table 6). Albumin, Hg^{2+}, PO_4^{3-}, SO_4^{2-}, and CN^- cause considerable inhibition of this enzyme. A similar enzyme has also been described in human skin fibroblasts (Elliott and Hopwood, 1984). With *N*-acetylglucosamine 6-sulfate, the enzyme shows optimal activity at pH 6.5 and has an apparent K_m value of 0.33 m*M*. Like human *N*-acetylglucosamine 6-sulfate sulfohydrolase, the fibro-

TABLE 6
KINETIC PROPERTIES OF CARBOHYDRATE AND NUCLEOTIDE SULFOHYDROLASES

Enzyme	Substrate used	pH optimum	K_m value (m*M*)	V_{max} (nmol/min/mg protein)
Iduronate 2-sulfate sulfohydrolase	*O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1→4)-2,5-anhydro-D-mannitol 6-sulfate	4.0	0.01	1.0
N-Acetylglucosamine 6-sulfate sulfohydrolase	Monosulfated trisaccharide of heparan sulfate	5.5	0.15	10.0
N-Acetylgalactosamine 6-sulfate sulfohydrolase	*N*-Acetylgalactosamine 6-sulfate, glucuronic acid, *N*-acetylgalactosaminitol 6-sulfate	4.5	0.12	200.0
Heparan sulfate *N*-sulfohydrolase	Heparan sulfate	4.5	0.1	1.0
Steroid sulfate sulfohydrolase	Dehydroepiandrosterone sulfate, pregnenolone sulfate, estrone sulfate	6.5	0.06	150.0
PAPS sulfohydrolase	3′-Phosphoadenosine 5′-phosphosulfate	6.0	0.017	0.73
APS sulfohydrolase I	Adenosine 5′-phosphosulfate	5.4	0.95	6.0
APS sulfohydrolase II	Adenosine 5′-phosphosulfate	6.0	0.04	42.0

blast enzyme is also strongly inhibited by phosphate and sulfate ions (Elliott and Hopwood, 1984). It has been suggested that this sulfohydrolase is also localized in lysosomes (Habuchi *et al.*, 1979; Nakanishi *et al.*, 1979a). At present nothing is known about the physicochemical properties or amino acid and carbohydrate composition of this enzyme.

3.6. *N*-Acetylgalactosamine 6-Sulfate Sulfohydrolase

This enzyme cleaves sulfate group from *N*-acetylgalactosamine 6-sulfate and keratan sulfate (Dorfman and Matalon, 1976). *N*-Acetylgalactosamine 6-sulfate sulfohydrolase is a glycoprotein with a molecular weight of 90,000 to 100,000 (Table 5) as determined by gel filtration (Glossl *et al.*, 1979; Lim and Horwitz, 1981). SDS-gel electrophoresis has indicated a major band having a molecular weight between 78,000 and 85,000. The purified enzyme does not hydrolyze *N*-acetylglucosamine 6-sulfate and galactitol 6-sulfate (Glossl *et al.*, 1979; Lim and Horwitz, 1981). It shows optimal activity at pH 4.5, with a K_m value of 0.12 m*M* for the trisaccharide derived from chondroitin 6-sulfate (Lim and Horwitz, 1981). Hyaluronic acid, chondroitin 6-sulfate, keratan sulfate, dextran sulfate, heparan sulfate, and heparin competitively inhibit the enzymic activity. The K_i value for heparan sulfate was 50 n*M*. Freeze drying of the purified enzyme preparation results in complete inactivation. *N*-Acetylgalactosamine 6-sulfate sulfohydrolase is strongly inhibited by sulfate, phosphate, and pyrophosphate ions, and chloride ions are mildly inhibitory (Lim and Horwitz, 1981). Human placental *N*-acetylgalactosamine sulfate sulfohydrolase resembles the *N*-acetylgalactosamine 6-sulfate sulfohydrolase from quail oviduct (Nakanishi *et al.*, 1979a), which hydrolyzes sulfate from UDP-*N*-acetylgalactosamine 6-sulfate (Nakanishi *et al.*, 1979b), in many properties. It has also been suggested that this enzyme displays heterogeneity in various tissues and may occur in low and high uptake forms (Lim and Horwitz, 1981).

3.7. Heparan Sulfate *N*-Sulfohydrolase (Sulfamidase)

This enzyme removes sulfate groups from the *N*-sulfonyl glucosaminyl residues from heparin and heparan sulfate. It has been purified from human placenta by using chromatography on concanavalin A and heparin Sepharose (Paschke and Kresse, 1979). The purified heparan sulfate *N*-sulfohydrolase is an acidic glycoprotein with an apparent molecular weight of 100,000. It has an isoelectric point of 4.7 (Table 5). Heparin Sepharose affinity chromatography (Farooqui, 1980a; Farooqui and Horrocks, 1984a) resulted in the separation of two forms of this enzyme. With heparan sulfate as substrate, the low-affinity form has a K_m value of 0.20 m*M*, whereas for the high-affinity form

the K_m value was 0.03 m*M* (Table 6). The two forms can also be distinguished by their pH optima and by the influence of KCl (Paschke and Kresse, 1979). Heparan sulfate *N*-sulfohydrolase is associated with the lysosomal fraction (Inove and Nagasawa, 1976). Cyanide ions have no effect on enzymic activity, but sulfate, sulfite, and phosphate at 5 m*M* produce 20, 50, and 30% inhibition. Heparan *N*-sulfate sulfohydrolase is strongly inhibited by divalent metal ions such as Hg^{2+}, Pb^{2+}, Cu^{2+}, Ca^{2+}, and Mg^{2+} (Friedman and Arsenis, 1974; Inove and Nagasawa, 1976). Freeman and Hopwood (1986) have achieved 20,000-fold purification of human liver sulfamate sulfohydrolase by using concanavalin A-Sepharose and Blue A agarose chromatographies. The purified enzyme has a subunit molecular weight of 56,000 and a native molecular weight of 100,000 to 125,000. Based on kinetic data, it has been suggested that the aglycone structure of GlcNS substrates considerably influences the catalytic efficiency of this enzyme.

3.8. PAPS Sulfohydrolase

3′-Phosphoadenosine 5′-phosphosulfate (PAPS) is the well-known sulfate donor that occupies a central position in sulfate biochemistry (Balasubramanian and Bachhawat, 1970; DeMeoi, 1975; Farooqui, 1980b). It is hydrolyzed into PAP and sulfate by PAPS sulfohydrolase (Balasubramanian and Bachhawat, 1962). Nothing has been reported on the purification of this enzyme, but the crude enzyme preparation shows optimal activity at pH 6.0. It is activated by Co^{2+} and Mn^{2+} and inhibited by ADP, fluoride, and sulfhydryl compounds (Balasubramanian and Bachhawat, 1962). The desulfation of PAPS is a specific reaction. Arylsulfohydrolase A from chicken brain and rabbit kidney cortex does not hydrolyze PAPS to PAP (Farooqui and Bachhawat, 1972; Farooqui and Helwig, unpublished). Because PAPS sulfohydrolase has never been purified from any source, nothing is known about its physicochemical properties.

3.9. APS Sulfohydrolase

This enzyme hydrolyzes the sulfate group from adenosine 5′-phosphosulfate (APS) and is widely distributed in the animal kingdom (Bailey-Wood *et al.*, 1969). Among various mammalian tissues, brain is the richest source. Intracellular distribution studies of APS sulfohydrolase have indicated a bimodal distribution of this enzyme between lysosomal and cytosolic fractions of bovine liver and pig kidney (Bailey-Wood *et al.*, 1970; Armstrong *et al.*, 1970). Cytosolic APS sulfohydrolase has been purified to homogeneity from bovine liver (Stokes *et al.*, 1973a). The purified enzyme has a molecular weight of 69,000 (Table 5). It has optimal activity at pH 5.4 (Table 6).

Inorganic sulfate and AMP, the products of the enzymic reaction, inhibit the enzyme noncompetitively and competitively, respectively (Stokes *et al.*, 1973b). The pattern of product inhibition is consistent with an ordered uni–bi-reaction sequence with sulfate as the last-released product. The enzyme is irreversibly inhibited by ethoxyformic anhydride and Rose Bengal. The extent of inhibition is reduced in the presence of ATP, which is a competitive inhibitor of APS-sulfohydrolase activity ($K_i = 7.5 \times 10^{-6}$ *M*); ADP and AMP inhibit the enzyme activity noncompetitively ($K_i = 2.5 \times 10^{-5}$ *M* and 1.5×10^{-3} *M*, respectively). On the basis of various kinetic data, Stokes *et al.* (1973b) have deduced the presence of an essential histidine residue at the active site of this enzyme. Roger *et al.* (1978) purified lysosomal bovine liver APS sulfohydrolase to apparent homogeneity by using ion exchange and 5′-AMP Sepharose affinity chromatography. The purified lysosomal enzyme differs from the corresponding cytosolic enzyme in many of its kinetic and physicochemical properties. The lysosomal enzyme is a basic protein (p*I* = 8–10) with a molecular weight of 56,000. The cytosolic enzyme is relatively acidic in nature. The K_m value (0.04 m*M*) for the lysosomal enzyme is 20 times lower than that for the cytosol enzyme.

The role of the APS sulfohydrolases in sulfate metabolism is not understood precisely. The lysosomal APS sulfohydrolase can hydrolyze bis(4-nitrophenyl) phosphate and 4-nitrophenyl 5′-phosphothymidine (Roger *et al.*, 1978). Thus the lysosomal APS sulfohydrolase is less specific than its cytosolic counterpart, which does not hydrolyze these nitrophenyl derivatives. The apparent role of the lysosomal enzyme is to hydrolyze the acid anhydrides of such compounds as FAD, ATP, and ADP in secondary lysosomes. Thus lysosomal APS sulfohydrolase is an acid anhydride hydrolase that helps the cell in the recovery of nucleoside monophosphates from acid anhydrides. The APS sulfohydrolase in the cytosolic fraction probably regulates the concentrations of PAPS and therefore plays an important role in the control of sulfate conjugation.

4. Genetic Abnormalities of Sulfohydrolases

In recent years sulfohydrolases have attracted considerable attention because of their involvement in several human genetic disorders (Table 7). We will now discuss the enzymic aspects of diseases associated with the abnormal activities of sulfohydrolases.

4.1. Metachromatic Leukodystrophy

Metachromatic leukodystrophy (MLD) is a human sphingolipidosis characterized by marked disintegration of the myelin sheath of the nerve cell and

TABLE 7
METABOLIC DISORDERS CAUSED BY THE DEFICIENCY OF SULFOHYDROLASES

Disorder	Accumulated product	Enzyme deficiency	Primary tissue involved
Metachromatic leukodystrophy	Cerebroside 3-sulfate	Arylsulfohydrolase A	Brain
Maroteaux–Lamy syndrome	Dermatan sulfate	Arylsulfohydrolase B	Brain, bone
Hunter's syndrome	Dermatan sulfate	Iduronate sulfohydrolase	Brain, bone
Morquio's syndrome	Keratan sulfate	*N*-Acetylgalactosamine 6-sulfohydrolase	Brain, bone
Sanfilippo's syndrome	Heparan sulfate	Heparan sulfate *N*-sulfohydrolase	Brain, bone
Placental sulfohydrolase deficiency	Steroid sulfate	Steroid sulfate sulfohydrolase	Placenta
Multiple sulfohydrolase deficiency	Cerebroside 3-sulfate, steroid sulfate, and heparan sulfate	Arylsulphohydrolases A, B, C, iduronate sulfate sulfohydrolase, *N*-acetyl galactosamine 6-sulfohydrolase	Brain, bone

the accumulation of cerebroside 3-sulfate in brain, kidney, and other visceral organs. The neurological symptoms of this disease include disturbed motor function, ataxia, nystagmus, and intellectual deterioration (Dulaney and Moser, 1978; Kolodny and Moser, 1983; Farooqui and Horrocks, 1984b). This disease is transmitted as an autosomal recessive trait and is known to occur in at least three clinically and genetically distinct forms, which are designated as (1) late infantile form (appearing between 1 and 3 years of age) (2) juvenile form (appearing between 5 and 7 years of age), and (3) adult form (appearing after 14 years or later in adult life). Austin *et al.* (1963, 1965) were the first to report the deficiency of arylsulfohydrolase A in the brain, liver, and kidney of metachromatic leukodystrophy patients. Studies from several laboratories indicate that metachromatic leukodystrophy patients have a mutant arylsulfohydrolase A that cross-reacts with the monospecific antibody to normal arylsulfohydrolase A, but has very little or no residual enzymic activity (Shapira and Nadler, 1975b). Variants of arylsulfohydrolase A that differ from each other in kinetic properties are found in human urine (Ishibashi *et al.*, 1980; Kosugi *et al.*, 1983). Different forms of metachromatic leukodystrophy might be caused by the lack of a particular variant of human arylsulfohydrolase A. Thus Suzuki and Mizuno (1974) suggest that

arylsulfohydrolase A occurs in at least two components, A_1 and A_2. They postulate that both arylsulfohydrolase A_1 and A_2 are deficient in the juvenile form, whereas only arylsulfohydrolase A_1 is missing in the late infantile form. In addition to variant forms, arylsulfohydrolase A also exhibits microheterogeneity (Stevens *et al.*, 1973). The relation between variant forms and microforms of arylsulfohydrolase A and their role in the pathogenesis of various forms of MLD is not known.

Farrell *et al.* (1979) found three main bands of arylsulfohydrolase A activity after isoelectric focusing of extracts from cultured fibroblasts of normal human subjects. The residual arylsulfohydrolase A activity in the extracts from cultured fibroblasts of patients with the late infantile form also shows three main arylsulfohydrolase A bands of activity with isoelectric points of 5.4 to 5.8. The juvenile metachromatic leukodystrophy, however, is quite distinct and has only one main arylsulfohydrolase A activity band with an isoelectric point of 5.5. The abnormal arylsulfohydrolase A of late infantile metachromatic leukodystrophy, although deficient in enzyme activity, seems to retain the ability to undergo the postribosomal modification that results in the three-band pattern of the enzyme on cellulose acetate membrane isoelectric focusing (Farrell *et al.*, 1979). The mutation responsible for juvenile metachromatic leukodystrophy not only reduces the enzyme activity, but also the abnormal enzyme apparently does not undergo further postribosomal modification (Farrell *et al.*, 1979). Studies by Waheed *et al.* (1983) indeed indicate that human skin fibroblasts synthesize two arylsulfohydrolase A polypeptides of allelic nature that differ from each other in the number of asparagine-linked oligosaccharides. These polypeptides after postribosomal modification may give rise to arylsulfohydrolases A_1 and A_2.

Several cases of a new form of metachromatic leukodystrophy have been reported (Hahn *et al.*, 1981, 1982; Inui *et al.*, 1983). These cases have clinical symptoms resembling juvenile metachromatic leukodystrophy, but have only about half of the normal arylsulfohydrolase A activity in leukocytes and fibroblasts. The kinetic properties of the arylsulfohydrolase A from fibroblasts are normal. However, the cerebroside 3-sulfate loading test (Porter *et al.*, 1971a) with growing fibroblasts shows an abnormal response, indicating a disturbance in arylsulfatase A activity. Supplementation with the activator (Stevens *et al.*, 1981) of arylsulfatase A results in a normal cerebroside 3-sulfate loading test. This indicates that the new form of metachromatic leukodystrophy is not caused by the deficiency of arylsulfohydrolase A, but rather is caused by a deficiency of the activator protein (Shapiro *et al.*, 1979). Fujibayashi and Wenger (1986) have studied the biosynthesis of sulfatide/GM_1 activator protein in control and mutant cultured skin fibroblasts. Their results indicate that patients with variant form of metachromatic leu-

kodystrophy make lesser amounts of sulfatide activator protein than the normal cells. Dewji *et al.* (1986) have done the molecular cloning of the sulfatide activator protein. This cDNA can now be used for understanding the basis of genetic mutation in variant form of metachromatic leukodystrophy. Studies indicate that several other sphingolipidoses are also caused by a deficiency of activator proteins (Conzelman and Sandhoff, 1978). The role of these activator proteins in the degradation of cerebroside 3-sulfate is discussed in detail elsewhere (Li and Li, 1983). Prenatal diagnoses of several cases of metachromatic leukodystrophy have been reported (Dulaney and Moser, 1978; Kolodny and Moser, 1983).

4.2. Maroteaux–Lamy Syndrome

Stumpf *et al.* (1973) were the first to report the deficiency of arylsulfohydrolase B in Maroteaux–Lamy syndrome, a mucopolysaccharidosis characterized by severe skeletal deformities, gross corneal opacity, hepatosplenomegaly, marked retardation of growth, and increased urinary excretion of dermatan sulfate (Maroteaux *et al.*, 1963; Dorfman and Matalon, 1976). The kinetic properties of the residual arylsulfohydrolase B in Maroteaux–Lamy fibroblasts are identical to those of the enzyme derived from normal human fibroblasts. Further, the decreased turnover of glycosaminoglycans in this disease can be normalized by supplementation with purified arylsulfohydrolase B (Shapira *et al.*, 1975b). Using arylsulfohydrolase B antibody, it has been shown that Maroteaux–Lamy fibroblasts contain a protein (mutant) that cross-reacts (Shapira *et al.*, 1975b). In normal human fibroblast, arylsulfohydrolase B is synthesized and secreted as a 64,000 M_r precursor (Steckel *et al.*, 1983). This precursor is processed within 24 hours via short-lived intermediates into two different forms. Form I (chains of 47,000 and 11,500) is labeled first and is twice as stable as form II (chains of 40,000 and 31,000). Arylsulfohydrolase B activity is associated with the 64,000 M_r precursor and with form I but not with form II. According to Steckel *et al.* (1983), the genetic mutation in Maroteaux–Lamy syndrome causes a nearly complete absence of form I, whereas it elevates the amount of form II by about 50%. Patients with Maroteaux–Lamy syndrome are diagnosed by determining arylsulfohydrolase B activity in skin fibroblasts, leukocytes, and urine (Farooqui, 1980c; Hopwood *et al.*, 1986).

A marked increase in arylsulfohydrolase B activity is also observed in Hurler's syndrome and Sanfilippo's syndrome (Farooqui and Bachhawat, 1971; Farooqui and Mandel, 1977), which are characterized by a general alteration in glycosaminoglycan metabolism. The significance of these observations in relation to glycosaminoglycan metabolism is not understood at

present, and much further work is required to establish the relation between increased arylsulfohydrolase B activity and glycosaminoglycan metabolism.

4.3. Multiple Sulfohydrolase Deficiency

This disease is characterized by the accumulation of cerebroside 3-sulfate, glycosaminoglycans, and steroid sulfates (Austin, 1973; Murphy *et al.*, 1971; Eto *et al.*, 1974a; Farooqui, 1980c) and a profound deficiency of activity of arylsulfohydrolases A, B, and C, iduronate 2-sulfohydrolase, and heparan sulfate *N*-sulfohydrolase in various tissues (Basner *et al.*, 1979b). The genes for two of sulfohydrolases (iduronate 2-sulfohydrolase and arylsulfohydrolase C) are located on the chromosomes (Bach *et al.*, 1973; Mohandas *et al.*, 1979), whereas genes for other sulfohydrolases are on autosomes (DeLuca *et al.*, 1979; Hors-Cayla *et al.*, 1979). It is difficult to explain how the deficiency of four or five different sulfohydrolases fits together; therefore the nature of the primary defect in multiple sulfohydrolase deficiency remains obscure. However, Moser *et al.* (1972) have suggested that sulfohydrolases A, B, and C may have a common subunit that is controlled by a single gene. In this respect it must be mentioned here that sulfohydrolase A is made up of two similar subunits (Steven *et al.*, 1975). Arylsulfohydrolase C is composed of subunits of molecular weight 47,000 and 25,000 (Perumal and Robins, 1971). The molecular weight of arylsulfohydrolase B is about 50,000–60,000 (Farooqui and Roy, 1976). So it seems that arylsulfohydrolases may have a common polypeptide with a molecular weight of about 50,000. The possibility of occurrence of a common polypeptide is also supported by the following observations. Arylsulfohydrolase B (Farooqui, 1976c), like enzyme A (Jerfy and Roy, 1974), contains an essential histidyl residue in its active site. Further, like arylsulfohydrolase A, enzyme B also has a rather large amount of proline (Farooqui and Roy, 1976). Thus arylsulfohydrolases may have a common catalytic polypeptide (molecular weight 50,000) at the time of translation from mRNA; however, this common polypeptide may be modified substantially during posttranslation and assembly stages to give native and catalytically different arylsulfohydrolases A, B, and C. But this hypothesis is not supported by studies that indicate that the residual arylsulfohydrolase A activity in multiple sulfohydrolase deficiency is due to a reduced amount of an otherwise normal enzyme (Fiddler *et al.*, 1979) and that its level can be modulated by the pH (Fluharty *et al.*, 1979b) and by the presence of thiosulfate in culture medium (Kresse and Holtfrerich, 1980). Further, it has been established that structural genes of arylsulfohydrolases A and B are located on different chromosomes. Thus arylsulfohydrolase A has concordant segregation with mitochondrial aconitase encoded by a gene assigned to chromosome 22, whereas arylsulfohydrolase B segregated with β-*N*-acetylhexosami-

nidase encoded by a gene assigned to chromosome 5 (DeLuca *et al.*, 1979; Hors-Cayla *et al.*, 1979). This strongly suggests that arylsulfohydrolases A, B, and C are very different proteins that are controlled by different genes.

It is well known that the activities of various lysosomal hydrolases are strongly inhibited by heparan sulfate and other glycosaminoglycans (Avila and Convit, 1976). A possibility therefore exists that the primary defect in multiple sulfohydrolase deficiency is a mutation of hydrolase acting on heparan sulfate. An accumulation of this glycosaminoglycan may then inhibit other sulfohydrolases (Farooqui and Horrocks, 1984b). This possibility has not been tested.

Waheed *et al.* (1982b) have provided very interesting information about the rate of synthesis and half-life of arylsulfohydrolase A in multiple sulfohydrolase deficiency. In fibroblasts from these patients, the apparent rate of synthesis and half-life of arylsulfohydrolase A are two to five times lower and four to nine times shorter, respectively, compared to the control fibroblasts. They propose that in normal cells a gene product (protein) is responsible for the stability of sulfohydrolases and that multiple sulfohydrolase deficiency is caused by a mutation in this gene. Hoogeveen *et al.* (1983) have found a stabilizing protein for lysosomal glycosidases in human skin fibroblasts. The genetic deficiency of this protein in galactosialidosis results in decreased activities of β-galactosidases and neuraminidases. Horwitz *et al.* (1986) have studied the biosynthesis of microsomal steroid sulfatase in skin fibroblasts of normal individual and multiple sulfatase deficiency patients. Pulse-chase labeling of normal and multiple sulfatase deficiency (MSD) fibroblast shows a normal rate of synthesis of steroid sulfatase in MSD during a 3-hour pulse, but during the chase steroid sulfatase of MSD cells disappears with a half-life of 4 to 6 hours and approximately 25% of the material remains after 24 hours. In normal fibroblasts steroid sulfatase has a half-life of 6 days. Mutant steroid sulfatase has the same molecular weight and the same amount of endoglycosidase-sensitive carbohydrate as the normal enzyme. It has been suggested that the defect in MSD is caused by a rapid degradation of steroid sulfatase. These findings are similar to those reported for arylsulfohydrolases A and B in multiple sulfatase deficiency (Waheed *et al.*, 1982b; Steckel *et al.*, 1983).

4.4. Pseudoarylsulfohydrolase A Deficiency

Lott *et al.* (1976), Dubois *et al.* (1977), and Butterworth *et al.* (1978) reported low arylsulfohydrolase A activities in leukocytes and skin fibroblasts from healthy members of a family having a metachromatic leukodystrophy patient. Kihara (1982) called this condition "pseudoarylsulfohydrolase A deficiency." During a cerebroside 3-sulfate loading test fibroblasts from this

deficiency hydrolyzed cerebroside 3-sulfate-like fibroblasts from normal subjects (Porter *et al.*, 1971a). This observation indicates that the cerebroside 3-sulfate loading test (Porter *et al.*, 1971a), if performed on cells cultivated from amniotic fluid, can be used as a diagnostic test for distinguishing a fetus with metachromatic leukodystrophy from a pseudo-arylsulfohydrolase A-deficient fetus. There are several suggestions as to the cause of reduced arylsulfohydrolase A activity in pseudo-arylsulfohydrolase A deficiency. Dubois *et al.* (1977) postulated a defect in a regulatory gene leading to underproduction of normal arylsulfohydrolase A. Langenbeck *et al.* (1977) proposed a mutation at the arylsulfohydrolase A locus giving rise to an altered form of arylsulfohydrolase A with attenuated catalytic activity. Studies of Fluharty *et al.* (1983) provide direct evidence for the occurrence of structurally altered arylsulfohydrolase A in pseudo-arylsulfohydrolase A deficiency. They suggested that altered enzyme must be a product of the pseudodeficiency gene because a similar immunoreactive product was not detected in normal or MLD fibroblasts. It is still not clear from this work whether the attenuated arylsulfohydrolase A activity in pseudo-arylsulfohydrolase deficiency is caused by a decreased rate of synthesis or an increased lability of the mutant enzyme.

4.5. Hunter's Syndrome

Hunter's syndrome, a sex-linked recessive trait, is characterized by abnormal accumulation of heparan sulfate and dermatan sulfate and by the deficiency of L-iduronate 2-sulfate sulfohydrolase (Bach *et al.*, 1973; Sjoberg *et al.*, 1973; Lim *et al.*, 1974). These patients have short stature, large head, skeletal deformities, hepatosplenomegaly, intellectual impairment, skin lesions, and umbilical hernia. Hunter's syndrome is known to occur only in hemizygous males. The heterozygous females have two populations of cells, one of which is as deficient in L-iduronate 2-sulfate sulfohydrolase as that of the affected patient (Neufeld *et al.*, 1975). Females are probably protected from the disease by a transfer of enzyme activity from normal cell population to the abnormal (Neufeld *et al.*, 1975). The heterozygotes are diagnosed by assaying iduronate sulfate sulfohydrolase activity in serum lymphocytes, fibroblasts, and hair roots (Migeon *et al.*, 1977; Liebaers and Neufeld, 1976; *Yutaka et al.*, 1978).

4.6. Morquio's Syndrome

Morquio's syndrome is an inherited disorder of glycosaminoglycan catabolism characterized by marked skeletal deformities, corneal clouding, normal intellect, and excessive urinary excretion of heparan sulfate. Matalon *et*

al. (1974a) were the first to report the deficiency of hexosamine 6-sulfate sulfohydrolase. Using a 6-sulfated tetrasaccharide prepared from chondroitin 6-sulfate, Singh *et al.* (1976) later reported that the defective enzyme was an *N*-acetylgalactosamine 6-sulfate sulfohydrolase. The deficiency of this sulfohydrolase was confirmed in several tissues (Horwitz and Dorfman, 1978; Koto *et al.*, 1978; Minami *et al.*, 1980; Yuen and Fensom, 1985). Glossl *et al.* (1980) prepared an antiserum in guinea pigs against normal human placental *N*-acetylgalactosamine 6-sulfate sulfohydrolase. The antiserum precipitated *N*-acetylgalactosamine 6-sulfate sulfohydrolase from a concentrate of normal human urine. The antigen–antibody complex was enzymatically active. Urine concentrates from five patients with Morquio's syndrome did not contain material cross-reacting with antibodies of *N*-acetylgalactosamine 6-sulfate sulfohydrolase. From the sensitivity of the indirect immunoassay, it was concluded that the urine of the five patients contained less then 5% of the normal amount of cross-reacting material (Glossl *et al.*, 1980), indicating that the amount of defective enzyme is decreased in this syndrome.

4.7. Sanfilippo's Syndrome (Type A)

Sanfilippo's syndrome is an inherited genetic disorder of heparan sulfate catabolism, characterized by progressive mental retardation, mild skeletal deformities, and excretion of excessive amounts of heparan sulfate in the urine (Dorfman and Matalon, 1976; McKusick, 1972). A deficiency of heparan sulfate *N*-sulfohydrolase is responsible for the pathogenesis of Sanfilippo's syndrome (type A) (Kresse and Neufeld, 1972; Kresse, 1973; Hopwood and Elliott, 1981). The patients and heterozygotes are diagnosed by estimating heparan sulfate *N*-sulfohydrolase activity in fibroblasts and peripheral leukocytes (Schmidt *et al.*, 1977).

4.8. Placental Sulfohydrolase Deficiency

This condition is characterized by an absence of the increase in estrogen excretion found in normal pregnancy. France and Liggins (1969) were the first to report this condition and according to them placenta is deficient in the ability to hydrolyze those steroids that serve as estrogen precursors. Many cases of steroid sulfohydrolase deficiency have been reported (France *et al.*, 1973; France, 1979; Piraud *et al.*, 1984; Marinkovie-Ilsen and Williams, 1984). This disease can be distinguished from other sulfohydrolase deficiencies both by being sex linked and by causing pregnancy complications (Oakey *et al.*, 1974; Oakey, 1978). The babies delivered in these pregnancies have been male, and there are no reports of infant mortality (Bedin *et al.* 1980; Migl *et al.* 1980; Attenburrow *et al.*, 1984). Studies made by Shapiro *et al.*

(1977) have indicated that steroid sulfohydrolase deficiency is not only localized to placenta, but also generalized to other somatic tissues. The reason for the inactivity of placenta sulfohydrolase is obscure. The absence of an activator or the presence of an inhibitor seems an unlikely explanation (McNaught and France, 1980). The possibility that abnormal microsomal phospholipid composition, which is known to influence many enzymic activities (Wang *et al.*, 1974; Tukey *et al.*, 1979), as a cause of placental sulfatase deficiency has also been considered (McKee and France, 1983), but there were no significant differences in the phospholipid compositions of microsomes from normal and steroid sulfohydrolase-deficient tissues. Immunological studies by Van der Loos *et al.* (1983) and Epstein and Bonifas (1985) have clearly established that X-linked steroid sulfohydrolase deficiency is caused by a decrease in the amount of steroid sulfohydrolase protein.

All family members with a deficiency of steroid sulfate sulfohydrolase in their fibroblasts have a specific dermatologic condition called "ichthyosis" (Shapiro *et al.*, 1978; Shapiro, 1979; Migl *et al.*, 1980; Harkness, 1982; Crawfurd, 1982; Andria *et al.*, 1984a,b). Several papers firmly assign the steroid sulfate sulfohydrolase–X-linked ichthyosis locus to the distal region of the short arm of the X chromosome (Mohandas *et al.*, 1979; Muller *et al.*, 1980; Tiepolo *et al.*, 1980; Chance and Gartler, 1983; Ropers and Wiberg, 1982). It has been reported that short arms of X and Y chromosomes may be homologous and may undergo true pairing (Ferguson-Smith, 1966). Whether this is so and any unique features of the DNA in these regions of the X and Y chromosomes should eventually be determined when their nucleotides are sequenced.

4.9. Cytosolic Sulfohydrolase Deficiencies

No deficiency of PAPS or APS sulfohydrolases has been reported. The facts that PAPS sulfohydrolase has never been purified and that only two reports on APS sulfohydrolases have appeared (Stokes *et al.*, 1973a,b; Roger *et al.*, 1978) make it difficult to assign a physiological role to these enzymes. However, there have been several studies (Fukui *et al.*, 1981; Skidmore and Trans, 1970; Tran-Thi *et al.*, 1981) indicating that PAPS and APS sulfohydrolases have many properties in common with nucleotide pyrophosphatase (Yoshida *et al.*, 1983). Yamashina *et al.* (1983) and Yoshida *et al.* (1982) have indicated that phosphosulfate linkage of APS is elevated in fibroblasts from Lowe's syndrome patient in parallel with elevation of the PAPS degrading activity. It will be of considerable interest to analyze these enzymes in various types of mucopolysaccharidoses.

5. Sulfohydrolases in Pathological States of Nongenetic Origin

Pronounced changes of arylsulfohydrolases have been observed in many pathological states. Many diseases are associated with increased excretion of arylsulfohydrolases in urine. A 30-fold increase of arylsulfohydrolase activity is observed in myeloid leukemia. Rather substantial increases in sulfohydrolases are also noticed in diseases of the bladder, testes, and uterus and in cancer of the breast and the prostate gland (Dzialozynski and Gniot-Szulzycka, 1967; Posey and Morgan, 1977; Peltonen *et al.*, 1981).

In a series of studies on human lung tumors, Gasa and his associates (Gasa *et al.*, 1980; Gasa *et al.*, 1981; Gasa and Makita, 1983; Nakamura *et al.*, 1984) demonstrated elevated levels of arylsulfohydrolases A and B in human lung tumors. Further, an acidic variant of arylsulfohydrolase B (designated as B_1) appeared as a minor component in lung tumor. The tumor B_1 variant can be clearly distinguished from normal arylsulfohydrolase B in its isoelectric point. Variant B_1 has an isoelectric point of 7.4, compared with normal human lung arylsulfohydrolase B (p*I* 9.4). The treatment of tumor variant B_1 with phosphomonoesterase and neuraminidase results in formation of arylsulfohydrolase B, indicating that the B_1 variant contains bound phosphate and neuraminic acid (Gasa *et al.*, 1981; Gasa and Makita, 1983). Two forms of arylsulfatase B were also found in leukocytes of chronic myelogenous leukemia (CML) patients (Uehara *et al.*, 1983). The amounts of the variant form (B_1) relative to the basic form (B) are considerably increased in leukocytes of CML. It has been demonstrated (Uehara *et al.*, 1985) that chemotherapy of CML patients results in a marked decrease in variant form (B_1) with a concomitant increase in basic form (B). This observation suggests that the relative amount of the variant form (B_1) can be used as a potential prognostic indicator for the therapy of chronic myelogenous leukemia. Nakamura *et al.* (1984) purified arylsulfohydrolase A from normal human lung and lung tumors. The negative-charge heterogeneity of arylsulfohydrolase A, which is more extensive in lung adenocarcinoma, most probably stems from variations in the number of neuraminyl and phosphate residues bound to this enzyme. Increased levels of arylsulfohydrolase B are also found in colorectal cancer (Morgan *et al.*, 1975). Ueno *et al.* (1983) suggested that urinary arylsulfohydrolase activity can be used as a laboratory test for monitoring the activity of malignant diseases.

Kwashiorkor patients have also shown increased urinary excretion of arylsulfohydrolase A (Ittyerah *et al.*, 1967; Latif *et al.*, 1979, 1981). The fatty infiltration of the liver or the cloudy swelling and tubular degeneration of the kidney in kwashiorkor (Gillan, 1934) may be partially responsible for the

increased urinary excretion of arylsulfohydrolases. Rinderknecht *et al.* (1970) have shown that salivary arylsulfohydrolase activity of sufferers from pyorrhea is increased 300% above the normal level, but returned to a normal level upon treatment. A considerable increase of arylsulfohydrolase activity is also observed in the urine of patients with pulmonary and retinal tuberculosis (Boyland *et al.*, 1955). Patients with rheumatoid arthritis and osteoarthrosis (Peltonen *et al.*, 1981) have also show four to eight times higher activities of arylsulfate sulfohydrolases when compared to normal subjects.

Lykkesfeldt *et al.* (1983) have reported two cases in which steroid sulfate sulfohydrolase deficiency was associated with testis cancer. Manowitz *et al.* (1981) and Shah *et al.* (1985) have found a new variant of arylsulfohydrolase A in schizophrenic patients. According to Hulyalkar *et al.* (1984), new variants of arylsulfohydrolase A are synthesized in patients with alcoholism. A deficiency of arylsulfohydrolase A was also found in a case of nonprogressive psychomotor retardation (Denesino *et al.*, 1984). Activities of arylsulfohydrolases A and B are increased 2.5- and 8-fold, respectively, in the blister fluid of bullous pemphigoid (Higuchi *et al.*, 1982). Arysulfohydrolase activity can be remarkably reduced after treatment with prednisolone. This suggests that arylsulfohydrolases A and B may play a pathogenic role during blister formation in bullous pemphigoid.

6. Therapeutic Possibilities for Sulfohydrolase Deficiency Disorders

During the past decade considerable research has been focused on the development of strategies for the treatment of diseases caused by the deficiencies of lysosomal hydrolases. Attempts have been made to decrease the amounts of an accumulated product not only by dietary restriction, chelation, and administration of an appropriate metabolic inhibitor, but also by the direct administration of the specific active enzyme (Brady and Barranger, 1982; Desnick, 1983; Brady, 1983). Skin fibroblast and bone marrow transplants and organ transplants capable of producing normal enzymes have also been tried (Dean *et al.*, 1979; Hobbs *et al.*, 1981; Benson, 1982; Navari *et al.*, 1984). Enzyme administration therapy has shown some success in Gaucher's disease (Murray *et al.*, 1985) and Fabry's disease (Brady, 1975), but in genetic diseases where the central nervous system is involved, this approach has not been successful because the injected enzyme does not cross the blood–brain barrier (Austin, 1967; Greene *et al.*, 1969; Rattazzi *et al.*, 1980; Neuwelt *et al.*, 1981; Brown *et al.*, 1982; Umezawa *et al.*, 1985). Attempts are now being made to open the blood–brain barrier of various animals by using hyperosmotic solutions of mannitol and arabinose (Bar-

ranger *et al.*, 1979) and hyperbaric oxygen (Rattazzi *et al.*, 1980, 1981). Both these procedures are dangerous for human subjects, and a safe and effective procedure is needed for the temporary opening of the human blood–brain barrier.

It is now fairly certain that cultured fibroblasts (Porter *et al.*, 1971b; Wiesman *et al.*, 1972) and dissociated brain cells from MLD patients can ingest added arylsulfohydrolase A from growth medium and can hydrolyze the stored cerebroside 3-sulfate as in normal cells. Furthermore, the abnormal metabolism of sulfated glycosaminoglycans in various types of mucopolysaccharidosis can be corrected by the addition of various sulfohydrolases to the growth medium (Kint, 1974; Eliahu *et al.*, 1981). This suggests that if the injected enzyme can safely cross the blood–brain barrier, the replacement enzyme therapy may work in sulfohydrolase deficiencies. The entrapment of sulfohydrolases into a general carrier (liposomes and erythrocyte ghosts) may provide another valuable strategy for the treatment of these diseases (Gregoriadis, 1976; Ihler *et al.*, 1973; Lloyd and Griffiths, 1979; Hudson *et al.*, 1980). It has been shown that certain enzymes can be delivered to rat brain by means of liposomes without disturbing the blood–brain barrier (Naoi and Yagi, 1980; Takada *et al.*, 1982; Onodera *et al.*, 1983; Umezawa *et al.*, 1985). These are very important findings that must be tried with nonhuman primates and in animal models of human genetic disorders (Winchester, 1982). Thus the development and trial of new types of enzyme delivery techniques are necessary for safe and effective clinical enzyme replacement.

Skin fibroblast or bone marrow transplantation has been used in treating Hurler's and Sanfilippo's syndromes (Dean *et al.*, 1979; Hobbs *et al.*, 1981; Benson, 1982; Navari *et al.*, 1984) as an alternative to enzyme infusion therapy. At 13 months after grafting, the enzymic activity in the leukocytes and serum of the recipients reached heterozygote levels and some patients showed a remarkable clinical improvement (Rappeport and Ginns, 1984; Barranger, 1984). Only a few patients have responded to this treatment, and in others there was no evidence of clinical improvement (Munnich *et al.*, 1982; Dean *et al.*, 1982). Thus more trials of skin fibroblast and bone marrow transplantation are required for judging the usefulness of these procedures.

7. Direction of Future Research on Sulfohydrolases

Several sulfohydrolases have been characterized. Methods that have been used for the preparation of these enzymes are complicated and time consuming. New and rapid methods are required for obtaining large quantities of sulfohydrolases.

The success of sulfohydrolase studies will ultimately depend upon se-

quencing and constructing accurate genetic maps for the normal and mutant sulfohydrolases. Attempts should be made to isolate and characterize mutant sulfohydrolases. A better understanding of the genetic defect can be obtained by cloning the gene for defective enzymes (Gusella *et al.*, 1984). cDNA obtained during gene cloning of normal enzymes can be used to probe the DNA of patients with sulfohydrolase deficiencies. This will show whether the genes for sulfohydrolases in these defects are deleted or substantially altered.

Much work is needed on the treatment of sulfohydrolase deficiencies in humans. For successful enzyme replacement therapy, safe and effective procedures are needed for opening the blood–brain barrier and for delivering the active enzyme to the central nervous system. The ideal cure of human genetic disorders can be obtained by appropriate use of recombinant DNA technology (Gusella *et al.*, 1984; Asghar *et al.*, 1985; Ginns, 1985). This suggests that the treatment of sulfohydrolase deficiency will require the insertion in the diseased cell of the normal segment of DNA for the synthesis of the normal sulfohydrolase. Further development of recombinant DNA technology and gene transfer methodology will be necessary for the future treatment of these human genetic disorders.

8. Conclusion

Remarkable advancements have been made in studies of the physicochemical properties of sulfohydrolases during the past 25 years. The enzymic defects of almost all disorders associated with a deficiency of sulfohydrolases have been demonstrated. This information can be used for developing new diagnostic tests using physiological substrates and easily available human body materials such as leukocytes, serum, skin fibroblasts, urine, tears, and hair roots. The analysis of these materials will help in the detection of heterozygote carriers of genetic disorders associated with the deficiency of sulfohydrolases. The detection of heterozygotes will be of great value, not only for improved genetic analysis and knowledge of the etiology of these diseases, but also for optimal genetic counseling. Once these heterozygote carriers have been detected, one can monitor the pregnancies at risk of these diseases (Eto *et al.*, 1982; Baier and Harzer, 1983) by using cells cultivated from amniotic fluid for the assay of sulfohydrolases. In this way the genetic disorder can be predicted and the parents can be advised about termination of the pregnancy. Thus, prenatal diagnosis offers a promising alternative for parents who risk having a child with a genetic disorder. Further, there is considerable interest in new variants of sulfohydrolases in lung cancer and in routine assays of sulfohydrolase activities for detection of malignant diseases.

All these observations indicate that sulfohydrolases, a group of lysosomal enzymes, have emerged as an important group of enzymes involved in a variety of human disorders of genetic and nongenetic origins.

ACKNOWLEDGMENTS

I am indebted to Prof. Lloyd A. Horrocks, Department of Physiological Chemistry, The Ohio State University, for his advice during the preparation of this article. This review is supported by Grants NS-08291 and NS-10165 from the U.S. Public Health Service.

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THYMIC PHYSIOLOGY AND BIOCHEMISTRY

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1. Introduction

Although the presence of the thymus gland in the body has been recognized since the beginning of recorded scientific observation (cf. Duckworth, 1962), its important role as a central endocrine organ for the immune system has only been elucidated within the past several decades. Indeed, prior to 1961 the thymus was often listed together with the pineal gland and appendix as probably being vestigial in nature. This point of view was altered dramatically in 1961 with the demonstration that removal of the thymus in mice (Miller, 1961) and rabbits (Archer and Pierce, 1961) within 24 hours after birth was followed by a severe impairment of immunological responsiveness, atrophy of lymphoid tissue, and early death due to infection. This discovery in the neonate was rapidly confirmed and expanded to include most animal species studied. It was thus realized for the first time that the thymus gland contributes significantly to normal mammalian physiology and by some poorly understood mechanism controls the development and expression of immunity.

The interaction between lymphocytes found within the thymus (thymocytes) and the epithelial elements of the thymic stromal tissue was first suggested by Maximow (1909), who observed that the thymic epithelium seemed to attract "lymphoid wandering cells" and to induce their proliferation in that organ. However, it was not until the past two decades that we have begun to understand the complex biochemical events by which the thymus exerts its influence on the immune system. It is now established that thymic epithelial cells have the capacity for synthesizing and secreting a variety of maturation–differentiation-inducing polypeptides and that the

normal expansion and differentiation of one class of lymphocytes, termed T (for thymic-dependent) cells occurs *in situ*, within the thymus. It has also now been established that the thymus is a true endocrine organ and that at least some of the differentiation factors produced by its epithelial cells enter the bloodstream and affect target cells at distant sites.

In this chapter we will review the anatomy and physiology of the thymus gland as currently conceived. We will pay particular attention to the variety of polypeptides that are produced by thymic epithelial cells and that have been well characterized biochemically. Finally, we will discuss the various primary and secondary immunodeficiency disorders that are associated with abnormalities of the endocrine thymus.

2. Overview of the Thymic-Dependent Immune System

In order to fully understand the complex biochemical mechanisms by which the thymus and its hormones control the expression of immunity, it is first necessary to review briefly the organization of the thymus-dependent (T cell) immune system (Fig. 1). The predominant cells of the peripheral lymphoid tissue (i.e., spleen, lymph nodes) includes both B and T lymphocytes. In the presence of foreign antigens, B lymphocytes differentiate into plasma cells, which in turn synthesize antibody; thus, B cells make up the *humoral* arm of the immune system. In contrast, T lymphocytes are responsible for mediating all the classical *cellular* immune responses such as delayed type hypersensitivity skin responses, organ transplant rejections, and sensitized antitumor immunity as well as immunity toward various viral, fungal, and protozoal pathogens (Reinherz and Schlossman, 1980).

Although both B cells and T cells appear to be similar morphologically (small lymphocytes), they can be distinguished with the use of various serologic reagents such as fluorescent monoclonal antibodies that detect specific surface antigens. The monoclonal antibody methodology has also helped to establish that different subsets of mature T cells exist, and whereas some T cells function as effector cells (e.g., killer cells), others have been categorized as immunoregulatory cells (e.g., helper or suppressor cells) on the basis of their ability to modulate (augment or inhibit) the production of immunoglobulins by B cells as well as various functions of T cells (Reinherz and Schlossman, 1980). Thus, T cells are not only important for mediating all classical cellular immune responses, but they also serve an important homeostatic mechanism by which immunological responsiveness to a wide variety of antigens is controlled.

The thymus gland is required for the normal maturation and differentia-

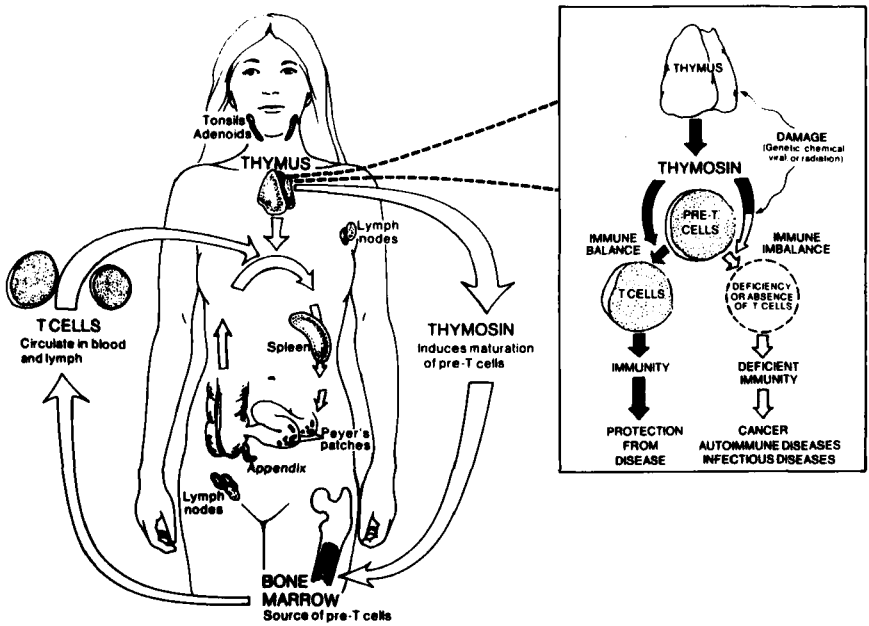


FIG. 1. Organization of the immune system.

tion of all the various T cell subsets that have been described. During fetal and neonatal life, the thymus serves as a microenvironment for the differentiation and expansion of progenitor lymphoid cells, which have migrated from the yolk sac, fetal liver, and ultimately the bone marrow. The expansion and maturation of progenitor lymphoid cells occur within the thymus under the influence of various differentiation factors released locally from epithelial cells found within the thymic stromal tissue. Once generated, immunologically competent mature T cells leave the thymus to seed the peripheral lymphoid tissues (i.e., lymph nodes and spleen) and ultimately to circulate in the blood and lymph as small lymphocytes.

In adults, the thymus continues to play an important role with regard to maintaining immune balance among the various subsets of T cells that are under its control. Although the thymus undergoes a gradual age-dependent involution beginning at puberty, its epithelial cells and endocrine capacities persist, to some degree, into adulthood. Nevertheless, the physiologic involution of the thymus precedes the well-documented deterioration of T cell immunity that is associated with old age and that reflects the gradual turnover of immunologically competent T lymphocytes.

3. Phylogenetic Origins of the Thymus

A thymus is found in all jawed vertebrates (Manning, 1981), and it is the first organ of the immune system to become infiltrated with lymphocytes during the development of the lymphoid tissues. The most primitive vertebrate to possess a thymuslike structure is the lamprey. These primitive animals, which first appeared over 400 million years ago, possess nonencapsulated thymiclike tissue consisting primarily of clusters of lymphoid and epithelial cells scattered along the pharyngeal region of the gills (Finstad *et al.*, 1964). Lampreys are capable of eliciting adaptive immune responses including both cell-mediated and humoral immunity (Finstad *et al.*, 1964). The closest phylogenetic relative of the lamprey, the hagfish, is similarly capable of manifesting both humoral and cell-mediated immune responses (Hildemann and Thoenes, 1969), although no well-defined thymuslike structure has been identified in this vertebrate.

The first encapsulated thymus encountered is present in the cartilaginous fishes (Romer, 1962), which appeared approximately 250 million years ago. The most common members of this class (chrondrichthyes), the sharks and rays, have a thymus that is found over the gills and consists of both a cortex and medulla with numerous lymphocytes (Good *et al.*, 1966). The chondrostean fish, such as the sturgeon and puddlefish, which first appeared approximately 160 million years ago, have a well-defined thymus gland organized into lobulated structures characteristic of mammals and show, for the first time in the evolutionary scale, the presence of distinct Hassall's corpuscles (Good *et al.*, 1966). The later fishes, such as the holosteans and the teleosts, also possess well-defined thymus glands that involute with age (Good *et al.*, 1966). However, none of the primitive or later fishes possesses recognizable peripheral lymphoid structures such as lymph nodes or tonsils, although many have well-developed spleens (Good, *et al.*, 1966).

The lymphoid system of the amphibians is more complex than that of fishes. In addition to a well-defined thymus gland, primitive lymph node tissue is also present (Kent *et al.*, 1964). In frogs and toads for the first time marked seasonal changes in thymic size are noted (Dustin, 1911). Reptiles have a lymphoid system that is quite similar to that seen in amphibians, including a well-developed thymus that also involutes with age (Dustin, 1911).

The evolutionary development of the lymphoid system in birds is unique. In addition to the presence of discrete lobulated thymic tissue, there is a second discrete lymphoid organ found near the cloaca, the bursa of Fabricius, which functions to direct specifically the maturation of B cells and the capacity to produce humoral antibody (Glick, 1964). In contrast, the role of

the thymus is to control the development of cell-mediated immune processes ascribed to T cells (Cooper *et al.*, 1965).

In most mammals the thymus is similar in histological appearance to the gland of lower vertebrates. The structure is usually paired and develops embryologically from proliferating endodermal cells evaginating from the region of the third pharyngeal pouch on each side of the midline (Maximow, 1909). In higher species, these cells grow in a chordlike manner, migrating caudally into the region of the thorax, where both thymic anlage join. The organ is encapsulated and each lobe consists of many lobules separated by sheets of connective tissue. Thus, the appearance of a discrete thymus gland together with the development of complex secondary lymphoid structures occurred simultaneously and progressively with evolution of the species to higher forms and appears to have been a consequence of the requirement for a diversified and effective immune system for protection of the host in new and more challenging environments.

3.1. Thymic Hormonelike Activity in the Blood of Invertebrates and Primitive Vertebrates

During the past few years several molecules have been identified in invertebrates and primitive vertebrates which share properties with analogous molecules in mammals. For example, immunoglobulins are known to exist in evolution as early as the fish. The serum and blood fluid from frogs (*Rana pipiens*), toads (*Xenopus laevis*), tunicates (*Styela clava*), and earthworms (*Lumbricus terrestins*) were analyzed by radioimmunoassay (see Section 7.2) for the presence of several of the thymosin polypeptides (thymosin α_1 and thymosin β_4) (M. D. Cooper *et al.*, in preparation). Significant levels of thymosin α_1 and thymosin β_4-like activity were found in both the invertebrates and primitive vertebrates studied; thus the thymosins are added to the growing list of biologically active molecules that may have been conserved during evolution.

4. Anatomy of the Thymus Gland

4.1. Gross Anatomy

In man the thymus is a bilobed gland located predominantly in the anterosuperior mediastinum in proximity to the heart (Rosai and Levine, 1976; Kendall, 1981). Its base lies upon the pericardium and great vessels. It is a midline, pyramid-shaped organ (Fig. 2) formed by two fused, identical lobes

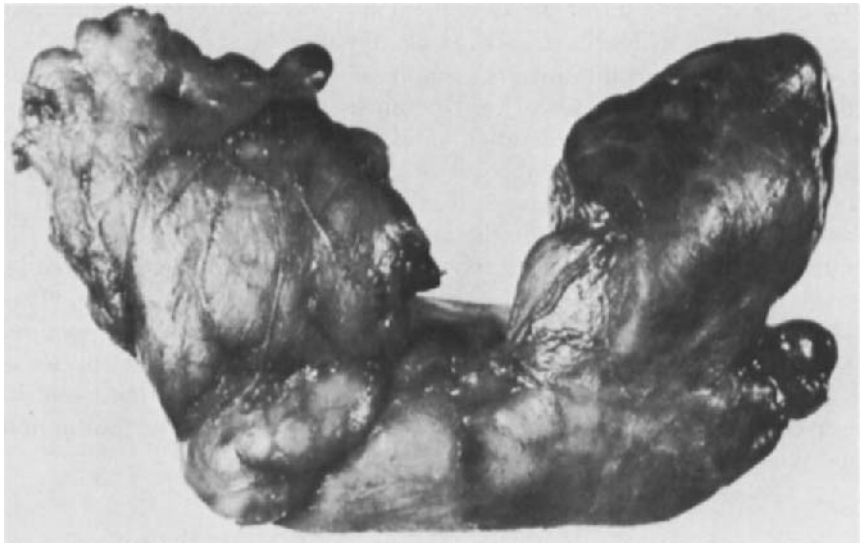


FIG. 2. Normal infant thymus. The external appearance reveals that the thymus is formed by two identical lobes that partially fuse in the midline. [From Rosai and Levine (1976). With permission of the Armed Forces Institute of Pathology.]

and enclosed in a fibrous capsule. In addition, aberrant nodules of thymic tissue can be found in as many as 20% of humans, the majority of which are in the neck in association with the thyroid or parathyroid glands (Gilmour and Bridges, 1941).

The size of the human thymus varies widely, but under normal circumstances is related primarily to age. The fully developed thymus of an infant is pink, but in adults the thymus gradually involutes and eventually turns yellow, reflecting the increased deposition of mature fat. In addition to the age-related decrease in thymic size, a certain degree of thymic atrophy is associated with increased steroid production due to either acute stress or the stress of terminal illness. The discrepancies in children between small thymic size seen with chronic illnesses and the relatively larger thymic size seen with acute crib deaths culminated in the late 1800s (Paltauf, 1889) with the identification of an erroneous condition termed "status thymico-lymphaticus" that ascribed enlarged thymus glands to sudden crib death. Radiation of the thymus was widely applied for such "thymic enlargement," a therapy that subsequently proved to be unnecessary as well as dangerous in view of the resultant marked increase in tumors of the thyroid and other sites within the prior irradiated portals (Pifer *et al.*, 1963).

4.2. Microscopic Anatomy

4.2.1. *Major Cells of the Thymus*

The basic microscopic anatomy of the thymus is remarkedly similar in all animal species (Rosai and Levine, 1976; Kendall, 1981). The thymus is essentially an epithelial organ infiltrated by lymphocytes and other mesenchymal cells. A thin fibrous capsule covers the organ and penetrates into the parenchyma, dividing it into lobules (Fig. 3). The lobule, which varies from 0.5 to 2.0 mm in size, constitutes the histologic unit of the thymus. Each lobule is divided into cortex and medulla (Fig. 3). There is a much greater density of lymphocytes found within the cortex than in the medulla and the boundary between the two zones is quite distinct (Fig. 4). The medullary portions are continuous from lobule to lobule, thus resulting in a highly branched network.

4.2.1.1. *The Capsule and Connective Tissue Septae.* Regardless of whether the thymus gland lies totally within the mediastinum, as in man, or is composed of chains of lobes that extend bilaterally into the neck, as in birds, reptiles, and guinea pigs, the lymphoid tissue is completely surrounded by a connective tissue capsule of fibroblasts and collagen. The capsule increases in thickness during life in most animals. Even though the capsule is composed of fibrous connective tissue, it can be permeable to lymphoid cells so that during development in reptiles, birds, and mammals the initial entry of stem cells into the thymus occurs through the capsule, before the gland is vascularized.

The connective tissue of the capsule permeates the gland along with the developing blood vessels so that the capsule constitutes a perivascular space that is extraparenchymal. Once developed the thymus is a highly vascularized organ and the arteries enter via fibrous trabeculae from the capsule. Arterioles at the corticomedullary junction give rise to capillaries that form the cortical arcades that eventually drain into postcapillary venules in the medulla. Each blood vessel is surrounded by endothelial cells and a basal lamina, and on the thymus side the space is lined by epithelial cells of the stroma, which also bear a basal lamina. The endothelial cells and basal lamina of the associated epithelial cells constitute a potential blood–thymic barrier (Marshall and White, 1961; Clark, 1963). Such a barrier to the penetration of macromolecules is to some degree only relative, although it appears that the cortical capillaries are more impermeable than the medullary capillaries. Nevertheless, the connective tissue of the perivascular spaces, in both the capsule and the septae, provides a potential avenue for movement of cells in and out of the thymus.

No afferent lymphatic vessels have been found in the thymus, although

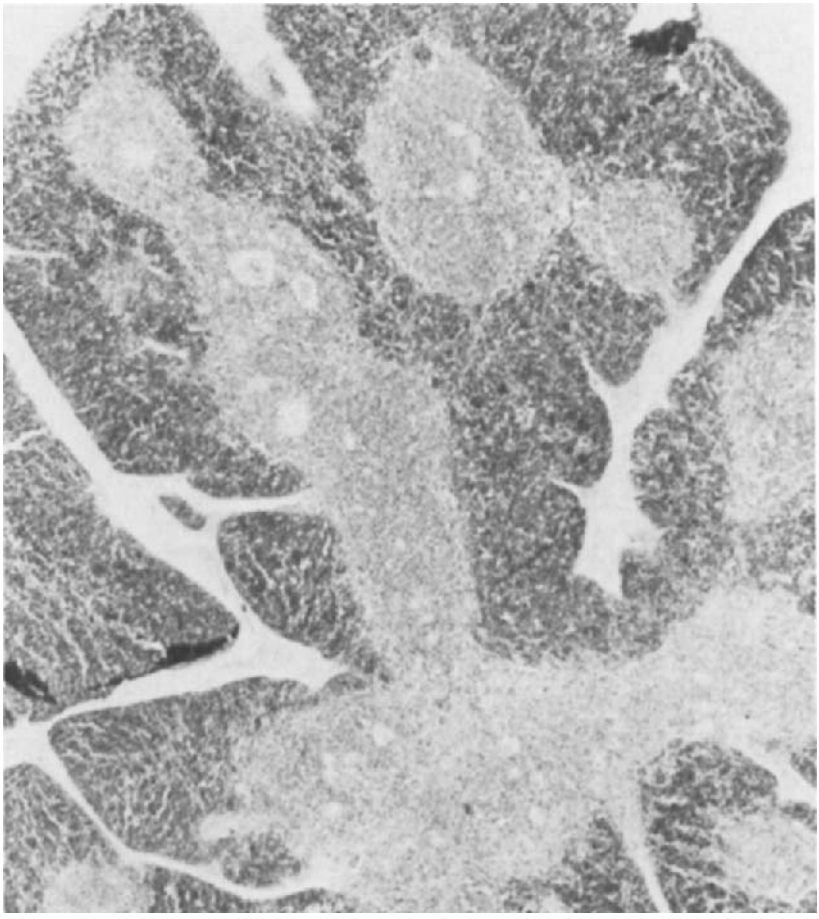


FIG. 3. Normal thymus. The normal thymus in this infant shows the lobulation and sharp separation of cortex from the stalklike medulla. ×40. [From Rosai and Levine (1976). With permission of the Armed Forces Institute of Pathology.]

small efferent vessels are present in the medulla that drain into mediastinal and lower cervical lymph nodes. Thus, the major route of emigration of precursor T cells into the thymic stroma occurs via blood-borne spread and into the perivascular space of the connective tissue stroma.

4.2.1.2. *Epithelial Cells.* The stroma of the thymus is composed of epithelial cells that can be distinguished from mesenchymal cells in that they possess desmosomes and tonofilaments, whereas mesenchymal cells do not.

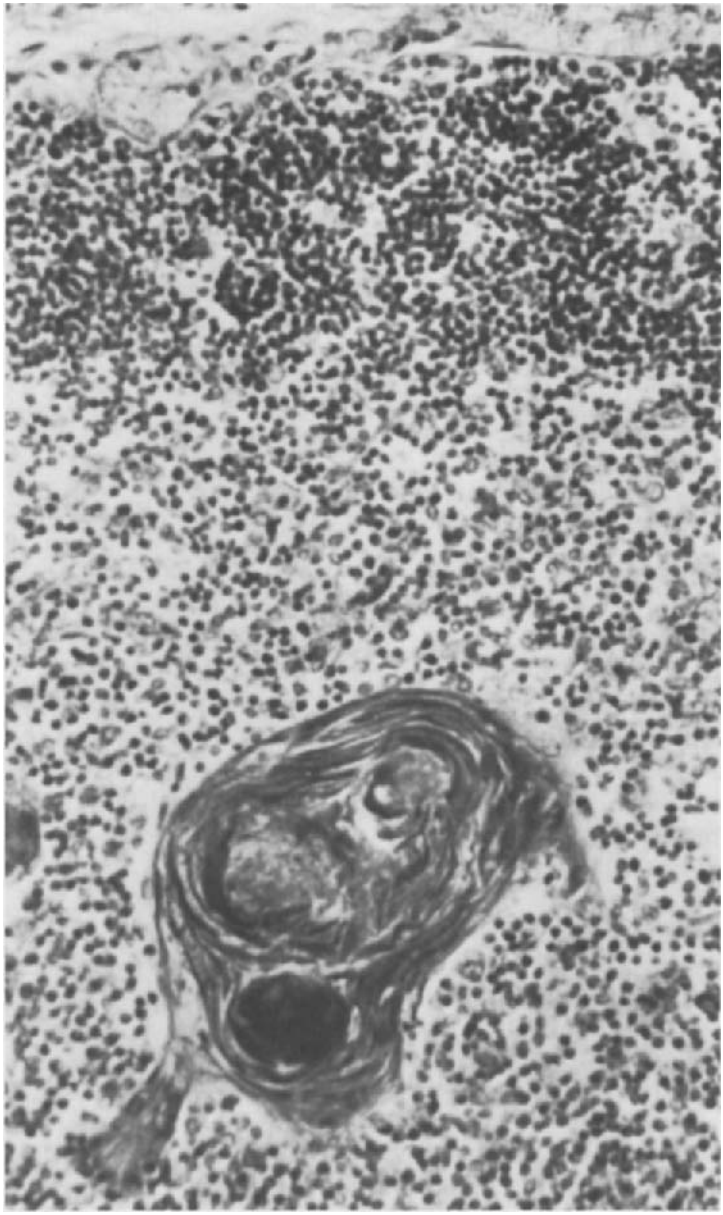


FIG. 4. Normal thymus. This child's thymus shows the dense cortex composed predominantly of lymphocytes and the less dense medulla with fewer lymphcoytes. Note the Hassall's corpuscle. ×40. [From Rosai and Levine (1976). With permission of the Armed Forces Institute of Pathology.]

Thymic epithelial cells possess long cytoplasmic processes and adjacent cell membranes held together by desmosomes and interdigitations enabling them to form intertwined three-dimensional lattices. In man, thymic epithelial cells are concentrated primarily in two separate regions, the subcapsular cortex and the medulla (Ito and Hoshino, 1966; Goldstein and MacKay, 1969; Bearman *et al.*, 1978; Hirokawa *et al.*, 1982). In the subcapsular area, thymic epithelial cells form a thin circumferential sheet that encompasses the entire surface of the thymic lobules in the superficial layer of the cortex, whereas in the medullary region they form a meshlike interconnected network of cells extending in various directions throughout the parenchyma (Fig. 5).

Numerous ultrastructural and histochemical studies have now demonstrated conclusively that many of the epithelial cells in the thymus have the characteristics of secretory cells (Singh, 1981). Cortical as well as medullary thymic epithelial cells may exhibit membrane-bound, electron-dense granules, which are also found in a variety of endocrine organs. Epithelial cells containing membrane-bound, electron-dense granules have now been identified in avian (Frazier, 1973; Hakason *et al.*, 1974; Kendall and Frazier, 1979) and various mammalian thymus glands (Kohnen and Weiss, 1964; Weakley *et al.*, 1964; Clark, 1966; Chapman and Allen, 1971; Jordan, 1975) including human thymus glands (Pinkel, 1968; Vetters and Macadam, 1973; Bloodworth *et al.*, 1975). The thymic medulla exclusively contains a population of large epithelial cells that can be distinguished by the presence of numerous rough endoplasmic reticulum as well as by the presence of numerous small, electron-dense granules that can be discerned around the Golgi apparatus and elsewhere in the cytoplasm (Singh, 1980). The morphology of these granules closely resembles the secretory granules of other polypeptide-secreting cells (Bloodworth *et al.*, 1975).

There now have been several different monoclonal antibodies prepared to antigens identified on thymic epithelial cells (Haynes, 1984; Haynes *et al.*, 1983a,b; 1984). One of these, termed A2B5, reacts with a complex neuronal ganglioside expressed on the cell surface of neurons, neural crest-derived cells, and peptide-secreting endocrine cells (Eisenbarth *et al.*, 1979, 1982). The A2B5 antigen is also found in high concentrations on thymic medullary as well as subcapsular cortical epithelial cells in human and rodent thymus (Haynes *et al.*, 1983b). A thymic epithelial cell-specific antigen termed TE-4 stains only thymic endocrine epithelial cells in a pattern similar to that described for monoclonal antibody A2B5 and not nonneuroendocrine thymic stromal cells (Haynes, 1984). The TE-4 antigen is not found on any other normal human tissue except the basal layer of squamous epithelium in the skin, esophagus, tonsil, and conjunctiva. A monoclonal antibody has been developed that detects an antigen (TE-3) present on cortical but not medullary epithelial cells (McFarland and Haynes, in preparation).

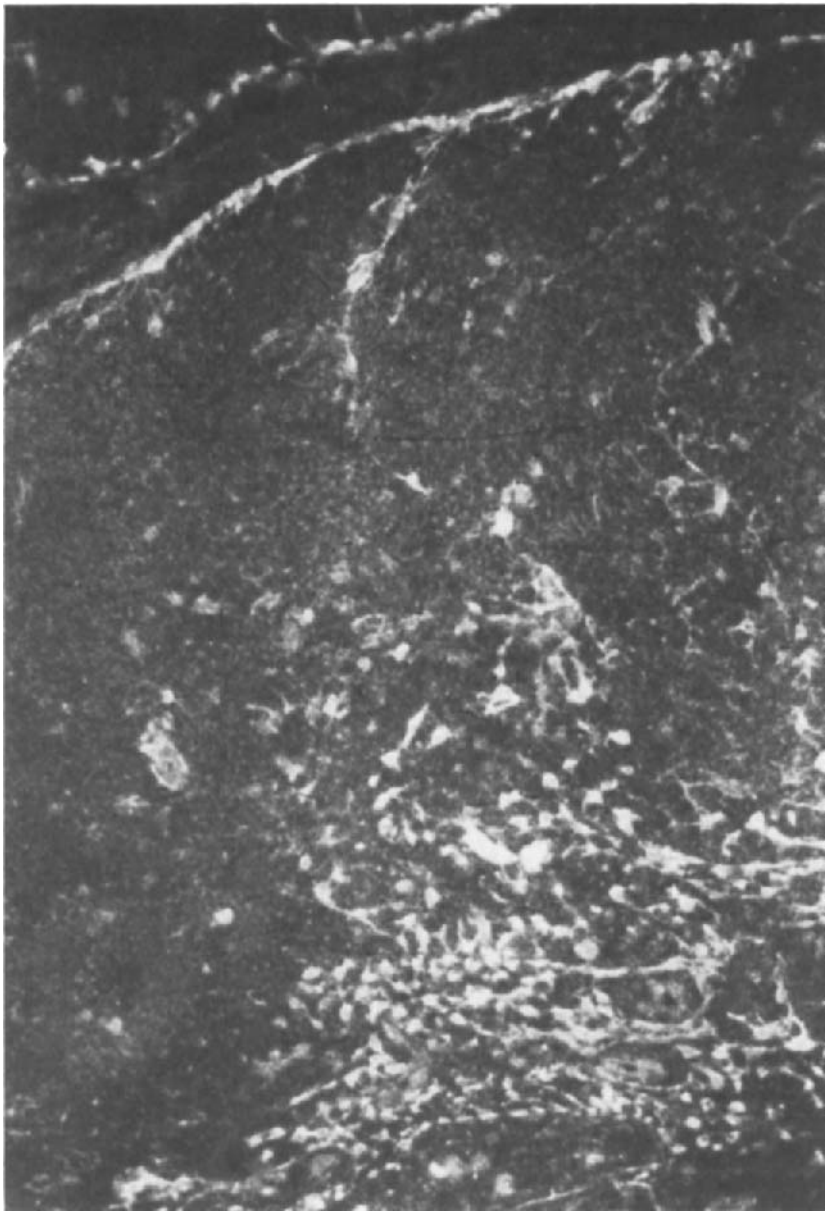


FIG. 5. Thymic epithelial cells. Both the superficial cortical and medullary thymic epithelial cells of this infant's thymus gland stain positively with an immunofluorescent antibody to thymosin α_1. [From Goldstein *et al.* (1981). With permission of Academic Press.]

Immunohistochemical studies using specific antisera to a number of well-characterized thymic polypeptides have confirmed that it is the thymic epithelial cells that are the major hormone-producing cells of the thymus. Although most thymic polypeptides are found in both medullary and subcapsular cortical epithelial cells, some are produced almost exclusively by epithelial cells localized in the subcapsular region (Hirokawa *et al.*, 1982) (Fig. 5). The precise cells of origin for each of the well-characterized thymic polypeptides will be discussed in detail later in this article (see Section 6).

4.2.1.3. *Hassall's Corpuscles.* Hassall's corpuscles are a characteristic feature of the thymic medulla (Fig. 4). They are formed by a mass of mature epithelial cells that layer concentrically upon one another and keratinize. The periphery of the Hassall's corpuscle is continuous with the epithelium of the medulla from which it originates (Kohnen and Weiss, 1964). Most corpuscles are intimately related to blood vessels; however, the precise role of Hassall's corpuscles has not been established. Several theories suggest that Hassall's corpuscles may represent "graveyards" for dead thymic lymphocytes (Blau, 1967a) or regions of accumulation of antigens (Gitlin *et al.*, 1953; Blau, 1967b; Kouvalainen and Gitlin, 1967; Kater, 1973). A monoclonal antibody has been produced that detects an antigen (TE-8) found in Hassall's corpuscles but not on thymic epithelial cells or other stromal cells (Haynes, 1984). In contrast the TE-4, antigen found on thymic epithelial cells is not present in Hassall's corpuscles. At least one thymic polypeptide (thymosin α_7; see Section 6.1.2.2) is localized exclusively to Hassall's corpuscles (Haynes, 1984). Nevertheless, the currently available information has failed to delineate the role or precise derivation of this well-known thymic structure.

4.2.1.4. *Lymphoid Elements.* The thymus plays a central role in the differentiation of T lymphocytes (Cantor and Weissman, 1976), and although much recent emphasis has focused on the thymic epithelial cells, it must be emphasized that the thymus is a lymphoid structure composed predominantly of thymic lymphocytes or thymocytes. The thymus can be viewed as a solid epithelial organ, penetrated by blood vessels and infiltrated with thymocytes in four discrete areas: the subcapsular cortex, the inner cortex, the medulla, and the perivascular connective tissue.

The subcapsular cortex is the primary site of thymic lymphocytopoiesis and a major zone for colonization of stem cells (or pre-T cells) emigrating from the yolk sac, fetal liver, or other sites. During embryogenesis, the first thymic lymphocytes, large basophilic blast cells, are observed in this area and a certain number of such cells are usually found here in the normal thymus. Most cells in the subcapsular cortex are large lymphocytes and they

are in an active state of proliferation. Mitosis is common in the subcapsular region, and it is thought that cells are forced deeper into the cortex by mitotic pressure (Kendall, 1981). It is currently believed that the close association between subcapsular cortical epithelial cells and these young thymocytes is responsible for this initial intrathymic expansion of thymocyte numbers.

The inner cortex is crowded with small, nonproliferating lymphocytes interspersed with scattered, large mitotic cells. The cells move slowly from cortex to medulla and require approximately 3 days to reach the corticomedullary border in adult animals (Steel and Lamerton, 1965; Metcalf and Wiadrowski, 1966). Apparently the inner cortex is an area where nonproliferating thymocytes begin a series of maturational events that are associated, for example, with changes of various surface antigenic determinants, as well as in the synthesis of various intracellular enzyme products, such as terminal deoxynucleotidyltransferase (TdT). These aspects of thymocyte differentiation will be discussed in Section 4.3.3. The mechanisms responsible for the changes occurring in the inner cortex have not as yet been defined, but it is possible that secretory products of either the subcapsular cortical or the medullary epithelial cells could diffuse into the inner cortex through the interstices of the epithelial matrix (Clark, 1973).

It is clear that the most important area for thymocyte maturation is the thymic medulla. It is here that thymocytes complete their final differentiation steps and ultimately leave the thymus via medullary venules to seed the peripheral lymphoid tissues. Either just prior to leaving the thymus or shortly after entering the periphery, these matured thymocytes acquire the immunological competence and antigenic characteristics that have been ascribed to mature T cells. The change from cortical to medullary thymocyte is associated with loss of sensitivity to glucocorticoids and alterations of various surface antigens and intracellular enzymes (see Section 4.3.3). Whereas inner cortical thymocytes are small and relatively deficient in the organelles that denote cellular activity, medullary thymocytes are larger and have a variety of well-developed organelles (Abe and Ito, 1970).

Early in the ontogeny of the lymphoid system when death and phagocytosis of thymocytes are minimal, the enormous number of lymphocytes produced in the thymus appear destined entirely for export (Weissman, 1967; Clark, 1968; Michalke *et al.*, 1969). Emigrating cells appear in thymic veins and lymphatics on their way to colonize peripheral lymphoid organs (Kotani *et al.*, 1966; Larsson, 1966; Williams *et al.*, 1971). However, during the major period of ontogenesis most of the rapidly proliferating cortical thymocytes are destroyed *in situ* and only a minority of thymic lymphocytes mature completely and ultimately migrate to the peripheral lymphoid tissues (Matsuyama *et al.*, 1966; McPhee *et al.*, 1979). The predominant sites

for emigration of matured thymocytes are the medullary venules (Clark, 1963, 1968; Tovo and Olah, 1967; Goldstein, 1968). The perivascular connective tissue appears to be the final common path for emigrating thymocytes and it may provide their first encounter with the extrathymic environment (Raviola and Karnovsky, 1972).

4.2.1.5. *Myoid Cells.* Because of the association between the thymus and the neuromuscular disorder myasthenia gravis, much attention has focused on the possible relationships between the thymus and the neuromuscular system. Indeed, the identification of one of the thymic polypeptide hormones (thymopoietin) was based on its ability to induce the neuromuscular blockade characteristic of myasthenia gravis in animals (see Section 6.2). Myoid cells have been identified in the thymus gland of all vertebrate classes above the osteichthytes and are especially numerous in the anurans and vertebrates (Bockman and Winborn, 1967; Raviola and Raviola, 1967; Ito *et al.*, 1969; Tovo *et al.*, 1969; Gilmore and Bridges, 1974; Pauchtler *et al.*, 1975). Although the origin of thymic myoid cells is uncertain, they do contain striated filaments with actin and myosin as found in striated muscle, whereas the epithelial cells of the thymus contain musclelike myosin only (Kendall, 1981). The myoid cells of the thymus are not innervated (Tovo *et al.*, 1969; Gilmore and Bridges, 1974) and their function, if any, is currently not well understood.

4.2.1.6. *Interdigitating Reticulum Cells.* Interdigitating reticulum cells are structural phagocytic cells of mesenchymal origin that were first identified in peripheral lymphoid tissues such as the lymph nodes and spleen in rodents (Steinman *et al.*, 1979) as well as in man (Rausch *et al.*, 1977). A characteristic feature of such cells is their long cytoplasmic processes, which can wrap around cells and form complex surface patterns (Kendall, 1978). Interdigitating reticulum cells are found only within specific areas of peripheral lymphoid tissues, such as the paracortical regions of lymph nodes, where mature T cells reside. They are felt to serve an important accessory cell role by virtue of their ability for phagocytosis of foreign antigens and presentation of processed antigen to T cells. Interdigitating reticulum cells have also been identified within the deep cortex and medulla of the thymus (Kendall, 1981). However, since the thymus exhibits a relative blood barrier to foreign antigens (Marshall and White, 1961; Clark, 1963), the precise role, other than a structural role, of such cells in the thymus is still unclear.

4.2.1.7. *Thymic Nurse Cells.* Another cell described in both mouse and human thymus glands has been termed the thymic nurse cell (Wekerle, 1980; Ritter *et al.*, 1981; Kyewski and Kaplan, 1982; van de Wijngaert *et al.*,

1983). This cell is quite large and appears to have engulfed large numbers of thymocytes. It is located primarily in the subcapsular cortex. Although it has been referred to as an epithelial cell, its precise relationship to thymic epithelial cells and thymic interdigitating reticulum cells remains to be established. It has been suggested that thymic nurse cells may play an important role with regard to thymocyte differentiation. Because of the intimate membrane contact between thymic nurse cells and thymocytes, it has been postulated that these cells are responsible for "educating" T cells to recognize self- and nonself-antigens.

4.2.1.8. *Macrophages.* Besides thymocytes, cells of the mononuclear phagocyte series constitute the other hematopoietic population of cells that are present in the thymus. Significant numbers of macrophages are present in the thymus in the adult (Kostowiecki, 1963; Bearman *et al.*, 1978) and they are even present early in gestation (Jordan *et al.*, 1979). The fact that cells of the mononuclear phagocytic series colonize the thymus at the same stage as the first lymphoid progenitors suggests that they may play an important role in intrathymic T cell maturation. It is possible that thymic macrophages may induce the differentiation and/or proliferation of thymocytes by releasing various soluble mediators such as lymphocyte activating factor (LAF) termed interleukin-1 (IL-1). However, the precise role of thymic macrophages, if any, in T cell development remains to be defined.

4.3. The Ontogeny of the Thymus

4.3.1. *Stem Cell Colonization*

The development of the thymus has been a subject of active investigation since the late 1950s, and extensive studies have been performed in mice, amphibians, and avian species. In mice and chicks, an epithelial thymic rudiment is discernible at 10–11 days of fetal gestation, with lymphoid blasts first appearing at approximately 12–14 days. Initially it was believed that the first lymphocytes arose from the epithelial components (Auerbach, 1961). However, later experiments clearly demonstrated that the initial lymphoid development of the thymus occurs via the colonization by blood-borne stem cells from the yolk sac, fetal liver, and/or blood islands (Moore and Owen, 1967; Owen and Ritter, 1969; Le Douarin and Jotereau, 1975, 1981).

In the chick/quail model system as well as in the mouse, colonization seems to occur in several waves, with cycles of 5 to 6 days. There is an initial period of stem cell influx, which may depend on the epithelial thymus elaborating an attractant factor, followed by a refractory phase, during which there is little stem cell influx, followed by another wave of colonization (Le

Douarin and Jotereau, 1975, 1981). Initial studies of human thymus development also suggest that the production of an attractant factor promotes stem cell colonization (Auerbach, 1960).

4.3.2. *Microenvironmental Influences*

The thymic microenvironment is a complex, specialized tissue derived from at least three sources: endoderm of the third pharyngeal pouch, ectoderm derived from the third brachial cleft, and mesenchymal stromal cells derived from embryonic mesoderm (Weller, 1933; Norris, 1938; Auerbach, 1960, 1961, Patten, 1968; Cordier and Haumont, 1980). Pharyngeal pouch endoderm and brachial cleft ectoderm give rise to epithelial components, while mesodermal-derived mesenchymal cells form the fibrous capsule, vessels, and interlobular septa. Early in thymic ontogeny mesodermal-derived connective tissue induces epithelial cell maturation and fetal thymic lobulation. The cortical epithelial component of the thymic microenvironment is thought to be derived from the ectodermal brachial cleft, while the medullary epithelium is thought to be derived from the third pharyngeal pouch endoderm (Weller, 1933; Norris, 1938; Patten, 1968; Cordier and Haumont, 1980).

In man, the mesodermal component of the thymus microenvironment (fibroblasts and vessels) surrounds the thymic primordia at 6 to 7 weeks of gestation. Thymic epithelial rudiments are devoid of lymphoid cells entirely until 9 to 11 weeks of gestation (Patten, 1968; Papiernik, 1970). By 7 weeks of gestation thymic epithelial cells expressing both the A2B5 and TE-4 antigens are present (Haynes *et al.*, 1984; see Section 4.2.1.2). From 9 to 13 weeks the TE-4$^+$, A2B5$^+$ epithelium is arranged in lobulated zones surrounded by mesenchymal tissue and without a discernible corticomedullary junction. By 9 weeks numerous thymocytes are present that express various T cell antigens. By 15 weeks of gestation a corticomedullary junction is present and the thymic epithelium has compartmentalized into the subcapsular cortical and medullary areas. At 15 weeks of gestation Hassall's bodies are present.

Although it appears that the key microenvironmental influence in the embryonic thymus derives from its epithelial cells, other nonlymphoid cells are present early in gestation in the thymus, which may possess important inductive capabilities for thymocytes including the thymic nurse cell, thymic macrophages, and interdigitating reticulum cells (see Sections 4.2.1.6–4.2.1.8).

4.3.3. *T Cell Differentiation*

Following the inflow of stem cells, which are nondividing, the lymphoblasts undergo several cycles of proliferation and maturation. An epithelial

thymus rudiment from a 13-day-old mouse embryo will have only a scattering of lymphoblastlike cells, but when placed into organ culture will proceed to develop into a lymphoid thymus containing increasing numbers of small thymocytes, whose phenotypic and functional characteristics resemble those of cells isolated from the 18–20-day gestation thymus. The *in vitro* development of the thymus confirms the notion that a continuous inflow of stem cells is not necessary, but rather that stem cells entering the thymus have proliferative potential and will develop over a short period of time into recognizable T cell populations.

In man, the discrete stages of intrathymic differentiation have been analyzed by using a panel of monoclonal antibodies that detect specific surface differentiation antigens. Many different antigens have now been identified, but for simplicity only the major ones will be discussed. Mature peripheral blood T cells are characterized by having surface receptors for sheep erythrocytes (OKT11) and various mature T cell antigens such as OKT3 (Reinherz and Schlossman, 1980). Peripheral blood T cells can be further subdivided into those that function as helper T cells and exhibit the OKT4 (helper) antigen and that include approximately two-thirds of all T cells and those that express the OKT8 (cytotoxic-suppressor) antigen and function as either suppressor cells or killer cells. It has not been possible with the monoclonal antibody methodology to further distinguish killer T cells from suppressor T cells. The OKT8 population makes up approximately one-third of peripheral blood T cells. In mature T cells, the OKT4 and OKT8 phenotypes are mutually exclusive so that one or the other is expressed but not both.

From a number of studies using both suspensions of human thymocytes as well as using fixed thymus tissue specimens, a schema has been developed to account for the phenotypic changes that occur during thymocyte differentiation (Reinherz and Schlossman, 1980). Some surface antigens, such as OKT10, are found on all thymocytes as well as on a subpopulation of activated (i.e., by mitogens) mature T cells. Other markers such as OKT6 are found exclusively on cortical (or common) thymocytes. In contrast to peripheral blood T cells, most cortical thymocytes are biphenotypic in that they express *both* OKT4 and OKT8 markers in addition to OKT6. Medullary (late, mature) thymocytes still possess OKT10 but have lost OKT6 expression. They are now $OKT3^+$ and have segregated to express either OKT4 or OKT8 but not both.

From such analyses a hypothetical scheme of human thymocyte differentiation has been proposed, which is summarized in Fig. 10 later in this chapter. Such a schema is similar to that which had been obtained in murine systems (Cantor and Weissman, 1976). In both cases, it has been presumed that thymocytes differentiate as they migrate from cortex to medulla, where they ultimately leave the thymus. However, recent studies in mice suggest

that this may be an oversimplification of the stages of intrathymic differentiation. Early in gestation in mice a population of thymocytes is detected that expresses a thymocyte antigen (Thy-1^+) as well as a helper cell antigen (Lyt-1^+) that precedes by at least 1 day the appearance of cells that express the biphenotype OKT4–OKT8 equivalent phenotype (Lyt-1^+,2^+,3^+ in mice) (Mathieson *et al.*, 1981; Ceredig *et al.*, 1983). In addition, there is now evidence in animals that stem cells may enter the thymus at two locations including both the subcapsular cortex as well as the corticomedullary junction (Le Douarin and Jotereau, 1981; Goldschneider *et al.*, 1982). In man, it has not been possible to explore the developmental pathways of thymocytes experimentally in as much detail. Nevertheless, while it is clear that a major intrathymic differentiation pathway involves the maturation from immature cortical to mature medullary thymocyte, other pathways of differentiation are probably active as well (Scollay, 1983).

In the mouse, several additional characteristics have been elucidated that distinguish immature cortical thymocytes from the more mature medullary thymocytes. For example, whereas cortical thymocytes have receptors for peanut lectin (PNA^+) and are sensitive to lysis in the presence of corticosteroids, medullary thymocytes are PNA^- and relatively resistant to steroids (London *et al.*, 1978). A number of intracellular enzymes have also been defined that have been useful for distinguishing different stages of intrathymic differentiation. The best known of these is terminal deoxynucleotidyltransferase (TdT), a DNA polymerizing enzyme that catalyzes the addition of deoxyribonucleotide primers without requiring a template. Terminal deoxynucleotidyltransferase activity can be identified in a lymphocyte population by preparing a cell homogenate and then performing a quantitative enzyme assay, and it can be detected in individual cells by using specific immunofluorescent antibodies (Bollum, 1979). It has been found to be associated with immature and proliferating cells including approximately 0.5% of normal bone marrow cells, but it is not found in either mature T cells or B cells. In the human fetus the first TdT^+ tissue is the liver and at 13 to 16 weeks of fetal life, 30 to 60% of lymphoid cells are TdT^+. Thereafter the bone marrow and subsequently the thymus shows TdT^+ cells. Although the thymus is first populated by a TdT^- population at around 11 to 12 weeks, TdT^+ thymocytes become demonstrable beginning at about 17 weeks of gestation (Janossy *et al.*, 1980) and remain TdT^+ throughout life. The highest levels of TdT activity are found in cortical thymocytes, and activity gradually diminishes and is no longer detectable in the most mature medullary thymocytes. The biologic function of TdT remains uncertain, although it may be involved in the production of the multiple somatic mutations necessary for the generation of immunological diversity.

A number of different enzymes of purine metabolism including adenosine

deaminase (ADA), purine nucleoside phosphorylase (PNP), and ecto 5'-nucleotidase (5'-NT) have been identified in both mature T cells as well as mature B cells (Blatt *et al.*, 1980; Hoffbrand *et al.*, 1982). Deficiencies of each of these enzymes have been associated with specific immunodeficiency states, suggesting that purine metabolism is important in lymphocyte differentiation and proliferation. Among T cells, 5'-NT activity has been found to correlate with the degree of cell maturity and medullary thymocytes have been found to exhibit much greater activity than cortical thymocytes. ADA is essential for the differentiation of early T cell precursors and is found in highest concentrations in cortical thymocytes. PNP acts sequentially with ADA in purine metabolism, and cortical thymocytes appear to have diminished activity compared to either prothymocytes, medullary thymocytes, or mature T cells. In Section 9.2.2, a review of the influence of thymic factors on the intracellular enzyme and phenotypic characteristics of T cell precursors will be presented. A summary of these changes and the postulated differentiation steps that the well-defined thymic hormones appear to induce is summarized later in Fig. 10.

4.4. Thymic Involution

It is now well established that in most vertebrates the thymus undergoes a gradual age-dependent atrophy or involution in which the thymic parenchymal tissue is infiltrated with adipose cells and fat (Seyle, 1936). In man, maximal thymic size occurs just prior to puberty, and it then begins to gradually decrease in size and weight (Fig. 6) (Hammar, 1906, 1909, 1914, 1921).

Thymic involution is characterized by an infiltration of thymic tissue with adipose cells. This infiltration begins around blood vessels in connective tissue and eventually adipose tissue replaces large areas of cortex and to some extent the medulla as well (Fig. 7). The process of involution actually begins quite early, and single adipose cells can be found in the thymus of man around birth and their numbers increase during the period of rapid thymic growth (Singh, 1980). Superimposed on this pattern of age-dependent involution are the atrophic changes ascribed to the effects of various hormones including adrenocortical steroids, androgens, and estrogens (Dougherty, 1952; Soboon and Jirasatthan, 1974). Although thymic involution appears to be accelerated at puberty, the precise mechanisms responsible for physiological thymic involution have not been identified.

As age involution progresses, lymphatic tissue gradually decreases, the cortex thins, Hassall's corpuscles become more prominent, and fatty replacement of the organ ensues. Many of the Hassall's corpuscles become calcified or cystic. Some of the epithelial cells may acquire a spiral shape. In

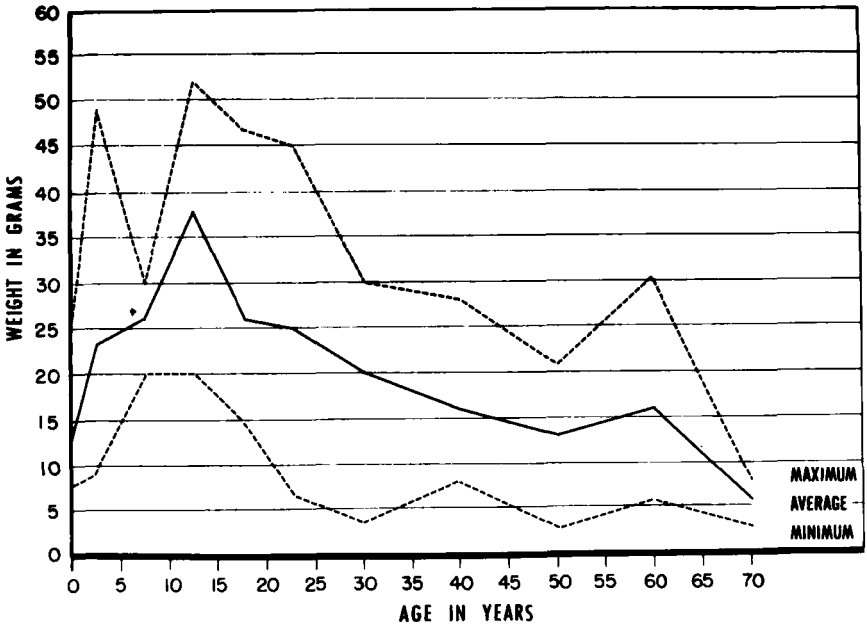


FIG. 6. Weight of human thymus. Variations in the weight of normal thymuses at any given age are shown. [From Rosai and Levine (1976). With permission of the Armed Forces Institute of Pathology.]

elderly individuals the thymus may be impossible to identify on gross inspection. However, it is now appreciated that the thymus does not completely disappear in older animals (Andrew, 1952), and in man strands of thymic parenchyma persist even in elderly individuals (Hammar, 1926). It has also been shown that the ratio of cortex to medulla decreases with age; it is 60% at birth, but only 30% at 70 years of age (Goldstein and MacKay, 1969).

The loss of hormone-producing epithelial cells begins early in life. In man, by the second decade the number of hormone-containing medullary thymic epithelial cells has decreased dramatically, whereas the number of hormone-containing cortical epithelial cells appears to decrease more gradually and can still be observed even in the fifth decade of life (Hirokawa *et al.*, 1982). The age-associated decrease in absolute numbers of hormone-containing thymic epithelial cells correlates with the gradual decrease in thymic hormonelike bioactivity measured in the blood of both animals (Dardenne *et al.*, 1974a,b; Savino *et al.*, 1983a) and in man (Bach and Dardenne, 1972a; Twomey *et al.*, 1979; Lewis *et al.*, 1978; Iwata *et al.*, 1981).

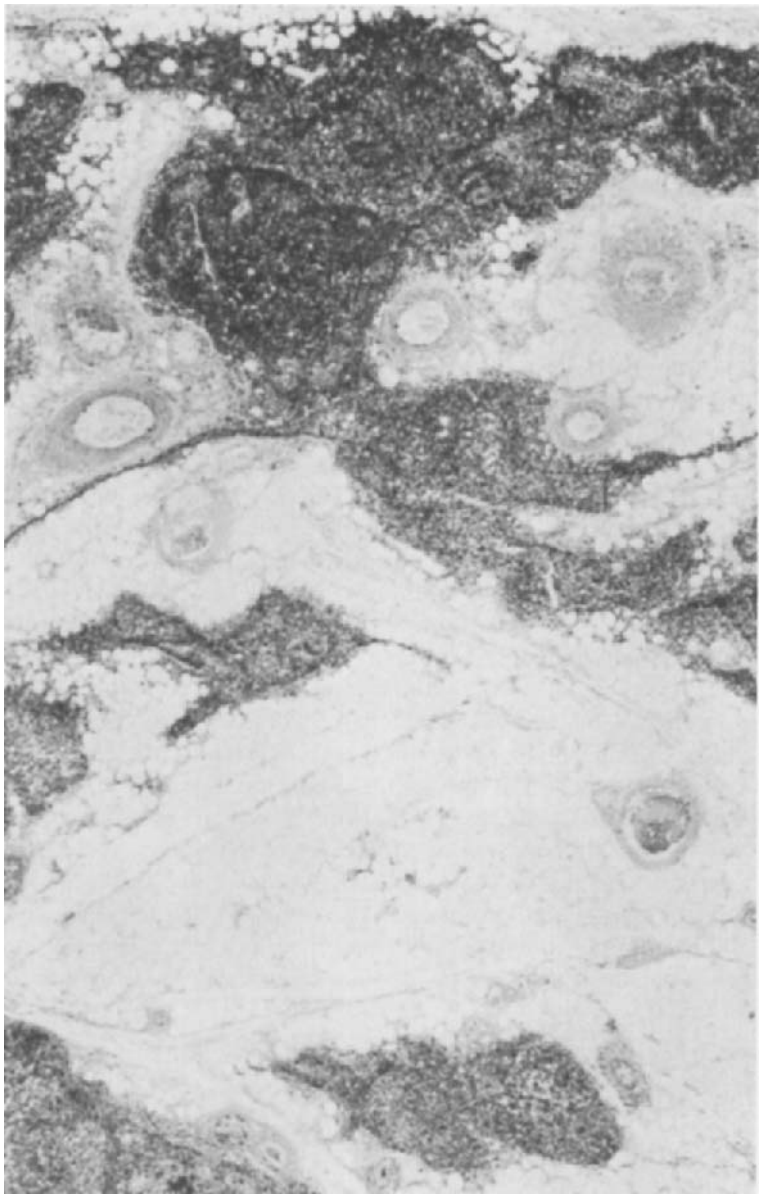


FIG. 7. Involuted thymus. This adult thymus shows involution with fat replacement. However, epithelial elements are still present to some degree. ×20. [From Rosai and Levine (1976). With permission of the Armed Forces Institute of Pathology.]

5. Endocrine Role of the Thymus in Historical Perspective

5.1. Neonatal Thymecty and Restoration with Thymic Grafts

The important role of the thymus in the development of immunological responsiveness was not fully appreciated until the early 1960s, when it was reported individually from several laboratories (Miller, 1961; Archer and Pierce, 1961; Good *et al.*, 1962) that animals thymectomized in the perinatal period exhibited severe defects including a depletion of lymphocytes in the blood, lymph nodes, and spleen. Neonatally thymectomized animals developed a wasting syndrome that was characterized by slowing of growth, recurrent infection, and premature mortality. Immunologically, the animals exhibited defective T cell immunity including an impaired ability to reject foreign skin grafts and to manifest delayed type hypersensitivity skin tests.

Shortly after the neonatal thymectomy studies, it was demonstrated that thymus glands implanted into neonatally thymectomized animals could prevent wasting, reverse lymph node atrophy, and completely abolish the immunological incompetence that would otherwise develop (Good *et al.*, 1962; East and Parrot, 1964). Further investigation focused on establishing whether the thymus contributed to the restoration of immunity by providing a source of lymphocytes or via a humoral mechanism, or both. It was thus demonstrated that the implantation of thymus tissue within cell impermeable Millipore diffusion chambers could partially reverse the effects of neonatal thymectomy, including the wasting syndrome (Levey *et al.*, 1963; Law *et al.*, 1964; Osoba and Miller, 1964). Further support of a possible endocrine role for the thymus was provided by the observations that thymic epitheliomas, consisting only of epithelial–stromal cells, exhibited similar immunorestorative effects (Stutman *et al.*, 1969) and that female thymectomized mice were immunologically restored when they became pregnant, presumably by the transplacental passage of an embryonic thymus factor (Osoba, 1965).

5.2. Studies with Crude Thymic Extracts

Attempts at preparing extracts of thymus glands in order to isolate putative thymic hormones date back as far as 1896 (cf. White and Goldstein, 1968; Goldstein and White, 1971). It was not until 1935 that an attempt was made to demonstrate the endocrine function of the thymus as it pertains to the lymphoid system (Gregoire, 1935). At that time it was reported that the regeneration of the cortical regions of the irradiated thymus occurred only if circulating lymphocytes were allowed to reach the epithelial anlage of the

thymus; if not the thymus remained epithelial. Such observations were not pursued further until the late 1960s, when following the thymectomy–thymus graft experiments, efforts were initiated to prepare thymic extracts with immune-reconstituting properties. Pioneering efforts in this field were made by Drs. A. White and A. L. Goldstein and colleagues, who demonstrated that treatment of neonatally thymectomized mice with a crude thymic extract, termed thymosin, decreased the incidence of wasting disease, improved survival, and restored cell-mediated immunity such as the ability to reject skin grafts (Law *et al.*, 1968; Asanuma *et al.*, 1970; Goldstein *et al.*, 1970). Over the ensuing two decades more than 20 different factors with thymic-hormonelike activity have been isolated from thymus tissue and blood. The detailed biochemical and physiological properties of the best-characterized thymic factors will be discussed in Section 6.

5.3. Thymectomy in Adult Animals

For many years it was thought that the thymus gland functioned primarily in fetal and neonatal life and no longer played an important role by the time adulthood was reached. This erroneous conclusion was based on two observations, namely, (1) that adult thymectomy in animals had no immediately obvious effect on the physiology of the thymus-deprived animal and (2) that beginning at puberty the thymus atrophies, leaving only a trace of its former self as a stromal–epithelial structure. However, it is now appreciated that thymic hormone-secreting epithelial cells persist well into adulthood (see Section 4.4). In addition, it is now understood that the failure to detect changes in immune competence shortly after thymectomy of the adult was due to the fact that mature T cells have a long half-life and that, even in the absence of a thymus, they continue to function with a half-life of 100 days in rodents and perhaps several years in humans (Little *et al.*, 1962). Reevaluation of the effects of adult thymectomy in rodents revealed that immunologic competence decreases only gradually after removal of the thymus and becomes apparent only after a period of 6 to 9 months, a quarter to a half of the animals' life span (Metcalf, 1965; Miller, 1965).

With the development of more sophisticated immunologic assays, many changes in immunologic competence were demonstrable in animals soon after adult thymectomy. These included a decrease in proliferative responses of lymphocytes to T cell mitogens (Johnston and Wilson, 1970) and in mixed leukocyte response (MLR) (Robson and Schwarz, 1971). One major postthymic T cell subset that remains under the control of the thymus in the adult is the T suppressor cell. Within 2 to 4 weeks after adult thymectomy there is a decrease in suppressor T cell activity, which can be restored by administration of various thymic factors (Zatz and Goldstein, 1972; Simpson

and Cantor, 1975; Asherson *et al.*, 1976; Reinisch *et al.*, 1977; Erard *et al.*, 1979). This decline is most likely attributed to a turnover of a short-lived postthymic T cell (Kappler *et al.*, 1974; Rocha *et al.*, 1983).

Probably the earliest cellular change that can be detected by 1 week following thymectomy in adult mice is the disappearance of splenic lymphocytes that form azathioprine-sensitive E rosettes (Bach and Dardenne, 1973). This observation enabled Bach and Dardenne to subsequently develop an *in vitro* bioassay that was utilized for the detection of circulating levels of thymic hormonelike bioactivity (see Section 7.1.1). Thus, there is now ample evidence that in the adult the thymus continues to manifest an important long-term role with regard to the maintenance and regulation of the immune system.

6. Comparative Biochemistry of Thymic Factors

The thymus appears to be an organ with the capacity to synthesize many different products that differ in chemical structure. A number of factors with thymic hormonelike activity have been prepared from thymus tissue and blood and these preparations are in various stages of characterization. Among the thymic preparations, thymosin fraction 5 (TF5), thymosin α_1, thymulin [facteur thymique serique (FTS-Zn)], thymopoietin, thymostimulin (TS), thymic humoral factor (THF), and thymic factor X (TFX) are the best-characterized, most thoroughly studied thymic preparations and the ones that have currently been entered in widespread clinical trials. TF5, TFX, and TS are partially purified extracts of calf thymus glands and include a number of different biologically active peptides. Several of the active polypeptides identified in TF5, such as thymosin α_1 and thymosin β_4, have been purified to homogeneity and sequenced. Thymulin is a nonapeptide that was isolated initially from porcine blood but is also found in high concentrations in thymic tissue. Four thymic peptides have been synthesized (thymosin α_1, MW 3108, thymosin β_4, MW 4982; thymopoietin II, MW 5562; and thymulin, MW 857) and these peptides appear to be unrelated chemically.

All of the thymic preparations that will be discussed in this chapter were first identified as crude thymic extracts with the ability to restore or enhance various parameters of thymic-dependent immunity either *in vitro* or *in vivo* by using lymphoid cells isolated from various immunodeficient animal models. Purified products were then isolated from the crude extracts and exhibited biologic effects similar to the crude preparations. The demonstrations that a number of biochemically unique thymic products exhibited similar biologic activities have been difficult to rationalize (cf. Stutman, 1983). In

order to explain such overlap it was suggested (Goldstein *et al.*, 1981) that the crude bioassays that have been employed to monitor the purification of thymic extracts do not accurately reflect the subtle *in vivo* physiological effects of the individual peptides, which may act to regulate different, selective aspects of T cell differentiation in a sequential fashion that is similar to the sequential interactions within the complement or coagulation cascase systems.

In this section we will review the biochemical and biologic properties of the well-characterized thymic preparations. It should be stressed that although many different thymic factors have been described that can induce T cell differentiation *in vitro* and/or *in vivo* in various experimental systems, very few have satisfied all of the requirements for categorization as true thymic hormones. Indeed, detailed thymectomy and thymus reimplantation studies to establish the absolute thymus dependency of circulating bioactivity have only been performed for thymulin (Bach *et al.*, 1972; Bach and Dardenne, 1973). Although thymosin α_1, thymosin β_4, and thymopoietin are all detectable in serum, strict thymus dependency has not, at this time, been completely established.

6.1. Thymosin and Its Component Polypeptides

Thymosin was first prepared as a crude extract of mouse or rat thymus glands by Goldstein and White in 1966 and was originally assayed by its "lymphocytopoietic" properties when injected into mice (Klein *et al.*, 1965, 1966; Goldstein *et al.*, 1966). Goldstein and colleagues demonstrated that treatment of neonatally thymectomized mice with a crude thymosin preparation decreased the incidence of wasting disease, improved survival, and restored cell-mediated immunity such as the ability to reject skin grafts (Law *et al.*, 1968; Asanuma *et al.*, 1970; Goldstein *et al.*, 1970). During the next decade a number of different *in vitro* and *in vivo* murine bioassays were employed, none of them completely satisfactory (cf. Stutman, 1983), with which the final purification procedures for thymosin were developed.

6.1.1. *Thymosin Fraction 5*

Thymosin fraction 5 (TF5) is a partially purified mixture of polypeptides prepared from calf thymus glands as starting material (Hooper *et al.*, 1975). The crude thymus extract is purified by a heat step, acetone precipitation, and fractionation with ammonium sulfate. The 25%–50% ammonium sulfate precipitate is further subjected to ultrafiltration using an Amicon DC-2 hollow fiber system to yield fraction 5 that is lyophilized. Fraction 5 consists of 10 major and at least 30 minor polypeptides on analytical isoelectric gel focusing (Fig. 8), with molecular weights ranging from 1000 to 15,000, and is

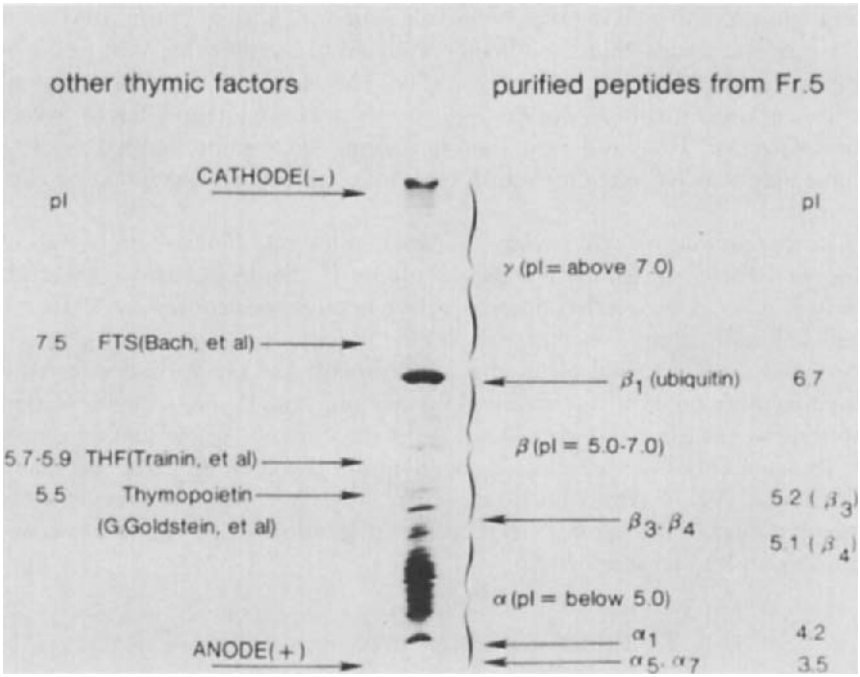


FIG. 8. Isoelectric focusing of thymosin fraction 5 in LKB PAG plate (pH 3.5–9.5). Purified thymosin peptides from the α, β, and γ regions are identified. The isoelectric points of several other well-characterized thymic factors are illustrated for comparison. [From Goldstein *et al.* (1981). With permission of Academic Press.]

free of lipids, carbohydrates, and endotoxin. Thymosin fraction 5 has become a standard preparation in that it has demonstrated a wide range of biologic activities (see Section 9) both *in vitro* and *in vivo*. It was the first partially purified thymic extract to enter clinical trials in primary immunodeficiency patients in the United States (cf. Schulof and Goldstein, 1983).

6.1.2. *Nomenclature of Thymosin Polypeptides*

Analytic isoelectric focusing of TF5 has revealed the presence of a number of components in the preparation. A nomenclature based on the isoelectric focusing pattern of thymosin fraction 5 in the pH range 3.5–9.5 has been described (Low and Goldstein, 1979) and is illustrated in Fig. 8. The separated polypeptides are divided into three regions, the α region consists of polypeptides with isoelectric points below 5.0; the β region, 5.0–7.0; and the γ region, above 7.0. The subscript numbers α_1, α_2, β_1, β_2, etc., are used to identify the polypeptides from each region as they are individually isolated.

Over the past decade Goldstein and colleagues have focused on isolating, characterizing, and evaluating the biologic properties of the major polypeptides found in TF5. Because multiple different cell types are found in thymus tissue including thymic lymphocytes (thymocytes), hormone-secreting thymic epithelial cells, and other structural components, it was expected that TF5 would contain a mixture of (1) thymic differentiation factors produced by thymic epithelial cells, (2) lymphokinelike molecules or other intracellular products originating within thymocytes, and (3) nonspecific peptides reflecting cellular debris and/or resulting from the isolation procedures. It is also possible that the isolation procedures themselves may have altered some of the biologically active thymus-specific polypeptides, leading to the isolation of various partly degraded peptides that originally belonged to larger molecules. Goldstein and colleagues have attempted to isolate and evaluate the biologic activity of as many of the polypeptides as possible from TF5. If an individual polypeptide was found to exhibit relevant biologic activity, it was given a prefix "thymosin," whereas components of TF5 that did not appear specifically related to T cell maturation or function were given the prefix "polypeptide."

6.1.2.1. *Thymosin α_1 ($T\alpha_1$).* The first thymosin polypeptide isolated from the highly acidic region of bovine fraction 5 has been termed thymosin α_1 ($T\alpha_1$). Thymosin α_1 is a polypeptide consisting of 28 amino acid residues with a molecular weight of 3108. Thymosin α_1 was isolated by ion-exchange chromatography on CM-cellulose and DEAE-cellulose as well as gel filtration on Sephadex G-75 (Goldstein *et al.*, 1977; Low *et al.*, 1979). The yield of $T\alpha_1$ from TF5 is only about 0.6%. The complete amino acid sequence (Low and Goldstein, 1979) of this peptide is shown in Fig. 9. The amino terminus of $T\alpha_1$ is blocked by an acetyl group. Human, porcine, and ovine $T\alpha_1$ all appear to have identical amino acid sequences to bovine $T\alpha_1$.

Histochemical and immunofluorescence studies have documented the localization of $T\alpha_1$-producing cells primarily to the epithelial cells of the thymus medulla but also to a lesser degree within the ring of subcapsular cortical surface epithelial cells (Kater *et al.*, 1979; Dalakas *et al.*, 1980; Hirokawa *et al.*, 1982; Haynes *et al.*, 1983a,b,c; Haynes, 1984). Using a cell-free wheat germ system to assess the translation of messenger RNA from calf thymus, it was demonstrated that a larger-molecular-weight radioactive product was immunoprecipitable with antisera against $T\alpha_1$ and the tryptic isolated peptide products were identical to those expected of tryptic peptides from thymosin α_1 (Freire *et al.*, 1978). Thus, these results suggest that $T\alpha_1$ is indeed synthesized in the thymus. In addition, it has been demonstrated (Low *et al.*, 1983) that thymus tissue extracted in guanidine–HCl did not express $T\alpha_1$ immunoactivity, whereas tissue extracted in saline did. This finding suggested that a precursor molecule was cleaved during the saline

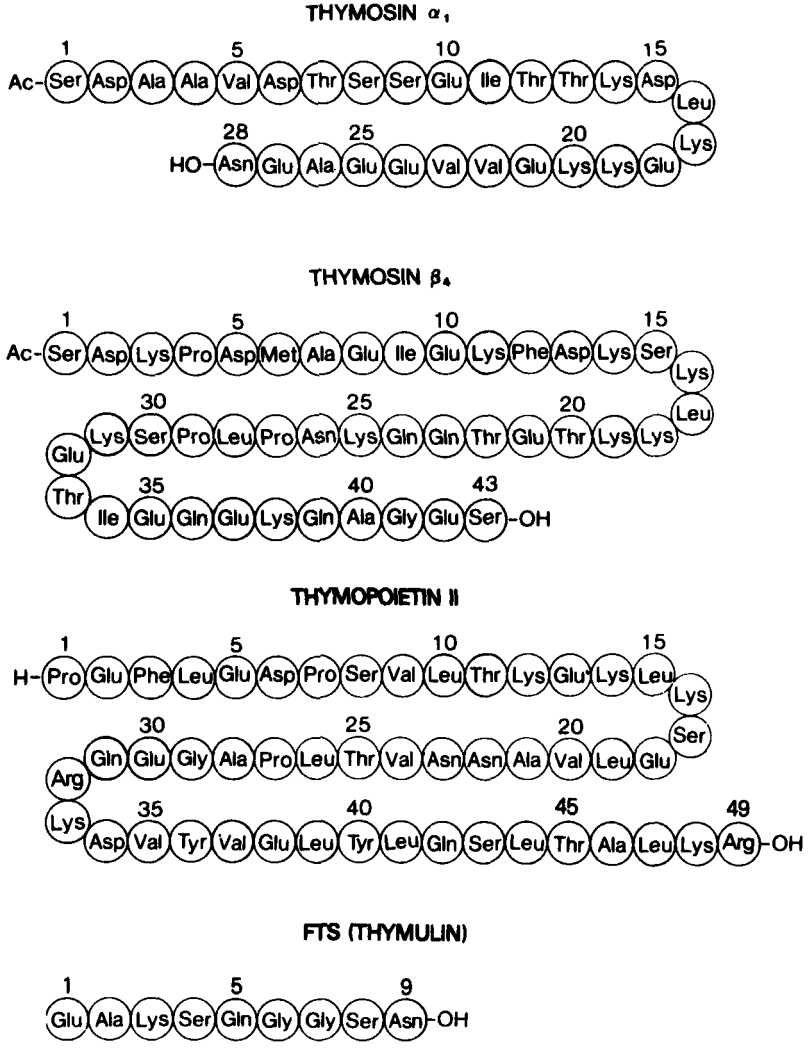


FIG. 9. Sequence analysis of well-characterized thymic hormones: thymosin α_1, thymosin β_4, thymopoietin II, and FTS (thymulin). [From Goldstein *et al.* (1981). With permission of Academic Press.]

extraction procedure to produce $T\alpha_1$. In most recent studies a major immunoreactive form of $T\alpha_1$ has been isolated from rat thymus that appears to represent the prohormone from which $T\alpha_1$ and other fragments are generated during the extraction procedures (Haritos *et al.*, 1984). The purified

prohormone consists of 112 amino acid residues with a p*I* in the range of 3.55 to 3.85. Although the complete amino acid sequence of this molecule has not yet been established, the $T\alpha_1$ sequence appears at its NH_2 terminus.

It has also been demonstrated by using a specific radioimmunoassay (RIA) that $T\alpha_1$ is present in serum and that its serum levels exhibit a circadian rhythm (McGillis *et al.*, 1983; Bershot *et al.*, 1983) and may be high or low in various disease states (see Section 7.2.1). Thus, $T\alpha_1$ appears to satisfy most criteria for categorization as a true thymic hormone. However, the heterologous rabbit antibody used in the RIA recognizes significant cross-reactivity in fraction 5 equivalent preparations isolated from other bovine tissues (see Section 7.2.1). In addition, by using RIA, it has not been possible to establish a strict thymus dependency for circulating levels of $T\alpha_1$ since serum levels are detectable even in elderly subjects and since individuals who have undergone therapeutic thymectomy for myasthenia gravis often do not have a decreased level following surgery (P. H. Naylor *et al.*, unpublished). It is possible that the background serum levels of $T\alpha_1$ detected in thymectomized or elderly humans may reflect either a nonthymic source of $T\alpha_1$ such as the epithelial layer of the skin (known to produce thymic hormonelike peptides) (cf. Chu *et al.*, 1983) or the presence of a biologically inactive $T\alpha_1$-like cross-reacting material. A variety of monoclonal antibodies specific for $T\alpha_1$ have been prepared (Stahli *et al.*, 1983) and a simple procedure described for removing the material found in human and fetal calf serum, which cross-reacts with the monoclonal antibodies. It is hoped that such advances will allow for the development of a more specific RIA.

Biologically active $T\alpha_1$ has been chemically synthesized by both solution (Wang *et al.*, 1978; Birr and Stollenwerk, 1979) and solid-phase (Wang *et al.*, 1980; Folkers, 1983) procedures. The gene for $T\alpha_1$ was synthesized and inserted into a plasmid, and biologically active N^a-deacetyl $T\alpha_1$ was isolated from *Escherichia coli* by DNA recombinant techniques (Wetzel *et al.*, 1980). At the current time, all of the clinical trials using $T\alpha_1$ have employed the chemically synthesized material (Baskies *et al.*, 1982; Dillman *et al.*, 1982; Schulof *et al.*, 1982, 1983a, 1984).

6.1.2.2. *Thymosin α_5 ($T\alpha_5$) and Thymosin α_7 ($T\alpha_7$).* Both of these partially purified peptides have been isolated from fraction 5 by ion-exchange chromatography on CM-cellulose and DEAE-cellulose and gel filtration on Sephadex G-75 (T. L. K. Low and A. L. Goldstein, unpublished). They are highly acidic, with isoelectric points around 3.5. The molecular weights of $T\alpha_5$ and $T\alpha_7$ are approximately 3000 and 2200, respectively. With the use of heterologous antibodies raised against $T\alpha_7$, this peptide was shown to be present primarily in thymic epithelial cells surrounding Hassall's corpuscles and in a few isolated cells in the medullary epithelium (Haynes *et al.*, 1983a,b,c; Haynes, 1984).

6.1.2.3. *Thymosin α_{11} ($T\alpha_{11}$).* This peptide was isolated from TF5 by preparative isoelectric focusing and high-performance liquid chromatography (Caldarella *et al.*, 1983). $T\alpha_{11}$ is homologous to $T\alpha_1$ through its first 28 amino acids and contains 7 additional amino acid residues at the carboxy terminus. Preliminary biological studies indicate that $T\alpha_{11}$ is 30 times as active as TF5 and equivalent in biological activity to $T\alpha_1$ with regard to protecting susceptible mice against opportunistic infections.

6.1.2.4. *Polypeptide β_1.* In general, the peptides isolated from the β region of thymosin fraction 5 do not appear to be thymus-specific products. The most predominant band on isoelectric focusing of TF5 is polypeptide β_1 (Fig. 8). It is composed of 74 amino acids and has an isoelectric point of 6.7 and a molecular weight of 4851 (Low *et al.*, 1979). This peptide does not possess significant biological activity in any of the *in vitro* or *in vivo* bioassays used to monitor the thymosin purification procedures. The sequence of β_1 (Low and Goldstein, 1979) was found to be identical to a protein isolated originally from calf thymus glands but also found in many different tissues and termed ubiquitin by G. Goldstein and colleagues (Schlesinger *et al.*, 1975b). Both β_1 and ubiquitin appear to represent the N-terminal 74 amino acids of the nuclear chromosomal protein A24 (Olson *et al.*, 1976). Thus, it has been postulated that β_1 (ubiquitin) is a degradation product of A24 (Hunt and Dayhoff, 1977). The β_1 peptide has been shown to be homologous to an ATP-dependent coupling factor (AFP) involved in proteolysis (Wilkinson *et al.*, 1980). This observation may account for the ubiquitous distribution of this peptide.

6.1.2.5. *Thymosin β_3 ($T\beta_3$) and Thymosin β_4 ($T\beta_4$).* Both of these polypeptides were isolated from TF5 by chromatography on DEAE–cellulose and gel filtration on Sephadex G-75 (Low *et al.*, 1981). The isoelectric points and molecular weights of $T\beta_3$ and $T\beta_4$ are 5.2 and 5.1 and approximately 5500 and 4982, respectively. Thymosin β_4 was the second thymosin peptide to be sequenced (Low *et al.*, 1981) and chemically synthesized (Wang *et al.*, 1981) (Fig. 8). $T\beta_3$ and $T\beta_4$ appear to share an identical sequence through most of their amino-terminal part and differ in the carboxyl-terminal ends. With the use of immunofluorescent techniques and heterologous antisera to $T\beta_3$ and $T\beta_4$, these peptides were found to localize almost exclusively to the subcapsular thymic epithelial cells covering the thymic cortex, and unlike $T\alpha_1$, they were not present within thymic medullary epithelial cells (Hirokawa *et al.*, 1982; Haynes *et al.*, 1983a,b,c; Haynes, 1984).

A radioimmunoassay for $T\beta_4$ has been developed (see Section 7.2.1.2) and it has been identified in both animal and human sera at concentrations much higher than that of $T\alpha_1$ (Naylor and Goldstein, 1984). However, $T\beta_4$ does

not appear to be a thymus-specific product in that it is also synthesized by peritoneal as well as splenic macrophages (Xu *et al.*, 1982). Thus, the peptides of the β region appear to have more widespread origins in the body than those of the α region.

6.1.2.6. *Thymosin β_8 ($T\beta_8$), Thymosin β_9 ($T\beta_9$), and Thymosin β_{10} ($T\beta_{10}$).* Three additional thymosin peptides termed thymosin β_8, thymosin β_9, and thymosin β_{10} have been sequenced (Hannappel *et al.*, 1982; Caldarella *et al.*, 1983). Thymosins β_8 and β_9 appear chemically related to $T\beta_4$. However, the cells of origin, as well as the biological properties of these polypeptides, have not as yet been studied in detail.

6.2. Thymopoietin

The isolation of thymopoietin (initially termed thymin) by G. Goldstein and colleagues resulted from their interest in myasthenia gravis, a disorder characterized by deficits in neuromuscular transmission and often associated with abnormalities of the thymus gland (Goldstein and Mananaro, 1971). The original purification procedures were monitored with a bioassay that assessed the ability of the thymic extracts to induce a neuromuscular blockade similar to that seen in myasthenia gravis. Subsequently, the biologically active polypeptides were also found to be capable of inducing the differentiation of bone marrow stem cells into mature T cells *in vitro*, so that an important physiological role of the preparation was postulated (Goldstein, 1978a; Goldstein *et al.*, 1979; Basch and Goldstein, 1974).

The purification procedure for thymopoietin includes a heat step, two passages on Sephades G-50, and fractionation on hydroxyapatite and QAE-Sephadex columns (Goldstein, 1974). Two isopeptides were identified that were related by peptide mapping and immunologic cross-reactions. To avoid confusion with the pyrimidine base, thymine, Goldstein changed the nomenclature of his products from thymin to thymopoietins I and II (Goldstein, 1978a,b). These two preparations appeared to be closely related polypeptides that differed by only two amino acid residues (Goldstein, 1978a,b). Thymopoietin II has a molecular weight of 5562 and a p*I* of 5.5. The amino acid sequence of the molecule has been delineated, although a retraction of the original sequence has been reported (Audhya *et al.*, 1981); the corrected sequence is shown in Fig. 9. Comparisons of the available data suggest that thymopoietins I and II are distinct from the other thymic factors. At the present time there is no evidence of homology of thymopoietins I or II with any of the established structures of the polypeptides in calf thymosin fraction 5.

Goldstein and colleagues also purified a third polypeptide that was ini-

tially thought to be a precursor of the thymopoietins. However, this molecule was subsequently found to have a wide distribution in nature in tissues other than the thymus and was termed ubiquitin (Schlesinger *et al.*, 1975b). The sequence of ubiquitin has turned out to be identical to that of a peptide isolated from thymosin fraction 5 by Low *et al.* (1979), termed polypeptide β_1. (See Section 6.1.2.4.)

Fugino *et al.* (1977) reported the synthesis of the entire 49-amino acid chain of thymopoietin II and established that the product had biological activity similar to native thymopoietin II. A tridecapeptide fragment of thymopoietin, corresponding to residues 29 through 41, was synthesized by solid-phase methodology and was shown to have 3% of the biological activity of the entire molecule. In addition, a biologically active pentapeptide (Arg-Lys-Asp-Val-Tyr) corresponding to residues 32 through 36 of the 40-amino acid sequence of thymopoietin has been synthesized (Goldstein, *et al.*, 1979) and termed thymopentin (TP-5) (Zaruba *et al.*, 1983). Thymopentin has been the preparation that has been investigated clinically in immunodeficiency patients.

The cells of origin of thymopoietin appear to be the thymic epithelial cells. By the use of a heteroantiserum and immunofluorescent techniques, the peptide was shown to localize to thymic epithelial cells (Goldstein, 1977). In more recent studies it has been demonstrated that both the subcapsular cortical as well as the medullary thymic epithelial cells react with anti-thymopoietin antibodies (Haynes, 1984). A molecule that is indistinguishable from thymopoietin by immunoassay is also present in one major extrathymic site, the epidermis. Two to fifteen percent of human epidermal cells appear to endogenously produce thymopoietin in tissue culture. These cells have been characterized as basal keratinocytes of the epidermis (Chu *et al.*, 1983).

A radioimmunoassay for thymopoietin has been developed (Goldstein, 1976; Lisi *et al.*, 1980). However, the assay has not as yet been adapted to study serum levels of thymopoietin. A bioassay has been developed by Twomey and colleagues (Twomey *et al.*, 1977) that uses thymopoietin as a standard and measures the induction of a thymus-derived membrane surface antigen (Thy-1.2) on lymphocytes obtained from the spleens of nude (genetically thymus-deficient) mice. This bioassay has been applied to evaluating serum levels in man (see Section 7.1.2) and serum levels have been shown to decrease significantly with age and following therapeutic thymectomy for myasthenia gravis (Twomey *et al.*, 1979).

6.3. Thymic Humoral Factor

The isolation of thymic humoral factor (THF) by Trainin and colleagues was the culmination of studies to explain their observation that thymus tissue

in Millipore chambers implanted into neonatally thymectomized mice led to the restoration of specific immunologic competence in these animals (Levey *et al.*, 1963). Subsequently, Trainin and his colleagues pursued their observations by preparing cell-free extracts that conferred immune competence to spleen cells from neonatally thymectomized mice *in vitro* (Umiel and Trainin, 1975). The initial bioassay was an *in vitro* model of the graft-versus-host reaction. In this assay the immunocompetence of isolated lymphoid cell populations was assessed by the ability of these populations to induce an increase in weight or size of an allogeneic spleen explant. It was observed that spleen cells from neonatally thymectomized mice did not achieve this competence unless they were previously exposed to the thymic extracts.

The initial product was generally obtained from calf thymus, but syngeneic mouse extracts were also shown to be active in the assay. The method used for the isolation of purified THF involves prolonged dialysis of crude thymic homogenates against cold distilled water. The further purification of THF to homogeneity has been achieved (Shohat *et al.*, 1978). The procedure involves successive chromatographic steps on Sephadex G-10 and G-25 and DEAE-Sephadex A-25. The homogeneity of THF has been established by isoelectric focusing on polyacrylamide gels. The isoelectric point of THF is 5.6. On the basis of leucine as unity, the minimal molecular weight is 3220 (Kook *et al.*, 1975).

There is no apparent relationship between the amino acid composition of THF and other purified thymic factors. At the present time the primary amino acid sequence of THF has not been established, nor have the cells of origin of THF been identified.

6.4. Thymulin (Facteur Thymique Serique)

The isolation of thymulin (facteur thymique serique) (FTS) was the culmination of a number of studies by Bach, Dardenne, and colleagues aimed at assessing the immunological status and likelihood of kidney rejection in patients with renal transplants. These investigators developed the azathioprine rosette bioassay (see Section 7.2) that detected thymic hormonelike biologic activity in the serum of animals and man (Bach *et al.*, 1971; Dardenne and Bach, 1973; Bach and Dardenne, 1972a,b). Although several different thymic extracts were active in their system (Dardenne and Bach, 1973), these investigators sought to isolate the active agents directly from pig serum (Pleau *et al.*, 1977; Dardenne *et al.*, 1977; Bricas *et al.*, 1977; Bach *et al.*, 1978). The active factor was initially termed facteur thymique serique because of its origin in serum. It has been characterized as a nonapeptide with a molecular weight of 847. The amino acid sequence of FTS is shown in Fig. 9.

The extraction procedure employs large quantities of pig serum as starting

material. Defibrinated serum is ultrafiltered on a hemodialyzer and concentrated on Amicon membranes. Amicon concentrates are then subjected to four consecutive chromatographic steps: Sephadex G-25, carboxymethyl cellulose, Sephadex G-25 in acetic acid medium, and Sephadex G-10. In every case, the active fractions are detected with the bioassay described earlier. From an initial 15 liters of normal pig serum containing 1200 g of total protein, the yield of FTS is about 3 μg, and the biological activity of the purified product is increased 100,000-fold.

There is no apparent species specificity, since the amino acid analysis of calf and human FTS is identical to that of porcine FTS. This sequence does not show any homology with any of the other thymic polypeptides (e.g., thymosin α_1) that have been described. FTS has now been synthesized by both classical solution (Bricas *et al.*, 1977) and solid-phase (Strachan *et al.*, 1979) procedures. The synthetic material showed full biological activity and chromatographically displayed characteristics identical to those of natural FTS. It has been demonstrated that the biologically active form of FTS is coupled to zinc, whereas the inactive form lacks metal (Dardenne *et al.*, 1982a). After the presence of zinc in the molecule was revealed (Dardenne *et al.*, 1982a) and when its production by thymic epithelial cells was directly demonstrated by immunofluorescence techniques (Savino *et al.*, 1982), the name of the nonapeptide–Zn complex was changed to thymulin. For the remainder of this chapter the name thymulin will be used interchangeably with FTS.

In detailed studies, thymulin has been shown to satisfy more criteria for categorization as a true thymic hormone than any of the other well-characterized thymic polypeptides. The physiology of thymulin production has been studied by several different methodologies including (1) measurement of serum levels using the murine rosette bioassay (see Section 7.1) and (2) immunofluorescent studies using antithymulin antibodies produced in rabbits (Pleau *et al.*, 1978; Monier *et al.*, 1980) and monoclonal antibodies produced in mice. (Dardenne *et al.*, 1982b). In addition, a radioimmunoassay (RIA) using an antiserum raised in rabbits (Pleau *et al.*, 1978), as well as an RIA and an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody (Ohga *et al.*, 1982, 1983), have been developed.

One of the requirements for establishing the thymic origin of any putative thymic hormone is to evaluate the effects of thymectomy and thymus grafting on circulating levels of the putative hormone. In a series of classic experiments, Bach, Dardenne, and colleagues demonstrated that circulating "FTS-like" bioactivity (see Section 7.1.1) is absent in genetically athymic (nude) mice and disappears following thymectomy in mice (Bach and Dardenne, 1973), pigs (Lacombe *et al.*, 1974), and man (Bach *et al.*, 1972). Furthermore, serum FTS-like bioactivity reappears in thymectomized mice

after grafting of a thymus gland (Bach and Dardenne, 1973) or an epithelial thymoma (Dardenne *et al.*, 1974b), but not after thymocyte administration or lymph node grafting.

The murine rosette bioassay that has been utilized extensively to evaluate serum thymulin levels suffers from one major drawback: it is a nonspecific assay and at least several of the other thymic peptides that have been described, thymosin α_1 (Wong and Merrifield, 1980; Ciardelli *et al.*, 1982) and thymopoietin (Twomey and Kouttab, 1982) exhibit activity in the assay in addition to thymulin (see Section 7.1.1). Thus, the active chemical species that produce FTS-like bioactivity in serum cannot be determined by utilizing the bioassay alone. The thymulin RIA and ELISA assay have been applied to studying tissue extracts as well as serum in order to definitively establish the presence of FTS in both the thymus and blood. Significant amounts of thymulin have been detected by using the RIA in thymic but not splenic extracts (Bach *et al.*, 1978). The presence of immunoreactive thymulin in normal serum and its absence in the serum of thymectomized animals have been demonstrated by RIA using Amicon filtration, concentration, and G-25 Sephadex chromatography (Bach, 1983). It has been demonstrated by using the ELISA assay (Ohga *et al.*, 1982) that the monoclonal antibody against FTS could completely absorb the FTS-like bioactivity in human serum that is detected with the murine rosette bioassay. These results support the view that the FTS-like bioactivity in normal human serum is due solely to the activity of thymulin or an FTS-like molecule that is immunologically indistinguishable from porcine FTS.

The most direct evidence for establishing the thymic origin of thymulin has come from immunofluorescence studies with antithymulin antibodies. By using conventional antithymulin rabbit antibodies, thymulin localization to thymic epithelial cells was independently obtained in several different laboratories (Monier *et al.*, 1980; Schmitt *et al.*, 1980; Jambon *et al.*, 1981; Kato *et al.*, 1981). These results were confirmed and expanded upon with a panel of antithymulin monoclonal antibodies (Savino *et al.*, 1982). In each of these studies the fixation of anti-FTS antibodies to thymic epithelial cells was abolished by preincubation of the antibodies with synthetic FTS. Thymulin localization in the thymus was demonstrated both in human (Jambon *et al.*, 1981) and in mouse thymus (Monier *et al.*, 1980; Schmitt *et al.*, 1980; Kato *et al.*, 1981; Savino *et al.*, 1982), in frozen sections (Jambon *et al.*, 1981; Savino *et al.*, 1982), and in thymic epithelial cell cultures (Savino *et al.*, 1982). In general, the antithymulin antibodies appeared to be specific for epithelial cells of thymic origin, and although both medullary and cortical epithelial cells contained FTS, the overwhelming majority of FTS-containing cells were found in the thymic medulla. Nevertheless, only a minority of all thymic epithelial cells were found to contain FTS, amounting to approx-

imately 1% of all medullary epithelial cells. In addition, thymus glands from old mice were almost totally devoid of thymulin-containing cells (Savino *et al.*, 1983a), which is consistent with the finding that serum FTS levels decline dramatically with age (Bach and Dardenne, 1973). Antibodies to both $T\alpha_1$ and FTS were simultaneously compared for binding to thymic epithelial cells by using indirect immunofluorescence. Identical cells were labeled with both antibodies, suggesting that the same epithelial cells produce both peptides (J. F. Bach *et al.*, personal communication).

More recent studies have also suggested that thymulin may be carried in serum by carrier molecules, such as prealbumin (Dardenne *et al.*, 1980a,b), and that there may be a feedback mechanism by which thymulin secretion is regulated (Bach, 1983). As a result of studies that evaluated different fractions of serum in the murine bioassay, it was felt that inhibitors of thymulin activity are normally present in the circulation (Bach and Dardenne, 1973). It was also noted, however, that a second peak of biological activity analogous to native FTS was found in the region corresponding to that of prealbumin. It had been previously reported that prealbumin isolated from serum was active in the murine rosette bioassay (Burton *et al.*, 1978). However, since prealbumin levels are not altered in thymectomized or aged mice, when serum FTS levels are low, it has been postulated that this molecule serves as a carrier for FTS.

The initial insights into the mechanisms of feedback control of thymulin secretion resulted from experiments in which the grafting of thymuses into normal mice led to a transient increase in circulating FTS levels that were directly proportional to the number of thymic lobes grafted (Dardenne and Tubiana, 1979). However, several weeks after thymus grafting there was a diminution of serum FTS levels back to baseline. Conversely, depletion of serum thymulin levels by repeated injections of antithymulin monoclonal antibody or by immunization against thymulin induced an increase by fivefold in the number of thymulin-containing cells (Savino *et al.*, 1983b). Thus, it has been postulated that feedback regulatory control mechanisms based on circulating serum FTS levels control the number of thymic epithelial cells capable of producing the hormone.

6.5. Thymic Factor X

The role of immunological mechanisms in the pathogenesis of leukemia and their effects on the course of the disease have been a subject of study in Poland by Aleksandrowicz and colleagues (Rzepecki *et al.*, 1974) since 1948. Between 1972 and 1974, this group of investigators focused its attention on assessing the clinical usefulness of thymus fragments taken from myasthenia gravis patients and transplanted into selected patients with acute and chron-

ic leukemia and Hodgkin's disease (Aleksandrowicz *et al.*, 1973; Rzepecki *et al.*, 1973, 1974; Szmigiel *et al.*, 1975). With this treatment more than 50% of the patients exhibited some clinical improvement, although the effects tended to be transient, lasting usually only 4 to 8 weeks. Nevertheless, the appearance of immunological enhancement a short time after thymus transplantation led the group in Krakow to begin work on isolating a calf thymus extract, which was termed thymic factor X (TFX).

In the early studies a crude aqueous extract of calf thymus tissue was employed. Evidence that the administration of TFX resulted in enhancement of humoral and cell-mediated immunity (Szmigiel *et al.*, 1975; Rzepecki *et al.*, 1974) led to attempts to purify the crude aqueous extract. The purification procedure that was developed involves ammonium sulfate fractionation, desalting through a Sephadex G-25 molecular sieve, and ion-exchange chromatography (Aleksandrowicz *et al.*, 1973; Czarnecki and Jaskolski, 1978). Thymic factor X is a nucleotide and lipid-free polypeptide mixture, with a major component having a molecular weight of 4200, accompanied by traces of several other peptides with molecular weights ranging from 2000 to 18,000 (Skotnicki, 1978; Staroscik *et al.*, 1978). The final purification of TFX has not yet been achieved. At the present time there is no information available concerning the relationship between TFX and any of the other purified or partially purified (e.g., thymosin fraction 5) thymic preparations. Many of the biological properties of TFX, both *in vitro* and *in vivo*, are similar to those reported with other thymic preparations. The Polish group has had a broad experience in treating patients with a variety of primary and secondary immunodeficiencies with this preparation.

6.6 Thymostimulin

Thymostimulin (TS) is an extract of calf thymus glands that has been partially purified by Falchetti *et al.* (1977) in Italy. Calf thymus tissue is first minced and extracted with ammonium acetate. This extract is then fractionated with ammonium sulfate precipitation. The 0–25% ammonium sulfate cut is further purified by ultrafiltration on an Amicon PM-10 membrane, desalted on Sephadex G-25, and gel filtered on Sephadex G-50. The biologically active preparation exhibits two predominant bands on polyacrylamide gels at pH 8.6. At the present time there have been no attempts to further define the constituents of this partially purified preparation. Although TS is similar to thymosin fraction 5 in its purification schema, a 0–25% ammonium sulfate precipitation step is used, whereas a 25–50% saturation fractionation cut is employed for the isolation of thymosin fraction 5. In addition, the purification procedure for TF5 includes an acetone precipitation step, whereas this procedure is not included in the preparation of TS. Thus, there

should be some differences in the components of TS, as compared to those isolated from thymosin fraction 5. In recent years many studies have been performed with TS to characterize its biological activity as well as its possible therapeutic potential as an immune-modifying agent for patients with primary or secondary immunodeficiency disorders. Results of these studies will be summarized in Section 9.

6.7. Other Thymic Factors

Many other extracts of both thymus tissue and blood have been described that exhibit thymic hormonelike activity in various bioassays. These preparations include homeostatic thymic hormone (HTH) (Comsa, 1973), lymphocytopoietic factors (LSH) (Luckey *et al.*, 1973), hypocalcemic and lymphocytopoietic substances (TP) (Mizutani, 1973), thymic polypeptide preparation (Milcu and Potop, 1973), thymosterin (Potop and Milcu, 1973), leucotrophina (Brunetti and Bellotti, 1978), prealbumin fraction of human plasma (Burton *et al.*, 1978), thymus-dependent human serum factor (SF) (Astaldi *et al.*, 1976), porcine thymic hormone (Jin *et al.*, 1979), as well as thymic epithelial supernatants (Kruisbeek *et al.*, 1977). All of these preparations are described in a review article (Goldstein *et al.*, 1982). Several of the products, in retrospect, are clearly not involved in the normal thymic physiological mechanisms. For example, the prealbumin fraction of human plasma probably exhibits biologic activity in the murine bioassay of Bach and Dardenne because it contains the carrier molecule for FTS (Bach and Dardenne, 1973). The SF described by Astaldi *et al.* (1976) was found to be identical with adenosine (Astaldi *et al.*, 1980; Facchini *et al.*, 1982), and so its thymus dependency is unlikely even though it does exhibit biologic properties that are similar to other thymic preparations (see Section 9.3.3).

7. Circulating Thymic Hormone Levels in Animals and Man

One easily applied method for monitoring the endocrine functions of the thymus in health and disease is by quantitating blood levels of the various thymic-specific peptides for which either bioassays or RIA have been developed. On the basis of whether serum levels of the various thymic hormones are high or low, immunodeficiency diseases could be characterized as being associated with either a hyperfunctioning or a hypofunctioning endocrine thymus gland. Most of the bioassays that have been utilized to monitor the purification procedures of the various thymic peptides are not applicable to studying serum levels, either because they are not sensitive enough to detect the various peptides in serum or because interfering substances exist in

serum and render the assays useless. However, two bioassays—namely, the murine rosette bioassay of Bach and Dardenne (1973) and a bioassay developed by Twomey and colleagues (Twomey *et al.*, 1977)—have been adapted to study serum specimens (see Sections 6.2 and 6.4). In addition to these two bioassays, the RIA for thymosin α_1, thymosin β_4, and thymulin have been applied to evaluating serum levels of these peptides. Various other RIA, ELISA, and target cell binding assays have been reported but have not as yet been applied successfully to quantitating serum levels.

In this section we will summarize the status of the currently available methodologies for determining serum levels of products that reflect the endocrine function of the thymus. It had been hoped that specific abnormalities in blood levels of thymic hormones would provide important information for diagnosing specific immunodeficiency disorders. However, this has not turned out to be the case, and even though the currently available assays can frequently detect abnormalities on the part of the endocrine thymus, there have been no specific serum alterations that are diagnostic of any particular immunodeficiency disorder, and no strong correlations exist between low or high serum thymic hormone levels and the immune status of the patient as defined in T cell functional assays.

7.1. Bioassays

It must be reemphasized that a major problem in interpreting the results of thymic hormone serum levels from currently available bioassyas is that various thymic-derived products may exhibit activity in the assay so that bioactivity detected in serum may reflect the presence of one or more thymic or nonthymic (e.g., adenosine) products. Therefore, such techniques provide only a semiquantitative estimate of serum thymic hormone activity and they do not identify the presence of any specific hormone in the blood. The only definitive mechanism for establishing that the observed serum bioactivity reflects the presence of a particular thymic hormone is either to demonstrate that all of the biologic activity is lost in the presence of a highly specific antibody raised against the hormone or to isolate and identify the peptide in each sample. This practice has generally not been employed in evaluating clinical specimens.

7.1.1. *Bach–Dardenne Bioassay*

The most widely applied serum bioassay is the murine rosette–azathioprine assay developed by Bach and Dardenne (1972a). This is the only currently available bioassay that has generated reproducible results in laboratories around the world (Bach and Dardenne, 1972a; Dardenne and Bach, 1973; Garaci *et al.*, 1978; Burton *et al.*, 1978; Imaizumi *et al.*, 1981; Iwata *et*

al., 1981; Franceschi *et al.*, 1981). It is based upon the observation that spleen cells from adult thymectomized mice (as early as 1 week after thymectomy) are not inhibited, as normal spleen cells are, in their capacity to form rosettes with sheep red blood cells by low concentrations of the drug azathioprine or by xenogeneic antilymphocyte antibodies or anti-T cell antibodies. This population of T cells can be regenerated *in vitro* by incubating spleen cells from adult thymectomized mice with preparations possessing thymic hormonelike activity. The assay consists of detecting the greatest dilution of serum or lowest concentration of standard preparation that restores azathioprine sensitivity of splenic E-rosette-forming cells (E-RFC). Sera to be tested are filtered through a CF50 Amicon membrane in order to eliminate high-molecular-weight serum factors that have been shown to nonspecifically interfere in the assay (Bach *et al.*, 1978). The ultrafiltrates are incubated for 90 minutes at 37°C with spleen cells together with 10 μg/ml azathioprine, a concentration that inhibits rosette formation of splenic lymphocytes from normal mice, but not from thymectomized mice. Rosettes are then formed by centrifugation with sheep red blood cells and enumerated in a hemocytometer after gentle resuspension. In the presence of FTS or other thymic factors, rosette formation is inhibited in the presence of azathioprine. Results are expressed in terms of serum dilutions, with the higher the active dilution, the greater the concentration of FTS in the specimen.

With this assay, both natural and synthetic FTS standard preparations exhibit activity at concentrations as low as 0.6×10^{-6} ng/ml. Partially purified thymic preparations, such as thymosin fraction 5, are active in this assay at much higher concentrations than FTS. For example, TF5 was found to be active at final concentrations between 0.35 and 350 ng/ml (Twomey and Kouttab, 1982). Thymopoietin was active in the rosette bioassay at concentrations between 2 and 70 ng/ml (Twomey and Kouttab, 1982), whereas $T\alpha_1$ was active at concentrations between 10^{-6} and 10^{-7} *M* (Wong and Merrifield, 1980; Ciardelli *et al.*, 1982). Several synthetic C-terminal $T\alpha_1$ peptides appeared to be almost as active as FTS itself (Ciardelli *et al.*, 1982), although no apparent sequence homology is present between the C-terminal region of $T\alpha_1$ and the pentapeptide sequence of FTS essential for activity in this assay (Imaizumi *et al.*, 1981). Because of such overlapping activity it is impossible, with the Bach–Dardenne assay alone, to establish which thymic agent(s) give rise to detectable biologic activity in serum. Therefore, these investigators in selective cases have demonstrated that the serum bioactivity could be completely removed by addition of antithymulin antibodies, thereby suggesting that the major molecular species responsible for serum activity in their assay is indeed FTS (see Section 6.4). Nevertheless, for the remainder of this chapter when the Bach–Dardenne bioassay is used to detect serum thymic hormone bioactivity, it will be catagorized as FTS-like bioactivity.

The murine rosette bioassay was initially employed to monitor the purification of FTS as well as to delineate the thymic dependency of its serum levels (see Section 6.4). Subsequent studies focused on defining the age dependency and alterations of serum FTS levels associated with various immunodeficiency states (see Section 7.1.3). It has been demonstrated both in mice (Dardenne, 1983) and man (Bach and Dardenne, 1972a) that serum FTS-like bioactivity declines gradually with age, in parallel with thymic involution and with the loss of FTS-containing thymic epithelial cells (Savino *et al.*, 1983a). In man serum FTS-like bioactivity is maximal at birth, is stable until the age of 15 to 25 years, and then declines progressively until it becomes barely detectable by the fifth and sixth decades of life (Bach *et al.*, 1978; Iwata *et al.*, 1981). There is an approximate 30-fold decrease in circulating bioactivity between children (mean level $\frac{1}{64}$) and elderly subjects (mean level equal to or less than $\frac{1}{2}$ (Iwata *et al.*, 1981). For this reason serum FTS-like bioactivity is generally referenced to age-matched normal values.

7.1.2. *Twomey Bioassay*

The bioassay of Twomey and colleagues (Twomey *et al.*, 1977) uses thymopoietin as the standard and measures the induction of a thymus-derived membrane surface antigen (Thy-1.2) on lymphocytes obtained from the spleens of germ-free, genetically athymic (nude) mice. Serum samples are filtered through PM 30 membranes prior to testing and are incubated for 18 hours at 37°C with indicator cells (spleen cells depleted of macrophages and B lymphocytes) in the presence of ubiquitin, which inhibits nonspecific T cell induction and increases the sensitivity of the assay. Activity is determined from the percentage of indicator cells induced to express the Thy-1.2 antigen using a sensitive enzyme cytotoxicity test, and results are expressed as the equivalent inductive capacity per milliliter of serum compared to thymopoietin standard preparations. The assay is capable of detecting as little as 0.2 ng of thymopoietin. Various other thymic preparations are also active in this assay. For example, TF5 was active at a concentration of 25 ng/ml. $T\alpha_1$ was found to be the most active purified thymic peptide in the assay and approximately 40 times more potent on a weight basis than thymopoietin, whereas FTS was somewhat less potent (Twomey and Kouttab, 1982). The bioassay of Twomey and colleagues appears to be comparable to the Bach–Dardenne method in that the Twomey assay also demonstrates an age-associated decline in serum thymic hormone activity as well as a loss of such activity following therapeutic thymectomy for myasthenia gravis (Twomey *et al.*, 1979). In the Twomey assay normal serum thymic hormone activity levels range from 10 to 20 ng equivalents of thymopoietin per milliliter from birth until the fourth decade of life and then drops to less than 7 ng equivalents/ml.

7.1.3. *Comparative Studies with Bioassays in Human Diseases*

Serum thymic hormone levels, based on either the bioassay of Bach and Dardenne or the method of Twomey and colleagues, have now been determined for a variety of primary and secondary immunodeficiency disorders (summarized in Table 1).

7.1.3.1. *Diseases Associated with Depressed Thymic-Hormone Bioactivity.* Primary immunodeficiency disorders may be either congenital or acquired and are generally classified according to the mode of inheritance and whether the defect involves T cells, B cells, or both. Three major classes of primary immunodeficiency disorders have been identified. In patients with Bruton's (sex-linked) agammaglobulinemia the primary defect is characterized by the absence of B lymphocytes or by a defect in the differentiation of B lymphocytes, which results in a profound impairment of antibody synthesis. In patients with severe combined immunodeficiency (SCID) the absence of lymphoid stem cells is the main cause of the severe immunological abnormalities that affect cell-mediated as well as humoral immunity. The classic example of SCID, Swiss-type agammaglobulinemia, is characterized by severe lymphopenia involving both B and T cells and is inherited with an autosomal recessive pattern. Affected infants rarely survive a year. DiGeorge's syndrome is a developmental abnormality of the third to sixth pharyngeal pouches that results in varying degrees of thymus and parathyroid gland dysgenesis. In this disorder the primary immune abnormalities reflect the absence of functioning T lymphocytes and patients exhibit marked deficiencies of cell-mediated immunity. Carefully performed autopsy studies have been revealed a tiny but otherwise histologically normal thymus, usually in an ectopic location.

A great variety of patients with primary immunodeficiency disorders have now been studied with both bioassay procedures (Bach *et al.*, 1972, 1975; Incefy *et al.*, 1977; Lewis *et al.*, 1978; Iwata *et al.*, 1981). Serum thymic hormone levels are lower than age-matched normal levels for all patients studied with DiGeorge's syndrome, and a high proportion of patients with severe combined immunodeficiency. In patients with the complete DiGeorge's syndrome, thymic hormone levels are always undetectable, whereas in the partial form, FTS-like bioactivity is measurable but lower than normal. In some cases in which thymus grafting was utilized as a therapeutic modality, serum FTS-like bioactivity became detectable as early as 1 day after transplantation (Incefy *et al.*, 1977) and eventually returned to normal (Lewis *et al.*, 1978). These results have suggested that the increase in serum thymic hormone levels resulted from production by the transplanted thymus. In patients with SCID, regardless of the bioassay employed, thymic hormone levels were found to be either undetectable or much lower than that of age-matched normal donors. In rare cases serum FTS-like bioactivity

TABLE 1

SERUM THYMIC HORMONE BIOACTIVITY IN VARIOUS HUMAN DISEASES

Disease	Serum levels	References
Immunodeficiency syndromes		
DiGeorge's syndrome	Always low or absent	Bach *et al.* (1972); Incefy *et al.* (1977); Iwata *et al.* (1981); Lewis *et al.* (1978)
Severe combined immunodeficiencies	Mostly low or absent	Incefy *et al.* (1977); Iwata *et al.* (1981); Lewis *et al.* (1978)
Wiskott–Aldrich syndrome		
Ataxia telangiectasia		
Common variable immunodeficiencies	Decreased in $\frac{1}{3}$ to $\frac{1}{2}$ of cases	Iwata *et al.* (1981); Cunningham-Rundles *et al.* (1981); Bordigoni *et al.* (1982); Bousquet *et al.* (1979); Iwata *et al.* (1981)
IgA deficiency		
Bruton's agammaglobulinemia	Normal	Iwata *et al.* (1981)
AIDS	Decreased in $\frac{3}{4}$ of cases	Dardenne *et al.* (1983)
Chronic mucocutaneous candidiasis	Usually normal	Iwata *et al.* (1981); Kirkpatrick *et al.* (1978)
Autoimmune diseases		
Systemic lupus erythematosus	Decreased in $\frac{1}{4}$ to $\frac{1}{2}$ of cases	Bach *et al.* (1975); Lewis *et al.* (1981); Iwata *et al.* (1981)
Myasthenia gravis	Normal, except in some older patients (increased)	Bach *et al.* (1972); Shore *et al.* (1979); Twomey *et al.* (1979)
Other diseases		
Mycosis fungoides and Sezary's syndrome	Increased	Safai *et al.* (1979)
Zinc deficiency	Decreased	Cunningham-Rundles *et al.* (1981); Iwata *et al.* (1979b)
Thymoma	Normal or increased	Chollet *et al.* (1981); Kirkpatrick *et al.* (1978)
Hodgkin's disease and acute lymphoblastic leukemia	Decreased in $\frac{1}{2}$ of cases	Schulof *et al.* (1981); Twomey *et al.* (1980)
Atopic infantile asthma	Decreased	Garaci *et al.* (1978)
Chronic graft-versus-host disease	Decreased	Atkinson *et al.* (1982)
Osteopetrosis	Normal or decreased	Iwata *et al.* (1981)
Chronic granulomatous disease	Normal or decreased	Iwata *et al.* (1981)
Cockayne's syndrome	Decreased	Bensman *et al.* (1982)
Protein energy malnutrition	Decreased or normal	Chandra (1979)
Down's syndrome	Usually decreased	Franceschi *et al.* (1981)
Progeria	Normal	Iwata *et al.* (1981)

was near normal, but it did not change significantly following bone marrow transplantation. However, patients with low serum FTS-like bioactivity prior to transplantation developed sustained increments after successful bone marrow transplantation that paralleled the appearance of a thymic shadow early in the course of their immunologic reconstitution. These results have been interpreted to indicate that the humoral function of the epithelial thymus may have been stimulated by contact with colonizing cells originating in the bone marrow that was transplanted.

Other less severe forms of primary immunodeficiency disorders include ataxia telangiectasia and Wiscott–Aldrich syndrome. Wiskott–Aldrich syndrome is a sex-linked genetic disease characterized by eczema, thrombocytopenia, and repeated infections. The immune defects of this disease involve both arms of the immune system. Ataxia–telangiectasia is an autosomal recessive genetic disorder characterized by cerebellar ataxia, oculocutaneous telangiectasia, and immunodeficiency. The immune defects include varying degrees of thymus hypoplasia and abnormalities of thymic-dependent immunity. The majority of patients with both of these syndromes have been shown to have abnormally low levels of circulating FTS-like bioactivity (Iwata *et al.*, 1981) consistent with the observed T cell defects in thymic-dependent immunity.

Other immunodeficiency states that may be associated with depressed serum FTS-like bioactivity include selective IgA deficiency, common variable immunodeficiency (CVI), and acquired immunodeficiency syndrome (AIDS). Common variable immunodeficiency represents a heterogeneous group of syndromes that may be congenital or acquired, sporadic or familial, and that occur in males and females. The patients have in common the clinical manifestations of antibody deficiency with panhypogammaglobulinemia. Surprisingly, thymic hormone bioactivity was found to be low in approximately one-half of young patients with CVI (Iwata *et al.*, 1981; Cunningham-Rundles *et al.*, 1981). In several patients with CVI there was evidence for an inhibitor of FTS-like bioactivity in serum rather than a depression of absolute FTS activity. Noteworthy, however, was that even though serum FTS-like bioactivity was frequently depressed in patients with CVI, there were no clear-cut associations between low serum thymic hormone activity and depressed blood lymphocyte proliferative responses to T-cell mitogens or antigens. It has been reported that 15 of 20 male homosexuals with Kaposi's sarcoma or opportunistic infections or both and diagnosed as having AIDS also exhibited depressions of serum FTS-like bioactivity (Dardenne *et al.*, 1983). The precise role of the thymus gland in the etiology of this acquired T cell immunodeficiency disorder is currently under intense investigation (see Section 7.2.2).

Several authors have reported low thymic hormone serum bioactivity in

some patients with selective IgA deficiency, whether or not the antibody deficiency was associated with T cell abnormalities (Bousquet *et al.*, 1979; Iwata *et al.*, 1981; Bordigoni *et al.*, 1982). In five children with ataxia–telangiectasia and total absence of IgA in serum, with low serum FTS-like bioactivity, treatment with synthetic thymulin induced a decrease in the frequency of infections and an improvement in cell-mediated immunity. In addition, serum IgA appeared in serum by 4 weeks after treatment was initiated and eventually attained normal levels (Bordigoni *et al.*, 1982). At the present time, the mechanism by which FTS administration normalizes this selective defect in B cell immunity is unclear.

There are a number of other diseases that have been associated with aberrations of serum thymic hormone bioactivity, and these are listed in Table 1. It is noteworthy that serum thymic hormone bioactivity has been found to be low in 25% to 50% of patients with systemic lupus erythematosus (Bach *et al.*, 1975; Twomey *et al.*, 1979; Iwata *et al.*, 1981; Lewis *et al.*, 1981), Hodgkin's disease (Schulof *et al.*, 1981), and acute lymphoblastic leukemia (Twomey *et al.*, 1980). In all of these studies it has not been possible to correlate abnormalities of serum thymic hormone bioactivity with specific defects of T cell immunity. Thus, it remains to be established whether low serum thymic hormone levels in autoimmune or neoplastic disorders reflect an etiologic role for thymus dysfunction in these disease processes or merely a secondary manifestation of the diseases themselves. In patients with acute lymphoblastic leukemia it was demonstrated that the low bioactivity detected in the Twomey assay was related to a circulating inhibitor of "thymopoietinlike" bioactivity (Twomey *et al.*, 1980). Thus, it is possible that other secondary immunodeficiencies that are associated with depressed serum thymic-hormonelike bioactivity may also reflect the presence of circulating inhibitors to thymic hormones rather than an absolute deficiency in their production.

Another major problem with the currently available serum bioassays is that they do not detect significant serum bioactivity in subjects beyond the fifth decade of life. This makes it impossible to establish whether serum thymic hormone levels in patients with diseases more commonly associated with old age, such as rheumatoid arthritis, or the more common cancers are lower than those of age-matched healthy donors.

7.1.3.2. *Diseases Associated with Normal Serum Thymic Hormone Bioactivity.* In contrast to patients with SCID or DiGeorge's syndrome, infants with Bruton's agammaglobulinemia all have exhibited normal serum thymic hormone bioactivity. This result is consistent with the concept that primary disorders of B cell development are not associated with dysfunction of the endocrine thymus. There are several other human diseases that are unex-

pectedly associated with normal serum thymic hormone bioactivity. These include myasthenia gravis and progeria. In myasthenia gravis, serum thymic hormone activity detected by both of the available bioassays was generally normal in young patients, although levels tended to be higher than normal in older patients (Bach *et al.*, 1972; Twomey *et al.*, 1979). Nevertheless, therapeutic thymectomy for myasthenia gravis is usually associated with a dramatic decrease in serum thymic hormone activity, thus suggesting that thymic-derived products do contribute to the pathophysiology of the disease. All of the three patients reported with progeria exhibited normal serum FTS-like bioactivity (Iwata *et al.*, 1981). Since such individuals exhibit the stigmata of markedly accelerated aging, this finding is not consistent with the observation that serum FTS-like bioactivity falls and becomes barely detectable in old age. Nevertheless, it suggests that the premature aging associated with progeria is not related to dysfunction of the endocrine thymus.

7.1.3.3. *Diseases Associated with Elevated Serum Thymic Hormone Bioactivity.* There are several diseases in man associated with elevations rather than depressions of serum FTS-like bioactivity. Two cases have been reported in the literature of thymomas secreting inappropriate amounts of thymic hormones (Kirkpatrick *et al.*, 1978; Chollet *et al.*, 1981). In addition, increased thymopoietin-like activity and serum FTS-like activity have both been observed in the sera of patients with cutaneous T cell lymphoproliferative disorders such as mycosis fungoides and Sezary's syndrome (Safai *et al.*, 1979). The site of production and role of the hormones in the pathogenesis of these diseases have not been established. However, since the thymuses in patients with mycosis fungoides and Sezary's syndrome have been reported to be normal in size and histology, it is possible that the increased serum levels of thymic hormones resulted from production at a nonthymic site, namely, the epidermal layer of the skin. Substantial evidence now suggests a close relationship between the thymus and skin. Structurally the thymus and skin have certain similarities. Epidermal keratinocytes and epithelial cells of Hassall's corpuscles express cross-reactive antigens (Sun *et al.*, 1979) as do squamous and thymic epithelial cells (Haynes, 1984). A possible genetic link between thymic and cutaneous epithelium is observed in the genetically athymic (nude) mouse, which has both deficient thymic development and impaired differentiation of hair, an epidermal appendage (Flanagan, 1966). Indeed, the only other body tissue that on immunofluorescent staining possesses cytoplasmic thymic peptides besides thymic epithelial cells is the basal layer of squamous epithelium in the skin (Chu *et al.*, 1983). Thus, it is possible that such cells may be induced to secrete thymic hormonelike products in the presence of the malignant infiltrating T cells. This would be

analogous to the induction of FTS secretion by thymic epithelial cells in patients with SCID following bone marrow transplantation and colonization of the thymus gland with donor stem cells.

7.2. Radioimmunoassays

Radioimmunoassays (RIA) have been developed for thymosin α_1 (McClure *et al.*, 1981; D. Wara *et al.*, 1982; Stahli *et al.*, 1983), for FTS (Bach *et al.*, 1978; Pleau *et al.*, 1978; Ohga *et al.*, 1983), and for thymopoietin (Goldstein, 1976; Lisi *et al.*, 1980). However, very few data are available concerning serum levels in either normal subjects or patients with immune disorders.

7.2.1. *Thymosin*

7.2.1.1. *Thymosin α_1.* Most of the clinical information available at the present time has come from studies employing a radioimmunoassay for thymosin α_1 (McClure *et al.*, 1981; D. Wara *et al.*, 1982). This $T\alpha_1$ RIA utilizes a rabbit antiserum prepared against synthetic $T\alpha_1$ coupled to keyhole limpet hemocyanin as an immunogenic carrier. Prior to use, the antiserum is absorbed with an immobilized preparation of a bovine kidney fraction 5 preparation. A synthetic tyrosine-containing thymosin α_1 analog is radiolabeled with ^{125}I and used as the tracer. The $T\alpha_1$ RIA is capable of detecting as little as 40 pg of $T\alpha_1$ in serum or plasma. The RIA appears to be specific for $T\alpha_1$ and shows no cross-reactions with FTS or thymopoietin. With this technique, the concentration of $T\alpha_1$ in the blood was shown to be highest *in utero*, to decrease after birth, and to remain fairly constant during adulthood. Because of subtle technical differences in the assay procedures, serum levels detected in the two laboratories differ. Normal serum values reported by Wara *et al.* (1982) varied from a high of 727 ± 82 pg/ml (mean ± SE, n = 15) for children aged 1–5 years to a low of 252 ± 21 pg/ml (mean ± SE, n = 29) for individuals aged 20–45 years. More recent normal adult levels reported by Goldstein and colleagues (Naylor *et al.*, 1984) ranged from 400 to 1000 pg/ml. In both murine and human serum, levels of $T\alpha_1$ have also been shown to exhibit a circadian rhythm that was inversely correlated to serum corticosteroid levels (McGillis *et al.*, 1983; Bershot *et al.*, 1983).

Serum thymosin α_1 levels detected by RIA are approximately 10 times higher than those of circulating thymulin levels as determined by RIA (see Section 7.2.3). In addition, there are several differences between serum $T\alpha_1$ levels and thymulin levels in association with aging. First, the 3-fold decline of serum $T\alpha_1$ levels with age was not as dramatic as the 30-fold decline of serum thymic hormone activity determined in the various bioassays or in the thymulin RIA. In addition, serum $T\alpha_1$ levels tend to drop abruptly in childhood and remain constant after age 20 and well past the sixth decade of life,

whereas serum thymic hormone bioactivity tends to decrease gradually beginning at puberty and nadirs by the fourth or fifth decade. The reasons for such discrepancies are unclear. It is possible that the $T\alpha_1$ RIA also detects other serum peptides that are not biologically active or are not products of the thymus gland. In fact, cross-reactivity has been detected in fraction 5 equivalent preparations isolated from other bovine tissues, including spleen (67.6% cross-reactivity), liver (12.4% cross-reactivity), and brain (5.9% cross-reactivity). In addition, the RIA for rodent $T\alpha_1$ does not give parallel inhibition curves to $T\alpha_1$ standards, presumably due to the presence of interfering substances in mouse serum. With this assay it has not been possible to establish a strict thymus dependency for circulating levels of $T\alpha_1$ since there was no change in serum levels following thymectomy in mice (McGillis *et al.*, 1983). It is possible that the background serum $T\alpha_1$ levels detected in thymectomized animals or elderly humans may reflect a nonthymic source of $T\alpha_1$ such as the epithelial layer of the skin or the presence of a biologically inactive material that cross-reacts in the immunoassay. The concept that $T\alpha_1$ may have multiple sources is not a new one. A large number of peptides (e.g., insulin) are now being detected in other than the original tissue from which they were isolated, a finding that may necessitate a redefining of the terms hormone and endocrine (Roth *et al.*, 1982). Thus, the possibility that thymosin peptides may have multiple sources and diverse biologic functions is consistent with our understanding of how a variety of other hormonal peptides contribute to the maintenance of homeostasis.

An RIA has been developed for $T\alpha_1$ employing a mouse monoclonal antibody against the peptide (Stahli *et al.*, 1983). This RIA was not as sensitive as the one employing a rabbit heteroantiserum and has a sensitivity of 1 to 3 ng/ml. In this assay cross-reactive serum materials must be removed by precipitation with 0.1 *M* Na-acetate at pH 4.5. With this RIA TF5 was found to exhibit approximately 1% $T\alpha_1$ immunoactivity on a weight basis. Studies using serum specimens, however, could not be performed with this more specific $T\alpha_1$ RIA because the assay was not sensitive enough to detect $T\alpha_1$ in serum.

7.2.1.2. *Thymosin β_4.* The $T\beta_4$ radioimmunoassay utilizes an antibody raised in rabbits against synthetic thymosin β_4 conjugated by glutaraldehyde to keyhole limpet hemocyanin (Naylor *et al.*, 1984). A ^{125}I-labeled tyrosine-C13 analog of the biologically active C-terminal fragment is used as the radioactive tracer. The radioimmunoassay is sensitive in the nanogram range and no cross-reactivity with common serum proteins is demonstrable. High-performance liquid chromatography of serum samples indicates that two thymosin β_4 cross-reactive species are present in human serum. This is in contrast to $T\alpha_1$, which is present as a single peak. Since the assay measures

immunoreactive epitopes of the biologically active site, it is probable that both peaks have biological activity.

Thymosin β_4 levels are high in cord blood compared to adult serum. In rodents, $T\beta_4$ continues to decline with age for a period that is later in life than $T\alpha_1$. Levels decline in both postmenopausal and functionally castrate females (Rebar *et al.*, 1983). Estrogen supplementation in these women relieves the symptoms of the endocrine failure, but thymosin β_4 levels are even lower after long-term estrogen treatment.

7.2.2. *Abnormal Serum Thymosin Levels in Human Diseases*

The initial $T\alpha_1$ RIA that employs rabbit heteroantiserum has been used to evaluate serum levels in children with primary immunodeficiency disorders (D. Wara *et al.*, 1982). With this assay, 11 of 13 children with combined immunodeficiency, 5 to 7 children with ataxia–telangiectasia and all 3 patients with Wiscott–Aldrich syndrome exhibited serum $T\alpha_1$ levels below the mean of the normal age-matched donors. Such findings were similar to those reported using the Bach–Dardenne bioassay (Iwata *et al.*, 1981). A major inconsistency, however, was in children with DiGeorge's syndrome. Whereas all such children manifested low serum thymic hormone bioactivity, only 3 of 6 exhibited levels below the mean of normals using the $T\alpha_1$ RIA and serum levels were actually quite high in 2.

Although initially perplexing, the discordant results described may be explained by the recent findings in patients with acquired immunodeficiency syndrome (AIDS). Although it was expected that serum thymosin α_1 levels would be low in patients with AIDS, it was found that 60% of AIDS patients with Kaposi's sarcoma, 54% of AIDS patients with *Pneumocystis carinii* pneumonia, as well as 10–60% of asymptomatic male homosexuals (Hersh *et al.*, 1983, Reuben *et al.*, 1983; Biggar *et al.*, 1983; Naylor *et al.*, 1983, 1984) or hemophiliacs (Kriess *et al.*, 1984; Kessler *et al.*, 1984) exhibited elevated serum thymosin α_1 levels that were greater than two standard deviations above the normal mean (Naylor *et al.*, 1983). In addition, similar findings have been observed in pediatric patients with AIDS (Naylor *et al.*, 1984). Thus, it is possible that the previously described pediatric immunodeficiency patients with elevated serum thymosin α_1 levels (all of whom had received numerous blood transfusions and lived in the San Francisco area) may have also had AIDS or an AIDS-associated infection at the time serum thymosin α_1 levels were assessed.

Several hypotheses could account for the frequently elevated serum levels of thymosin α_1 seen in AIDS that would fit the epidemiologic data suggesting a viral etiology for AIDS. One possibility is that the elevated levels reflect end organ failure resulting from a virus-induced destruction of helper T cells and a feedback compensatory increase in thymic release of thymosin

α_1. A second possibility is that a virus could invade thymic epithelial cells and viral modified epithelium would be subject to an "autoimmune" attack resulting in cell death and release of thymosin α_1. In this regard it has been demonstrated that histological sections of thymus tissue in autopsy specimens from patients dying of AIDS exhibited changes characteristic of autoimmune destruction (Seemayer *et al.*, 1984; Davis, 1984). Alternatively, the virus itself could cause thymic epithelial cells or nonthymic cells to increase production of a variety of proteins, some of which could cross-react in the thymosin α_1 assay. Finally, the increased serum thymosin α_1 reactivity could reflect cross-reactivity to the viral agent of AIDS.

This last possibility is supported by the observations that the thymic epithelial cells that produce thymosin α_1 also express an antigen similar or identical to the core protein (P19) of the human T cell leukemia–lymphoma virus (HTLV) (Haynes *et al.*, 1983c) and that elevated serum thymosin α_1 levels may accompany T cell leukemias including those that are HTLV positive (Zatz *et al.*, 1984b). Elevated Tα_1-like immunoactivity has also been observed in patients with malignant brain tumors and head and neck cancer (Wara *et al.*, 1982), as well as in patients with chronic progressive multiple sclerosis (Salk *et al.*, 1982). On the other hand, patients with locally advanced lung cancer exhibited normal serum levels compared to age-matched controls (Schulof *et al.*, 1983a, 1984).

7.2.3. *Thymulin*

Several different RIAs for thymulin have now been developed (Pleau *et al.*, 1978; Bach *et al.*, 1978; Monier *et al.*, 1980; Ohga *et al.*, 1983). These assays employ either a polyclonal rabbit heteroantiserum generated by injecting FTS coupled with gluteraldehyde to a fragment of rabbit IgG or a mouse monoclonal antibody against FTS. The ^{125}I-labeled FTS is prepared either by the Bolton–Hunter method using FTS or by employing a tyrosine-containing analog. The FTS immunoassays do not show significant cross-reactivity with other unrelated peptides or with other purified thymic hormones, such as thymosin α_1 (Ciardelli *et al.*, 1982). Whereas the initial FTS RIA detected what appeared to be trace amounts of FTS in partially purified thymic preparations such as TF5 (Dardenne *et al.*, 1980a), the more recently developed FTS RIA did not detect any FTS cross-reactivity in several TF5 preparations (G. Incefy, personal communication).

An RIA using a rabbit heteroantiserum was the most sensitive and was capable of detecting as little as 1 pg of FTS, allowing for the quantitation of FTS in human peripheral blood. However, in order to detect FTS in serum it was first necessary to filter all specimens through an Amicon CF-50A membrane to remove large plasma proteases that would otherwise degrade the molecule. However, such a procedure could also remove any thymulin

that was bound to a larger-molecular-weight carrier protein (i.e., prealbumin). With this method normal serum levels were reported as varying from 20 to 44 pg/ml in several subjects ranging in age from 5 to 63 years.

A second FTS RIA has been utilized to detect thymulin in extracts of pig and calf thymus (350–500 pg/g tissue) glands but not pig spleens (less than 2 pg/g tissue), which is consistent with a thymic origin for FTS. This assay has also been used to detect FTS in serum specimens. However, the assay is very cumbersome to perform since in order for it to be applicable to serum, samples must be filtered with an Amicon membrane, concentrated, and chromatographed on Sephadex G-25. Nevertheless, serum specimens obtained from thymectomized mice or pigs were found to be almost totally devoid (less than 2 pg/ml) of FTS immunoactivity and the greater than 25-fold decrease of serum FTS immunoactivity correlated with a 60-fold (from $\frac{1}{28}$ to $\frac{1}{2}$) decrease in FTS-like bioactivity as detected in the rosette–azathioprine assay.

At the present time studies are in progress to establish serum FTS levels in healthy subjects of varying ages and in patients with T cell immune deficiencies.

7.2.4. *Thymopoietin*

An RIA has been reported for thymopoietin that can detect as little as 20 pg of the polypeptide (Goldstein, 1976; Lisi *et al.*, 1980). The assay utilizes an antithymopoietin serum raised in rabbits and ^{125}I-labeled thymopoietin as tracer. The RIA is specific for thymopoietin and no cross-reactions were observed with FTS or prealbumin. At present no studies have been reported concerning thymopoietin levels in patients with primary or secondary immunodeficiency disorders.

7.3. Target Cell Binding Assays

Target cell binding assays have found widespread application in the field of endocrinology as a simple means for detecting the presence of various polypeptide hormones (Korenman and Sanborn, 1971). Binding to target cells occurs at the biologically active site of the molecule, and so only biologically active hormones are quantitated by this method. It would seem that binding assays could be easily adapted to the study of thymic hormones. However, because of the difficulty in preparing cell populations enriched for precursor cells of thymic hormones, no such assays have yet been described with normal lymphoid cells. However, the presence of high-affinity receptors for [^{3}H]FTS have been identified on two human T-lymphoblastoid cell lines (Pleau *et al.*, 1980). Steady-state binding was reached within 120 minutes, and the binding was specific for FTS in that it was reduced to 10 to 20%

in the presence of excess unlabeled FTS but not in the presence of unrelated peptides or FTS analogs. No FTS binding could be detected on B or null-cell lines or on several other T cell lines. At the present time the binding assay has not been employed to evaluate serum bioactivity.

Several recent studies have also focused on the binding of various thymosin peptides to putative target cells. By using thymosin fraction 5 labeled with colloidal gold, it was demonstrated that several of the peptides bound to thymic lymphocytes (Brelensha and Warchol, 1982). However, only 2.8% of the cells were positive when this mixture of labeled peptides was used. Dalakas *et al.* (1983) reported an increase in antithymosin α_1 binding to peripheral blood lymphocytes in individuals with myasthenia gravis. Normal individuals had between 0.5 and 2% positive cells. In these studies, the investigators were presumably detecting lymphocytes in the peripheral blood that had thymosin α_1 bound to surface receptors for the peptide. In all the studies to date, neither FTS nor thymosin α_1 was found in significant numbers on thymocytes. Weak binding of antithymosin α_1 antibody has been observed on 90% of peripheral blood lymphocytes by using indirect immunofluorescence (M. M. Zatz *et al.*, unpublished).

8. Effects of Thymus Grafting in Human Primary Immunodeficiency Disorders

The first clinical attempts to reconstitute patients with impaired T cell immunity utilized fetal thymus transplants. Such transplants have met with varying degrees of success, with the best results being obtained in patients with DiGeorge's syndrome. In general, the thymic tissue has been implanted subcutaneously or into a muscle of the anterior abdominal wall. Thymus transplantation has resulted in immunological reconstitution of nearly half the patients with DiGeorge's syndrome (reviewed in Pahwa *et al.*, 1979), and recovery of thymic-dependent immunity was rapid in most instances, occurring within 10 days and suggesting that humoral products released from the transplanted thymus tissue were responsible for the reconstitution. Such a hypothesis was supported by the observation that serum thymic hormone bioactivity that was low prior to transplantation was restored to normal following immunologic recovery (Lewis *et al.*, 1977; Incefy, 1983).

In contrast to DiGeorge's syndrome, thymus transplantation has usually not been successful in patients with SCID (van Bekkum, 1973; Hong, 1976), although occasional T cell reconstitution has been noted (Murphy *et al.*, 1976). In a more rational approach, thymus tissue has been transplanted in conjunction with fetal liver as a source of stem cells for treatment of SCID,

and in these instances therapy has been successful in about 20 to 30% of cases (Pahwa *et al.*, 1978).

Thymus transplantation has also been employed experimentally in a variety of conditions including chronic mucocutaneous candidiasis, ataxia–telangiectsia, and Wiscott–Aldrich syndrome, but in these cases results have generally been disappointing (reviewed in Pahwa *et al.*, 1979; Skotnicki *et al.*, 1984). Thus, the only immunodeficiency state that has clearly demonstrated a lasting improvement of T cell immunity following thymus transplantation is DiGeorge's syndrome.

9. Biologic Activity of Thymic Factors

The concept that thymic hormones exist is now well accepted. However, many controversies still persist because of the multiplicity of different thymic products that have been isolated from thymus tissue over the past several decades. Many of the peptides appear to fulfill at least some of the accepted criteria for categorization as true thymic hormones. However, it is still unclear whether the various thymic polypeptides are components of a single thymic hormone (prohormone) that is capable of exhibiting the complete gamut of biologic properties ascribed to all of the different thymic peptides or whether each peptide alone or in certain combinations with other factors, at both intrathymic and extrathymic locations, regulates specific steps of T cell maturation. In this section we will review the biologic properties attributable to thymic factors in both animals and man.

9.1. Effects in Animals

9.1.1. *Immunodeficient Animal Models*

The nude mouse is the best experimental model for primary T cell immunodeficiency. This mutant strain has a congenital thymic aplasia resulting in absence of functional T cells and severely impaired immunity (De Sousa *et al.*, 1969). Attempts to fully reconstitute such animals with thymic factors have been mostly unsuccessful, with the exception of one report (Ikehara *et al.*, 1975). It is likely that full immunologic reconstitution requires an intact thymus and development of progenitor T cells in the thymic microenvironment.

Nevertheless, the influence of thymic factors on components of the immune response has still been analyzed by using this model. Thus, it has been shown that $T\beta_3$ and $T\beta_4$ can induce expression of TdT in bone marrow and spleen cells from nude mice (Pazmino *et al.*, 1978a,b) and that $T\alpha_1$ can

induce the expression of the Lyt-1,2,3$^+$ phenotype on bone marrow and spleen pre-T cells from such animals (Ahmed *et al.*, 1979; Goldschneider *et al.*, 1981). A variety of thymic factors, including TP-5, TF5, and Tα_1, were capable of inducing T cell antigens on spleen cells from nude mice (Twomey and Kouttab, 1981, 1982).

When a less severely immunocomprised host, that is, an adult thymectomized animal was used, it has been shown that suppressor cell activity can be restored with injections of thymosin or FTS and that phenotypic changes that occur in splenic T cells after thymectomy can be reversed by TP5 or FTS (Nash *et al.*, 1981; Bach, 1977a). In an irradiated, thymectomized, and bone marrow-restored host, both bone marrow and spleen cells were induced with TF5 and Tα_1 to provide helper cell activity for antibody production (Ahmed *et al.*, 1979).

9.1.2. *Induction of T Cell Differentiation Markers*

Another advantage of employing animal systems for evaluating the biologic effects of thymic factors is the ease in preparing precursor T cells (e.g., bone marrow cells) from normal animals. One of the earliest studies to demonstrate committed T cell precursors employed normal murine bone marrow stem cells (Scheid *et al.*, 1973). In these early experiments, a variety of thymic preparations including thymosin and thymopoietin were used to induce expression of T cell markers (Thy-1 and TL antigen) *in vitro* in a low-density subpopulation of murine bone marrow cells. Interestingly, other nonthymic preparations such as dibutyryl cAMP could also induce such phenotypic changes, suggesting a second messenger pathway for the action of thymic hormones (see Section 9.3).

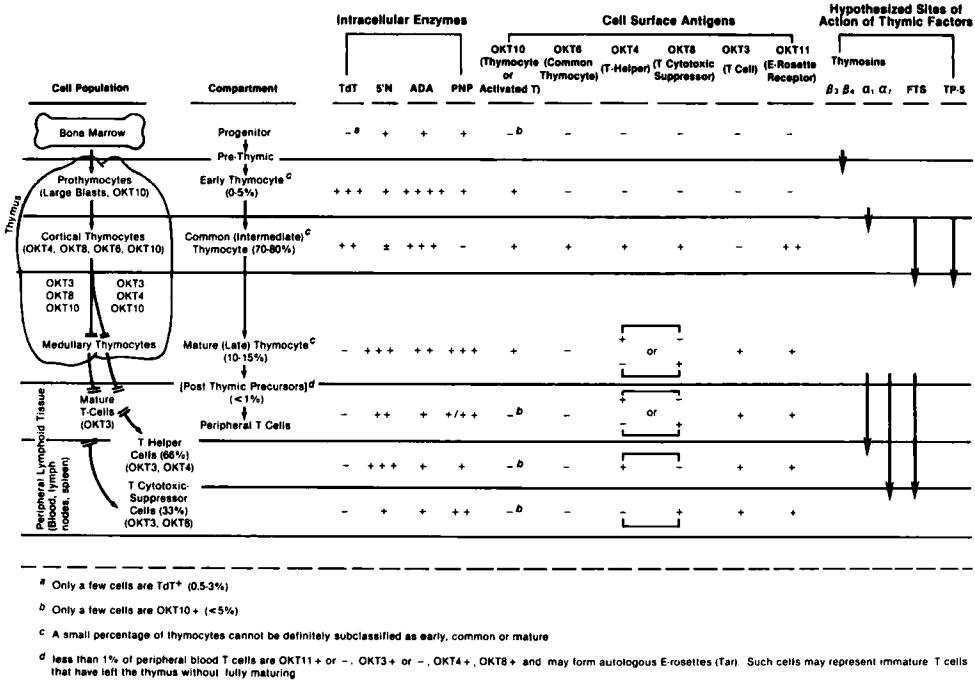
In addition to inducing T cell antigens, TF5, Tβ_3, Tβ_4, and Tα_1 have been shown to induce the expression of TdT in normal mouse bone marrow cells. Of particular interest are studies demonstrating that only 50% of Lyt-1,2,3$^+$ induced cells are TdT$^+$, suggesting the existence of TdT$^-$ and TdT$^+$ T-cell precursors in bone marrow, the latter of which might give rise to a medullary thymocyte population (Goldschneider *et al.*, 1981).

Conversely, the *in vitro* treatment of normal murine thymocytes with Tα_1 reduced their TdT content (Hu *et al.*, 1982). In addition, both TF5 and Tα_1 have been shown to reduce the density of corticosteroid receptors on murine cortical thymocytes (Osheroff, 1981). These inductive capacities of thymosin α_1, β_3, and β_4 are consistent with their intrathymic localization within thymic epithelial cells (see Section 6.1). Thymosin α_1 is found within both medullary and subcapsular cortical epithelial cells. Thus, Tα_1 released *in situ* could be responsible for inducing both early and late stages of thymocyte differentiation and for promoting the subsequent loss of TdT activity and corticosteroid sensitivity. On the other hand, Tβ_3 and Tβ_4 are localized

almost exclusively in subcapsular cortical epithelial cells and appear to act solely on immature cortical precursor cells and to be responsible for the appearance of TdT activity observed in cortical thymocytes. These observations are included in the summary schema illustrated in Fig. 10.

9.1.3. *Induction of T Cell Functions*

A broad spectrum of T cell functions is modulated by thymic factors in various animal models and in some systems identical effects of several different factors have been observed (cf. Kruisbeek, 1975). Many of the functional assays employed are susceptible to extrinsic perturbations, thus making it difficult at times to establish a true inductive effect of the thymic factor as opposed to the nonspecific alteration of the optimal assay condition. Such pleiotropic effects of thymic factors with a variety of influences on the functions of both immature as well as mature T cells has created more controversy than clarity with regard to the precise role the thymus plays in the



[a] Only a few cells are TdT+ (0.5-3%)

[b] Only a few cells are OKT10+ (<5%)

[c] A small percentage of thymocytes cannot be definitely subclassified as early, common or mature

[d] less than 1% of peripheral blood T cells are OKT11+ or −, OKT3+ or −, OKT4+, OKT8+ and may form autologous E-rosettes (Tar). Such cells may represent immature T cells that have left the thymus without fully maturing

FIG. 10. T-cell Differentiation in man. This figure summarizes the data presented in Sections 4.3.3 and 10.2 regarding the influence of well-defined thymic factors on T cell differentiation. The hypothesized sites of action of several thymic polypeptides are shown at the right.

generation and maintenance of immunologically competent T cells (cf. Stutman, 1983). An example of the difficulties in interpreting paradoxical results can be illustrated with thymulin, which can enhance delayed type hypersensitivity in adult thymectomized mice and depress it in normal mice (Bach, 1983). In order to account for such findings it has been rationalized that thymic factors can potentially stimulate both helper and suppressor T cells, and depending upon the dose used or the immune status of the recipient, an effect upon one of the two subsets dominates. Several reviews have summarized the multiplicity of biologic effects of thymosin (Schulof and Goldstein, 1983), thymulin (Bach, 1983), THF (Trainin *et al.*, 1982), and TFX (Skotnicki *et al.*, 1984).

A select group of studies has helped to shed light on the possible physiological mechanisms by which thymic factors control the expression of functional aspects of immunity. Probably the most consistent effect of thymic factors is their ability to induce suppressor T cell activity in various animal models. For example, TF5 has been shown to induce suppressor cell activity in spleen cells of nude mice for both antibody and cytotoxic T cell responses (Marshall *et al.*, 1981; Ahmed *et al.*, 1978, 1979). Similar effects have been observed with several purified thymic peptides including $T\alpha_7$ (Ahmed, 1978, 1979) and thymulin (summarized in Bach, 1983). Other thymic peptides, such as $T\alpha_1$, appear to be more associated with the generation of functional helper T cells. Similar helper inductive effects have also been observed with thymulin in both normal thymocytes and nude mouse spleen cells, which may result from its ability to enhance the production of T cell growth factor (interleukin-2, IL-2) (Palacios *et al.*, 1982; Palacios 1983).

Thus, the bulk of evidence available to date would suggest at least in mice that the spleen contains a population of thymic hormone-responsive lymphoid cells that functions mainly in the suppression of immune responses, perhaps masking concomitant helper effects. In the thymus, the predominant effects of thymic peptides appear to be the induction of functional helper cells, possibly by the enhancement of lymphokine production. The effects of thymulin, thymosin, or other thymic factors on IL-2 production may indeed represent a major function of the endocrine thymus, since IL-2 has been shown to be a potent physiological promoter of T cell maturation (Ruscetti and Gallo, 1981).

9.1.4. *Experimental Models of Human Diseases*

In the preceding section, the effects of thymic hormones in animal models of primary immunodeficiency were discussed. In addition, there are several experimental models for secondary immunodeficiencies, such as neoplasia, aging, autoimmune disease, and infectious diseases, that may provide useful information regarding the eventual clinical use of the thymic hormones.

9.1.4.1. *Infectious Diseases.* Resistance to a variety of organisms, including BCG, cryptococcus, and candida, has been shown to be increased in immunosuppressed animals following *in vivo* treatment with TF5 (Collins and Auclair, 1979; Collins and Morrison, 1979; Bistoni *et al.*, 1982; Ishitsuka *et al.*, 1983). The basis for this increased resistance may be explained by studies showing that mouse strains with low resistance to candida or BCG have increased resistance and elevated production of two lymphokines, MIF and γ interferon following *in vivo* treatment with TF5 (Neta and Salvin, 1983; Salvin and Neta, 1983). Similar results were seen with $T\alpha_1$. In another model, interferon production in response to Newcastle's disease virus infection was also increased by *in vivo* treatment with TF5 or $T\alpha_1$ (Huang *et al.*, 1982).

9.1.4.2. *Autoimmune Disease.* For many years the NZB mouse has been studied as a model of human autoimmune disease. The autoimmune phenomena have been attributed to the development of an immunoregulatory T cell imbalance resulting from the loss of suppressor T cell activity leading to excessive antibody production by unregulated B cells (Gershwin *et al.*, 1974; Dauphinee *et al.*, 1974). Several studies have been performed that indicate that aberrant T cell responses in NZB mice can be normalized by administration of thymic factors. Thus, increased suppressor cell responses have been reported in NZB mice after thymulin (Bach *et al.*, 1978), TP-5 (Lau and Goldstein, 1980), or TF5 treatment (Dauphinee *et al.*, 1974), and decreased autoantibody production has been shown. However, no consistent improvement in the survival or clinical state of these experimental animals has been noted thus far.

9.1.4.3. *Aging.* It has been postulated that waning thymic function precedes the decrease in immunocompetence that occurs in aging animals and in man (Mankinodan, 1978). It is logical therefore to test whether the thymic factors will be useful in improving the immune status of aged subjects. The increase in autologous rosette-forming cells that occurs in the spleens of aged mice can be reversed by exposure to TP-5 (Nash *et al.*, 1981). *In vivo* treatment with TP-5 or TF5 has resulted in improved T-cell help and antibody-forming capacity in aged mice (Weksler *et al.*, 1978; D'Agostaro *et al.*, 1980). It has been reported that THF induces an increase in IL-2 production by lymphocytes from old mice. (Grinblat *et al.*, 1983).

9.1.4.4. *Tumor Immunity.* At the present time it has not been resolved as to whether the primary *in vivo* mechanisms of lymphoid-mediated tumor rejection results from the activity of sensitized cytotoxic T cells or non-T natural killer cells (NK cells) (Herberman, 1981) or both. Several of the well-

characterized thymic factors have been shown to enhance NK activity, including thymulin (Bardos and Bach, 1982) and TS (Fiorilli *et al.*, 1981), and most thymic factors enhance the generation of cytotoxic T cells in suitable model systems (cf. Stutman, 1983; Bach, 1983; Zatz *et al.*, 1983).

It has also been reasonably well established in animal models that increased susceptibility to tumor transplantation and carcinogenesis follows neonatal thymectomy and, conversely, that thymic grafts in diffusion chambers can restore the ability to reject lethal xenogeneic tumor transplants (Halenbeck *et al.*, 1969). In syngeneic systems it has been demonstrated that mice painted with methylcholanthrene developed significantly fewer tumors if grafted monthly with syngeneic thymus tissue (Maisin, 1964) and the mortality rates from syngeneic leukemia grafts in neonatally thymectomized mice could be reversed by thymic grafts within Millipore chambers (Abdou and McKenna, 1969). Thymosin (Hardy *et al.*, 1971; Carlsson *et al.*, 1981), FTS (Bach *et al.*, 1978), and THF (Trainin *et al.*, 1967a) have all been shown to accelerate the rejection of various syngeneic tumors and/or to enhance the generation of cytotoxic T cells in newborn, neonatally thymectomized or adult thymectomized mice, thus suggesting that the mechanism of tumor destruction was via the augmentation of cytotoxic T cell activity.

However, since it is difficult to extrapolate results of studies in neonatal or adult thymectomized mice to man, more clinically relevant animal tumor models have been sought to evaluate the therapeutic efficacy of thymic hormone administration. Two such models have been developed that employ thymosin along with concurrent chemotherapy as the primary cytoreductive modality (Chirigos, 1977; Zatz *et al.*, 1981). These latter two approaches are more likely to be employed in clinical cancer trials, in which thymic hormones will be administered as adjuncts to conventional chemotherapy or radiation therapy.

9.2. Effects in Man

As might be expected, all of the well-characterized thymic hormones have been shown to augment T cell numbers and modulate various T cell functions in man. The earliest investigations were restricted to studying the *in vitro* effects on lymphoid cells isolated either from the blood or bone marrow. Regardless of whether the studies were performed with thymosin, thymopoietin, THF, FTS, TFX, or TS, the results were similar and are summarized in this section.

9.2.1. *In Vitro Effects*

9.2.1.1. *Effects on Peripheral Blood Lymphocytes from Healthy Subjects.* In general, studies utilizing peripheral blood lymphocytes were

aimed at assessing the influence of thymic factors either on T cell numbers or on T cell functions. When such studies were first initiated in the early 1960s, the methodologies available were crude and assessments of T cell numbers were usually restricted to determining the percentage of lymphocytes that formed rosettes in the presence of sheep erythrocytes (E-RFC). The functional assays employed in these initial studies were generally restricted to the evaluation of lymphoproliferative responses to T cell mitogens, such as phytohemagglutinin (PHA), as well as to foreign histocompatability antigens in mixed luekocyte reactions (MLR). More recently, the monoclonal antibody methodology has been utilized to quantitate the percentages of circulating T cells, T cell subsets (e.g., helper or suppressor T cells), and various other populations of lymphoid cells that are found both in blood and in bone marrow. Emphasis has shifted more to the effects of thymic factors on the numbers and functions of killer cells as demonstrated by cytotoxicity assays.

The utilization of peripheral blood lymphoid cells as target cells for evaluating the immunomodulatory effects of thymic factors is based on the assumption that circulating thymic hormones continue to interact with mature T cells and play a role in the maintenance of thymic-dependent immunity. Because of the ease in isolating peripheral blood lymphocytes from venipuncture specimens, this population of cells has frequently been employed experimentally. It must be emphasized, however, that when unfractionated peripheral blood lymphocytes are used as target cells, the predominant cells present are mature T lymphocytes. Thus, when unfractionated peripheral blood lymphocytes are used as targets, it would be difficult to discern any effects on T cell precursors since they would make up, at best, only a very minor component of the lymphoid population being studied.

In general, it has been found that when peripheral blood lymphocytes from healthy donors are used as target cells, thymic factors do not increase T cell percentages to supraphysiological levels (cf. Schulof and Goldstein, 1981; Schulof *et al.*, 1981). It has been difficult to establish precisely how thymic factors influence T cell functions *in vitro* since, for example, mitogen or MLR responses can *either* be augmented or suppressed by incubation with thymosin (Wolf, 1979; Kaufman, 1980), THF (Shoham and Eshel, 1980), thymopoietin (Shoham and Eshel, 1980), or FTS (Kaufman, 1980). As observed in animals, the difficulties in interpreting such findings in man probably result from the varying influences of the various thymic preparations on immunomodulatory T cell subpopulations under different experimental conditions. Thus, studies of the *in vitro* effects of thymic factors on gross T cell numbers or functions have not made a significant contribution toward understanding the mechanism(s) by which the thymus controls the expression of immunity.

One recent area of intense research interest is the effects of various thymic factors on the production of soluble mediators (lymphokines) by T cells, most notably T cell growth factor [TCGF or interleukin-2 (IL-2)] and γ interferon (IFN-γ). Interleukin-2 is released by activated T cells and plays a pivotal role in sustaining both proliferative and cytotoxic immune responses. Interferon, on the other hand, augments T cell cytotoxic activity but exhibits antiproliferative effects. It has been demonstrated that $T\alpha_1$ can increase IFN-γ production *in vitro* in human peripheral blood lymphocytes (Svedersky *et al.*, 1982), whereas TF5, but not $T\alpha_1$, has a pronounced effect on increasing IL-2 production (Zatz *et al.*, 1984a). Similar effects have been demonstrated with thymulin in animals (Palacios, 1983). Thus, it is possible that the variable effects of thymic hormones observed to date in human functional assays may be related to differences in their ability to influence lymphokine production.

9.2.1.2. *Effects on Precursor T Cell Populations.* In order to more rationally study the effects of thymic hormones on the normal differentiation of human pre-T cells, it was necessary to perform *in vitro* incubations with cell populations enriched for precursor cells found within the bone marrow or thymus or at low concentrations in the blood. In man, the earliest such studies were performed by using bone marrow stem cells fractioned by discontinuous density gradients. Crude thymic extracts were initially employed, and it was found that after a 2–14-hour incubation period such preparations could induce the expression of E-rosette receptors and a T cell surface antigen, HTLA (human T lymphocyte antigen), as detected in a microcytotoxicity test (Touraine *et al.*, 1974; Incefy *et al.*, 1975), whereas splenic extracts could not. Similar results were obtained with normal marrow T cell precursors using thymopoietins I and II (Incefy *et al.*, 1975). In addition, various thymosin preparations, FTS, and synthetic TP-5 were also active in such a system (Kagan *et al.*, 1979; Incefy *et al.*, 1980, 1981). Since in man, 95%–99% of thymocytes express HTLA and E-rosette receptors, it has been concluded that the appearance of these markers in bone marrow populations reflected the differentiation of precursor cells to thymocytes. Similarly, incubation of human bone marrow cells with FTS, thymopoietin, and TP5 enabled them to respond to T cell mitogens and in MLR (Incefy *et al.*, 1980, 1981). When marrow cells were fractionated by discontinuous density gradients, the T-cell precursors that could be induced to respond in MLR were found in different fractions from those that were induced to respond to phytomitogens, suggesting that distinct populations of T cell precursors exist in normal marrow.

It has also been possible to induce a small number of human adult peripheral blood lymphoid cells to express T cell surface markers following

incubation with thymic extracts. However, because the number of precursor T cells found in the peripheral blood is small, in these studies it was usually necessary to enrich for precursor cells by using discontinuous bovine serum albumin gradients (Vogel *et al.*, 1975; Horowitz and Goldstein, 1978) or by depleting mature T cells and B cells using cytotoxic antisera (Kaplan, 1978; Kaplan and Peterson, 1978). With such procedures, various thymic preparations, including thymosin, FTS, and thymopoietin, were capable of inducing peripheral blood precursor cells to express T cell surface markers and functions.

The ability to induce T cell surface markers in bone marrow precursor cells was shown to be dependent upon RNA and protein synthesis, but not to involve DNA synthesis (Incefy *et al.*, 1975; Incefy and Good, 1976). In contrast, RNA and protein synthesis were not necessary for inducing the expression of T cell markers using fractionated peripheral blood precursor cells (Vogel *et al.*, 1975). These findings would suggest that bone marrow and peripheral blood precursor cells are not equivalent and probably reflect different stages of differentiation requiring different metabolic events to acquire mature T cell characteristics under the influence of thymic extracts. Indeed, a unique peripheral blood "postthymic" precursor cell has been demonstrated in man that includes less than 1% of peripheral blood mononuclear cells, and it has been shown that such cells are targets for thymic hormones. Theoretically, postthymic precursors represent T cells that have left the thymus before they have fully matured. Several such populations have been described. One population fails to form E rosettes and is phenotypically either OKT11(−), OKT4(+), OKT3(−) or OKT11(−), OKT8(+), OKT3(−). These low-density cells can be concentrated by using discontinuous gradient fractionation procedures. It has been demonstrated that incubation with FTS induces them to become OKT11(+), OKT3(+) to form E rosettes and in some cases switch from OKT4(+), OKT8(−) to OKT4(−), OKT8(−) or OKT8(+), OKT4(−) (Levai and Utermohlen, 1983). Another property that postthymic precursor cells develop following exposure to FTS is the ability to form rosettes with autologous erythrocytes (Tar cells) (Palacios and Alarcon-Segovia, 1981). In man, Tar cells have been shown to bear the OKT3, OKT4, and OKT8 antigens and to mediate autologous MLR (AMLR) (Palacios *et al.*, 1980). Following incubation with FTS, Tar cells are induced to proliferate and to further differentiate (Palacios and Alarcon-Segovia, 1981).

Probably the most revealing study into the sequential maturational events induced by thymic peptides in precursor cells employed human thymocytes obtained from children undergoing cardiac surgery (Ho *et al.*, 1983). During maturation, human thymocytes exhibit a series of sequential changes in the expression of various intracellular purine degradative enzymes including

adenosine deaminase (ADA), 5-ecto nucleotidase (5'NT), and purine nucleoside phosphorylase (PNP), as well as in the expression of terminal deoxynucleotidyltransferase (TdT). As discussed in Section 4.3.3, during maturation from cortical to medullary thymocytes, there is a fall in TdT and ADA activity and a rise in PNP and 5'NT activity. In man, the effects of thymosin fraction 5, $T\alpha_1$, and TP5 on intracellular enzyme activities, as well as on the expression of various surface antigens defined by monoclonal antibodies, were delineated. Each of the three preparations caused one or more maturational changes in human thymocytes. Whereas $T\alpha_1$ induced a fall in TdT and ADA activities, TP5 induced an increase in PNP. Thymosin fraction 5 as well as $T\alpha_1$ both induced the expression of the OKT3 surface antigen, which is a membrane marker of mature T cells found only in a small percentage of medullary thymocytes. Nevertheless, none of the thymic factors studied could induce all of the changes that characterize the differentiation of cortical thymocytes. For example, $T\alpha_1$ did not influence PNP or 5'NT activity or the expression of the cortical thymocyte surface antigen (OKT6). Thus, as has been postulated in animal models (see Section 9.1), results in man also suggest that the well-defined thymic peptides may act sequentially to induce all of the maturational changes that occur physiologically in thymocytes. Thus, whereas $T\alpha_1$ may be required for both an early step of critical thymocyte maturation, leading to the loss of TdT and ADA activity, as well as a late step in medullary thymocyte maturation, leading to the expression of OKT3 positivity, TP5 appears to be involved in relatively later stages of thymocyte maturation that are associated with the induction of PNP and 5'NT. A summary of the different steps at which the various thymic polypeptides may influence T cell maturation, based on the studies in both animals and man, is presented in Section 11 and schematically depicted in Fig. 10.

9.2.1.3. *Effects on Peripheral Blood Lymphocytes Isolated from Patients.* Most of the early studies assessing the *in vitro* influence of various thymic factors on T cells were performed prior to the widespread utilization of monoclonal antibody reagents and therefore employed E-rosetting procedures for determining T cell numbers. The effects of thymic factors on the percentage of peripheral blood lymphocytes expressing E-rosette receptors have been studied in a wide range of disorders associated with depressed T cell numbers. The greatest number of studies have been performed with TF5. Thymosin has been shown to increase the percentage and absolute numbers of E-RFC formed by peripheral blood lymphocytes from patients with primary immunodeficiencies such as DiGeorge's syndrome, ataxia–telangiectasia, and Wiskott–Aldrich syndrome (Wara and Ammann, 1975; Steele *et al.*, 1976; Astaldi *et al.*, 1978; Ammann *et al.*, 1978; Rubenstein *et al.*, 1979; Wara *et al.*, 1980; Barrett *et al.*, 1980; Wara, 1983). Presumably,

null cells obtained from infants with these diseases contained prethymic precursor cells that could be induced in the presence of thymosin to differentiate to mature T cells. In contrast, lymphocytes obtained from children with severe combined immunodeficiency (SCID), who presumably lack a stem cell population capable of differentiating to mature T cells, had no induction of E-RFC following incubation with TF5.

In other diseases, TF5 has been shown to increase the percentages of E-RFC formed by peripheral blood lymphocytes from patients with cancer (Sakai *et al.*, 1975; Schafer *et al.*, 1976; Constanzi *et al.*, 1977; Kenady *et al.*, 1977, Chretien *et al.*, 1978; Byrom *et al.*, 1978a,c,d), allergies (Byrom *et al.*, 1978b), asthma (Byrom *et al.*, 1978a), severe burns (Ishizawa *et al.*, 1978), viral infections (Scheinberg *et al.*, 1976), liver disease (Mutchnick and Goldstein, 1979), uremia (Harris *et al.*, 1975), tuberculosis (Vladiminsky *et al.*, 1978), kwashiorkor (Olusi *et al.*, 1980), systemic lupus erythematosus, and rheumatoid arthritis (Scheinberg *et al.*, 1976; Moutsopoulos *et al.*, 1976). In general, although E-RFC percentages increased, they did not totally normalize. Similar results have been observed with THF by using lymphocytes from patients with primary immunodeficiency, neoplastic, infectious, and autoimmune diseases (Handzel *et al.*, 1975, 1977, 1979; Dolfin *et al.*, 1976; Varsano *et al.*, 1977; Michalevicz *et al.*, 1978; Ramot *et al.*, 1981); with thymopoietin in patients with primary immunodeficiency, rheumatoid arthritis, uremia, malnutrition, and infections (Aiuti *et al.*, 1980; Auteri *et al.*, 1980; Abiko *et al.*, 1980; Jackson and Zaman, 1980); with thymulin in patients with primary immunodeficiency (Incefy *et al.*, 1975; Bach *et al.*, 1980; Incefy *et al.*, 1980; Bene *et al.*, 1982); and with TFX (Dabrowski *et al.*, 1980; Skotnicki *et al.*, 1984) and TS (Aiuti *et al.*, 1979; Aiuti and Businco, 1983; Martelli *et al.*, 1982a; Bernengo *et al.*, 1979) in patients with primary immunodeficiency, neoplastic and, infectious disorders.

The studies have clearly demonstrated that in many clinical conditions thymic factors can induce *in vitro* surface markers characteristic of mature T cells on target cells isolated from peripheral blood. In general, only one or two different concentrations of thymic factors were employed and only short incubation times (5–30 minutes) were required for the expression of E-rosette receptors. In all cases, however, positive effects were only observed if preincubation E-RFC percentages were low. Most recent studies with peripheral blood lymphocytes (PBL) from patients with various primary immunodeficiency disorders have shown that thymulin at a concentration as low as 0.25 ng/ml could also induce monoclonal antibody-defined T cell surface antigens (Bene *et al.*, 1982). In this investigation there was an inverse correlation between sensitivity of the target cells *in vitro* to thymulin and circulating FTS-like bioactivity for individual patients.

Bone marrow stem cells from patients with primary immunodeficiency

diseases have also been used as target cells for incubations with various thymic factors. For example, marrow cells of three patients with DiGeorge's syndrome were induced to express T cell surface antigens by various thymic extracts, including thymopoietin and TP5, although the induction of E-rosette receptors was variable (Touraine *et al.*, 1974; Pahwa *et al.*, 1979). Marrow cells from patients with SCID showed a variable pattern of induction of T cell antigens and E-rosette receptors (Incefy *et al.*, 1981), but cells from most patients were refractory to such inductive effects.

In addition to the effects of thymic hormones on total T cell numbers in a wide variety of patients, a number of studies have suggested that thymic hormones can influence the expression of various subpopulations of T cells in several clinical conditions. For example, with lymphocytes from patients with inflammatory bowel disease, TF5 has been shown to enhance *in vitro* a subpopulation of T cells capable of forming E rosettes under suboptimal conditions ("active" E rosettes) (Dopp *et al.*, 1980), and TP5 has exhibited similar effects (Verhagen *et al.*, 1980). Thymosin α_1 has been shown to increase the percentage of Tar cells with the use of lymphocytes obtained from cancer patients (Caraux *et al.*, 1979), whereas thymulin has exhibited similar effects with lymphocytes from patients with systemic lupus erythematosus (SLE) (Palacios and Alarcon-Segovia, 1981). It has also been shown that TP5 can normalize the abnormally elevated monoclonal antibody-defined helper–suppressor (OKT4–OKT8) T cell ratios observed in patients with rheumatoid arthritis (Veys *et al.*, 1981). With the widespread availability of monoclonal antibody reagents, it is to be expected that many similar studies will be performed with thymic factors in the near future.

There have not been as many reports on the *in vitro* effects of thymic hormones on the functional properties of T cells from patients with various diseases. Thymosin fraction 5 has been shown to enhance MLR reactivity of PBL from patients with primary immunodeficiency disorders (Wara *et al.*, 1980; Barrett *et al.*, 1980). The depressed autologous MLR (A-MLR) of PBL from patients with SLE showed a significant increase following incubation with FTS (Palacios and Alarcon-Segovia, 1981). Thymic humoral factor has been shown to increase the ability of PBL from patients with primary immunodeficiency, autoimmune, or infectious diseases to produce a lymphokine [leukocyte migration inhibition factor (LMIF)] and to exert graft-versus-host responses (Varsano *et al.*, 1976, 1977; Handzel *et al.*, 1979). Thymosin fraction 5 was also shown to enhance LMIF production by PBL of cancer patients (Wolf *et al.*, 1980). The proliferative responses of PBL from cancer patients to T-cell mitogens (e.g., PHA) could be augmented by incubation with thymosin fraction 5 (Hardy *et al.*, 1976). A similar effect was observed by using TS with PBL of untreated patients with Hodgkin's disease (Martelli *et al.*, 1982a,b). With most of the reported studies, although improvements

were noted in various T cell functions, they did not totally normalize. Thus, as observed with E-RFC numbers, the various thymic factors could produce *in vitro* only partial restoration of T-cell function.

Within the past few years there has been a great deal of interest in defining the functional abnormalities of immunoregulatory T cell subpopulations (i.e., helper of suppressor T cells) in various diseases, particularly the autoimmune diseases. In disorders associated with a relative lack of suppressor cell activity such as SLE (Horowitz *et al.*, 1977) and rheumatoid arthritis (Zatz *et al.*, 1984b), thymosin fraction 5 was found to increase suppressor cell activity. Similar results were observed using FTS and PBL from a patient who developed SLE following thymectomy for myasthenia gravis (Calabrese *et al.*, 1981). In contrast, in cancer patients with evidence of excessive suppressor cell activity, *in vitro* incubation of PBL with thymosin fraction 5 could diminish such activity (Serrou *et al.*, 1980; Hersh *et al.*, 1980). Thus, it would appear that thymic factors may be capable of playing a homeostatic role in diseases associated with an imbalance of immunoregulatory T cell activity.

Because of the potential usefulness of thymic hormones in treating various infectious diseases, several studies have now focused on the *in vitro* effects of thymic factors in various human models of infectious diseases. It has been demonstrated (Lowell *et al.*, 1980) that PBL from individuals immunized with tetanus toxoid (TT), group C meningococcal polysaccharide (MgC), or gonococcal pilus (GP) vaccines exhibited significant enhancement of specific anti-TT, anti-MgC, and anti-GP antibodies when incubated with pokeweed mitogen in the presence of thymosin fraction 5 or $T\alpha_1$. The total amount of polyclonal antibody secreted (measured by RIA), however, was not necessarily enhanced and was often suppressed. Thus, these results suggest that thymosin can potentiate the development of antigen-specific helper cells against antigens associated with infectious agents. In another study, it has been reported that the *in vitro* incubation of thymosin fraction 5 with PBL from patients with chronic mucocutaneous candidiasis did not alter their proliferative responses to candida antigen (Akhter *et al.*, 1980). However, a lack of effectiveness in this disorder was not surprising since such patients do not, in general, manifest abnormal circulating thymic hormone levels (Kirkpatrick *et al.*, 1978; Iwata *et al.*, 1981). Thus, the underlying basis for this disease does not appear to be related to a defect of the endocrine thymus.

9.2.2. *Thymic Factor Therapy for T Cell Immunodeficiency*

The rapidity of immune reconstitution in patients with DiGeorge's syndrome following fetal thymic transplants suggested that humoral products released from the transplanted tissues were responsible for the beneficial

effects (see Section 8). Thus, a logical extension to thymus grafting was to employ cell-free thymic extracts therapeutically in patients with impaired T cell immunity. The rationale for administering thymic factors to children with primary immunodeficiencies has been to replace the activity of the endocrine thymus in an attempt to induce nonfunctional precursor cells to become immunologically competent T lymphocytes. The rationale for employing thymic factors in adults is based on the observations that circulating thymic hormone bioactivity decreases dramatically after puberty as the thymus undergoes its physiologic age involution. This age-associated decline of thymus function precedes the well-documented deterioration of immune function with age, and it has been proposed that the increased incidence of autoimmune and neoplastic diseases in the elderly population may reflect the loss of homeostatic control on the part of the endocrine thymus (Schulof and Goldstein, 1976).

The first patient treated with a thymic factor was a 4-year-old girl with thymic hypoplasia and abnormal immunoglobulin synthesis (Wara *et al.*, 1975; Wara, 1983). She was treated with TF5 at a dose of 20 mg/kg/week for a total of 33 months. After 1 month of thymosin therapy, she had conversion of delayed hypersensitivity skin tests as well as an increase in absolute lymphocyte count. In addition, T cell percentages increased from 10 to 60% and serum immunoglobulin levels increased from 220 to 1220 mg/dl. However, no effects were seen on T cell functions in lymphoproliferative responses to T cell mitogens or in MLR. The patient's clinical condition improved with a decrease in the number and severity of infections and diarrhea. She has continued off therapy and appears healthy at age 13. This initial success prompted the many subsequent therapeutic attempts with a variety of thymic preparations.

To the present time, the therapeutic trials with thymic preparations have employed either TF5, $T\alpha_1$, TS, TP5, thymulin, THF, or TFX. In general thymic factor therapy has been well tolerated with minimal side effects limited to local skin reactions in a minority of patients. Rare systemic anaphylactoid reactions have been reported with partially purified calf thymus extracts such as TF5 (reviewed in Schulof and Goldstein, 1983) as their only potential severe toxicity.

9.2.2.1. *Primary Immunodeficiency Diseases.* Immune reconstitution of patients with primary immunodeficiency disorders has been reported following therapy with TF5 (Wara *et al.*, 1975; Wara and Ammann, 1978; Astaldi *et al.*, 1978; Sharp and Peterson, 1978; Bamzai *et al.*, 1978; Barret *et al.*, 1980; Bonagura and Pitt, 1981; Wara, 1983), TS (Aiuti *et al.*, 1979; Davies and Levinsky, 1982; Aiuti and Businco, 1983), TP5 (Aiuti *et al.*, 1980; Fiorilli *et al.*, 1981, 1982; Aiuti and Businco, 1983), THF (Handzel *et al.*, 1979), and

thymulin (Bach *et al.*, 1980; Bordigoni *et al.*, 1982; Bach, 1983). Because of the low incidence of primary immunodeficiency diseases, all of the reported trials with thymic factors have involved small patient numbers. Nonetheless, the overwhelming evidence indicates that thymic factors exert a therapeutic role in selected patients with primary immunodeficiency disorders. In general, when immune reconstitution did occur, it required as much as 3 to 6 months of therapy. Patients with DiGeorge's syndrome responded the most consistently to TF5, TS, TP5, and thymulin and in general exhibited an increase in T cell numbers associated with increased T cell function and clinical stability after therapy. In contrast, no patient with SCID responded to treatment with TF5, probably due to the fact that such patients lack stem cells for thymic hormones. However, some patients with a less severe variant of SCID have improved immunologically and clinically after treatment with TS (Davies and Levinsky, 1982) or TP5 (Aiuti *et al.*, 1980; Fiorilli *et al.*, 1981, 1982). These results suggest that combined immunodeficiency disease represents a heterogeneous population of patients most likely requiring replacement of stem cells as part of their primary therapy, whereas patients with DiGeorge's syndrome form a more homogenous group who theoretically should be reconstituted with thymic hormones alone.

In clinical studies with TF5 a correlation has been observed between pretreatment *in vitro* enhancement of E-RFC numbers of MLR reactivity with thymosin and subsequent *in vivo* improvement of immune parameters (Wara *et al.*, 1980; Wara, 1983). In general, approximately 80% of patients whose peripheral blood T cell numbers or functions improved *in vitro* (using 50–200 μg/ml TF5) subsequently showed improvement after parenteral administration. Nevertheless, occasional patients have shown clinical improvement following therapy with thymic hormones even though pretreatment *in vitro* incubations did not result in enhanced T cell numbers or function (Aiuti *et al.*, 1979; Bonagura and Pitt, 1981). Thus, the predictability of *in vitro* incubation of peripheral blood lymphocytes with thymic factors for subsequent clinical efficacy is not perfect.

9.2.2.2. *Autoimmune and Infectious Diseases.* The successes in children with primary immunodeficiencies have culminated in an increasing number of attempts at thymic factor therapy for adult patients with more common disorders, including autoimmune diseases, cancer, and infectious disorders. Studies have suggested that various thymic preparations, such as THF (Varsano *et al.*, 1977, Trainin *et al.*, 1982), TFX (Dabrowski *et al.*, 1980; Skotnicki *et al.*, 1984), and TS (Businco *et al.*, 1980; Aiuti and Businco, 1983) can shorten the course of recurrent viral infections (including Herpes zoster, Herpes simplex, and cytomegalovirus) as well as accelerate the restoration of T cell immunity in such patients. Preliminary results of a trial of

TF5 in male homosexuals and hemophiliacs at risk for developing AIDS have also demonstrated a partial restoration of T cell function and normalization of IL-2 production by mitogen-activated lymphocytes (Naylor *et al.*, 1984). The clinical efficacy of TF5, TP5, THF, and thymulin is currently being investigated in patients with rheumatoid arthritis, sarcoidosis, and systemic lupus erythematosus (Shohat *et al.*, 1978; Lavastida *et al.*, 1981; DiPerri *et al.*, 1980; Auteri *et al.*, 1980; Jacobs *et al.*, 1984). The rationale for treating patients with autoimmune disorders with thymic factors is to determine whether such preparations can help to normalize the aberrant immunoregulatory cell activity that is characteristic of these diseases.

9.2.2.3. *Cancer.* In cancer patients it is hoped that thymic factors can be employed as adjuncts to conventional chemotherapy or radiation therapy to ameliorate the immunosuppressive side effects associated with such treatment. If T cell-dependent immunity were maintained and/or restored, the cancer patient would theoretically be more capable of mounting a specific host immune response against the tumor, as well as to the various viral, bacterial, and fungal pathogens to which he would otherwise be susceptible.

For the overwhelming majority of cancers, the administration of thymosin (either TF5 or $T\alpha_1$) or other thymic factors alone does not result in tumor regression (reviewed in Schulof and Goldstein, 1983; Dillman *et al.*, 1982). However, the administration of TF5 has caused regression in approximately 10 to 20% of patients with renal cancer and some with prostate cancer (Wara *et al.*, 1984; Schulof *et al.*, 1984). Since many of these patients exhibited normal pretreatment immune profiles and since each of these cancers is considered to be a hormonally responsive tumor, it has been suggested that thymosin may act by an undefined endocrine mechanism in these disorders.

In other cancer studies TF5 has been shown to accelerate the reconstitution of T cell function and improve survival in patients with head and neck cancer treated with radiotherapy (Wara *et al.*, 1978, 1981), and similar improvements were noted in patients with non-oat cell lung cancer who were treated with $T\alpha_1$ following radiotherapy (Schulof *et al.*, 1983a,b, 1984). TS has been shown to enhance T cell numbers and functions following administration to immunosuppressed patients with Hodgkin's disease (Martelli *et al.*, 1982b) and malignant melanoma (Bernengo *et al.*, 1983), and TFX has exerted similar effects in patients with leukemia and lymphoma (Skotnicki *et al.*, 1984). Nevertheless, when employed concurrently with chemotherapy, with the exception of one study of TF5 in patients with oat cell lung cancer (Cohen *et al.*, 1979), none of the thymic preparations has been shown to be capable of either enhancing immune function or improving overall survival. Thus, further investigation is necessary in order to learn how to best incorporate thymic factor therapy into the currently employed complex chemotherapeutic regimens.

From the studies described in this section, it is apparent that in selected clinical conditions associated with either primary or secondary immunodeficiency, various thymic preparations are capable of restoring T cell numbers and function. However, clinical trials with thymic hormones are in their infancy and a number of questions still must be resolved before thymic factor therapy can become an accepted form of treatment. Most of the reported studies to date, particularly in primary immunodeficiency disorders, were nonrandomized and involved small patient numbers, and so they need confirmation in large-scale, randomized trials. Both the dosage and schedule dependency of each of the thymic factors have been empirically established, and further emphasis is needed in defining the optimal dosage and frequency of administration. Nevertheless, because they are nontoxic preparations, the thymic factors hold promise as important new drugs for treating a wide variety of disorders associated with abnormalities of thymic-dependent immunity.

9.2.3. *Pharmacokinetics of Thymic Hormone Administration*

Since the various thymic hormones have only recently entered clinical trials, there is little information available on circulating levels following parenteral administration in man. In their initial description of the rosette–azathioprine bioassay, Dardenne and Bach (1973) reported that after the intravenous injection of a crude thymosin fraction to adult thymectomized mice, transient serum thymic hormone bioactivity was demonstrable and peaked at 2 hours postinjection and disappeared after 48 hours. More recent studies using the rosette–azathioprine bioassay have indicated that FTS itself disappears rapidly from blood after intravenous administration, with a half-life of 15 minutes (Bach *et al.*, 1978). The half-life of FTS could be prolonged by preincubation with serum from thymectomized mice or by binding to carboxymethyl cellulose.

Of the thymic hormones currently undergoing clinical evaluation, the most detailed pharmacokinetic studies have been performed with TF5 and $T\alpha_1$. The parenteral administration of TF5 has been associated with the generation of detectable serum thymic hormone bioactivity as detected in the Bach–Dardenne assay (Iwata *et al.*, 1981). A 10-year-old child with chronic mucocutaneous candidiasis treated with TF5 showed a prompt rise of serum FTS-like bioactivity that persisted within the normal range for several weeks following the discontinuation of treatment, but which eventually returned to low levels (Iwata *et al.*, 1981). These studies have clearly demonstrated that the parenteral administration of TF5 generates detectable serum bioactivity. However, it cannot be determined which of the peptides present in TF5 gave rise to the serum activity.

Studies utilizing the RIA for thymosin α_1 have provided additional pharmacokinetic data. It has been demonstrated (W. Wara *et al.*, 1982) that the

SQ administration of TF5 to cancer patients (60–150 mg/m^2) resulted in a 4–30-fold increase in circulating Tα_1 levels. In a randomized trial in lung cancer patients designed to compare the effects of thymosin α_1 (900 μg/m^2) administered SQ either twice weekly or via a loading dose schedule (daily for 14 days followed by twice weekly maintenance), it was shown that following a single injection of Tα_1, peak plasma levels reached extraordinarily high concentrations (approximately 27 ng/ml) by 6 hours postinjection and then returned to near baseline levels by 24 hours (Schulof *et al.*, 1982). The peak plasma levels achieved were 20–50 times higher than the highest physiological levels ever detected in newborn infants. Interestingly, either schedule of Tα_1 administration resulted in a gradual rise of baseline Tα_1 over the first 3 weeks of study to levels that were greater than two standard deviations above the mean of age-matched normals. Baseline levels began to drop over the next week but were maintained at more than one standard deviation above that of the normal donors. Patients receiving Tα_1 by either schedule maintained similar elevations of baseline Tα_1 levels when compared to patients who had received placebo over an 11-week study period. Thus, daily injections of Tα_1 may not be necessary in order to chronically elevate its circulating levels.

9.3. Mechanism of Action of Thymic Hormones

Possible second messenger mechanisms for thymic hormone-mediated differentiation of T cell precursors have been demonstrated both by pharmacological manipulation and by direct measurement of the putative second messenger. Many of the effects of thymic hormones can be mimicked by various cyclic nucleotides and/or other physiological and nonphysiological agents. For example, in mice the T cell differentiation antigens (Lyt or theta) can be induced on bone marrow or spleen cells from thymectomized mice not only by thymic hormones such as thymopoietin, thymosin α_1, and thymulin, but also by endotoxin, isoproterenol, dibutaryl cyclic AMP, phosphodiesterase inhibitors, prostaglandins, and poly(A)–poly(U) (Scheid *et al.*, 1973; Bach and Bach, 1973; Garaci *et al.*, 1981).

9.3.1. *Cyclic Nucleotides*

Several indirect arguments have suggested that cyclic AMP (cAMP) may be a second messenger for thymic hormone action. These include the observations that cAMP in the Bach–Dardenne bioassay acts synergistically upon rosette-forming cells with thymulin (Bach and Bach, 1973). In addition, the inductive effects of thymic hormones can readily be mimicked by a variety of products that increase cellular cAMP and conversely can be inhibited by products that decrease cAMP (Scheid *et al.*, 1973, 1975; Astaldi *et al.*, 1978).

It has also been demonstrated that dibutaryl cAMP or agents that increase intracellular levels of cAMP in lymphocytes (i.e., theophylline or prostaglandin E_2) mimicked the ability of THF to endow spleen cells from neonatally thymectomized mice to exhibit functional activity characteristic of mature T cells in an *in vitro* model of the graft-versus-host response (Kook and Trainin, 1975a,b). Although it has been demonstrated by using direct measurement that THF could elevate cAMP levels in murine T cell precursors (Kook and Trainin, 1974), other reports with thymosin, FTS, and thymopoietin have shown no clear-cut evidence of cAMP synthesis following incubation with thymic factors (Bach and Bach, 1973; Naylor *et al.*, 1976, 1978, 1979a,b). Thus, the status of cAMP as a second messenger for thymic hormone activity is controversial.

Another possible cyclic nucleotide second messenger for thymic hormones is cyclic GMP (cGMP). An increase in cGMP in murine thymocytes was demonstrated following incubation with TF5 (Naylor *et al.*, 1976, 1979c). Similar findings were reported in lymphocytes using thymopoietin (Sunshine *et al.*, 1978). By using their sensitive assay a subpopulation of thymocytes could be isolated in which the major elevation of cGMP was occurring (Naylor *et al.*, 1979a). Thymosin causes an influx of calcium into thymocytes, consistent with the fact that the increase in cGMP is calcium dependent (Naylor *et al.*, 1979b).

9.3.2. *Prostaglandins*

More recent investigations have suggested that prostaglandins may play an important role as possible second messengers for thymic hormones. Indomethacin, a synthetic prostaglandin inhibitor, could prevent thymulin induction of Thy antigen in mice (Bach *et al.*, 1972; Fournier and Bach, 1975) and thymulin incubation enhanced prostaglandin synthesis in human lymphocytes (Gualde, 1982). Prostaglandins were also capable of mimicking the action of thymic factors in the Bach–Dardenne bioassay (Rinaldi-Garaci, 1982; Rinaldi-Garaci *et al.*, 1983a), and the *in vivo* administration of a long-lived PGE_2 analog induced the appearance of serum thymic hormonelike bioactivity in adult thymectomized mice (Rinaldi-Garaci, 1982). TF5 and $T\alpha_1$ (Rinaldi-Garaci *et al.*, 1983b) were both shown to rapidly induce a dose-dependent stimulation of the release of PGE_2 in spleen cells derived from adult thymectomized mice.

9.3.3. *Adenosine and Related Metabolites*

A serum factor that was initially felt to represent a specific thymic hormone was termed SF (A. Astaldi *et al.*, 1976; G. Astaldi *et al.*, 1979, 1980; Facchini *et al.*, 1982). Serum factor was shown to increase cAMP, to decrease TdT activity, to induce changes in nonhistone nuclear proteins in

murine thymocytes, and to increase their resistance to steroids and their capacity to exhibit graft-versus-host responses. Furthermore, treatment of immunodeficient patients with TF5 induced the appearance of SF by as early as 3 hours postinjection, which continued to rise during 2 weeks of daily injections and which returned to pretreatment levels after discontinuation of treatment (A. Astaldi *et al.*, 1977). The increase in serum activity correlated to varying degrees with signs of immunologic reconstitution.

Although SF was erroneously hypothesized to be a unique thymic-dependent factor, biochemical analyses established that the active moiety was adenosine (G. Astaldi *et al.*, 1980; Facchini *et al.*, 1982). Adenosine appears to possess immunosuppressive properties for T cells in various functional assays (reviewed in Stutman, 1983) and can induce the phenotypic surface markers of suppressor cells. The congenital deficiency of the enzyme adenosine deaminase (ADA) leads to an increased intracellular concentration of adenosine and other purine intermediates and is associated with a severe combined immunodeficiency (Meuwissen and Pollara, 1978; Mitchell and Kelley, 1980). It is found predominantly in immature thymocytes (see Section 4.3.3) and levels of this enzyme are decreased *in vitro* by incubation with $T\alpha_1$ (Ho *et al.*, 1983) but not with TF5, TP5, or thymulin (Ramagopal and Reem, 1982; Ho *et al*, 1983). Thus, these studies suggest that thymic factors may affect the differentiation of thymocytes by inducing changes in the concentration of intracellular intermediates of metabolism. In a unique therapeutic trial, the administration of red blood cells (as a source of ADA) and TF5 was capable of inducing immune reconstitution in a child with congenital ADA deficiency (Rubenstein *et al.*, 1979).

From the second messenger studies performed to date, it can be concluded that a number of candidates exist for intermediates of thymic hormone action. However, it is clear that the intermediate events by which thymic factors induce T cell differentiation are complex and no doubt involve a wide spectrum of mechanisms. The most important of these still remain to be defined.

9.4. Interactions between Thymic Hormones and Trace Metals

The fortuitous synthesis of inactive or instable lots of thymulin suggested that the peptide could exist in two forms, one biologically active and the other inactive (Dardenne *et al.*, 1982b). Subsequent studies revealed that the active form of this thymic peptide contains a metal, probably zinc, whereas the inactive form lacks metal. In the presence of a metal-ion-chelating agent, the biologic activity of thymulin is lost and such activity can be restored by the addition of zinc salts and certain other metal salts, including

aluminum and gallium. Zinc salt alone was not biologically active. The interaction between thymulin and zinc was subsequently confirmed by using gel chromatography, and the interaction between zinc and thymulin was further supported by the observation by microanalysis of the metal in thymic epithelial cells. It is noteworthy that zinc deficiency in humans and mice is associated with a profound impairment of thymic-dependent immunity, thymic involution, and early loss of circulating FTS-like bioactivity (Iwata *et al.*, 1979a, 1981).

10. Interactions between the Thymus and Neuroendocrine System

10.1. Thymic Influence on the Endocrine System

Evidence is accumulating to indicate that there is a close relationship between the endocrine thymus gland and the other more classical endocrine systems of the body. In both neonatally thymectomized, day 3 thymectomized and nude (athymic) mice, severe endocrine abnormalities have been reported (Nishizuka and Sakakura, 1971; Pierpaoli and Besedovsky, 1975; Deschaux *et al.*, 1982; Rebar *et al.*, 1981a,b). Serum levels of hormones including estrogen, testosterone, corticosterone, leutinizing hormone (LH), and follicle-stimulating hormone (FSH) are altered (Deschaux *et al.*, 1979; Rabar *et al.*, 1981a,b). Ovarian dysgenesis occurs in day 3 thymectomized mice and rats even though these animals do not develop the severe wasting disease associated with neonatal thymectomy (Nishizuha and Sahahura, 1971; Hattori and Brandon, 1979). These thymus-impaired mice and rats also have decreased adrenal and testis weights. Thymic extracts such as thymosin and homeostatic thymic hormone (HTH) reversed the effect of neonatal thymectomy (Deschaux *et al.*, 1982). The alterations in FSH and LH in nude mice were also reversed by thymus grafts into female mice. Thus, it appears that an actively functioning thymus gland early in ontogeny is essential for normal development of the endocrine system.

10.2. Endocrine Influence on the Thymus

The close relationship between the thymus and the more classic endocrine organs is further illustrated by the results of studies designed to elucidate the effects of endocrine ablative surgery on thymus gland function. Prepubertal orchidectomy resulted in an increase in thymus weight and accelerated rejection of skin grafts (Castro, 1974). The effects are reversible by testosterone administration. Adrenalectomy also delayed the involution of the thymus (Dougherty, 1952; Ishidate and Metcalf, 1963).

Castration or ovariectomy (when associated with adrenalectomy) induced a decrease in serum FTS-like bioactivity (Bach, 1983) and genetically dwarf (Snell–Bagg) mice exhibited a premature decline in serum bioactivity (Pelletier *et al.*, 1976). High doses of corticosteroids induced a decrease in serum FTS-like bioactivity and serum $T\alpha_1$ levels also exhibited an inverse relationship with serum corticosterone levels as well as a circadian rhythm (McGillis *et al.*, 1983). Thus, current evidence indicates that the endocrine functions of the thymus gland are closely integrated to the other well-defined endocrine organs.

10.3. Relationship between the Thymus Gland and the Central Nervous System

A direct link between the immune (thymus) and endocrine system via the brain has been proposed (Rebar, 1983; Hall and Goldstein, 1983). Such a link is supported by the observations that (1) efferent pathways between the central nervous system (CNS) and the immune system exist, (2) hypophysectomy alters immune responsiveness (i.e., prolongs skin graft and lowers antibody production), (3) thymic hormones such as thymosin α_1 and thymosin β_4 are found in discrete areas of the brain, and (4) administration of thymic hormones alters endocrine hormone levels (Comsa *et al.*, 1975; Rebar *et al.*, 1980, 1981a,b; Hall and Goldstein, 1983; Palaszynski *et al.*, 1983; Healy *et al.*, 1983).

Of significant interest are the most recent studies in rats that evaluate the thymus–reproductive and thymus–stress axes. Both *in vitro* administration of thymosin β_4 into the medial basal hypothalamus and pituitary in chambers and *in vivo* administration into the cerebrovascular system have induced elevations of LH in media and serum, respectively (Rebar *et al.*, 1981b; Hall *et al.*, 1983). Localization studies have demonstrated that the rat olfactory bulb has the highest concentration of thymosin β_4, although it is also present in several other distinct sites (Hannappel *et al.*, 1982). On the other hand, $T\alpha_1$ did not influence levels of LH. Nevertheless, $T\alpha_1$ is also present in the brain (Hall *et al.*, 1982; Palaszynski *et al.*, 1983), with its highest concentrations in the subcortical nuclei involved with both the autonomic and neuroendocrine system. These sites include both the pituitary gland and the hypothalamus. When injected intracranially, thymosin α_1 stimulated a rise in serum corticosterone in mice (Hall *et al.*, 1982). The increase was rapid (<3 hours) and did not occur in experiments where the peptide was incubated with cultured adrenal fasciculata cells (Vahouny *et al.*, 1983).

Thymosin fraction 5 has been injected intravenously into primates (Healy *et al.*, 1983). There was a significant elevation of ACTH, cortisol, and β-

endorphin in the serum of the animals. These experiments clearly point to a thymus–pituitary connection mediated by one or more polypeptides in thymosin fraction 5. Whether the active factor is thymosin α_1 or one or more other peptides remains to be determined.

11. Summary of Thymus Gland Biochemistry and Physiology

A summary of the major characteristics of the well-defined thymic factors is shown in Table 2. It should be appreciated from the wealth of data presented in this chapter that all of the traditional criteria required for categorizing the thymus as a true endocrine organ have now been satisfied. Nevertheless, the complex mechanisms by which the thymus controls the development and expression of immunity are still not well defined, nor is our full understanding of T-cell development, function, and regulation.

Probably the two most perplexing and controversial issues in thymus endocrinology are the multiplicity of apparently unique thymic factors that have been described and the pleiotropic effects on precursor T cells, as well as on mature T cells, that each of the putative thymic hormones possess (cf. Stutman, 1983). Many of the biologic activities of thymic hormones can be mimicked by nonspecific agents such as endotoxin, prostaglandins, and cyclic AMP. In addition, it is not clear why multiple different thymic factors should exhibit identical effects in some of the biological assays.

It is probably unfair to expect that any single thymic polypeptide should have the capability of inducing the full maturation of precursor stem cells into mature functional T lymphocytes. It is perhaps more appropriate to hypothesize that the variety of well-defined thymic polypeptides act sequentially in the thymus to promote selected steps of T-cell differentiation similar to the sequential interactions seen in the complement and coagulation cascase systems. Thus, the most informative studies concerning the mechanisms of thymic hormone actions are those that have employed prethymic precursor cells or thymocytes and specific assays to detect the influences of the preparation on various intracellular enzymes or cell surface markers that reflect the state of differentiation of the cell. A detailed schematic representation of the different steps in T cell differentiation (in man) was shown in Fig. 10 earlier in this chapter. Figure 10 has been generated from studies performed with both murine and human thymic precursor cells, and on the basis of such studies the hypothesized sites of actions of each of the well-characterized thymic factors are indicated. It would appear from the currently available information that some thymic factors (such as thymosin β_3

TABLE 2
CHARACTERISTICS OF WELL-DEFINED THYMIC FACTORS[a]

	Agent	Usual source	Chemistry	Molecular weight(s)	Chemically synthesized	Present in thymic epithelial cells		Adapted to measure serum levels		Decreases in serum	
						Subcapsular cortical	Medullary	Bioassay	RIA	Postthymectomy	With age
I.	Thymosin	Calf thymus									
	Fraction 5		family of heat-stable acidic polypeptides	1000–15,000							
	Thymosin α_1		Polypeptide, 28 amino acid residues, pI = 4.2	3108	Yes[b]	+[f]	+	No	Yes	No (?)	±[c]
	Thymosin α_7		Polypeptide, pI = 3.5	2200	No	–	–[d]	No	No		
	Thymosin β_3		Polypeptide, pI = 5.2	5500	No	+[e]	–	No	No		
	Thymosin β_4		Polypeptide, pI = 5.1	4982	Yes	+[e]	–	No	Yes		
II.	Thymulin (FTS-Zn)	Pig serum	Nonapeptide, heat labile pI = 7.3	847	Yes	+	++	Yes	Yes	Yes	Yes
III.	Thymopoietin	Calf thymus									
	Thymopoietin II		Polypeptide, 49-amino acid residues, heat stable, pI = 5.2	5562	Yes	+[f]	+	Yes[g]	±[h]	Yes[g]	Yes[g]
	TP-5		Amino acid residues, 32–36 of thymopoietin		Yes						
IV.	Thymic humoral factor (THF)	Calf thymus	Polypeptide, 31-amino acid residues, heat labile, pI = 5.7–5.9	3200	No	?	?	—	—		

[a] Reproduced from

[b] Also synthesized by DNA recombinant techniques.

[c] Decrease demonstrated over the first 10 years of life, but not with further aging.

[d] primarily found in Hassall's corpuscles.

[e] Also synthesized by macrophages in various tissues.

[f] Also detected in squamous epithelium of skin.

[g] Assay uses thymopoietin as standard but may detect other thymic factors.

[h] RIA developed but not applied to serum.

and β_4) act primarily on early stages of thymocyte differentiation, consistent with their origins in subcapsular cortical epithelial cells. Some factors, such as TP-5, appear to act predominantly in intermediate steps, whereas others, such as thymosin α_1 and thymulin, promote both intermediate and late stages of T cell differentiation. Preliminary evidence would also suggest that whereas some thymic factors, such as thymosin α_7, promote the final differentiation of suppressor T cells, others, such as thymosin α_1, are involved in the maturation of helper T cells. By taking this simplistic view of T cell development, a framework can be developed to further identify the steps and mechanisms by which the endocrine thymus exerts its important physiological role. It is hoped that the next several decades will provide more sophisticated methodologies, which will result in further insight into the molecular basis for the function of the thymus gland.

Another major problem that has plagued the field of thymic hormone research is that specific bioassays for thymic hormones are not available. Indeed, the thymic hormones have been aptly termed "hormones in search of a bioassay" (Bach and Carnaud, 1976). It is quite simple to add a thymic factor to a routine immune assay and look for a positive or negative influence. However, unless the target cell population for such studies is well defined, the results are likely to be difficult to interpret in terms of normal physiological mechanisms. This latter point no doubt explains many of the inconsistencies reported in the thymic hormone field.

The ultimate significance of the thymic hormones is their potential clinical application for both the diagnosis and treatment of various immunodeficiency states. It had been hoped that the utilization of assays to detect low circulating thymic hormone levels would provide diagnostic tests for immunodeficiency diseases or more specifically for immunodeficiency diseases that would be responsive to thymic hormone administration. Unfortunately, since many different immunodeficiency states are characterized by abnormal circulating thymic hormone levels, neither high nor low levels are diagnostic of any specific disease entity. Furthermore, some diseases, such as SCID, may be associated with low serum thymic hormone activity but are not improved by parenteral thymic hormone administration.

By far the most important clinical application of the thymic hormones is their potential therapeutic use in selected patients with primary immunodeficiency disorders (e.g., DiGeorge's syndrome), infections, cancer, and autoimmune disorders. The overwhelming evidence available to date indicates that thymic hormones exert immunorestorative effects when administered to patients with impaired T cell immunity. However, it remains for future clinical investigations to identify the most active preparations, to firmly establish the optimal dosages and schedules for their administration, and to determine which clinical conditions will benefit from their use.

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THE PHYSIOLOGY AND BIOCHEMISTRY OF NORMAL AND DISEASED LUNG

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1. Introduction

There were several purposes for writing this article. A primary purpose was to familiarize the reader with the current state of knowledge of the cellular composition and anatomy, the physiological properties, and the biochemical mechanisms associated with normal lung function. This information then provides the basis for describing the pathophysiology associated with various obstructive, restrictive, and vascular disease states of the lung. A final goal of this chapter was to describe, where possible, the diagnostic tests used to detect the lung diseases discussed in this chapter and the biochemical mechanisms associated with the therapeutic treatment of these diseases. Because of space limitations and the broad nature of the subjects described, a detailed coverage of all these topics was not possible. Therefore, at the beginning of certain sections, the reader is referred to excellent review chapters and important books that provide more detailed coverage of the topics at hand. In other sections, where the information presented is so timely that it has not appeared in review form, original articles are referenced for the reader's information.

2. Anatomy of the Lungs

2.1. Overview

In man the lungs lie in the pleural portion of the thoracic cavity (Fig. 1) and extend from the diaphragm (base of the lungs) to a point slightly above

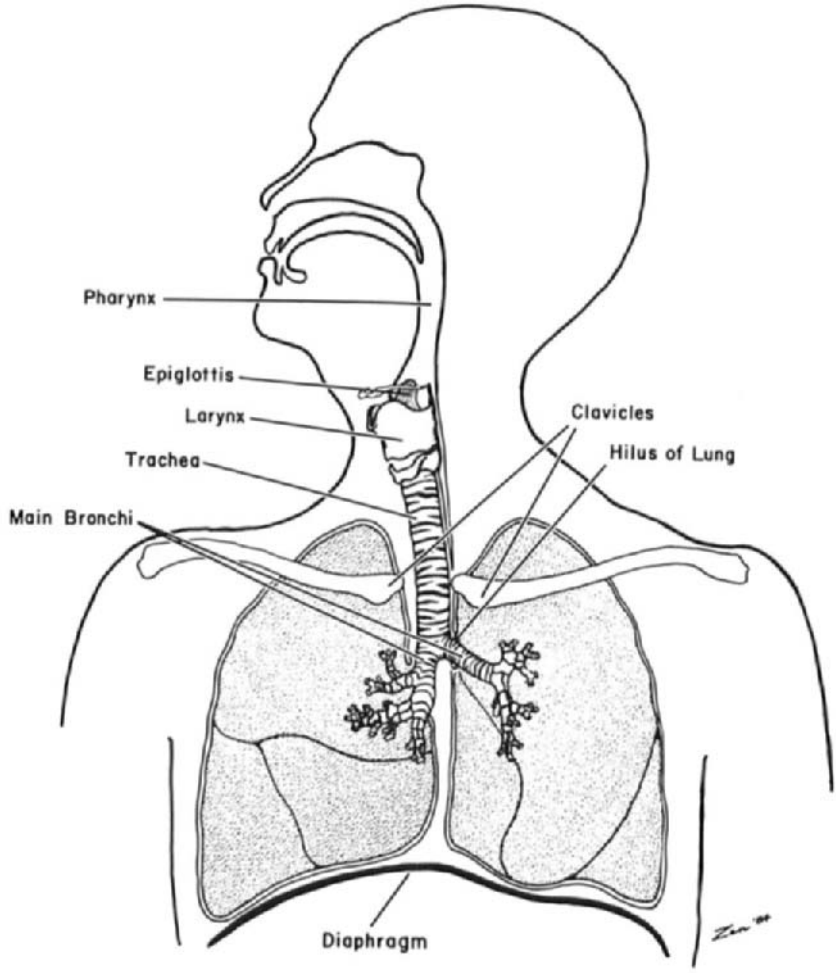

FIG. 1. The extrathoracic (nose and pharynx) and intrathoracic (trachea, bronchi, and lungs) respiratory system.

the clavicles (apex of the lungs). The lungs lie against the ribs both anteriorly and posteriorly and are covered on the outside by a pleural membrane.

It is common knowledge that the lungs are part of the respiratory system by which man exchanges oxygen and carbon dioxide between the blood and atmosphere. As depicted in Fig. 1, the respiratory system is made up, in total, of the external and internal nose, the pharynx (throat), which is a common passage for air and food, the larynx (or voice box), the trachea, the main bronchi, and ultimately, the airways and tissues of the lungs themselves. The epiglottis is an important movable cartilaginous cover that sepa-

rates the pharynx from the larynx and thus prevents the entry of food and liquid into the lungs. An excellent detailed description of the anatomy of the respiratory system can be found in Netter (1979). Within the context of this chapter, the discussion of lung anatomy that follows considers in brief both the gross anatomy and the cellular composition of the trachea, bronchi, secondary airways of the lung (bronchioles, alveolar ducts, and alveolar sacs), and pulmonary vascular system.

The trachea passes from the larynx to the level of the fourth thoracic vertebra, where it divides into the two main bronchi that enter the right and left lungs. The anterior and lateral walls of the trachea and main bronchi are composed of about 20 C-shaped plates of cartilage, which are joined together by a posterior wall composed of bundles of interlacing smooth-muscle fibers, epithelial cells, mucus glands, and elastic fibers. A schematic diagram of the trachea and main bronchi is presented in Fig. 2. Just after the tracheal

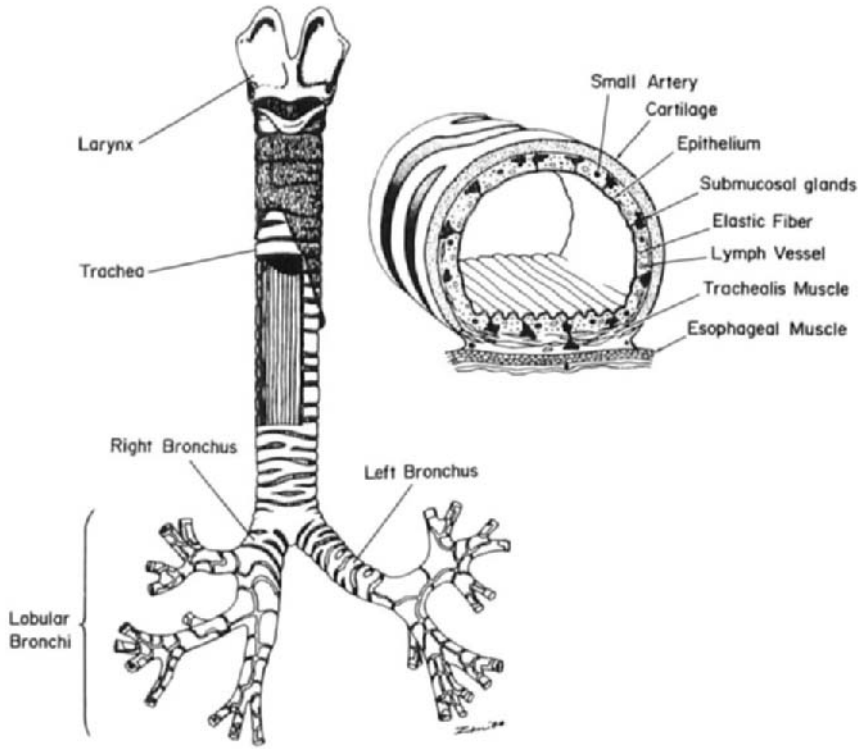

FIG. 2. The central respiratory passages: trachea and bronchi. (The inset is a cross section describing the cellular organization within the trachea.)

bifurcation into the main bronchi and just prior to the point where the main bronchi enter the lungs, the cartilage plates of the main bronchi come together and completely encircle the airways. At this point the smooth-muscle layer under the cartilage forms a complete layer of interlacing bundles that encircle the bronchi.

The right main bronchus is slightly larger than the left and angles away from the trachea less sharply, accounting for the fact that aspirated foreign objects more frequently lodge in the right main bronchus than the left. At the point where the main bronchi enter the right and left lungs—referred to as the hilus of the lung—they subdivide into the lobar bronchi, which provide the connections between the main bronchi and the three lobes of the right lung and two lobes of the left lung. Within the lung, the lobular bronchi continue branching into smaller airways, ultimately forming bronchioles, alveolar ducts, and alveolar sacs of the lung (Fig. 3). During this branching process the cartilaginous rings found in the bronchi eventually become irregular and ultimately disappear entirely in the formation of the bronchioles. Because of the decreasing presence of cartilaginous rings in the smaller bronchi and in the bronchioles, these structures have less inherent rigidity and can collapse or constrict during extreme conditions of lung dysfunction.

At the terminal point of the airways, the terminal bronchiole becomes the acinus, or the respiratory unit, of the lung. The acini vary in size but in general contain several generations of respiratory bronchioles (bronchioles that have alveolar sacs directly opening to their lumen), which lead to alveolar ducts and ultimately alveolar sacs. Connective tissue lines the airways and forms septa that separate acinus from acinus. Collateral ventilation can occur, however, between acini through the pores (the pores of Kohn) that are located in the adjacent alveolar walls.

The lungs are innervated along the tracheobronchial tree by the autonomic nervous system. Afferent autonomic nerve fibers from "stretch receptors" in the alveoli, "irritant receptors" in the bronchi, bronchioles, and trachea, and "cough receptors" in the larynx transmit signals to the central nervous system via the vagus nerve. Conversely, parasympathetic efferent nerve fibers that originate in cells in the dorsal vagal nuclei that are closely related to the central medullary respiratory centers travel to the tracheobronchial tree via the vagus nerve. These fibers innervate short postganglionic nerves in the vicinity of, or within, the walls of the tracheobronchial tree. The nerves of the parasympathetic pathway initiate impulses to the smooth muscle and glands of the tracheobronchial tree, which are cholinergically mediated and which are termed "excitatory" since they elicit smooth-muscle contraction and mucosal gland secretion. The lung is also innervated by adrenergic sympathetic efferent nerves. The preganglionic efferent fibers of

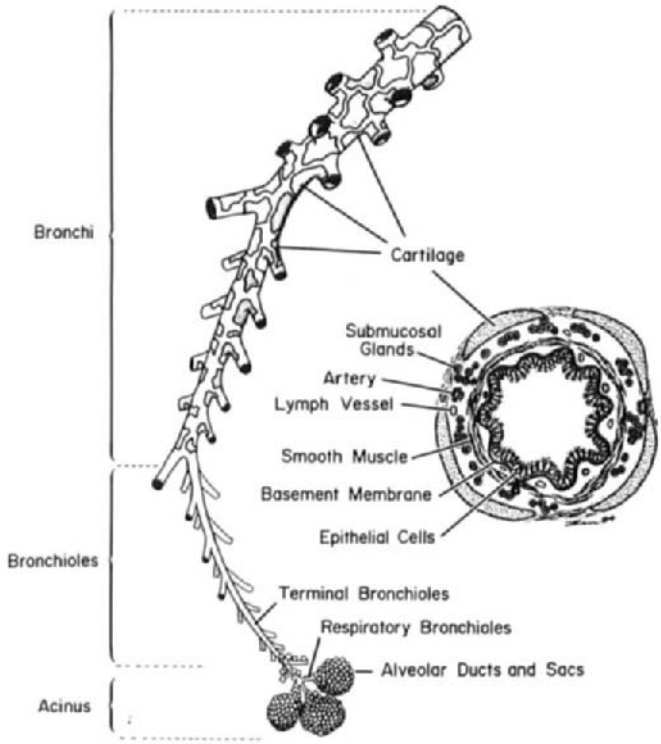

FIG. 3. The branching of the peripheral respiratory airways from bronchus to bronchiole to acinus. (The inset is a cross-section describing the cellular organization within a typical bronchus.)

this pathway originate in the spinal cord and travel to the sympathetic trunks via white rami communicates. There they stimulate fibers that innervate the tracheobronchial tree. These sympathetic fibers can be either "excitatory" through the release of catecholamines that elicit airway smooth-muscle contraction and mucus secretion via α receptors or "inhibitory" by mediating bronchial smooth-muscle relaxation and inhibiting mucosal gland secretion through the release of catecholamines, which interact with β_2 receptors within the lung. A nonadrenergic "inhibitory" pathway has also been described as mediating tracheobronchial smooth-muscle relaxation in the human lung (Richardson, 1981). This system has not been as well characterized as other efferent nerve pathways, however, and the mediator of this nonadrenergic pathway is not currently known.

Because of the unique function of the lung to supply oxygen to the circulatory system of the body, the lung is equipped with two arterial blood

systems. One comes from the pulmonary artery, which pumps blood for oxygenation from the right ventricle of the heart to the alveolar spaces of the lung; the second arterial system is supplied by the bronchial arteries, which arise from the aorta and which supply the lung with oxygenated blood for the metabolic processes that are carried out within this organ itself (Fig. 4). The bronchial arteries enter the lung at the hilus (also the entry point for the main bronchus, which was described above in Fig. 1) and transverse the airway walls to the respiratory bronchioles. The pulmonary artery also enters at the hilus and runs with the airways and their accompanying bronchial arteries in a connective tissue sheath to the respiratory bronchioles, but the

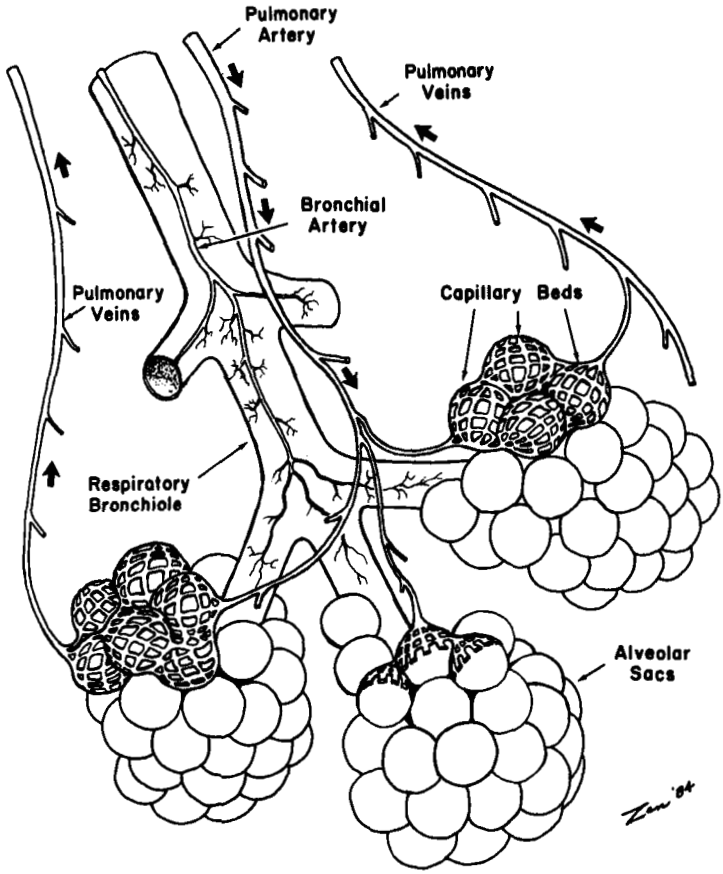

FIG. 4. The vascular systems of the lung: one originating from the pulmonary artery and supplying the alveolar spaces of the lung and the second originating from the bronchial arteries and supplying the central airways and respiratory bronchioles.

vessels from the pulmonary artery subsequently continue on and segment into capillaries, which supply the alveolar walls with blood for oxygenation. These capillaries then interconnect with pulmonary veins that lie in the outer periphery of the lung and return the oxygenated blood to the heart. Unlike the systemic circulatory system, the pulmonary circulation (which is made up of the pulmonary artery, alveolar capillary beds, and pulmonary vein) is a very low pressure system having typical systolic, mean and diastolic pulmonary artery pressures of 22, 13, and 8 mm Hg, respectively, in the normal human being. In addition, the pulmonary vascular resistance is only one-twentieth of the systemic vascular resistance.

2.2. Cellular Components of the Bronchi, Bronchioles, Alveoli, and Pulmonary Vasculature

Structurally, the total cellular composition of the lung is approximately 24% epithelial cells, 42% interstitial cells, 4% macrophages, and 30% vascular endothelial cells (Gail and Lenfant, 1983). The bronchi and bronchioles are hollow tubes that are lined throughout their length with a layer of the epithelial cells consisting of eight different cell types. As described in Fig. 3, the epithelial cells of the bronchi are supported by a basement layer that overlies a layer of loose connective interstitial tissue containing bundles of smooth-muscle cells that encircle the airways, submucosal mucus-secreting glands, elastic fibers that run longitudinally in the airways, and accessory blood vessels and nerves. In the bronchi, cartilage is also present in the connective tissue layer to provide additional support.

The cells of the epithelial layer have been extensively studied and a review article by Gail and Lenfant (1983), which provided the background information for this section of the chapter, describes their properties in greater detail. These cells are schematically depicted in Fig. 5 and include ciliated cells, intermediate cells, brush cells, mucous (or goblet) cells, serous cells, Clara cells, basal cells, and Kulchitsky cells. Unlike most of the epithelial cells, the last two cell types are attached to the basement membrane but do not permeate through the epithelial layer to the lumen. The major cellular component in the epithelium is the ciliated columnar cell; however, in smaller peripheral airways these may be cuboidal. This cell is present in the airway epithelium from the upper trachea to the respiratory bronchioles but not in the alveoli. Proportionately more ciliated cells are present in the large airways than in the small bronchioles. The ciliated cells contribute to the lung mucociliary transport system, which traps and removes foreign particles from the lung. This transport apparatus is described in more detail in a later section of this chapter, which describes lung defense mechanisms.

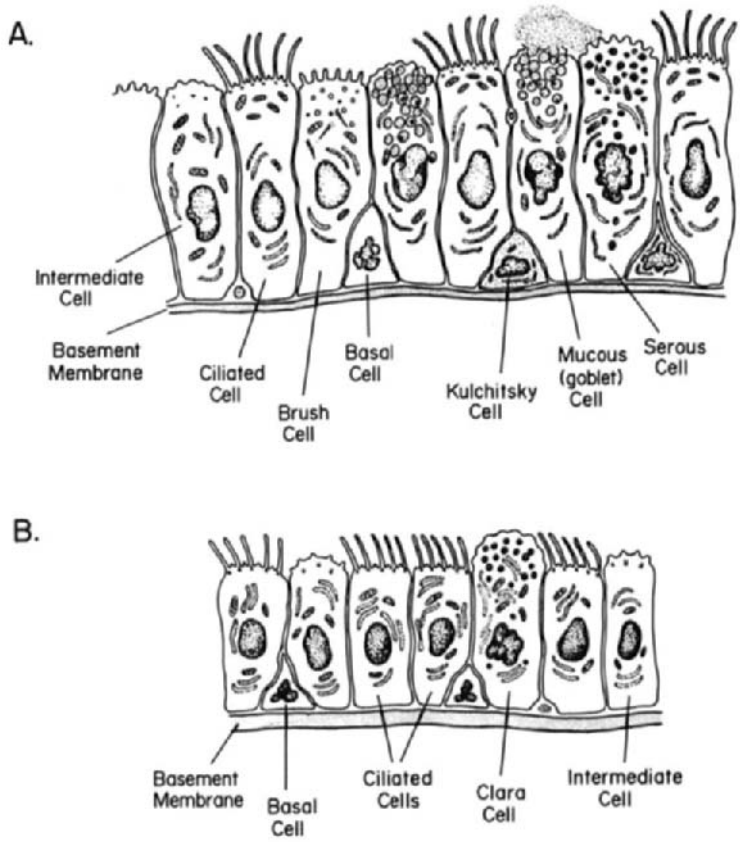

FIG. 5. (a) Cell types found in the epithelium of central airways and (b) cells of the epithelial layer of the respiratory bronchiole.

Instead of forming a continuous lining in the airways, the ciliated cells are grouped in fields that are separated by areas of nonciliated cells or by submucosal gland openings. All closely packed cilia in each field appear to beat in the same direction in a coordinated fashion. The ciliary function of these cells can be stimulated by cholinergic and adrenergic agents and inhibited by exposure to smoke and other air pollutants.

The epithelial basal cell is characterized by its small amount of cytoplasm in relationship to its relatively large nucleus. It is present mainly in large airways and is believed to be the stem cell or precursor cell of the tracheal epithelium and able to differentiate into other epithelial cell types. The intermediate cell, which is columnar and extends from the basement mem-

brane to the airway lumen, is believed to have a similar function. The function of the brush cell in the epithelium is unknown, although it resembles a similar cell type found in the gut, which has absorptive functions. This cell type is somewhat uncommon in the lung, but when it is found it is present throughout both the peripheral and central airways.

The exact function of the Kulchitsky cell is unknown, but there are suggestions that this cell may be a type of endocrine cell. These cells have a roughly triangular shape and contain a round or an oval nucleus, an abundant Golgi apparatus and smooth endoplasmic reticulum, and numerous amine- and peptide-containing neurosecretory granules. This type of cell is more abundant in the human lung before birth than in the adult and is found most frequently in middle-size bronchi. The exact function of these cells is not known, but it is possible that such cells could perform a variety of functions through the release of biologic amines and peptides. The function could include regulating bronchial smooth-muscle tone or controlling the pulmonary circulation. They may also, as part of an endocrine of the lung function, influence metabolic processes occurring in other organs of the body.

Three of the cell types in the epithelium have secretory functions. These cells are the mucous (goblet) cells, serous cells, and Clara cells. These cells contribute to the secretion of airway mucus, a complex mixture of water, glycoproteins, immunoglobulins, lipids, and salts. The secretion of mucus is a defense function that contributes to the removal of foreign objects from lung airways via the mucociliary transport process, as described in later sections of this chapter. Excess mucus secretion can be detrimental, however, since it can obstruct the movement of air through the airways and is a component of certain pulmonary diseases such as asthma, emphysema, chronic bronchitis, and cystic fibrosis. A hypertrophy of secretory cells in the epithelial layer of the airways is often characteristic of these diseases.

Morphologically, the mucous cells contain numerous secretory granules, ribosomes, a prominent Golgi apparatus, a well-developed endoplasmic reticulum, and microvilli. The secretory granules of the mucous cells are often large, confluent, and contain electron-translucent material. The serous cells differ from mucous cells in that the secretory granules they contain are smaller and discrete, in contrast to the large confluent granules found in the mucous cells. The difference in the types of secretory granules suggests that the serous and mucous cells secrete chemically different components of the mucus. Serous and mucous cells are more prominent in the trachea and large airways than in the small airways.

The Clara cells, unlike the mucous and serous cells, are located predominantly in the smaller airways (e.g., the bronchioles). They are columnar in shape and appear to contain more abundant smooth endoplasmic than other

secretory cells. Like the serous cells, the Clara cells also contain distinct electron-dense secretory granules but in smaller numbers than the serous cells. The granules of the Clara cell do not appear to contain mucin but instead have a lipid protein and possibly neutral glycoprotein matrix. Conditions such as lung injury, irritation, or disease (e.g., asthma or bronchitis) that lead to an excess of mucus production also seem to be associated with a differentiation in the epithelial layer of the airway of Clara cells and serous cells into mucous cells.

Clara cells have also been shown to have additional functions besides secretion. That is, these cells appear to be prime sites of xenobiotic metabolism in the lung via cytochrome P_{450}-dependent hydroxylation reactions (Serabjit-Singh *et al.*, 1980; Devereaux *et al.*, 1984). Thus, the Clara cells may also be important in the detoxification of inhaled foreign substances.

In considerations of the mucus-secreting cells of the airways, it should be remembered that the major source of the mucus in the large airways is not the individual mucous, serous, or Clara cells of the epithelial layer, but instead is the submucosal glands that are found in the interstitial connective tissue below the basement membranes of the epithelium of the trachea and large- and medium-size bronchi (see Figs. 2 and 3). These glands consist of a series of mucus-secreting tubules that empty into a central collecting duct that ultimately opens to the bronchial epithelial layer of the airways. The secretory cells lining the tubules of these glands are similar to the mucous and serous cells found in the epithelium. Mucous cells line the part of the tubules closest to the epithelial layer, whereas serous cells are present more distally. Submucosal glands are present in a greater density in the trachea than in the peripheral airways.

As the bronchi segment into bronchioles, the cellular composition of the epithelium lining changes composition as ciliated cells, basal cells, Kulchitsky cells, mucous cells, and serous cells become less prominent, whereas Clara cells become more numerous in the peripheral airways. With the absence of basal cells and Kulchitsky cells, the epithelium layer becomes a single layer and more cuboidal in appearance. The interstitial matrix surrounding the airways becomes less dense in the peripheral airways, and submucosal glands are no longer present. As the bronchioles become alveolar ducts and ultimately alveolar sacs, even more dramatic changes occur. The cellular composition of the alveolar spaces thus ultimately becomes predominantly type I and type II alveolar cells and macrophages (Fig. 6).

The type I alveolar cell constitutes over 90% of the alveolar surface. This cell is highly differentiated and very flat. It provides the thin surface through which gas exchange with the pulmonary capillaries occurs. It is separated from the endothelial cells that line the capillary lumen by a common basement membrane.

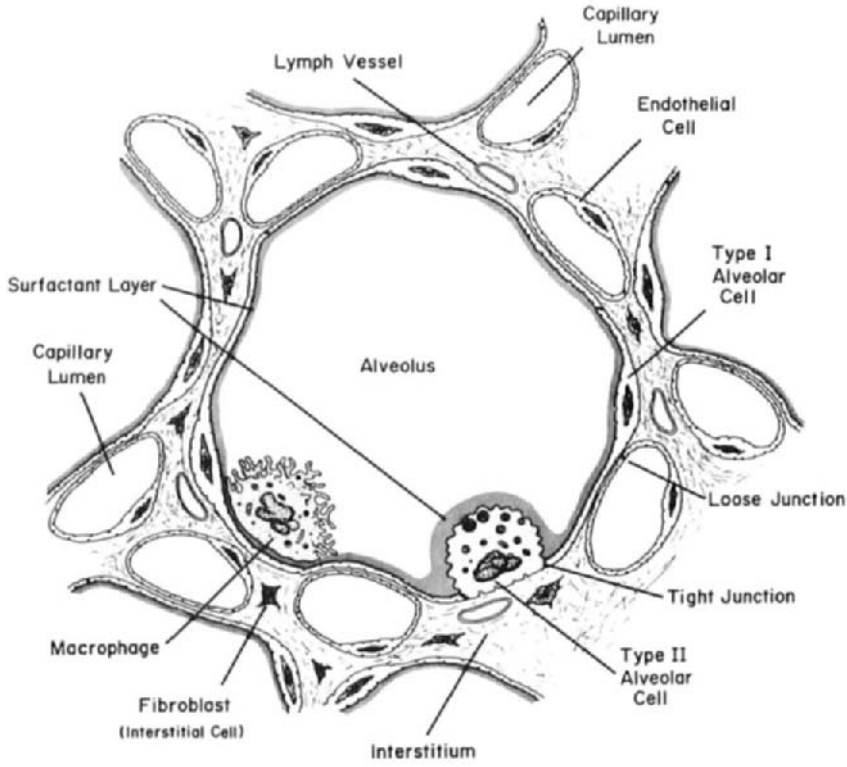

FIG. 6. Cross-section of the alveolar space in the lungs.

The type II alveolar cells are larger polygonal cells that are interspersed among the type I cells and are connected to the type I cells by tight junctions. These junctions make the alveolar epithelium quite impermeable to most large molecules and salt solutions. The surface of the type II alveolar cell that is exposed to the alveolar lumen is covered with microvilli, and the cytoplasm contains a number of lamellar inclusion bodies thought to be associated with the synthesis, storage, and release of the pulmonary surfactant that lines the alveolar epithelium. Thus, current thought is that a prime function of the type II cell is to produce pulmonary surfactant, which is a chemically complex mixture of saturated phosphatidylcholine, various other unsaturated lipids, cholesterol, phosphatidyl glycerol, and specific apoproteins. Pulmonary surfactant functions in a detergentlike fashion to lower the surface tension present in the alveoli and thus to reduce the inflation pressures needed to keep alveoli open at low lung volumes and to facilitate alveolar expansion during inspiration.

In addition to its role in surfactant production, the type II cell also has a progenitor function, acting as the stem cell of the alveolar epithelium. The alveolar type I cells are highly susceptible to injury by oxygen and a variety of noxious substances. In such situations, type II cells can differentiate into type I cells and thus regenerate the gas-exchanging alveolar surface. Other functions of the type II cell are also believed to be the metabolism of foreign compounds (xenobiotic metabolism) and fluid and electrolyte absorption from the alveolar sacs into the interstitial matrix that surrounds the alveoli.

The other cell found in the alveolar sac is the alveolar macrophage. These are migratory cells that are free in the alveolar space and are characterized by the presence of irregular cytoplasmic projections and large numbers of lysosomes. As will be described in subsequent sections of this chapter, these cells have an important function in the defense mechanisms of the lung.

The alveolar sacs are surrounded by capillary beds (Figs. 4 and 6). At the points of gas exchange these capillaries come in close contact with the alveolar wall such that, as described previously, the alveolar type I cells and capillary endothelial cells contain a common basement membrane. Within the capillary, the endothelial cells are joined by intracellular tight junctions. The cytoplasm surrounding the nucleus in the endothelial cells contains a number of intracellular organelles such as mitochondria, the Golgi apparatus, and ribosomes, but the cells also exhibit long cytoplasmic extensions that may be less than 0.1 μm wide and lack specialized organelles. These cytoplasmic extentions contain many pinocytotic vesicles called "caveolae intracellulares," some of which open to the capillary lumen. These caveolae are not evenly distributed over the endothelial surfaces but instead are organized in groups. It has been postulated that the caveolae may be the site of many of the metabolic processes conducted by the pulmonary vascular endothelium. These processes will be described in greater detail in Section 3. The surface of the pulmonary endothelial cells also contains many projections, which serve to increase the cellular surface area available to contact the blood and may also slow down the flow of plasma along the cellular surface to increase the time allowed for the exchange of metabolites between the blood and the endothelium.

3. Functions of the Lung

The primary function of the lung is to exchange oxygen and carbon dioxide between the gaseous atmosphere in which man lives and the blood that transmits oxygen to, and removes carbon dioxide from, the cells of the body. In addition to its primary function, the lung also has other important nonrespiratory functions for the body. For example, it is an important metabolic

organ. Furthermore because the lung is one interface between the outside environment and the rest of the body, the lung must be capable of providing important defense mechanisms for the body. Thus the functions of the lung are actually threefold: to provide a means of efficient gas exchange while at the same time providing important metabolic and defense mechanisms to the body.

3.1. Gas Exchange

The principles of gas exchange by the lung are extensively detailed in several key books and reference articles to which the reader is referred for more comprehensive information (Netter, 1979; Burrows *et al.*, 1983; West, 1979, 1983). Summarized here are the fundamental points.

The key units of oxygen and carbon dioxide gas exchange in the lung are the alveoli. In the human lung there are nearly 300 million alveoli, each associated with as many as 1000 capillary segments. The conducting airways of the lung (the trachea, bronchi, and bronchioles) function as ducts to transport environmental air to the alveoli, to adjust the temperature of the air to 37°C, to properly humidify the air, and to remove large particles from the air before they reach the alveoli; but gas exchange itself is confined to the respiratory bronchioles and alveolar sacs. In man, the volume of a single normal breath is about 500 ml; of this approximately 150 ml remains in the conducting airways (described as anatomical dead space in relation to gas exchange). The remaining air (350 ml) occupies the alveolar gas compartment. Since a respiratory rate of approximately 15 breaths per minute is normally maintained in man, the volume of free gas entering the alveoli of a typical person each minute is approximately 5 liters (350 ml/breath × 15 breaths/minute).

The alveolar sacs are ideally designed for a gas exchange function since they are richly supplied by the pulmonary capillary circulation and contain a membrane composed primarily of a single layer of alveolar type I cells that is very thin (0.5 to 1 μm thick) and has a large total surface area throughout the whole lung of approximately 70 m^2. The alveolar membrane functions as the blood–gas interface. Since only a portion of the alveolar surface is in contact with the pulmonary capillaries and since at a given instant of time the capillaries are not totally occupied by red blood cells, the "effective" alveolar surface area for gas exchange is less than 70 m^2—probably only 35 to 40 m^2. Furthermore, the total blood volume that at any one time is in contact with the alveolar interface is approximately 100 ml, meaning that the blood contacts the alveolar gas-exchange surface as a very thin film that is only one red blood cell thick.

Although air flows under positive pressure through the conducting air-

ways of the lung, this force is dissipated as the cross-sectional area of the lung increases in the small airways such that by the time the air enters the alveolar ducts, its movement is governed solely by diffusion. The driving force for the movement of oxygen from the gas phase of alveoli into the liquid phase of the pulmonary capillaries is therefore simply the difference in the partial pressure of the gas in these two compartments. Although the partial pressure of oxygen in atmospheric air is approximately 159 mm Hg, the actual partial pressure in the alveolus is considerably lower than this (105 mg Hg) due to the fact that the partial pressure of oxygen in the respired air drops as it is saturated with water vapor in the conducting airways and mixes with oxygen-depleted, carbon dioxide-enriched gas, which remains in the alveoli at the end of the previous expiration. Since the oxygen tension in the mixed venous blood returning to the lung is 40 mg Hg, an oxygen gradient of approximately 65 mg Hg exists as the driving force across the alveolar–capillary membranes.

For oxygen to enter the capillaries, it must pass through a number of barriers including the surfactant lining of the alveoli, the alveolar epithelial type I cells, alveolar and capillary basement membranes, the capillary endothelium, and ultimately into the capillary blood. The actual solubility of oxygen in the blood is quite low (0.003 ml of oxygen per 100 ml of blood per mm Hg) and at the oxygen tensions reached in the alveolus (105 mm Hg) the solubility (0.3 ml of oxygen per 100 ml of blood) would be inadequate for the normal metabolic needs of the body. Once in the blood, however, oxygen ultimately migrates into the red blood cells, where it is carried in chemical combination with hemoglobin. Hemoglobin is capable of carrying 1.3 ml of oxygen per gram of hemoglobulin protein when fully saturated with oxygen. Thus assuming a hemoglobulin concentration of 15 g/100 ml of blood, the total amount of oxygen in combination with hemoglobulin can be approximately 20 ml of oxygen per 100 ml of blood under conditions of full oxygen saturation (over 60 times that which could be carried simply dissolved in blood alone).

The amount of oxygen carried by hemoglobulin is dependent, in sigmoidal fashion, on the partial pressure of oxygen in the blood, as depicted by a well-known oxygen–hemoglobulin dissociation curve (Fig. 7). The shape of this curve has great physiological significance since it means that at the oxygen tensions normally attained with alveolar air (105 mm Hg), hemoglobulin can become fully saturated at a very fast rate. The flat upper portion of the curve also implies that relatively constant quantities of oxygen can be maintained in the blood over a large range of oxygen tensions, which may vary due to pulmonary disorders or perhaps exposure of the body to high altitudes.

The normal transit time for blood passing through the pulmonary capillary network is about 0.75 seconds. During this time oxygen must pass through

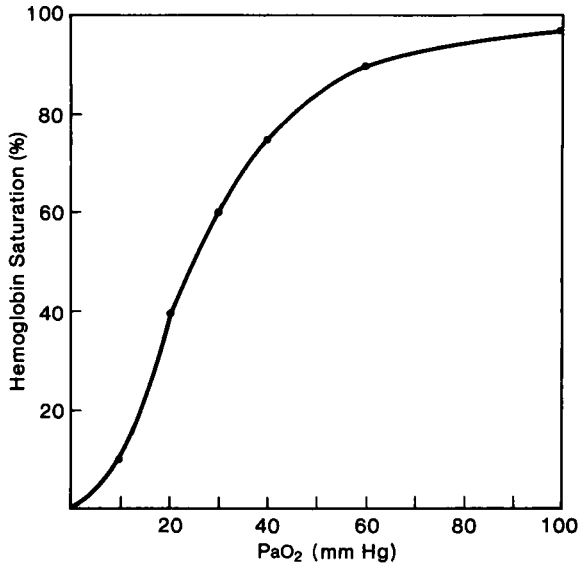

FIG. 7. Oxygen–hemoglobulin dissociation curve.

the alveolar–capillary membrane, enter the blood, and combine with the hemoglobulin molecule. Normally this process can occur within one-third of this transit time; however, under conditions where the diffusion capacity of oxygen is reduced due to changes in the membrane components of the alveoli (e.g., certain diseases such as interstitial fibrosis or pulmonary edema) or where a gas containing a low mixture of oxygen is breathed or under conditions of strenuous exercise where this transit time in the pulmonary capillary is decreased, complete equilibration may not be achieved and arterial hxpoxemia may occur.

Oxygen dissociates from hemoglobulin down a concentration gradient to tissues in the body to meet their metabolic needs. During this process the oxygen tension of venous blood drops to approximately 40 mm Hg, which normally corresponds to a 75% saturation of blood hemoglobulin. The release of oxygen from hemoglobulin can be regulated by a number of factors that accompany tissue metabolism. These factors include pH, the partial pressure of carbon dioxide in the blood, and temperature. Thus with a decrease in pH, an elevation in carbon dioxide levels, or an increase in temperature (all factors that accompany normal cell metabolism in tissues), the dissociation curve for oxygen shifts to the right, facilitating the unloading of oxygen within metabolically active tissues.

The amount of 2,3-diphosphoglycerate in red blood cells also influences

the oxygen–hemoglobulin dissociation curve. This compound, which is an intermediate product of anaerobic glycolysis, decreases the affinity of hemoglobulin for oxygen. The amount of this metabolite increases in red blood cells under conditions of anemia or hypoxemia, and it thus serves an important adaptive function in maintaining adequate tissue oxygenation by facilitating the dissociation of oxygen under conditions of decreased oxygen transport.

The other gas exchange function of the lung is the elimination from the body of carbon dioxide created during cellular metabolism. Arterial blood normally has a carbon dioxide tension of 40 mm Hg. Since metabolizing tissues normally have a high concentration of carbon dioxide, during the transit of the blood through the tissues of the body the carbon dioxide tension is raised to about 46 mm Hg. Like oxygen, the transport of carbon dioxide in the blood involves several steps (Fig. 8). Carbon dioxide from the tissues enters the plasma, where a small portion slowly combines with water to form carbonic acid but the majority enters the red blood cell. In the erythrocyte, conversion of the carbon dioxide to carbonic acid also occurs, but this is a much more rapid enzymatic process (catalyzed by carbonic anhydrase) than occurs in the blood plasma. The carbonic acid formed in the red blood cell and plasma eventually dissociates to form bicarbonate and hydrogen ions—the hydrogen ions being buffered primarily by hemoglobulin in the erythrocyte and blood proteins in the plasma.

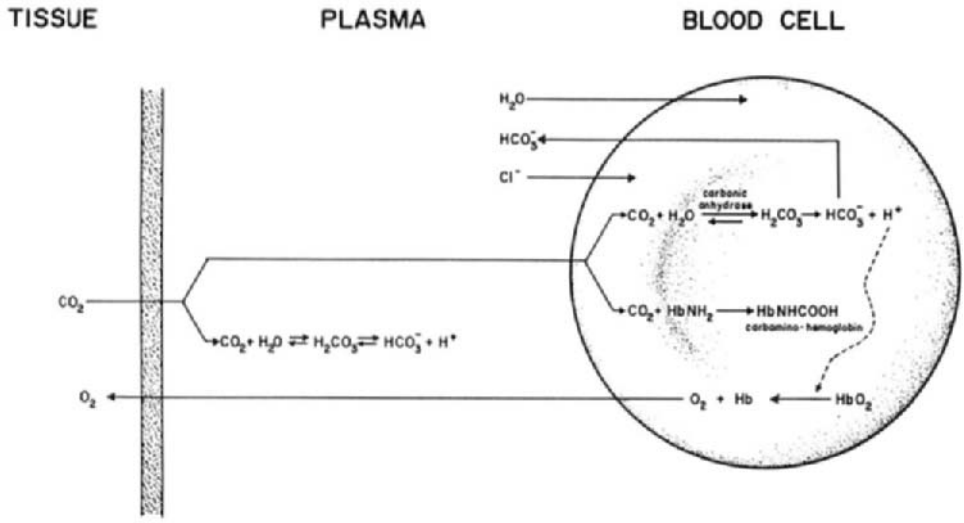

FIG. 8. Schematic view of the multiple mechanisms involved in CO_2 transport in the blood.

In the red blood cell the decrease in pH due to the formation of hydrogen ions from the dissociation of carbonic acid promotes release of oxygen from the hemoglobulin to the tissues. Also there is a concentration gradient formed between bicarbonate ion in the erythrocytes and in the plasma that results in the diffusion of bicarbonate ion out of the red blood cells and into the plasma. To maintain electronic neutrality, a shift of chloride ions (the chloride shift) occurs from the plasma into the red blood cells. Because of the cascade of events already described, most of the carbon dioxide produced during tissue metabolism is carried in the plasma to the lung in the form of bicarbonate ion. A small fraction of the carbon dioxide is carried via another mechanism. That is, when hemoglobulin loses oxygen, a small amount of carbon dioxide can react with the nonoxygenated hemoglobin to form carbamino–hemoglobin (also shown in Fig. 8).

In the pulmonary capillary bed, carbon dioxide is released to the alveolus by a reversal of the processes already described. Thus there is a "shift" of chloride ions out of the red cell and an influx of bicarbonate into the cell. Under these conditions, carbonic anhydrase, a readily reversible enzyme, promotes a rapid interconversion of carbonic acid to carbon dioxide and water. Also there is a reversal of the carbamino–hemoglobin association as hemoglobulin begins to take up oxygen from the alveolus. The carbon dioxide released in these reactions is driven from the capillary bed into the alveolus by the slight partial pressure gradient that exists between these two compartments (46 mm Hg in the capillary bed and 40 mm Hg in the alveolus). This slight gradient is apparently sufficient to drive the capillary–alveolar exchange since the diffusion rate for carbon dioxide is 20-fold more rapid than that for oxygen, due mainly to the greater solubility of carbon dioxide in blood.

The entire process of gas exchange is dependent upon a properly functioning and continual flow of both air and blood to the alveoli (i.e., adequate ventilation and perfusion of the alveolus). Normal rhythmic ventilation is under the control of central respiratory centers in the brain (the pons and medulla). The level of ventilation is also modulated by arterial carbon dioxide levels, the partial pressure of oxygen, and the pH of the blood. Chemoreceptors in the medulla are the primary sites of response to changes in the partial pressure of carbon dioxide and pH in the arterial blood. Decreases in oxygen tension in the blood can increase ventilation through actions on peripheral chemoreceptors in the carotid bodies, which then feed back to the medulla. This response is normally weaker than responses to increased carbon dioxide levels and decreases in pH, which are regulated centrally in the medulla. Under some conditions, reflex responses originating from the activation of stretch receptors in the alveoli or irritant receptors in the airways can also influence ventilation.

The ideal situation for gas exchange would appear to be to have each portion of the lung equally ventilated with air and equally perfused with venous blood. In fact this situation is never achieved. Thus, during normal upright posture, both blood flow per unit volume and ventilation decrease rapidly from the bottom to the top of the lung. The changes in blood flow are more dramatic than the changes in ventilation such that the ventilation/perfusion ratio varies from a high value of 3 at the top of the lung to a low value of 0.6 at the bottom. Under normal conditions, the effects of these uneven ventilation/perfusion ratios are trivial and result in a differential of arterial oxygen and carbon dioxide tension of only a few millimeters of Hg between the blood perfusing the upper and lower parts of the lung. In the diseased lung (e.g., in various chronic obstructive pulmonary diseases, pulmonary fibrosis, and pulmonary vascular diseases), however, much greater regional mismatching between lung ventilation and perfusion can occur owing to constricted or blocked airways (decreased ventilation) and vascular constriction or obstruction (decreased perfusion). Such conditions can lead to severe decreases in arterial oxygen tension (hypoxia) or increases in arterial carbon dioxide tension (hypercapia) and ultimately to respiratory failure.

3.2. Metabolic Functions of the Lung

Although it is convenient to consider the primary function of the lung to be gas exchange, it must be kept in mind that the lung is also an active metabolic organ that provides energy for its own needs and metabolic regulation of substances important to the homeostatic well-being of the body as a whole. Extensive coverage of this subject can be found in several reviews (Ryan, 1982; Fishman and Pietra, 1974; Ben-Harari and Youdim, 1983) and a book (Vane, 1980).

For its own metabolic needs the lung depends for the most part on aerobic metabolism. One to two percent of the total oxygen requirements of the body are directed to lung metabolism to provide energy for the act of breathing; for the synthesis of materials essential for the lung gas exchange function, such as pulmonary surfactant; for the synthesis of key structural components of the interstitial tissue of the lung, such as collagen and elastin; for phagocytic activities; and for the processing of important biologic regulatory substances for the body. This level of metabolic activity roughly approximates that of the liver but is less than that of contracting muscle or the brain. Most of these metabolic activities of the lung are described in other sections of this chapter; however, the processing of biologic regulatory substances does not fit into other categories in this chapter and therefore will be elaborated on more extensively in this section.

The lung is in a unique position to influence the substances that circulate throughout the body since its vascular bed (50% of the total capillary bed of

the body) receives the entire cardiac output from the heart. Thus the lung has the opportunity to remove or metabolize substances from the mixed venous circulation prior to their reaching the systemic arterial circulation. As would be expected, the endothelial cells in the pulmonary vascular system play an important role in these events. Also, it should be emphasized that a great deal of specificity appears to exist in the types of metabolic processes in which the lung can participate—primarily the uptake, processing, and/or metabolism of certain biologically active amines, peptides, nucleotides, and arachidonic acid metabolites. The regulation of these substances can have a profound effect on platelet aggregation, coagulation–anticoagulation hemostasis, and systemic blood pressure.

The specificity of the lung vasculature with regard to amine uptake is emphasized by the fact that the lung will only take up and metabolize serotonin and norepinephrine and not structurally similar substances such as tryptophan, epinephrine, and dopamine. In the case of both serotonin and norepinephrine, these substances are taken up by the pulmonary vasculature endothelial cells by active transport processes, metabolized to biologically inactive substances, and subsequently released once again to the systemic circulation. With both serotonin and norepinephrine this uptake mechanism in the lung is thought to play a significant part in the rapid inactivation of these substances that occurs within the body and to be at least as important, if not more important, than metabolism that may occur in the blood or liver. By providing a means by which serotonin and norepinephrine can be readily inactivated, the lung provides an important safeguard to the systemic circulation, where these endogenous substances could induce platelet aggregation (serotonin) or act as potent vasoconstrictors (serotonin and norepinephrine).

With regard to the processing of polypeptides, which is part of the metabolic function of the lung, the lung plays a central role in regulating the overall functioning of the renin–angiotensin–aldosterone system and the kallikrein–kinin system. One kinin, bradykinin, which is a potent vasodilator, is rapidly inactivated by an enzyme on the pulmonary endothelial surface. This same enzyme is also responsible for the conversion of angiotensin I (a decapeptide generated in the blood by the action of renin upon an α_2-globulin in plasma) to angiotensin II. Because of this latter activity, this enzyme is thus called angiotensin-converting enzyme. Angiotensin II is an active pressor agent in the systemic circulation acting initially by inducing the release of aldosterone from the adrenal cortex. This results in an increase in sodium retention, an increase in blood volume, and ultimately an increase in systemic blood pressure. Thus through regulating the metabolism of bradykinin and angiotensin I, the lung plays another important role in blood pressure homeostasis.

Another vasoactive substance that is actively metabolized by the lung is

the nucleotide adenosine diphosphate (ADP), an important aggregant of platelets. Enzymes on the luminal surface of the endothelial cells can convert ADP first to AMP and then to adenosine. In this process, not only is the platelet-aggregating agent ADP inactivated, but adenosine, a potent vasodilator, is released into the systemic circulation.

Other metabolic activities associated with the lung endothelial cells influence the regulation of blood coagulation and fibrinolysis. That is, endothelial cells in the pulmonary vasculature contain plasminogen activator that is capable of initiating fibrinolysis and another factor that has anticoagulant properties because of its ability to initiate a series of events with complement factors that decrease clot formation. Also, the endothelial cells have high-affinity binding sites for thrombin and can therefore act to rapidly clear this factor from the circulation and thereby also decrease the occurrence of thrombin-induced platelet aggregation and clot formation.

A final metabolic function of the lung is the production and regulation of arachidonic acid metabolites formed by either the cyclooxygenase (prostaglandins) or lipoxygenase pathways (5- and 12-hydroxyeicosatetrenoic acids and the leukotrienes). The generation of leukotrienes by the lung is important in certain pathophysiological mechanisms within the lung and is described in more detail in later sections of this chapter that concern asthma. The regulation of prostaglandin production can similarly be important in lung pathological conditions such as asthma but also is important in certain systemic processes such as platelet aggregation. With regard to the latter subject, it is important to note that not only is the lung an important synthesizer of prostaglandins, but the lung also has selective uptake and inactivation mechanisms for prostaglandins of the E and F series. Prostaglandins of the A series and the potent antiaggregant prostacyclin (PGI_2), however, pass intact through the lung into the systemic circulation. Thus through the regulation of arachidonic acid metabolism, the lung has another means by which to regulate those hemostatic mechanisms that influence platelet aggregation.

3.3. Defense Functions of the Lung

Because the lung functions in close contact with what is often a variable external environment, one of the important functions of the lung is to equilibrate inhaled air to a constant temperature (37°C) and humidity and to remove foreign objects and organisms from the air before they reach the systemic circulation. Most temperature equilibrium and humidification of the inhaled air occurs in the upper nasal passages, where it initially comes in contact with the rich, warm vascular supply of the nasal cavities and is humidified by nasal secretions. In addition, the hairs and mucous coating of

the nasal passages function to filter out most particles with sizes greater than 4.5 μm before they reach the level of the larynx.

Within the large airways, bronchi, and bronchioles, another mechanism—the mucociliary escalator—also exists to prevent particles of 2 μm and greater from reaching the level of the alveoli. The mucociliary escalator is the result of the presence in these airways of ciliated columnar cells that are covered by a mucous layer produced by mucous (goblet) cells and the tracheal submucosal glands. As stated in previous sections of this chapter, ciliated cells are present throughout the tracheobronchial tree down to the level of the alveoli, whereas mucous cells and the submucosal glands are more variable in their distribution, being present predominantly in the middle and large bronchi.

The mucociliary escalator functions when inhaled particles between 2 to 10 μm are deposited on the sticky mucous lining of the tracheobronchial tree and are propelled upward by the movement of this mucous layer in response to the beat of the cilia on the ciliated epithelial cells. This phenomenon is made possible because the mucous layer is biphasic, consisting of a watery solution in direct contact with the epithelia cells in which the cilia are free to beat. This watery solution is covered by a stickier, more adhesive gel layer that can trap and hold inhaled particles. The cilia beat in the wall layer at a rate between 1000 and 1500 strokes per minute such that at the point of their maximum upward velocity, the tips of the cilia come in contact with the gel layer to propel it upward. All recovery strokes subsequently occur in the water layer. This mechanism moves the mucus upward at a rate between 1 and 3 cm/minute. Ultimately, the mucus reaches the pharynx, where it is swallowed. Disease states that either alter the mucus-producing properties of cells in the tracheobronchial tree or decrease ciliary activity will obviously have a deleterious effect on this important clearance mechanism.

In addition to the mucociliary escalators, another mechanism also exists in the airways of the lung to more rapidly remove excess secretions or irritating particles from the lung. This is the cough reflex. The cough reflex may result from either mechanical irritation or chemical stimulation. Sensory organs located in the larynx, at the point where the trachea bifurcates to form the main bronchi or at the points of the branching of the main bronchi to lobar bronchi, are sensitive to mechanical stimulation and transfer impulses up the vagus to the medulla. The resulting response is the familiar cough sequence in which a rapid inspiration of air is followed by an expiratory effort against a closed epiglottis (the cartilaginous cover between the larynx and pharynx). When this expiratory effort peaks there is a rapid opening of the epiglottis, followed by an explosive expiration. Often after an initial inspiration there is a series of cough efforts, each occurring at progressively lower lung volumes. This mechanism then promotes the rapid expulsion of secretions and foreign particles from the tracheobronchial tree.

In spite of the presence in the respiratory system of a filtration apparatus in the nasal cavities, of the mucociliary escalator in the tracheobronchial tree, and of a cough reflex, inhaled particles and microorganisms smaller than 2 μm can often reach the lung alveoli. At this point, the alveolar macrophages are activated and function to scavenge these particles and remove them from the gas-exchange surface. The alveolar macrophages are formed from stem cells in the bone marrow and move to the interstitial spaces of the lung, where they mature and subsequently are transferred to the alveolar air-exchange surface. These cells are extremely mobile in nature and therefore can perform their scavenging functions by moving over a large surface area. These cells are quickly mobilized in response to the inhalation of large amounts of foreign debris. Once activated the cells endocytose this material into cellular vacuoles into which lysozomal hydrolytic enzymes (lysozyme, cathepsin, acid phosphatase, etc.) are released to digest the material. Oxidative processes are also stimulated during this process, resulting in the enzymatic formation of activated states of oxygen (such as superoxide), which also participate in the degradation of foreign material. As these degradative processes occur, the macrophages begin migrating out of the alveoli, either to the respiratory bronchioles, where they are cleared from the lung airways by the mucociliary escalator, or into the lymphatics, where they are subsequently cleared from the body.

The activation of alveolar macrophages also results in the release of peptides and lipid factors from these cells that are chemotactic for neutrophils. This process can initiate a secondary inflammatory response under conditions of severe activation, as might occur during an extensive and rapid multiplication of microbes present in the alveoli (e.g., as in bronchitis, cystic fibrosis, or infectious pneumonia). Under these conditions the release of chemotactic factors can rise to levels high enough to elicit the migration of neutrophils into the lung, where they can act with the alveolar macrophages to inactivate and degrade the foreign microbes.

More extensive information on the defense functions of the lung can be found in the excellent books and reviews (Netter, 1979; Burrows *et al.*, 1983; Brain *et al.*, 1977; Green *et al.*, 1977; Leith, 1968; Maklem, 1974) that provided the background for the subject in this article.

4. Physiological Measurements and Tests of Lung Function

In recent years, many new tests have been developed to assess pulmonary function in man. Here are presented the general principles, methodology, and clinical significance of the major lung function tests used in the diagnosis of obstructive and restrictive lung diseases. Obstructive diseases of the lung

are those diseases associated with a narrowing of the diameter of the airways leading to an increased resistance to airflow, whereas restrictive diseases are associated with a reduction in the expansive properties of the lung. For more detailed information on pulmonary function tests the reader is referred to several excellent references (West, 1979, 1982; Comroe, 1974; Macklem, 1975).

4.1. Lung Volumes and Capacities

Four primary lung volumes and capacities are usually measured (Fig. 9). Each capacity includes two or more of the primary volumes. The lung volumes and capacities are defined as follows.

Tidal volume (TV): The volume of gas inhaled or exhaled with each normal breath.

Inspiratory reserve volume (IRV): The maximum amount of gas that can be forcefully inspired from the end-inspiratory position in tidal volume.

Expiratory reserve volume (ERV): The maximum amount of gas that can be expired from the end-expiratory position in tidal volume.

Residual volume (RV): The volume of gas remaining in the lung at the end of the most forceful expiration.

Total lung capacity (TLC): The amount of gas contained in the lung at the end of a maximal inspiration.

Vital capacity (VC): The maximal amount of gas that can be expelled by forceful effort after a maximal inspiration.

Inspiratory capacity (IC): The maximum volume of gas that can be inspired from the resting expiratory level.

Functional residual capacity (FRC): The volume of gas in the lungs at the end of a normal expiration.

All the static lung volumes and capacities except FRC and RV can be measured directly through use of a simple spirometer (an apparatus traditionally consisting of a cylindrical bell immersed in water and equipped with outlets that a person can breathe into, or inhale from, to measure expiratory or inspiratory volumes). Functional residual capacity and RV are measured indirectly by using several alveolar gas dilution techniques.

One dilution technique for measuring FRC involves having a subject breathe from a spirometer a fixed concentration of a gas, such as helium (He), which is not soluble in blood or lung tissue. In such a maneuver, the subject is connected in closed circuit to a spirometer and begins to breathe in the He from the spirometer at the end of a normal expiration. Both the initial volume of gas in the spirometer (V_s) and the concentration of He (C_s) added to the spirometer are measured before the start of the test. The

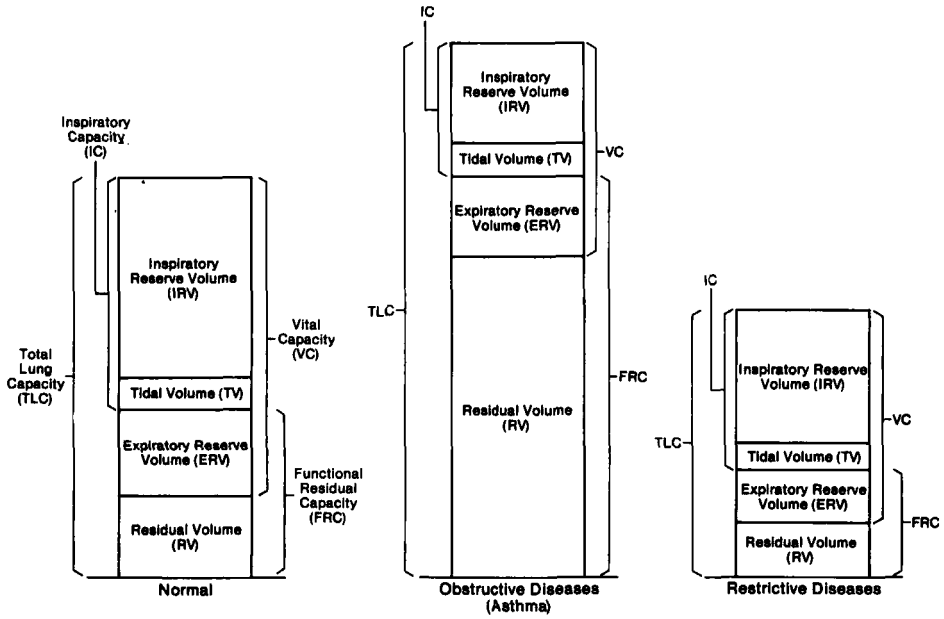

FIG. 9. Primary lung volumes and capacities and how they vary in obstructive or restrictive diseases.

subject continues breathing from the spirometer until the He concentration between the spirometer and the lungs has equilibrated. At this point the concentration of He in the subject's lungs (C_L) is the same as the He concentration measured in the spirometer. After this final concentration is known, the initial volume of gas in the lung (FRC) can be computed by solving the following algebraic equation:

$$\text{FRC} = \frac{V_s C_s}{C_L} - V_s$$

The residual volume (RV) is calculated by subtracting the expiratory reserve volume (measured directly with the spirometer) from the FRC.

Another way of measuring FRC is through the use of a body plethysmograph. A plethysmograph is an airtight box in which a man can sit. A subject sits in the box and breathes through a mouthpiece connected to the outside. As a person reaches end expiration, alveolar pressure (P_0) is equal to atmospheric pressure because there is no gas flow. Under these conditions FRC is measured by closing the mouthpiece with an electrically controlled shutter and having the patient continue to breathe against the obstruction. As

the subject tries to inhale, his thorax enlarges. This process decompresses the intrathoracic gas, thus creating a new thoracic gas volume (V') and a new pressure (P'). The increase in thoracic gas volume (ΔV) is measured as the rise in plethysmographic pressure and the new pressure (P') is measured with a gauge between the subject's mouth and the occluded mouthpiece. P' is assumed to equal alveolar gas pressure under conditions of no flow. Using Boyle's law relating pressure and volume of gases ($P_0V_0 = P'V'$), measuring P_0, P', and ΔV, and knowing $V' = V_0 + \Delta V$, one can calculate FRC (the original thoracic gas volume). The plethysmographic method of measuring FRC is very rapid (five determinations can be made in one subject in 5 minutes). In this case RV is again calculated by subtracting ERV from the calculated FRC.

4.2. Measurement of Airway Caliber

Tests of airway caliber are divided into two types: those that are measured at submaximal flow rates (e.g., airway resistance) and those that are measured at maximal flow rates (e.g., force expiratory volume and flow–volume curves).

Airway resistance is defined as the pressure difference between alveolar and mouth pressure per unit of airflow. To measure airway resistance in man, one needs to measure only two variables, alveolar pressure and airflow (since mouth pressure is approximated as being atmospheric). Alveolar pressure is measured directly with the body plethysmograph as described earlier. Airflow is measured by having the subject pant at slightly greater than normal FRC through a pneumotachograph (flowmeter) attached to the mouthpiece of a plethysmograph. Airway resistance is usually measured in the normal breathing range and a typical value for an adult is 2 cm H_2O/liter/second. Airway resistance varies inversely with lung volume (Briscoe and DuBois, 1958); however, the relationship can be made linear if resistance is converted to its reciprocal, conductance (see Fig. 10). Specific conductance is conductance normalized for lung volume.

The advantage of measuring airway resistance is that it is a simple, rapid, and extremely sensitive test of airway caliber, but the major disadvantage is that it cannot distinguish between resistance changes due to obstruction of the extrathoracic airway (pharynx, larynx, glottis, and trachea) or the intrathoracic airway (bronchi, bronchioles, and alveoli).

The best way to determine the caliber of the intrathoracic airways is to utilize pulmonary tests measured at maximal flow rates such as forced expiratory volume in 1 second (FEV_1) and maximal expiratory flow–volume curves. The forced expiratory volume maneuver requires a subject to inspire maximally and then exhale as hard as possible into a spirometer. The typical

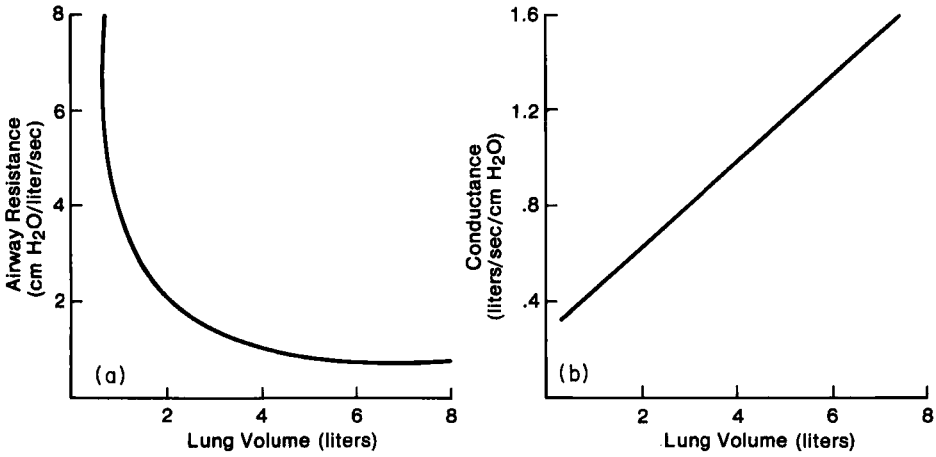

FIG. 10. Typical relationship between airway resistance and lung volume (a) and conductance (the reciprocal of resistance) and lung volume (b).

spirometric tracing obtained in this procedure is depicted in Fig. 11. The forced volume exhaled in the first second is the FEV_1. The total volume exhaled is the forced vital capacity or FVC. Normally, the FEV_1 is about 80% of the FVC. This maneuver is a useful way to separate restrictive and obstructive diseases. In restrictive diseases, both the FEV_1 and FVC are reduced, but the ratio FEV_1/FVC is normal. Conversely, in obstructive diseases, the FEV_1 is reduced to a greater extent than the FVC, and so the ratio FEV_1/FVC is decreased.

A second way of looking at forced expiration is with a maximum expiratory flow–volume (MEFV) curve, which describes maximum flow as a function of lung volume during a forced expiration (Fig. 12). In healthy human subjects, flow rates or flow–volume curves reach a maximum and will not increase with additional effort after the lungs have emptied 20–30% of their volume (Fry and Hyatt, 1960). This phenomenon of flow limitation is due to airway compression over most of the lung volume. Thus, flow rate is independent of effort and is determined by the elastic recoil force of the lung and the resistance of the airways upstream of the collapse point. In obstructive diseases of the lung this curve is shifted to the left, whereas restrictive diseases shift the curve in the opposite direction (also shown in Fig. 12).

Efforts have also been made to devise sensitive tests of small airway function. Such tests are necessary because the earliest lesions of chronic obstructive lung diesease occur in the peripheral airways and these airways normally contribute very little to flow resistance. Therefore measurements of lung function that depend solely upon resistance changes will not detect

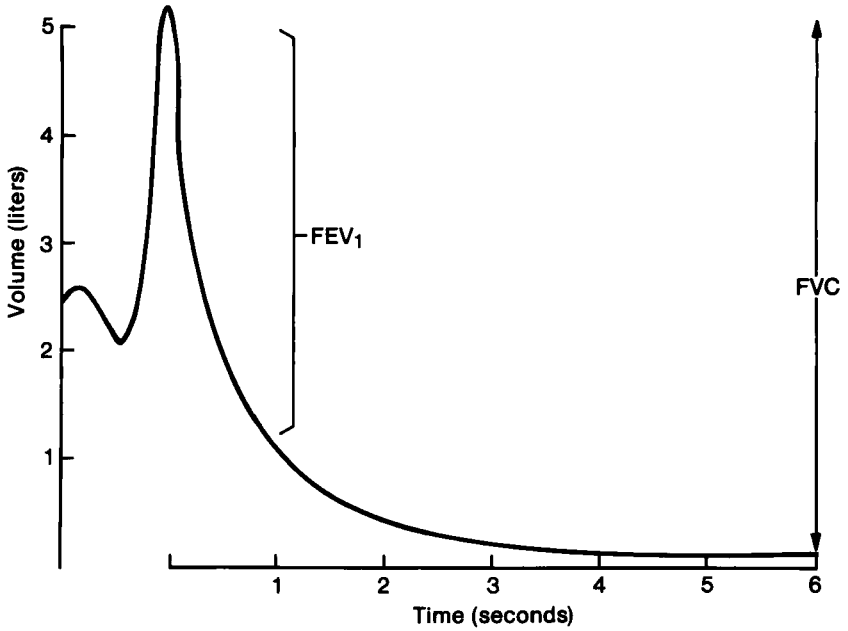

FIG. 11. Typical tracing obtained from a spirometer during a forced expiratory volume maneuver.

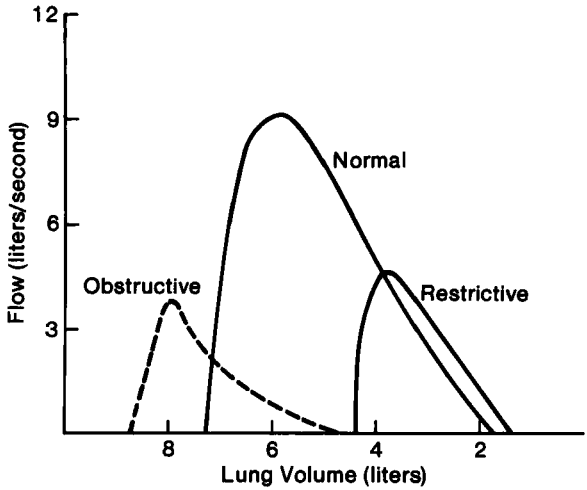

FIG. 12. Comparison of the typical maximal expiratory flow–volume curve in healthy subjects and those with obstructive or restrictive diseases.

lung disease at its earliest stages. One test that has been proposed as a test that might discriminate between obstruction of larger versus smaller airways compares MEFV curves obtained with the subject breathing air versus a 80% helium–20% oxygen gas mixture (Despas *et al.*, 1972). The basis of this test is the fact that laminar airflow, which predominates in small peripheral airways, is independent of gas density, whereas turbulent airflow, which occurs in the large central airways, is density dependent. The lung volume at which flow rates coincide between breathing air and breathing $He–O_2$ is called the volume of isoflow ($\dot{V}_{iso}$); in the normal lung, $\dot{V}_{iso}$ occurs at about 10% of the vital capacity. However, in patients with increased small airway disease, $\dot{V}_{iso}$ may increase to 20% of vital capacity or more.

4.3. Measurement of Static Lung Compliance

Compliance is defined as the change in volume produced by a unit change in lung elastic recoil pressure and is the most commonly measured index of the elastic properties of the lung. Elastic recoil pressure is the difference between the pressure in the alveolus and the pressure in the pleural space (intrapleural pressure).

It is too difficult and dangerous to routinely measure intrapleural pressure directly because a probe would have to be inserted into the thoracic cavity. However, a satisfactory approximation of changes in intrapleural pressure can be obtained by having a subject swallow an esophageal balloon such that pressure in the esophagus can be measured. The esophageal pressure is a valid reflection of the pleural pressure because the slight tone of the esophageal wall is negligible.

To measure compliance, the subject is intubated with an esophageal balloon and then told to breathe in or out of a spirometer in 500-ml increments. During breath holding, with the epiglottis open, the alveolar pressure is the same as the pressure at the mouth. Values of volume change and pleural pressure changes measured simultaneously produce a pressure–volume curve similar to that shown in Fig. 13.

The compliance is measured as the slope of the pressure–volume curve and the average normal value for an adult is 0.2 liter/cm H_2O. Compliance varies with the lung volume used, respiratory rate, body size, lung size, and whether it is measured during inflation to TLC or deflation to RV. A standardized methodology calculates compliance as the slope over 1 liter above FRC measured during deflation of the lungs (West, 1979). Specific compliance is compliance normalized by dividing by FRC and is independent of body and lung size.

The shape, position, and slope of the static deflation pressure–volume curve is characteristic of certain disease states. Obstructive diseases such as

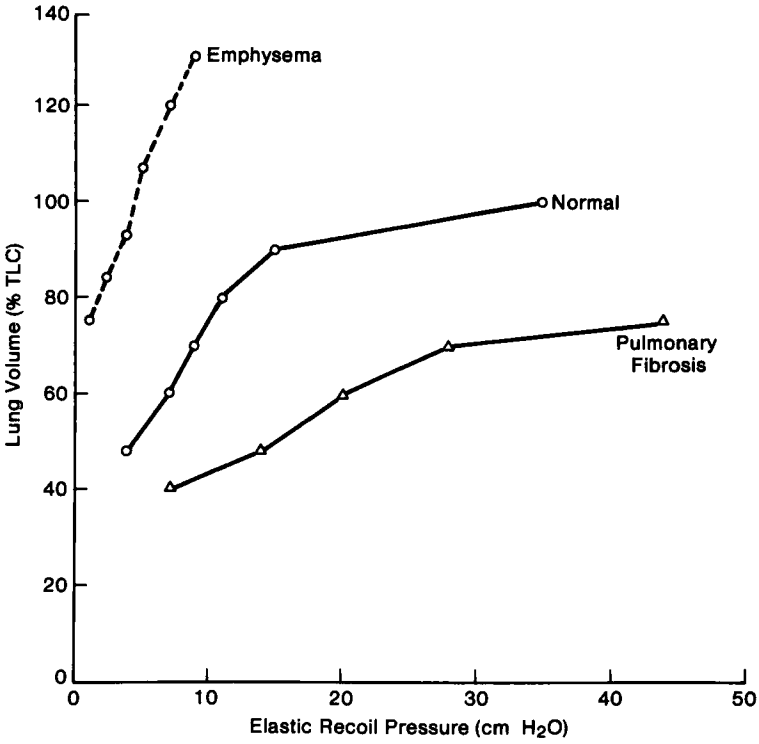

FIG. 13. Pressure–volume curve utilized for measuring lung compliance. Patients with obstructive diseases show a shift of the normal curve upward, whereas in restrictive diseases the curve shifts downward and to the right.

asthma and emphysema show a shift in the curve upward and to the left (see Fig. 13), whereas in restrictive diseases such as pulmonary fibrosis the pressure–volume curve is shifted downward and to the right. In general, compliance measurements are useful to assess abnormalities in the lung's elastic properties but are too nonspecific to be of conclusive diagnostic value.

4.4. DIFFUSING CAPACITY

The diffusing capacity of the lung for carbon monoxide (CO) is a measure of the ability of the alveolar capillary membrane to transfer or conduct gases from the alveoli to the blood. This transport process is entirely a passive one brought about by diffusion. As described previously in Section 2.2, the barriers for diffusion consist of surfactant, alveolar epithelium, interstitital fluid, capillary endothelium, plasma, and the red blood cell membrane.

Although CO is a nonphysiologic gas, it is used to measure diffusion because the concentration of CO in mixed venous blood is negligible and CO has a great affinity for hemoglobin. Thus, diffusion of CO into the blood is never limited by the ability of the blood to accept this gas.

There are two techniques used to measure diffusion capacity. In one procedure, the subject takes a single vital capacity inspiration of a dilute mixture of CO and holds his breath for 10 seconds. In the second, the subject breathes a low concentration of CO (about 0.1%) for 30 seconds until a steady state has been reached. In both methods, the rate of disappearance of the CO from the alveolar gas is calculated by measuring the concentrations of CO in the inspired and expired air with an infrared analyzer. The larger the diffusing capacity (D_LCO), the more CO enters the blood and the lower the amount of CO measured in the expired gas.

The normal diffusing capacity value for an adult at rest is about 25 ml/minute/mm Hg for CO. This value is reduced, however, when diffusion is impaired as a result of certain pathologic states that lengthen the barrier for diffusion (e.g., interstitial edema, alveolar edema, and fibrous tissue deposition) or decrease the area for diffusion (e.g., emphysema and nonventilated alveoli).

4.5. Single-Breath Nitrogen Test

The single-breath nitrogen (N_2) test is a simple, sensitive test whose major usefulness is in its diagnostic value in the early stages of airway obstruction. This test is also valuable in the assessment of anatomical dead space, uneven ventilation, and abnormal intrapulmonary gas mixing (Buist, 1975).

The basis of this test depends on the fact that airway closure occurs first in airways of the lower lobes not supported by cartilage because the intrapleural pressure is higher in these airways than in the airways of the upper lobes. The volume at which airway closure begins to occur can be detected by procedures that create a different composition of gas in the upper and lower lobes. Prior to beginning the test procedure, the patient breaths air so that his lungs contain gas that is approximately 80% N_2. The person then inspires one breath of pure oxygen from residual volume to his total lung capacity and breathes out. The N_2 concentration of the expired gas is continuously measured during a slow vital capacity expiration, and a curve such as that illustrated in Fig. 14 is produced. Phase I of the curve represents dead space gas, phase II represents a mixture of dead space gas and alveolar gas, phase III, the alveolar plateau, represents an almost uniform concentration of expired N_2 that comes almost exclusively from the alveoli, and phase IV represents gas from the upper-lung-zone alveoli.

Currently, three indices of airway collapse and air trapping are used. One

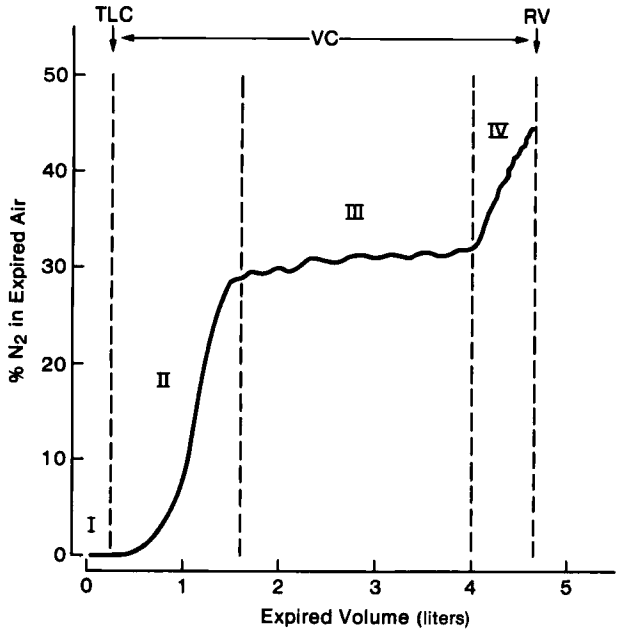

FIG. 14. Typical tracing obtained during the single-breath nitrogen test.

index is the closing volume, which is the volume above residual volume at which an abrupt change in expired N_2 concentration occurs and is usually expressed as a percentage of vital capacity. A second index is the closing capacity, which is the closing volume plus residual volume and is usually expressed as a percentage of total lung capacity. A third index is the slope of the alveolar N_2 plateau. For these three indices, a value that exceeds two standard errors above the published predicted value is considered abnormal. All three indices are elevated when there is premature airway closure, disease of the small airways, or uneven distribution of gas.

The major advantage of the single-breath nitrogen test is its sensitivity; however, the disadvantage is that none of the three indices (closing volume, closing capacity, and alveolar plateau slope) is specific for any lung abnormality. Thus, these indices cannot differentiate between diseases involving the airway or the parenchyma. Indeed, the closing volume and closing capacity can be influenced by a variety of pulmonary diseases, such as chronic bronchitis and emphysema, and even conditions in which there is an excess of interstitial fluid in the lungs due to kidney failure, liver failure, or postmyocardial infarction. In general, however, the single-breath nitrogen test is used as a simple, sensitive, early diagnostic test of general lung function.

4.6. Measurement of Ventilatory Drive

Clinical assessment of abnormalities in the regulation of respiration is usually limited to measurements of the concentration of carbon dioxide and oxygen in arterial blood (P_aCO_2 and P_aO_2). These measurements are made by the direct assay of blood samples with a blood gas analyzer (an instrument that utilizes special electrodes sensitive to CO_2 or O_2 to measure the levels of these gases in blood samples).

Additional routine clinical tests of respiratory drive control utilize relatively simple and rapid rebreathing methods to measure the ventilatory response to CO_2. The essential feature of the technique developed by Read (1967) to assess sensitivity to inspired CO_2 requires that a subject rebreathe for approximately 5 minutes from a bag containing 7% CO_2 in hyperoxic mixture. Under these conditions, the increase in ventilation that occurs is a linear function of the alveolar CO_2 concentration since any contribution to respiration by oxygen through arterial chemoreceptors is nullified by maintaining constant hyperoxic conditions. End-expired CO_2 is continuously monitored with an infrared CO_2 analyzer and for each millimeter of Hg rise in alveolar CO_2, ventilation should increase approximately 2 liters/minute. When averaged values for ventilation are plotted against alveolar CO_2, the slope of the response reflects the sensitivity to CO_2.

5. Obstructive Diseases of the Lung

As described earlier, obstructive diseases of the lung are those diseases associated with a narrowing of the diameter of the airways leading to an increased resistance to airflow. Such diseases can be caused by a variety of conditions, including constriction of the smooth muscles of the airways, occlusion of the airways by excessive mucus secretions, inflammation and edema (which lead to a thickening of the airway walls), and destruction of the lung parenchyma (which results in a loss of radial traction and concomitant narrowing of the airways). Common obstructive lung diseases discussed in this section include asthma, emphysema, chronic bronchitis, and cystic fibrosis.

5.1. Bronchial Asthma

Asthma is among the more common obstructive diseases of the lung. Nearly 3% of the population of the United States alone has some form of asthma. A number of excellent reviews (McFadden and Austen, 1983; Wilson and McPhillips, 1978; and McFadden and Feldman, 1977) and books

(Middleton *et al.*, 1983; Segal and Weiss, 1976) have been written to describe this disease state. The reader is referred to these references for more detailed information.

5.1.1. *Etiology*

Bronchial asthma is generally associated with a state of heightened sensitivity within the tracheobronchial tree to a number of bronchoconstrictive stimuli. The exact cause of this airway hypersensitivity is not known; however, it has been hypothesized that asthmatics have imbalances in the endogenous bronchodilating mechanisms that normally regulate airway tone. One endogenous bronchodilating mechanism that has been hypothesized to have decreased function in asthmatics is the β-adrenergic system by which endogenous epinephrine, interacting through β_2 receptors associated with adenylate cyclase systems in airway smooth muscle, increases intracellular cyclic AMP and thereby induces relaxation (Szentivanyi, 1968). In fact, it has been shown that some asthmatics have autoantibodies to their β-adrenergic receptors that have been hypothesized to bind to the receptors, thereby decreasing the availability of the receptors to mediate β-adrenergic bronchodilation (Fraser *et al.*, 1982). Alternatively, another bronchodilating system, the nonadrenergic inhibitory pathway (Richardson, 1981), has also been found to be defective in asthmatics. The mediator for the nonadrenergic system has not yet been elucidated. Thus, in the absence of normal bronchodilating function, cholinergic, and possibly α-adrenergic and other bronchoconstricting mechanisms, can predominate in asthmatics causing a widespread narrowing of the airways.

In addition to bronchoconstriction, other pathophysiological changes within the lung characteristic of asthma include a hypertrophy of mucosal glands and an increased secretion of thick, tenacious, and slow-moving mucus. During an asthmatic attack, the permeability of the capillaries within the pulmonary vasculature also increases. This can lead to edema of the bronchial wall and influx of eosinophils and neutrophils into the lung and can contribute to the development of a secondary inflammatory response. All of these effects lead to decreased gas exchange within the lung and are the predominant cause of the dyspnea, wheezing, coughing, and the increase in respiratory work widely associated with asthmatic attacks.

While asthma is a heterogenous disease and there is no single etiology that will account for all the various symptomatology experienced in this disease, asthma is commonly categorized into a number of different clinical subtypes that depend upon the provoking stimuli. The etiology of these subtypes is defined in some detail subsequently. It should be emphasized, however, that many patients will not clearly fit into any of the categories listed, but will fall into a mixed category that may incorporate features of several of these types of asthma.

5.1.1.1. *Extrinsic Asthma.* The most common and, hence, the best-defined form of asthma (25 to 35% of all asthmatic disease) is extrinsic (or allergic) asthma. This disease usually affects children and young adults. As with most allergies, a person is genetically predisposed to this form of asthma and at some point comes in contact with an antigenic substance (e.g., dust, dander, pollen) that initiates the production of a specific type of immunoglobulin (IgE) in the B lymphocytes found in the peripheral lymphoid tissue of lung; IgE subsequently binds to distinct receptor sites on mast cells within the bronchial mucosa and perivascular space and to receptor sites on alveolar macrophages. This process is called sensitization. Upon reexposure of the asthmatic to the antigenic substance a cross-linking of IgE antibody complexes on the mast cells and macrophages occurs that leads to the release of a number of different types of allergic mediators from cells, depicted in Fig. 15. These mediators then interact with receptors on other cell types within the lung to induce bronchoconstriction, increases in capillary permeability and mucus secretion, or the initiation of a chemotactic response that leads to a secondary inflammatory response.

In mast cells, both preformed mediators and those that are synthesized *de novo* after antigen challenge are released by a process that is believed to involve IgE-induced activation of a serine–esterase-like enzyme, the activation of methyltransferase activities within the plasma membrane, and an influx of calcium into the cell (Ishizaka, 1983). Inside the cell, calcium is believed to interact with the intracellular calcium-binding protein, calmodu-

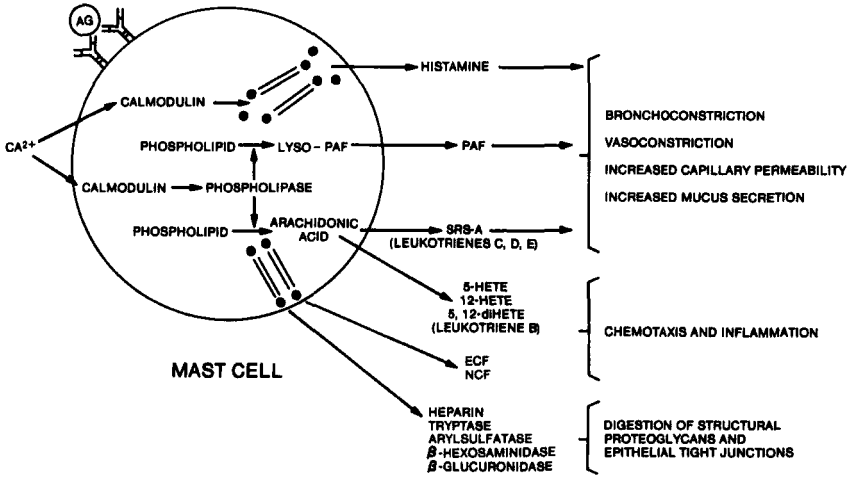

FIG. 15. IgE-mediated release of allergic mediators from lung mast cells.

lin, to induce the release of allergic mediators through a complex series of reactions (Douglas and Nemeth, 1982).

The preformed mediator of greatest familiarity that is released in this fashion from mast cells in human lung is the vasoactive amine histamine. This mediator is capable of inducing bronchoconstriction when administered to man by the aerosol route (Mathé *et al.*, 1973) and has been shown *in vitro* to initiate mucus secretion (Shelhamer *et al.*, 1980) and alter capillary permeability within the vascular tissue (Northover, 1975). But despite these pharmacological actions, histamine is not believed to be the primary mediator (if a primary mediator exists) in asthma since antihistaminic drugs have not demonstrated great use clinically (Karlin, 1972).

More recent studies have focused a great deal of attention on the role of two other mediators in allergic asthma: slow-reacting substance of anaphylaxis (SRS-A) and platelet-activating factor (PAF). SRS-A is now known to be a family of Δ^5-lipoxygenase derived arachidonic acid metabolites, leukotrienes C_4, D_4, and E_4 (Lewis and Austen, 1981; Samuelsson, 1983), whereas PAF has been structurally characterized as 1-alkyl-2-acetyl glycerol-3-phosphorylcholine (Vargaftig *et al.*, 1981). The steps involved in the biosynthesis of these mediators are described in Figs. 16 and 17. Both mediators are formed by the initial activation of phospholipase enzymes within the cells; however, the substrates for these enzymes differ. In the case of SRS-A, the substrate is a phospholipid, whereas the substrate for the formation of PAF is 1-*O*-alkyl-2-acyl-glycero-3-phosphorylcholine.

Studies in which the pharmacological actions of the leukotrienes and PAF have been characterized support the potential role of these mediators in asthma. Thus it has been experimentally demonstrated that leukotrienes C_4, D_4, and E_4 all induce many of the pathophysiological changes normally associated with an asthmatic attack. These include the ability to contract human bronchial smooth muscle (the leukotrienes are 2000- to 10,000-fold more active than histamine) and to induce vascular permeability changes and mucus secretion in lung tissue (Dahlen *et al.*, 1980; Weiss *et al.*, 1982; Sorter *et al.*, 1983; Marom *et al.*, 1982). Also, leukotrienes have been shown to be released from antigen-challenged human lung tissue in quantities sufficient to induce airway constriction (Dahlen *et al.*, 1983). Current evidence supporting a role for PAF in allergic asthma also includes the observations that this mediator is known to be synthesized and released from alveolar macrophages isolated from asthmatic patients (Arnoux *et al.*, 1983) and to induce pathophysiological changes in animals and man (Pinkard *et al.*, 1983; Gateau *et al.*, 1984) similar to those associated with an asthmatic attack. As with the leukotrienes, however, the evidence supporting a role for PAF in asthma is circumstantial and will not be conclusive until effective therapeutics have been developed that act to regulate the actions of this mediator.

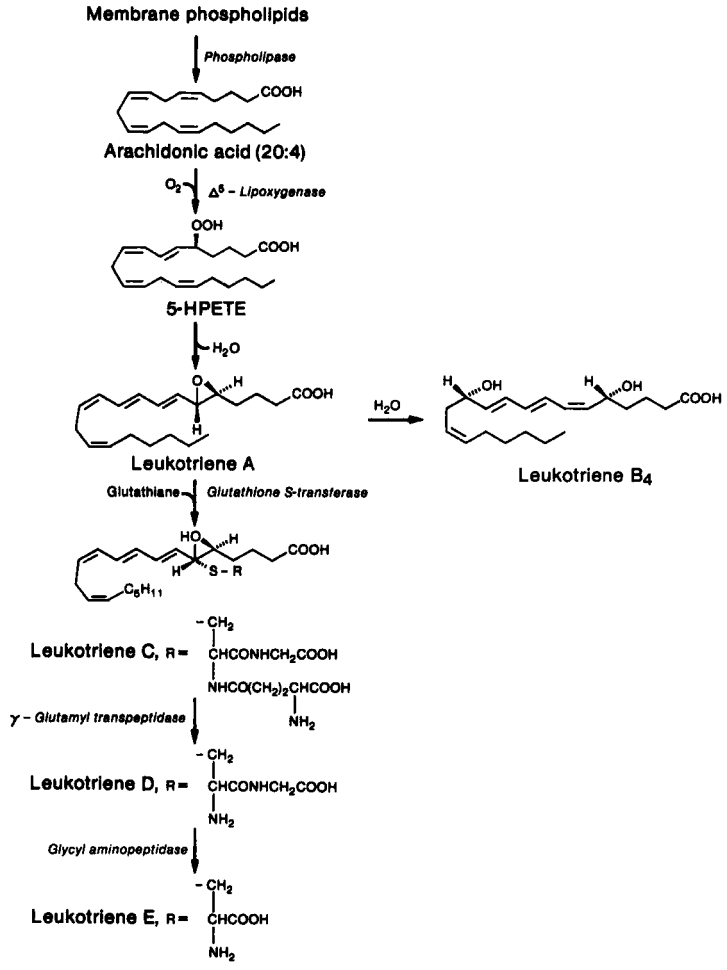

FIG. 16. The biosynthesis of leukotrienes via the Δ^5-lipoxygenase pathway.

Once released within the lung, the leukotrienes are hypothesized to induce bronchoconstriction of airway smooth muscle through a receptor-mediated process. Binding sites specific for the leukotrienes have been identified in human lung parenchyma (Nicosia *et al.*, 1984) and are believed to represent receptors on the plasma membrane of airway smooth-muscle cells. The interaction of the leukotrienes with a receptor is then hypothesized to induce the release of intracellular calcium in the smooth-muscle cell (Weichman *et al.*, 1983). As with mast cells, intracellular events within the smooth-muscle cells are believed to be modulated via the calcium-binding protein

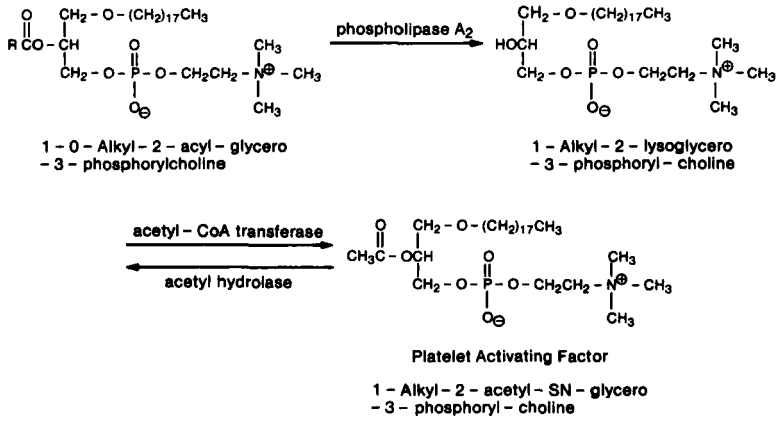

FIG. 17. The biosynthesis of platelet-activating factor.

calmodulin. Calmodulin initiates contraction through activation of the enzyme, myosin light chain kinase, which phosphorylates myosin light chain, allowing the interaction of myosin with actin and thereby initiating contraction of the actomyosin complex within the airway smooth-muscle cells (Adelstein *et al.*, 1982).

The bronchoconstrictive effects of PAF in animal models (primarily guinea pig) appear to be mediated primarily by the activation of platelets and release of a secondary bronchoconstrictive substance (Vargaftig *et al.*, 1982). A direct effect of PAF on guinea pig and human parenchymal tissue has also been reported, however (Stimler and O'Flaherty, 1983; Stimler *et al.*, 1983). One or both of these mechanisms of bronchoconstriction may occur in man.

In addition to histamine, SRS-A, and PAF, there are also other allergic mediators believed to be released in lung tissue during an asthmatic attack (Fig. 15). These included mast cell granule-associated peptide mediators that are chemotactic for neutrophils [neutrophil chemotactic factor (NCF)] or eosinophils [eosinophil chemotactic factor (ECF)], heparin proteoglycans, tryptase, a tryptic neutral peptide, arylsulfatase, and acid hydrolases such as β-hexosaminidase and β-glucuronidase. Also there are a variety of arachidonic acid metabolities (lipoxygenase-derived HETEs and cyclooxygenase-derived prostaglandins) that are synthesized de novo and released from a number of different types of cells in the lung. This is depicted schematically in Fig. 18. These arachidonic acid metabolites either are chemotactic or have direct effects on airway smooth muscle (i.e., induce bronchoconstriction or bronchodilation).

The release of ECF, NCF, and chemotactic HETEs (i.e. 5-HETE, 12-

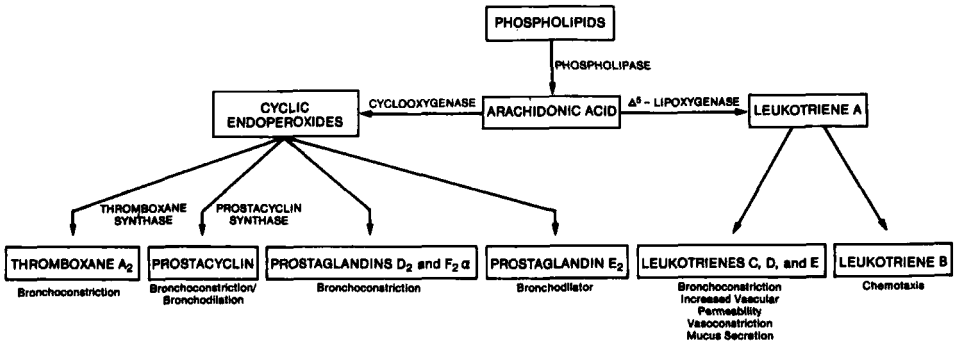

FIG. 18. The biosynthesis and biological effects of arachidonic acid metabolites in lung tissue.

HETE, and leukotriene B_4) in combination with increases in capillary permeability induced by other mediators such as histamine, the leukotrienes, and PAF leads to the influx of inflammatory cells (neutrophils, eosinophils, and macrophages) into the lung. These elicited cells may then be direct contributors to the secondary inflammatory response often associated with an attack of allergic asthma.

As mentioned earlier, the prostaglandins released in the lung during an asthmatic attack have both bronchoconstrictive ($PGF_{2\alpha}$, PGD_2, and thromboxane A_2) and bronchodilator properties (PGE_2 and PGI_2). An imbalance among these various arachidonic acid metabolites (including the leukotrienes) may have a role in inducing airway constriction. It should also be noted that an imbalance of prostaglandin metabolites has been hypothesized to contribute to other forms of asthma besides extrinsic (e.g., aspirin-sensitive asthma, as described later). A polypeptide factor of mast cell origin has been identified (prostaglandin-generating factor of anaphylaxis) that may have a role in regulating the production of prostaglandins during an asthmatic attack (Steel and Kaliner, 1981). Thus, some prostaglandins may be produced secondarily in response to the release of this factor from mast cells.

It should be emphasized that although a number of different bronchoconstrictive and inflammatory mediators of allergic asthma have been described individually in this section, it is also important to view their interactive and often synergistic actions in discussing their pharmacological profile and physiological role in asthma. One of the hallmarks of asthma is the hypersensitivity of the tracheobronchial tree of asthmatics to a variety of stimuli that may be the result of such synergistic actions. For example, it has been shown that the leukotrienes can increase the potency of histamine in airways (Creese and Bach, 1983). Also it has been shown in guinea pigs that

mediators such as the leukotrienes or histamine can induce the release of thromboxane and other bronchoconstrictive prostaglandins within the lung (Piper and Vane, 1971; Piper and Samhoun, 1982; Weichman *et al.*, 1982). An increased biosynthetic capability for such compounds in asthmatics may also play a role in hypersensitivity phenomenon. Finally, the release of inflammatory, chemotactic mediators (leukotriene B_4, ECF, and NCF) could induce the influx into the lung of neutrophils, eosinophils, and macrophages that in turn can release superoxide and lysosomal enzymes. This process in combination with the release of mast cell-derived neutral proteases and acid hydrolases has been hypothesized to lead to the destruction of the tight junctions between the epithelial layer of the airways (McFadden and Austen, 1983). This process could thereby facilitate the exposure of underlying smooth muscles to bronchoconstrictive mediators and account also, in part, for the hypersensitivity of asthmatic airways to bronchoconstrictive agents.

5.1.1.2. *Intrinsic Asthma.* This form of asthma is frequently found in middle-aged patients. Although the pathophysiological characteristics of intrinsic asthma are similar to those of extrinsic asthma, the causes of this condition are less clear. This form of asthma is not associated with a immunological release of bronchoconstrictive and inflammatory mediators from tissue mast cells. It may, however, be associated with respiratory tract infections. Such infections have been hypothesized to contribute to decreased β-adrenergic responses in this type of asthmatic (Busse, 1977). Infection could also contribute to airway hypersensitivity by inducing leaks in epithelial cell tight junctions within the airways and thereby increasing the exposure of airway smooth muscle to bronchoconstrictive agents.

5.1.1.3. *Exercise-Induced Asthma.* In some asthmatics (usually young, allergic patients) moderate to severe exercise can initiate an asthmatic attack. As with intrinsic asthma, the etiology of exercise-induced asthma has not been well defined. One theory (Deal *et al.*, 1979) suggests that cold-sensitive "irritant" receptors in the epithelia are associated with afferent nerve fibers within an autonomic reflex arc. Efferent motor fibers of this reflex then return to the airway smooth muscle via the vagus nerve. Thus, bronchoconstriction is induced through this reflex arc by stimulation of the cold-sensitive irritant receptors.

Alternatively, it has been suggested that exercise challenge may induce the release of allergic mediators from lung mast cells. Indeed, some supportive evidence exists for this type of mechanism since it has been shown that the levels of one mediator, NCF, increase in the serum of exercise-induced asthmatics in response to exercise challenge (Orehek, 1983). Additional sup-

portive evidence for the mediator release hypothesis is that therapeutic agents that are thought to act through inhibition of mediator release (e.g., disodium cromoglycate) are effective in treating exercise-induced asthma (Dahl and Henriksen, 1979).

5.1.1.4. *Aspirin-Sensitive Asthma.* This form of asthma develops in some patients a short time (~20 minutes) after the ingestion of aspirin or other nonsteroidal antiinflammatory drugs (e.g., indomethacin) or certain food additives (e.g., tartazine yellow) related to aspirin. While the actual patient population affected with this form of asthma is small (perhaps 10% of adult asthmatics) this particular side effect of nonsteroidal antiinflammatory drugs can be life threatening to those involved. Again, the etiology of this form of asthma is not certain; however, most hypotheses center around the known effect of aspirin to inhibit cyclooxygenase, the enzyme that initiates the cascade of reactions associated with the formation of prostaglandins, thromboxanes, and prostacyclin (see Fig. 17). Thus, aspirin-sensitive asthmatics may be extremely dependent on the natural bronchodilating effects of PGE_2 and upon ingestion of aspirin have a decreased ability to produce this prostaglandin. Alternatively, aspirin-sensitive asthmatics may have an increased capacity to produce bronchoconstrictive Δ^5-lipoxygenase products, and ingestion of aspirin might inhibit arachidonic acid metabolism through the cyclooxygenase pathway and promote metabolism through the Δ^5-lipoxygenase pathway. Both of these hypotheses remain to be proved, however.

5.1.1.5. *Environmental or Occupational Asthma.* With modern industrialization, the concentration of airborne pollutants in the environment has increased and led to the development of an environmental form of asthma, which can occur in individuals living in concentrated industrial areas. This type of asthma appears to result from exposure of individuals to toxic gases such as sulfur dioxide. Because of their hyperirritable airways, all types of asthmatics are effected by such environmental pollution, but there is one class that has no other underlying etiology besides an increased sensitivity to polluted air. In these individuals, exposure to environmental pollution is hypothesized to stimulate irritant receptors in the lung.

In addition, a variety of substances often encountered in an industrial working environment (e.g., metal salts, wood shavings, industrial chemicals, pharmaceutical agents, animal dander) have also been associated with asthmatic attacks. The underlying etiology of this type of "occupational asthma" can be immunologic (allergic) in nature and might be caused by the direct release of bronchoconstrictory substances or could be due to the direct stimulation of irritant receptors by the offending agent. This type of asthma is characterized by the fact that attacks occur at work; thus, while away from

the working environment, individuals with occupational asthma are symptom free.

5.1.1.6. *Status Asthmaticus.* This is not really a subtype of asthma such as those previously described, since it can be caused by any of the factors described for other types of asthma. Instead, status asthmaticus is categorized separately because of the severity of the reaction. Most asthmatic attacks are mild and can be quickly reversed by bronchodilating medication; however, status asthmaticus is a form of asthma refractory to usual drug therapies and of great enough severity to require immediate hospitalization. All characteristics of an asthmatic response including smooth-muscle contraction, mucosal and submucosal edema, basement membrane thickening, inflammatory cell infiltration, and mucosal plug formation are apparent in this extreme form of asthma. Without rapid treatment, status asthmaticus can quickly result in death due to hypoxia or respiratory acidosis.

5.1.2. *Diagnostic Measurements*

5.1.2.1. *Mild Forms of Asthma.* Mild cases of asthma are generally easy to diagnose. Initial symptomatology involves the occurrence of repeated but reversible attacks of wheezing and labored breathing. In patients with extrinsic asthma this can frequently be associated with a family history of allergic conditions or the previous existence of allergic rhinitis or urticaria in the patient. Often a specific allergen, such as dust, pollen, or animal dander, is suspected and can be readily identified.

In the absence of an attack, diagnosis can frequently be aided by performing a methylcholine challenge on the individual, followed by spirometric measurements. Asthmatics are characteristically hypersensitive to these test agents (Hargreave *et al.*, 1981). For example, an asthmatic will exhibit a 20% decrease in FEV_1 at a 10-fold lower dose than normal subjects. If the patient is suspected of having exercise-induced asthma, an exercise challenge can be performed in the physician's office and spirometric changes observed.

A patient examined during an asthmatic attack will exhibit definite changes in pulmonary function, most predominantly decreases in expiratory flow rate as assessed by decreases in FEV_1 and the maximal expiratory flow at 50 and 25% of vital capacity (see Figs. 11 and 12). Since a characteristic feature of mild asthma is its reversibility, the reversibility of these changes in pulmonary function by a 1% nebulized solution of isoproterenol administered to the patient is also a diagnostic characteristic of asthma.

Increased static lung volumes (FRC and TLC), as assessed by whole-body plethysmography, are also a diagnostic feature of asthma (Fig. 9). This is presumably due to premature closing of the airways during expiration be-

cause of the presence of constricted smooth muscle, edema, and increased secretions in the airways. The resulting hyperinflation can also be seen with chest X ray.

As part of the diagnosis of asthma, blood gas measurements are frequently made. In mild asthma, decreases in the P_aO_2 of arterial blood are observed due to ventilation–perfusion abnormalities that result from the presence of regions of reduced ventilation within the lung.

5.1.2.2. *Status Asthmaticus.* The diagnosis of status asthmaticus is frequently aided by a previous history of asthmatic attack. Altered pulmonary functions are observed in these patients, as described earlier, but they are not readily reversed by β_2 bronchodilators. Mucus secretion is frequently impaired in these individuals and is accompanied by a dry, nonproductive cough. Finally, diagnosis of this form of asthma is usually associated with alterations in blood gas levels of both O_2 (decreased P_aO_2) and CO_2 (increased P_aCO_2 leading to acidosis).

5.1.3. *Therapeutic Treatment*

5.1.3.1. *Therapeutic Treatment of Mild Asthma.* If the known precipitating factor of an asthmatic attack has been identified, (e.g., a specific antigen), a serious attempt should be made to avoid this agent. A number of different therapeutics can also be prescribed in the treatment of asthma, including theophylline preparations, β-adrenergic bronchodilators, corticosteroids, and allergic mediator release inhibitors. The first three drug types are useful in the relief of acute attacks, but, in addition, a goal of therapeutic treatment should be the prophylaxis of subsequent attacks. Mediator release inhibitors find special use in prophylactic regimens.

Theophylline preparations. Within the United States, where oral drugs are favored, mild attacks of asthma are usually treated initially with a theophylline preparation. Paramount to the use of this therapeutic is the need to monitor and maintain safe but efficacious blood levels (10–20 μg/ml). If blood levels exceed the safe limits, cardiovascular (tachycardia and hypotension), central nervous system (aggitation with eventual seizures), and gastrointestinal (nausea and vomiting) side effects can be life threatening. Because theophylline is metabolized at widely different rates in different individuals, it was once difficult to adjust therapeutic dosages to maintain appropriate blood levels. Now, setting dosages is much simpler because slow-release formulations of this drug (e.g., Theodur) are available that only require twice daily administration to maintain constant blood levels of the drug. Soon it is expected that once daily preparations will also be available.

The mechanism of action of theophylline has classically been attributed to inhibition of phosphodiesterase in airway smooth muscle and possibly also in

lung mast cells. In this manner cyclic AMP (cAMP) levels within cells would be increased—a state that favors bronchodilation of smooth-muscle cells and inhibition of the release of allergic mediators from mast cells. This mechanism is constantly debated, however, since the blood levels of theophylline required for a therapeutic effect are much lower (10^{-5} *M*) than the levels needed *in vitro* to demonstrate an effect on cAMP phosphodiesterase (10^{-4} to 10^{-3} *M*) (Jenne *et al.*, 1972; Isles *et al.*, 1982). Theophylline has been shown to be an adenosine receptor antagonist in a number of tissues (Fox and Kellye, 1978). This has led to speculation that this might be the mechanism by which theophylline acts on airway smooth muscle and lung mast cells (Fredholm, 1980). Initial reports that adenosine constricts airway smooth muscle (Fredholm *et al.*, 1979) and potentiates the release of allergic mediators such as histamine from lung mast cells (Marquardt *et al.*, 1978; Welton and Simko, 1980) supported this mechanism of action. Further studies have not confirmed the bronchoconstrictive actions of adenosine, however, making this mechanism of action for theophylline on airway smooth muscle less attractive. On the other hand, the ability of adenosine to potentiate allergic mediator release from mast cells and of theophylline to block this process has been verified in a number of laboratories; this finding suggests that this mechanism may account in part for the therapeutic actions of theophylline in asthma. For the most part, however, the biochemical mechanisms associated with the actions of theophylline on bronchial smooth muscle remain unclear.

β-*Adrenergic agents.* β-Adrenergic agents are also commonly prescribed (often in conjunction with oral theophylline preparations) for reversing the airway bronchoconstriction associated with a mild asthmatic attack. These drugs act primarily by stimulating B_2 receptors in airway smooth muscle, thereby activating adenylate cyclase in the smooth muscle, increasing cellular cAMP levels, and ultimately inducing bronchodilation. β_2 receptors are also located on lung mast cells, and their stimulation has been shown to inhibit allergic mediator release from lung tissue (Orange *et al.*, 1971).

β-Adrenergic agents (e.g., epinephrine, isoproterenol, metaproterenol, terbutaline, and salbutamol) are most commonly administered by the aerosol route. Oral preparations of metaproterenol and terbutaline are also available in the United States. The earliest members of this therapeutic class, epinephrine and isoproterenol, were not selective for the β_2-adrenergic receptors on airway smooth muscle since they also interacted with β_1-adrenergic receptors in the heart to cause side effects such as tachycardia and an increased force of contraction. Second-generation β agonists are more selective for β_2 receptors (metaproterenol, terbutaline, and salbutamol) and therefore cause fewer cardiovascular effects. These also have longer durations of action than epinephrine and isoproterenol. An additional side effect

often reported with β_2 agonists, especially after oral administration, is skeletal muscle tremor. This side effect is still present with the second-generation β_2-selective agents. A number of new β agonists are being developed by pharmaceutical companies and should be available for clinical use in the future.

Steroids. When asthmatics do not respond to the therapy described in the preceding section, they are commonly treated with steroids. Those usually prescribed include prednisone and beclomethasone. The clinical actions associated with steroid treatment include antiinflammatory effects, decreased mucus secretion, inhibition of antibody formation, and potentiation of the action of β_2-adrenergic bronchodilators. The cellular biochemical mechanisms associated with these effects of steroids are not completely understood, but one of the currently popular hypotheses concerning the mechanism of action of steroids is that these drugs act by inducing the intracellular synthesis of proteins that are endogenous inhibitors of cellular phospholipase activities (Flower and Blackwell, 1979; Hirata *et al.*, 1980). Thus, it is hypothesized that steroids might act, in part, by preventing arachidonic acid release and subsequent metabolism to prostaglandins and leukotrienes. The role that phospholipase inhibition could have in relieving the bronchoconstrictive and inflammatory events associated with an asthmatic attack is not difficult to envision since arachidonic acid metabolites (i.e., prostaglandins and leukotrienes) have been shown to affect these processes (see Section 5.1.1.1). The manner by which phospholipase inhibition influences antibody formation and potentiates the actions of β_2-adrenergic agents remains to be elucidated, however.

Orally administered steroids should only be administered for short periods of time since long-term therapy may lead to a variety of side effects, including suppression of adrenal function, hypertension, aggravation of diabetes, exacerbation of infections, osteoporosis, cataract formation, and peptic ulcer formation. The availability of aerosol formulations of beclomethasone has facilitated the long-term use of this type of therapeutic since this route of administration deposits the drug directly in the airways, where it can act therapeutically but is not absorbed into the systemic circulation.

Disodium cromoglycate (cromoglycate). Cromoglycate is especially useful in the prophylactic treatment of allergic and exercise-induced asthma. This drug is thought to act by preventing the release of allergic mediators from lung mast cells. The biochemical mechanisms associated with this effect of cromoglycate on mast cells are not well defined. This drug does not seem to act through elevating cAMP levels within the mast cell since it is neither a stimulator of adenylate cyclase nor an inhibitor of cAMP phosphodiesterase at doses in which inhibition of mediator release is observed. Instead it has been shown to act at a step that inhibits the antigen-induced influx of cal-

cium into the mast cell (Forman *et al.*, 1977; Spataro and Bosman, 1976). Cromoglycate appears to act via an extracellular receptor (Mazurek *et al.*, 1980) and has been shown to cause the phosphorylation of a 78,000-Da protein present in mast cells in conjunction with its inhibitory effects (Theoharides *et al.*, 1980). It is interesting to speculate that the site of phosphorylation may be a transport protein involved in "calcium gating"; however, such a relationship has not yet been proved.

In addition to inhibition of mediator release, cromoglycate has the property of inhibiting vagal reflex-mediated bronchoconstriction in dogs, which results from the stimulation of irritant nerve fibers with substances such as histamine and prostaglandins (Dixon *et al.*, 1980). The importance of this latter mechanism in the therapeutic actions of cromoglycate is unclear.

There are no predominant side effects associated with the use of cromoglycate. The drug is not orally active, however, and must be administered as a powder directly into the lungs through a special device called a spinhaler. A minimum trial period of 4 to 6 weeks of regular therapy is often required to obtain clinical activity. Under these conditions cromoglycate not only has been found to prevent allergic and exercise-induced asthma, but also decreases the airway hypersensitivity found to be a characteristic of asthma (Bernstein, 1981). Since the patients who have the greatest potential for therapeutic utility of this drug are children or young adults who have some difficulty using the spinhaler, a number of pharmaceutical companies have attempted the development of an orally active drug with a similar mechanism of action. There are no orally active drugs of this type currently available for clinical use, however.

Future therapeutics for the treatment of mild asthma. As knowledge of the basic biochemical mechanisms associated with asthma expands, the potential for new and unique therapeutic advances in the treatment of this disease also increases. Thus it is possible to hypothesize that in addition to more selective β_2 agonists and orally active cromoglycatelike mediator release inhibitors, drugs that specifically affect the actions of leukotrienes or platelet-activating factor (antagonist or synthesis inhibitors) or drugs that act through regulating the actions of calcium in airway smooth-muscle cells or mast cells will be available in the future.

5.1.3.2. *Therapeutic Treatment of Status Asthmaticus.* Status asthmaticus is life threatening and is therefore a medical emergency that requires hospitalization and much more aggressive treatment than do milder forms of asthmatic attack. The administration of intravenous theophylline or a β_2 agonist (epinephrine or isoproterenol) is an immediate requirement for treatment. Subsequent therapy concentrates on multiple approaches to rapidly mobilze secretions. These approaches can include maintaining ade-

quate hydration of the bronchial secretions, treatment of the patient with expectorant agents, mechanical suctioning and ventilation of the lungs, treatment with oxygen, and treatment with antibiotics if an infection exists. Intravenous steroid therapy is also begun if other therapeutic treatments are unsuccessful.

5.2. Emphysema

5.2.1. *Etiology*

Emphysema is a chronic medical problem of middle age that together with bronchitis (see Section 5.3) ranks second only to heart disease as a cause of disability compensation by the Social Security Administration. The yearly mortality rate due to this disease is large, being greater than 20,000 deaths in the United States alone (Hoidal and Niewoehner, 1983). The etiology, diagnosis, and therapeutic treatment of this disease have been extensively described in a number of review articles and books, to which the reader is referred for further information, (Hoidal and Niewoehner, 1983; Laros and Kuypner, 1976; Netter, 1979; Burrows *et al.*, 1983; West, 1982).

Morphologically, emphysema is associated with a destruction of the alveolar septum, which results in a dilation and consequent enlargement of the alveolar spaces (Fig. 19). This is apparently caused by a breakdown of the interstitial connective tissue proteins (primarily elastin) that provide the major structural framework of the lung parenchyma. Two types of emphysema have been defined on the basis of the types of destruction of the alveolar septa observed and the type of dilation of the terminal respiratory unit (the acini) that is observed. A typical acinus branches from a terminal bronchiole and consists of the respiratory bronchioles that have alveolated walls and lead to the alveolar ducts and ultimately to the alveolar sacs (see Fig. 3). In centrilobular (or centriacinar) emphysema, the sites of degradation and dilation are limited to the region of the terminal and respiratory bronchioles. In panlobular (or panacinar) emphysema, the entire acinus (including the alveolar ducts and sacs) is more uniformly affected.

The most widely accepted hypothesis for the development of emphysema is the "protease–antiprotease theory." According to this theory, there is a progressive destruction of the lung interstitium due to an excess of the enzyme elastase in the lung in relation to the availability of its endogenous inhibitor, α_1-protease inhibitor (α_1-PI). This inhibitor is synthesized in the liver and transported to the lung. A number of observations provide the basis for this hypothesis. For example, it has been observed that people who have a genetic deficiency in α_1-PI (serum levels less than 50 mg/100 ml in contrast to normal levels of 250 mg/100 ml) are prone to developing emphy-

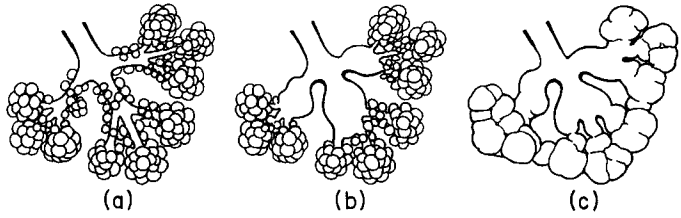

FIG. 19. Pathological destruction of the alveolar structure: (a) normal acinus, (b) centrilobular, and (c) panlobular emphysema.

sema at an early age (20–30 years of age) (Makino and Reed, 1970). Direct measurement of α_1-PI levels in the bronchial lavage of such patients has confirmed that such people have little or no antiprotease protection (Gadek *et al.*, 1981). Also, it has been observed that animals (dogs and hamsters) will develop emphysemalike symptomatology following the intratracheal administration of purified canine or human neutrophil elastase (Janoff *et al.*, 1977; Sloan *et al.*, 1981). Finally, smokers are known to develop more cases of emphysema than nonsmokers, and there is now convincing evidence that smoke can both induce the migration of elastase-containing cells (alveolar macrophages and neutrophils) into the lung (Harris *et al.*, 1970; Hunninghake *et al.*, 1979) and oxidatively inactivate α_1-PI (Carp *et al.*, 1982; Gadek *et al.*, 1979b), thus altering the protease–antiprotease balance in the lung.

As indicated earlier, two cell types appear to be important for the release of elastase into the lung—the alveolar macrophage and the neutrophil. The elastase in neutrophils is present in azurophilic granules and has been well characterized (Baugh and Travis, 1976). This elastase is inhibited by α_1-PI (Lonky and McCarren, 1983). Thus, it is believed that the neutrophil elastase is important in the pathogenesis of emphysema. The role of the alveolar macrophage in the increase of elastase load in the lung is not as well understood, however. For example, the majority of the elastase in human alveolar macrophages appears to be derived from neutrophils (McGowan *et al.*, 1983), suggesting that the primary function of the macrophage may be to clear and inactivate neutrophil elastase. It is likely, however, that the macrophage may also serve as a reservoir of neutrophil elastase and that in regions of high macrophage concentration, this cell could release a portion of the incorporated elastase and degrade lung elastin.

In addition to releasing elastase into the alveolar regions of the lung, macrophages and neutrophils also disrupt the potential protease–antiprotease balance of the lung through other functions. For example, both cells can secrete myeloperoxidase and hydrogen peroxide, which can cata-

lyze the inactivation of α_1-PI by oxidizing a methionine residue in the protein (Clark *et al.*, 1981; Cohen *et al.*, 1982). Also, activated human alveolar macrophages have been shown to release chemotactic factors for neutrophils (Cohen *et al.*, 1982), suggesting that an important action of the macrophage may also be to elicit the influx of neutrophils into the lung.

It should be pointed out that while the protease–antiprotease theory of emphysema is currently the dominant hypothesis used to explain the etiology of emphysema, other factors must also be considered as contributing to the development of this disease. For example, oxidants, present in smoke, probably contribute in other fashions besides inactivating α_1-PI. Such substances are capable of oxidizing a variety of cellular components (lipids, enzymes, and nucleic acids) and in this way may destroy parts of the alveolar epithelium, thus facilitating the exposure of elastin fragments present in the interstitial space to elastase.

Smoking may also have effects on the connective tissue of the lung that are independent of elastase and α_1-PI. For example, smoke has been shown to have inhibitory effects on connective tissue repair processes in the lung (Osman *et al.*, 1982), acting perhaps by inhibiting lysyl oxidase, an enzyme that cross-links desmosine in elastin. By affecting the general nutritional status of the body and making the lung more susceptible to oxidant injury, smoking could also contribute to the development of emphysema. For example, smoking is known to decrease the levels of vitamin C (Pelletier, 1975), which is an important antioxidant, and this fact has significant implications for the development of emphysema.

5.2.2. *Diagnosis*

Emphysema can only be diagnosed with certainty by a direct postmortum examination of sections of whole lung fixed after inflation. Fortunately, a number of other more circumstantial observations can also be combined and used to diagnose this disease. An obvious factor to consider is the general appearance of the patient. An emphysemic person is likely to complain of trouble breathing after exercise and can be observed in the resting situation to take slow deep breaths with an obvious use of accessory muscles of respiration. There is often a relatively prolonged period of expiration through pursed lips. In addition, the patient's neck vein may be extended during expiration but collapse quickly during inspiration.

As with other obstructive pulmonary diseases (e.g., asthma), spirometric tests will indicate a decrease in FEV_1 and an increase in the total forced expiratory time (the total time required to exhale the entire vital capacity of the lung). In emphysema, all of these pulmonary function parameters are altered due to a loss of the elastic recoil properties of the lung and a collapse of the intrathoracic airways during forced expiration, both of these phenomena being caused by the destruction of the interstitial connective tissue.

Measurement of static lung volumes by spirometry and through utilization of a plethysmograph (Fig. 9) may indicate a large increase in residual volume (RV) due to premature airway collapse and air trapping caused again by the loss of radial traction due to interstitial tissue destruction. A concomitant increase in total lung capacity (TLC) may also be evident due to a loss of elastic recoil in the lung. Because of this, an X-ray radiographic examination of an emphysemic's lungs often demonstrates marked lung hyperinflation.

Due to the alveolar tissue destruction occurring in the emphysemic lung, ventilation is apt to be impaired to areas of the lung in which blood perfusion is still adequate, leading to decreases in the ventilation/perfusion ratio of the lung. This will cause abnormalities in the N_2 washout curve observed during the single-breath nitrogen test, as evidenced by a constantly increasing nitrogen concentration in exhaled air (Fig. 20). This should be contrasted to the normal situation where the concentration of nitrogen increases very little after the exhalation of the first 0.8 liter of gas. Indequate ventilation will also be evident from blood gas measurements in which the partial pressure of oxygen in arterial blood (P_aO_2) may drop from approximately 100 mm Hg to around 70 mmHg. In emphysema, the lung's capacity to transfer gases to the blood is reduced, as measured by evaluation of the diffusing capacity for carbon monoxide (D_LCO).

Finally, in recent years analytical techniques have been developed for measuring elastin fragments or desmosine levels in serum (King *et al.*, 1980;

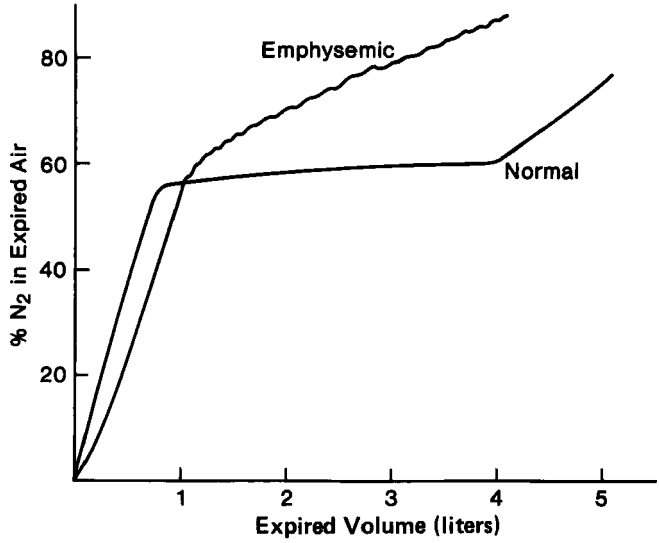

FIG. 20. N_2 washout curve of an emphysemic.

Turino *et al.*, 1980; Kucich *et al.*, 1980, Janoff *et al.*, 1983; Kucich *et al.*, 1983). There is evidence that emphysema development in animals can be correlated with increases in the serum levels of these components (Kucich *et al.*, 1980). Furthermore, preliminary studies indicate that serum levels of elastin fragments are increased in smokers and in patients with chronic obstructive pulmonary disease (Kucich *et al.*, 1983). It is hoped that further studies will also demonstrate a convincing correlation in man and that such analysis can then provide another quick diagnostic tool for emphysema.

5.2.3. *Therapeutic Treatment of Emphysema*

Initial treatment of emphysema should begin by the cessation of exposure to any exacerbating agent of the disease, such as smoke, air pollution, and other occupational factors. In some cases this may necessitate a move to another climate with a lower altitude, a warmer temperature, or a lower degree of pollution.

Any respiratory infections associated with the disease should be treated with antibiotics and followed by preventive measures (such as vaccination against influenza virus, avoidance of large crowds, and avoidance of people with obvious respiratory infections) in order to prevent the subsequent development of new infections. The patient should also be placed on a dietary regimen that will maintain adequate nutrition and hydration of bronchial secretions.

In cases where airways are blocked by secretions, these secretions should be removed. Initial attempts may be made to administer an expectorant agent to promote the expulsion of secretions via the cough reflex. If this is ineffective, postural (head-down) drainage or direct secretion removal by bronchoscope or a nasotracheal cannula should be attempted in the hospital.

More specific therapeutic measurements can include treatment with bronchodilators such as oral or aerosol β_2 agonists or oral theophylline preparations. The mechanism of action of these drugs has been thoroughly described in the section of this chapter that deals with the treatment of asthma (Section 5.1). Even if there is not an initial reversal of airway obstruction with these agents, they may be helpful over the long term and should be continued if side effects are not evident. Corticosteroid treatment may also be helpful in patients with a reversible component to their disease and should be given a 3- to 4-week trial to determine potential usefulness.

A moderate exercise program is often prescribed for the emphysemic patient unless contraindicated by an existing cardiac disorder. The purpose of this program is not necessarily to improve lung function, but more to improve the exercise tolerance of the patient and thereby his physical and emotional well-being. In some cases, specific breathing exercises to train the patient to breathe more slowly and to utilize abdominal mucles to a larger extent during exhalation are also useful.

When severe chronic hypoxemia is present, oxygen therapy is required. There is some debate, however, over how and when such therapy should be given. Controversial issues include the levels of hypoxemia at which oxygen therapy should be initiated, the levels of oxygenation needed to improve the patient's well-being, and the ultimate desirability of continuous or intermittent oxygen therapy. That is, in patients with severe exertional hypoxemia, low-flow oxygen by nasal cannula may be utilized intermittently during exercise. Similarly, if symptoms suggest that hypoxemia occurs only at night, low-flow oxygen treatment may be useful during sleep. When severe chronic hypoxemia is present (resting P_aO_2 at or below the mid-40 mm Hg range), continuous oxygen therapy should be utilized to bring P_aO_2 to near 55 mm Hg.

Edema and chronic congestive heart failure may accompany the later stages of emphysema. Mild edema should be controlled with diuretics. Treatment of heart failure is, obviously, much more difficult. The use of digitalis for this purpose should be specifically avoided, however, because digitalis toxicity with arrhythmias can result as a consequence of fluctuating blood gas values (Burrows, 1983).

Currently used therapeutic regimens treat only the symptoms associated with emphysema. There is considerable interest among medical researchers, however, in the future development of agents that will halt the progression of this disease, (e.g., elastase inhibitors). One possibility is that large-scale purification or biosynthetic procedures (via genetic engineering) might be developed to supply large quantities of α_1-PI. This α_1-PI might subsequently be given by intravenous treatment to patients with low endogenous levels of this inhibitor. Alternatively, other types of inhibitors synthesized by traditional organic synthetic procedures might also be developed. Inhibitors are currently being sought with bioavailability and low toxicity profiles appropriate for use in chronic emphysematous patients (Powers, 1983). The use of such inhibitor drugs in combination with early detection assays for elastin or desmosine fragments in human serum may permit future therapy to concentrate on the prevention of the disease in addition to relief of the ultimate symptomatology.

5.3. Chronic Bronchitis

5.3.1. *Pathogenesis and Etiology*

Chronic bronchitis is a pulmonary disease that often occurs in combination with emphysema, although the two conditions are distinct disease processes. Chronic bronchitis is characterized by excessive tracheobronchial mucus production, clinically described as mucus production sufficient to cause cough and expectoration for at least 3 months out of the year for more than 2

consecutive years (Ingram, 1983). Bronchitis often coexists with emphysema because the two diseases share common causative factors. namely, cigarette smoking and exposure to environmental pollution. These common causative factors may account for the increased prevalence of these diseases in men (20% of all men are believed to have bronchitis, whereas the incidence in women is much lower). Chronic obstructive pulmonary disease (COPD) consisting of some combination of chronic bronchitis and emphysema is the most common chronic disease of the lungs known, and a patient with this condition usually lives a number of years with increasing disability and multiple severe disease episodes. Although bronchitis and emphysema commonly exist concomitantly, it is easiest to describe the etiology, diagnosis, and therapeutic treatment of these diseases separately. Hence the preceding section of this article dealt singly with emphysema and this section will deal primarily with chronic bronchitis. The reader is referred to supplementary discussions of chronic bronchitis by Netter (1979) and Ingram (1983) for additional information on this disease.

Morphologically, bronchitis is associated with a hyperplasia and hypertrophy of the submucosal glands of the large airways and the mucous (goblet) cells in the small airways of the lung. In the large airways this pathology is manifested by a quantitative change in the Reid index, a measurement of the ratio of the thickness of the submucosal glands to that of the bronchial wall. This index is usually low in the bronchi of normal lungs (mean ratio of 0.44) but higher in the bronchi of patients with a history of the disease (mean ratio of 0.52). The hypertrophy of submucosal glands in the large airways is most likely the cause of most of the increased mucus secretion seen in patients with chronic bronchitis, since the mass of the submucosal glands is nearly 40 times greater than that of the single mucous cells. Although the exact normal volume of airway mucus secretion is unknown and may range between 10 and 100 ml per day, patients with chronic bronchitis may produce 200 to 300 ml per day (Clark and Pavia, 1980).

In addition to submucosal gland hypertrophy as a causative factor for the increased mucus production, it has also been observed that there is an increase in the rate of synthesis and secretion of mucus glycoproteins (Sturgess and Reid, 1972; Coles and Reid, 1978) in tracheal explants from the lungs of chronic bronchitics. Evidence also points to functional differences in the regulation of the rate of mucus secretion in normals and patients with chronic bronchitis (Coles *et al.*, 1981). Furthermore, it should be noted that changes in the morphological aspects of ciliated epithelium (e.g., loss of cilia, degeneration of cilia, and shortened cilia) have been observed in the large airways of chronic bronchitics (Wanner, 1977), suggesting that mucociliary transport is also impaired in this disease state.

Additional pathological changes that play a major role in the airway

obstruction observed in chronic bronchitis occur primarily in the small airways of these patients and include a narrowing of these airways by intraluminal mucus plugs, mucosal edema, smooth-muscle cell hypertrophy, and inflammatory cell proliferation.

The causes of bronchitis are largely environmental. Cigarette smoking is the most commonly identified correlate with the development of bronchitis. In fact, it has been shown in animal models that exposure to cigarette smoke can lead to hypertrophy and hyperplasia of the airway mucus-secreting glands (Jeffrey and Reid, 1981). In addition, exacerbations of bronchitis can be related to exposure to periods of heavy pollution (high sulfur dioxide levels in the air) and to certain occupations that expose individuals to inorganic/organic dusts or to noxious gases (e.g., work in plastics plants).

The role of infection in the development of bronchitis is actually quite circuitous. That is, there is some speculation that infections such as severe viral pneumonia early in life can contribute to the development of chronic bronchitis. For the most part, however, in adult years, the presence of much of the symptomatology associated with bronchitis (e.g., increased mucus production and decreased mucocilary transport) is thought to provide the basis for, rather than be the result of, an increased incidence of acute respiratory illnesses.

Although the causes of bronchitis are largely environmental, some studies with monozygotic twins have also suggested that in certain populations there is a genetic predisposition to the disease that cannot be accounted for simply on the basis of familial smoking habits (Ingram, 1983). The mechanism by which environmental, infectious, and genetic factors lead to the hypertrophy of submucosal glands and to the mucus hypersecretion characteristic of chronic bronchitis are largely unknown at the biochemical level. Such problems are currently active areas of research.

5.3.2. *Diagnosis*

A patient is usually diagnosed as having chronic bronchitis in the fifth decade of life. Symptom history is often an important factor in the diagnosis. It normally includes the presence of recurrent cough and sputum production over a number of years and a history of cigarette smoking. Initially, the cough and sputum production is most frequently noticed in the winter, but over time it may begin occurring with increased frequency and duration throughout the year. The patient usually appears for diagnosis after having experienced breathing problems after exertion. At that time, pulmonary function tests such as FEV_1 will indicate a severe degree of obstruction in the lungs. Respiratory rate and minute volume will appear normal in these patients, however, and there is no apparent breathing stress when the patient is at rest.

Examination of lung volumes often indicate that total lung capacity is normal but that there is a moderate elevation of residual volume and hence a mild decrease in vital capacity. Compliance and carbon monoxide diffusion capacity (D_LCO) measurements are usually normal.

The patient with chronic bronchitis is often overweight and cyanotic. Pulmonary hypertension, right ventricular failure, and peripheral pulmonary edema often appear in the latter stages of the disease. Arterial blood gas measurements may indicate severe ventilation–perfusion mismatching due to the failure of the patient to increase minute ventilations in the face of significant wasted ventilations due to altered blood flow through the lung. Arterial P_aCO_2 values may be chronically increased to the range of the high 40s to low 50s (mm Hg). P_aO_2 values may be decreased to the range of 45 to 60 mm Hg. This lowered P_aO_2 produces desaturation of hemoglobin (see Fig. 7), stimulates erythropoiesis, and can result in hypoxic pulmonary vasoconstriction. The resulting pulmonary vasoconstriction can further accentuate right-sided heart failure.

Radiographic examination of the chest of a patient with chronic bronchitis often reveals increased bronchovascular markings in the lower lung area and a somewhat enlarged cardiac silhouette. As right ventricular heart failure becomes more prominent, the cardiac silhouette may enlarge even further.

5.3.3. *Therapeutic Treatment*

The treatment of chronic bronchitis is quite similar to that for emphysema. Because cigarette smoking and air pollution are common causes of these two diseases, the therapeutic treatment of both diseases begins with discontinuing exposure to such exacerbating agents. This may necessitate a change of occupation or relocation to a more favorable climate.

As with emphysema, measures should also be readily initiated to clear up any concomitant bronchial infection with a 7- to 10-day course of antibiotics, such as tetracycline or ampicillin. Infections can usually be associated with the presence of pathogenic bacteria such as *Haemophilus influenza* or *Streptococcus pnemonia;* however, microscopic examination and sputum cultures may be necessary if the concomitant infection fails to respond to the usual antibiotics. After the acute stages of infection have subsided, preventive measures such as yearly vaccinations against common influenza virus strains should be instituted. The patient should also be supplied with a 7- to 10-day supply of antibiotics to keep at home and utilize at the first sign of the development of subsequent infections.

Pharmacotherapy, including the use of bronchodilatory drugs such as theophylline and β_2 agonists and corticosteroids, can be attempted to determine whether the patient will respond. It is hard to determine a priori those patients who may respond to such therapeutic measures. There is some

indication that the presence of eosinophils in a patient's sputum may help to identify a subgroup responsive to glucocorticoids. With this class of drugs, however, an objective improvement in lung function measurements should be obtained in the patients in order to support continued steroid treatment.

In patients among whom mucus hypersecretion is a problem, postural drainage in combination with liquification of mucus secretions through total-body hydration should be instituted. Finally, in patients with severe chronic bronchitis among whom arterial hypoxia is present in association with pulmonary hypertension and signs of right heart failure, oxygen therapy should be initiated. Such therapy should be carefully monitored and its beneficial effects objectively verified. If the patient suffers mainly from decreases in P_aO_2 during sleep, the oxygen supplementation may be instituted only at night. In severely hypoxic patients, however, continuous oxygen therapy is advised.

5.4. Cystic Fibrosis

Cystic fibrosis (CF) was first described clinically only 45 years ago and has subsequently been extensively characterized. Several reviews are available that supplement the information discussed in this section of the chapter (DiSant' Angese and Davis, 1976; Davis and DiSant' Angese, 1980; Wood *et al.*, 1976). The reader is referred to these for additional information on this disease.

Cystic fibrosis is generally recognized as a chronic obstructive pulmonary disease associated with abnormalities in mucus secretion resulting in obstruction of the airways, infection, and inflammation within the lung. The symptoms of CF usually become apparent in children between 3 and 5 years of age and initially include chronic cough associated with a thick, purulent mucus and wheezing and shortness of breath. Chronic respiratory infection and chronic inflammation then develop and increase in severity as the disease progresses, causing irreversible lung damage, respiratory failure, and death, usually in the second decade of life.

Cystic fibrosis is an autosomal, recessive genetic disease with an increase of 1/2000 in Caucasian births. It is the most common lethal, inherited disease among Caucasians; 50% of the victims of this disease survive to 16 years and only 2% to age 35. Heterozygotes carrying the CF gene make up about 5% of the United States population but do not express the disease.

Cystic fibrosis is recognized as a generalized exocrineopathy. While it causes progressive pulmonary disease in its victims, it also affects the functions of other organs. Thus, the intestine and exocrine organs, particularly the pancreas and sweat and salivary glands, are also affected by this disease. In general, the major pathology in CF is blockage of organ ducts by a thick,

tenacious mucus that causes obstruction of organ function and is responsible for the major clinical manifestations of the disease. In the pancreas, this obstruction leads to insufficient release of digestive enzymes, which results in maldigestion, malabsorption, and malnourishment. The disease is also associated with a relative state of dehydration throughout the body due to excessive secretion of electrolytes found in especially high concentrations in sweat.

5.4.1. *Etiology*

5.4.1.1. *General Pathogenesis in the Lung.* Little is known about the etiology of CF. As stated previously, the major processes occurring in the lungs of CF patients are obstruction of airways, chronic bacterial infection, and chronic inflammation. Yet, it remains unclear how these processes are interrelated and how they contribute to the fatal aspects of the disease. These symptoms do appear, however, to form a continuous, vicious cycle that contributes to the severe pathology associated with the disease.

The development of the lungs in CF patients appears to be normal since at birth the lungs of these people have normal macroscopic and microscopic appearance. Furthermore, no structural cellular defect has been detected in epithelia or secretory cells of the lungs of CF patients (Sturgess, 1982). As the disease progresses, however, there is a gradual accumulation of mucus in the glandular ducts and airway lumen accompanied by mucus cell hyperplasia and submucosal gland hypertrophy. A defect in mucociliary clearance also appears to occur in CF patients, possibly contributing to the accumulation of mucus. The accumulated mucus obstructs small airways and is accompanied by the infiltration of inflammatory cells into the lung, finally producing the generalized inflammatory condition associated with lungs of CF patients. Infection, caused especially by mucoid strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, ultimately occurs in the obstructed airways and plays a major role in the later stages of the pathogenesis of CF. The combined effects of infection, inflammation, and airway obstruction lead to the development of cysts, abscesses, and fibrosis in lung tissue. This ultimately leads to the irreversible destruction of the airway epithelia and a deterioration of the interstitium of the lung.

5.4.1.2. *Biochemical Mechanisms Associated with the Etiology of CF.* A number of approaches have been taken toward understanding the nature of the biochemical defects underlying CF. The most important include (1) analysis of the composition and properties of tracheobronchial mucus and fluid secretions, (2) identification of possible CF factors that might be responsible for the pathology, (3) identification of abnormalities in the regulation of pulmonary function by the autonomic nervous system, and (4) elucidation of the role of bacterial infection.

Mucus composition. Abnormalities in the concentration of various ionic components of exocrine secretions such as sodium, chloride, bicarbonate, potassium, and some charged macromolecules are consistent findings in CF patients. In fact, elevated concentrations of sodium and chloride in sweat are observed so repeatedly that they are used as a diagnostic indicator of the disease. On the other hand, the concentrations of these ions in tracheobronchial secretions are significantly lower than normal (Dearborn, 1976). Such observations have led to the hypothesis (Knowles *et al.*, 1981; Knowles *et al.*, 1983) that the ion transport processes of cell membranes are defective in CF patients. Evidence in support of this hypothesis has been obtained through measurements of the electrical potential difference (EPD) across the airway respiratory epithelium (Knowles *et al.*, 1981). In such studies, the EPD across the respiratory epithelium of CF patients was observed to be significantly greater than that found in healthy individuals used as controls. This greater EPD can be interpreted as being due to an abnormally high rate of ion transport into cells within the epithelia. Since the ion transport properties of the ductal cells of the submucosal glands and various secretory cells of the epithelium are important in the regulation of the composition of secretory products, abnormalities in the ion transport processes into these cells could profoundly influence the physiochemical properties of the mucus. For example, excessive ion transport into these cells could result in a dehydration of the mucus, since water follows passively with ions into the epithelia. This change in the characteristics of the mucus could in turn affect mucociliary transport by decreasing the clearance rate of mucus from the airways.

The association of CF with the occurrence of a thick, tenacious mucus has also led to intensive investigations of the major component of this material—the glycoproteins. A number of glycoproteins have been partially purified from tracheobronchial aspirates and sputum samples taken from patients with CF (Boat *et al.*, 1976; Boat *et al.*, 1977). In general, glycoproteins from CF patients have been observed to be more highly sulfated than those from healthy individuals. That is, biochemical analysis of the glycoproteins secreted from cultured respiratory epithelium and nasal polyps of CF patients (Boat *et al.*, 1974; Frates *et al.*, 1983), from healthy controls, or from patients having respiratory disorders other than CF such as chronic bronchitis or allergic rhinitis (Boat *et al.*, 1974; Lamblin *et al.*, 1977; and Roussel *et al.*, 1975) have revealed that CF glycoproteins are unique, more highly sulfated, and more acidic than the others. The greater acidic properties of CF glycoproteins could increase their tendency to precipitate when exposed to cations that are known to be present in mucus, such as calcium. This property could contribute to the altered properties of the mucus in CF patients. In addition, glycoproteins have been observed to be synthesized at a faster rate by cultured CF respiratory tissue than by tissue from normal controls (Frates

et al., 1983). These observations thus support the hypothesis that the obstruction of airways observed in patients with CF could result, in part, from an increased synthesis and secretion of mucus containing highly acidic glycoproteins.

The existence of CF factors. In genetic diseases, there is often a generalized chromosomal defect that is associated with the synthesis of some unique factor that causes the pathology of the disease. The search for such CF factors has been extensive (Bowman, 1979; Carson and Bowman, 1982; Dearborn, 1981). Evidence for a CF mucociliary inhibitor factor was initially presented by Spock *et al.* (1967), who first observed that sera from CF patients and their parents caused irregularities in ciliary movement of rabbit tracheal explants. Irregularities of beating of cilia in the airways could be the cause of the impaired mucus clearance and other lung pathology associated with CF. Several laboratories have subsequently reported partial purification of a mucociliary inhibitor from sera, urine, and saliva of homozygote carriers of the CF gene (Bowman, 1979; Dearborn, 1981; Carson and Bowman, 1982). In addition, the existence of other CF factors that cause mucus hypersecretion has been reported. These factors have been observed in studies *in vitro* with various mucus-secreting systems such as the rabbit tracheal epithelium (Boat *et al.*, 1982) and oyster gills (Czegledy-Nagy and Sturgess, 1978; Kurlandsky *et al.*, 1980). In general, however, the results from studies with CF mucociliar transport or secretion factors have been variable and the isolation of specific molecules has not yet been achieved.

Autonomic dysfunction. Abnormalities in autonomic function are also associated with CF (Davis and Kaliner, 1983). In exocrine tissue, for example, this is expressed as hypersensitivity to cholinergic and α-adrenergic stimulation and hyporesponsiveness to β stimulation. In the lung, the autonomic nervous system is closely associated with the regulation of airway smooth-muscle tone and mucus secretion. Thus a defect in autonomic function in the lung could lead to an increase in bronchial smooth-muscle tone and mucus secretion in response to cholinergic or α-adrenergic stimulation. While autonomic dysfunction in the lung has not, as of yet, been demonstrated experimentally in CF patients, research in this area currently represents a major approach in the study of the pathogenesis of this disease.

Infection of cystic fibrosis. About 90% of those with CF die from complications associated with chronic bacterial infection in the lung. There is little doubt that infection is secondary to the primary defect in CF since infection is absent in the lung in the early stages of this disease. Furthermore, other affected organs of the disease, such as the pancreas, salivary glands, and sweat glands, do not become infected. In addition, the immune system in CF patients appears to be normal and produces high titers of antibodies to infecting organisms (Talamo *et al.*, 1976).

It is believed that the lung infection results from impaired mucus clearance followed by colonization of bacteria in the mucus. The bacteria elaborate a number of toxins, polysaccharides, and enzymes including proteases, elastases, and exotoxin A, which may stimulate the production of additional mucus and further contribute to airway obstruction (Sam *et al.*, 1980; Adler *et al.*, 1983). *Pseudomonas aeruginosa* and *Staphylococcus aureus* are the most commonly found bacteria in the lungs of patients with CF, but *Klebsiella*, *Esherichia coli*, streptococci, and *Haemophilus influenza* can also be found. Of particular interest is the observation that mucoid strains of infectious bacteria, which are more pathogenic than nonmucoid strains, are most commonly found in patients with CF (Reynolds *et al.*, 1975, 1976). The mucoid strains are also more resistant to phagocytosis by alveolar macrophages and are impermeable to antibiotics because of their mucoid coats. Thus treatment of pulmonary infections in patients with CF can be unusually difficult.

5.4.2. *Diagnosis*

A young child who appears to have chronic respiratory impairment and is from a family with a genetic history of CF is, of course, suspect of having the disease. A number of diagnostic tests can then be applied. The single most accurate diagnostic procedure for CF is the sweat test, a test that measures the concentration of either sodium or chloride in sweat. The most reliable procedure was developed by Gibson and Cooke (1959) and uses pilocarpine applied to the skin to stimulate local production of sweat. The resulting sweat is then collected. Sodium or chloride is determined in 100 mg quantities of sweat. A value greater than 60 mEq/liter of electrolyte is considered a positive test. The results of the sweat test are considered in conjunction with clinical observation of the lung and pancreatic disease in the positive diagnosis of CF (Howell, 1976). Sweat testing is usually done on individuals between the ages of 3 and 5 years when pulmonary and pancreatic symptoms have become apparent. Currently lacking, however, are adequate screening methods for the identification of heterozygote carriers of the disease and other forms of prenatal screens for the disease. Current progress in this area has been discussed by Dearborn (1981).

5.4.3. *Therapy*

In general, the therapeutic regimen in CF patients is highly individualized and must take into account the diverse array of tissue systems affected by the disease. The major objectives of therapy for the pulmonary aspects of CF are to institute techniques to promote the clearance of mucus from the airways and to minimize the extent of bacterial infection in the lungs by administration of appropriate antibiotics (Marks, 1981; Wood *et al.*, 1976).

Airway clearance techniques may be initiated by postural drainage in combination with the administration of humidified air to hydrate and loosen the mucus. These procedures can be supplemented with pharmacotherapy, including the administration of bronchodilators such as aerosolized β_2 agonists or oral or intravenous theophylline. It is hoped that these drugs will dilate the airways and make medications such as mucolytics more accessible to the lungs. Mucolytics are believed to reduce the viscosity of mucus and dissolve mucus plugs by reducing disulfide bonds (—S—S—) between the glycoprotein components of the mucus. In severe cases, pharmacotherapy may be inadequate and endoscopy, lavage, or surgical intervention may be needed to physically remove the mucus.

Oral corticosteroids have also been used to treat the inflammatory process occurring in the lungs of CF patients. Antibiotics that are administered to treat bacterial infections include aerosolized gentamicin and tobramycine. These antibiotics are most effective against *P. aeruginosa* and *S. aureus* infections that occur in the CF patients.

6. Restrictive Diseases of the Lung

Restrictive diseases of the lung are associated with a restriction in the expansive properties of the lung either because of changes in the properties of the lung parenchyma or because of diseases of the pleura, chest wall, or the neuromuscular apparatus that regulates respiration. These diseases are characterized by a reduced vital capacity and reduced lung volumes (see Fig. 9). Airway resistance per se is not changed, however, in contrast to the obstructive lung diseases described in previous sections of this chapter. Fibrotic lung disease is the prominent classification for restrictive disease of parenchymal origin and is discussed in detail later. Because of space limitations, this chapter does not include discussions of restrictive diseases that affect the lung pleura (e.g., pneumothorax of various origins), the chest wall (scoliosis and spondylitis), or diseases of the neuromuscular systems associated with respiration (poliomylitis, myasthenia gravis, and muscular dystrophy). For information on these restrictive diseases the reader is referred to Netter (1979).

6.1. Fibrotic Lung Disease

Pulmonary fibrosis is the medical term given to an end-stage restrictive lung disorder characterized by excessive deposition of connective tissue (predominantly collagen) in the lung interstitium. This deposition is caused by some type of acute or subacute injury to the lung that triggers inflam-

matory responses, which then ultimately result in abnormal accumulation of intraalveolar and interstitial collagen fibers. Associated with this pathology is a progressive and eventually irreversible loss of lung volume and respiratory function. Interstitial pulmonary fibrosis affects slightly more men than women and most patients are between the ages of 40 and 70 years, although the disease is sometimes found in children and young adults. Unfortunately, in spite of the most aggressive medical treatment, pulmonary fibrosis is usually fatal and the average life span of a patient with this disease is only 47 months from the onset of symptoms (Crystal *et al.*, 1977).

6.1.1. *Etiology*

The term fibrotic lung disease actually encompasses more than 130 lung disorders. Table 1 presents a classification of the causes of the major interstitial fibrotic lung diseases. In only about one-third of the cases of pulmonary fibrosis is the cause known. The most prevalent inciting agents are occupational and environmental inhalants (notably inhaled organic dusts such as silica and the silicates), inhaled gases (ozone), oxygen in high concentrations, and oxides of sulfur and nitrogen. The remaining known causes of interstitial pulmonary fibrosis can be traced to cytotoxic drugs, poisons, ionizing radiation, infectious agents, and cardiac or metabolic diseases (Fulmer, 1982). But in the majority of cases of pulmonary fibrosis, no cause is discernible. Pulmonary fibrosis without known cause is termed idiopathic, cryptogenic, or more frequently primary pulmonary fibrosis.

Regardless of the initial known (or unknown) cause of pulmonary fibrosis, there is overwhelming evidence that the first manifestation of the disease is alveolitis or lung inflammation. Alveolitis is an accumulation of inflammatory and immune effector cells within the alveolar interstitium and on the epithelial surface of the alveoli (Keogh and Crystal, 1980). The neutrophil is the characteristic cellular component of fibrosing alveolitis and the numbers of macrophages and T or B lymphocytes remain unchanged in the interstitium and alveoli during this inflammatory reaction (Hunninghake *et al.*, 1981). The disease is reversible in the alveolitis stage; once fibrosis occurs, however, the parenchyma is damaged permanently. The intensity of the alveolitis can vary among patients and even within a single individual at different times during the course of the disease (Keogh and Crystal, 1980). Alveolitis gradually develops into alveolar septal fibrosis as the normally uniform alveolar architecture loses its anatomical regularity. The excessive deposition of collagen in the parenchymal interstitium surrounding the alveoli gradually becomes the prominent characteristic histological feature of all patients with pulmonary fibrosis.

Even in healthy lungs, collagen is the most abundant component of the lung interstitium (elastin and proteoglycan being the other primary compo-

TABLE 1
CLASSIFICATION OF THE CAUSES OF THE MAJOR INTERSTITIAL FIBROTIC LUNG DISEASES

Known causes
Occupational and environmental inhalants (inorganic dusts, organic dusts, gases)
Cytotoxic drugs
Drug reactions
Poisons
Radiation
Infectious disorders
Associated cardiac disorders
Associated metabolic disorders
Congenital disorders
Unknown causes
Diseases with characteristic morphology (e.g., sarcoid, eosinophilic granuloma)
Diseases without characteristic morphology
Idiopathic pulmonary fibrosis
Chronic interstitial diseases associated with the collagen vascular disorders

nents). In the normal lung there are two forms of collagen present, type I and type III. These forms of collagen have different amino acid sequences and are present in a ratio of 2:1, respectively (Hance *et al.*, 1976). The interstitial content of collagen is maintained by the continued biosynthesis, deposition, and degradation of this structural protein. The mechanism by which this ongoing process is altered to cause an excessive synthesis of abnormal collagen in pulmonary fibrotic disease is not known. What is observed is that as newly synthesized collagen accumulates in this disease state, it disorganizes the alveolar septa because the collagen fibers are not laid down in the regular arrangements of parallel crossbanded fibers found in healthy lungs. Instead, during fibrosis the fiber bundles become randomly oriented, thickened, twisted, and frayed. There is a shift from type III collagen toward fibrillar, less-yielding type I collagen. This change is consistent with the loss of compliance (lung extensibility) and the restrictive defect found on pulmonary function testing. The alveolar structures in fibrosis patients are also characterized by increases in the number of fibroblasts and increases in the amounts of fibronectin, a glycoprotein that mediates the attachment of fibroblasts to the extracellular matrix (Bitterman *et al.*, 1983). Once fibrosis is widespread throughout the lungs, the parenchymal tissue is irreversibly damaged and the alveolar capillary bed is markedly deranged, thus compromising gas exchange.

A number of theories have been advanced to explain the etiology of pulmonary fibrosis. Several of these are described below.

6.1.1.1. *Alveolar Macrophage–Neutrophil Interactions.* The most popular concept of the pathogenesis of pulmonary fibrosis hypothesizes that alveolar macrophages activated by a number of inciting agents (immune complexes, microorganisms, inorganic particles, or smoke) are stimulated to release neutrophil chemotactic factors. These factors induce an influx of neutrophils into the lung interstitial tissue. The chronic presence of neutrophils in the alveolar structures is then thought to mediate parenchymal injury through the release from the neutrophils of various enzymes, including collagenase, the primary enzyme responsible for collagen degradation (Hunninghake *et al.*, 1981). This theory is supported by the observations that although neutrophils are not normally present within the lung, they exist in large numbers in patients with fibrosis. Furthermore, the presence of active collagenase has been demonstrated in the lower respiratory tract of patients with fibrosis. Since collagenase activity may persist for periods of months to years (Gadek *et al.*, 1979a), collagen is under sustained enzymatic attack. Under these conditions the turnover of collagen could be accelerated and could result in an excessive production of alveolar interstitial collagen, altered in form, type, location, and amount.

6.1.1.2. *Chronic Mast Cell Degranulation.* It has been suggested that pulmonary parenchymal mast cells undergo a slow, chronic process of partial degranulation in patients with primary pulmonary fibrosis. Mast cells contain stored histamine, heparin, neutrophil chemotactic factor, and eosinophil chemotactic factor and can biosynthesize other mediators such as leukotrienes and platelet-activating factor. Chronic release of these products could thus sustain a prolonged inflammatory condition in the lung. Observations that the lungs of patients with fibrotic lung diseases have increased numbers of mast cells in alveolar structures would tend to support this hypothesis (Kawanami *et al.*, 1979).

6.1.1.3. *Autoimmune Basis of Fibrotic Disease.* The occurrence of increased numbers of inflammatory cells and increased amounts of immunoproteins (particularly immunoglobulins) and immune complexes in the bronchoalveolar lavage fluid of patients with primary pulmonary fibrosis has suggested that this form of pulmonary fibrosis may be an autoimmune disease (Lawrence *et al.*, 1980). Immune complexes have been shown to stimulate alveolar macrophages to secrete neutrophil chemotactic factors that elicit the migration of neutrophils into the lung. The chronic presence of neutrophils in the interstitial tissue could maintain the alveolar injury (Hunninghake *et al.*, 1981; Libby *et al.*, 1983). In addition, the circulating T lymphocytes of patients with pulmonary fibrosis appear to be sensitized to

type I collagen, implying that type I collagen is antigenic and recognized as non-self in these patients (Libby *et al.*, 1983).

6.1.1.4. *Genetic Basis for Pulmonary Fibrosis.* It has been suggested that some people may be genetically predisposed to pulmonary fibrosis because a familial clustering of the disease (73 definite cases in 19 families) has been correlated with autosomal dominant inheritance (Libby *et al.*, 1983). In addition, increased cell surface antigens (HLA genes) have been shown to be associated with primary pulmonary fibrosis (Libby *et al.*, 1983), and lowered levels of α_1-protease inhibitor, a broad-spectrum protease inhibitor, have been found in patients with fibrosing alveolitis (Geddes *et al.*, 1977). This latter observation is similar to the observed decrease in α_1-protease inhibitor found in genetic forms of emphysema.

6.1.1.5. *Elevated Fibronectin in Lung Interstitium.* Fibronectin is a large glycoprotein found in the lung interstitial connective tissue. It is secreted by fibroblasts, has binding sites for collagen, and is believed to function in the remodeling of interstitial structure. Since significantly elevated levels of this macromolecule have been found in the bronchoalveolar lavage of patients with pulmonary fibrosis (Rennard and Crystal, 1982), it has been hypothesized that elevation of fibronectin levels may be involved in the abnormal deposition of collagen in patients with fibrotic disease.

6.1.1.6. *Epithelial Cell Injury.* Fibroblast proliferation, and thus collagen production, is controlled by an intact epithelial cell layer. Thus it has been suggested that acute injury to epithelial cells could allow fibroblasts to grow undisturbed, producing excessive collagen that would lead to fibrosis (Haschek and Witschi, 1979).

6.1.2. *Diagnostic Measurements of Fibrotic Lung Disease*

The first symptoms of alveolitis in patients who ultimately develop pulmonary fibrosis normally occur at approximately 49 years of age. The time interval from the onset of these symptoms to the ultimate diagnosis of this disease averages 2.9 years (Thomas, 1978). The initial complaint patients usually express is a difficulty with breathing (dyspnea), sometimes apparently following a flulike illness or exercise. Indeed, progressive dyspnea is the cardinal symptom of fibrosing alveolitis (the stage of pulmonary fibrosis that is considered reversible). Dyspnea is first noted only on exertion but eventually occurs even at rest. A dry, hacking, nonproductive cough is common and may be troublesome. Finger clubbing occurs in 70 to 90% of the patients, and cyanosis is apparent in the advanced disease stages. Other vague symptoms include influenzalike symptoms, fatigue, and weight loss.

In some cases symptoms of arthritis may be present. On physical exam, loud inspiratory rales are heard over the lower third of the lungs.

It is imperative that the diagnosis of primary (idiopathic) pulmonary fibrosis not be made until all known and potentially treatable causes of the fibrotic disease have been excluded. This can be accomplished by a systematic, thorough analysis of the patient's occupational history, family history, history of recurrent pneumonias, and history of drug use, including the use of immunosuppressive agents, antimicrobials, and miscellaneous drugs such as hydrochlorthiazide, gold salts, chlorpropamide, sulfapyridine, hydralazine, and amiodarone.

Ideally, diagnosis and therapy of patients with pulmonary fibrosis would be improved if a simple noninvasive method were available to permit repeated assessment of the various stages of alveolitis leading to fibrosis. Unfortunately, however, the methods that are the mainstay of diagnosis of pulmonary fibrosis either cannot be used repeatedly or do not directly correlate with the intensity of the disease. The methods currently used in diagnosis of pulmonary fibrosis include chest roentgenograms, pulmonary function testing, lung biopsy, and a number of other more experimental techniques involving the evaluation of blood components and lung lavage composition. These diagnostic techniques are detailed next.

6.1.2.1. *Chest X Ray.* The evaluation of serial chest roentgenograms taken over the course of the development of a fibrotic lung disease demonstrates an evolving pattern of morphological changes. These begin with a ground-glass appearance, followed by a diffuse reticulonodular pattern predominantly in the lower lobes, which gradually changes to a coarser pattern with cystic areas and finally a honeycomb appearance. The honeycomb lung is characterized by well-formed cystic spaces less than 1 cm in diameter and generally reflects the end stage of pulmonary fibrosis. As the end-stage lung is approached, there may be elevation of the diaphragm, an unfolding of the aorta, kinking of the trachea, and signs of heart failure.

6.1.2.2. *Pulmonary Function Testing.* In fibrosis, pulmonary function abnormalities show the usual pattern of restrictive lung diseases due to the decreased distensibility of the alveoli. Lung volumes (forced vital capacity and total lung capacity; see Figs. 9 and 11) are diminished, as is the FEV_1. The most sensitive detectors of alveolitis in pulmonary fibrosis appear to be the reduced gas exchange properties of the lung, as measured by the lungs carbon monoxide diffusing capacity (D_LCO). Characteristic of pulmonary fibrosis is the flow–volume curve of restrictive disease described in Fig. 12. Typically, the patient with pulmonary fibrosis has a flow–volume curve shifted to the right from normal, illustrating that the patient with fibrosis

needs to generate large negative transpulmonary pressures in order to take in a small volume of air.

6.1.2.3. *Lung Biopsy.* Open lung biopsy is the best method available to determine the stage of the disease; however, problems such as discomfort, risk, and cost preclude its use more than once. Histological evaluation of a lung biopsy from a patient with pulmonary fibrosis reveals increased amounts of fibrous tissue in the alveolar septae, derangement of lung architecture with extensive fibrosis surrounding cystic structures, and focal areas of inflammatory cell infiltration adjoining the fibrotic tissue.

6.1.2.4. *Experimental Diagnostic Procedures.* Numerous blood studies have been proposed as monitors of the alveolitis stage of pulmonary fibrosis, including circulating immune complexes, γ-globulins, antinuclear antibodies, rheumatoid factor, complement-fixing antibodies, and angiotensin converting enzyme. In general, however, the systemic serum levels of these components do not reflect the state of chronic lung inflammation (Crystal *et al.*, 1977).

Recently, two additional methods have become available to aid in the diagnosis of primary pulmonary fibrosis. One method, fiberoptic bronchoscopy, has been used to obtain washings of bronchoalveolar cells. Theoretically, cells and proteins derived from the alveolar epithelial surface are similar in type, proportion, and function to components within the alveolar interstitium. Indeed, the cells obtained by lung lavage accurately reflect changes in pulmonary fibrosis. In one study (Ozaki *et al.*, 1982), the proportion of neutrophils in the washings averaged 33% in patients with fibrosing alveolitis, compared to 3% in controls. This technique has two advantages in that it can be used multiple times during the course of the patient's disease and the results can be quantitated.

Another new diagnostic technique involves nuclear scanning with gallium-67 (^{67}Ga) citrate, a radionuclide with a half-life of 78 hours. Gallium does not localize in normal lung tissue, but it accumulates in sites of acute and chronic inflammation; hence it can be used to quantitate lung inflammation and the intensity of alveolitis (Crystal *et al.*, 1981).

6.1.3. *Therapy*

Regardless of the pathogenic mechanism associated with pulmonary fibrosis, therapy should be directed toward attenuating the inflammatory and immune process (alveolitis) associated with the disease because therapy is much less effective once pulmonary fibrosis has developed. If possible, the agent inciting the alveolitis should be identified and removed, and steroid therapy initiated.

Steroids have been shown to reduce collagen synthesis and deposition in lungs of experimental animals. Corticosteroids are thought to inhibit total protein synthesis and preferentially collagen synthesis. Suggested mechanisms involve decreased neutrophil adherence, impaired phagocytosis and lysosomal fusion, suppressed macrophage phagocytic and chemoattractant activity, and reduced collagen synthesis by fibroblasts (Koenig *et al.*, 1983). Unfortunately, steroids have a wide range of adverse effects, and it has been reported that probably no more than 5 to 10% of patients with pulmonary fibrosis respond to corticosteroids.

Patients unresponsive to steroids are given immunosuppressive agents (azathioprine and methotrexate). These drugs have had limited success, and unfortunately, they also have a high incidence of side effects. Nonspecific therapies include oxygen supplementation, bronchodilator therapy (e.g., theophylline) if there is concomitant chronic obstructive lung disease, and prompt antibiotic therapy if an infection is associated with the disease.

The development of rational pharmacotherapy has been attempted by regulating a number of the biochemical mechanisms in collagen metabolism. Agents have been studied that selectively interfere with key steps in collagen biosynthesis. Other compounds (colchicine and vincristine) act to inhibit the secretion of collagen by disrupting the assembly of microtubules in secretory vesicles. Another approach has been the synthesis of inhibitors of collagen cross-linking (β-aminopropionitrile and penicillamine). Finally analogs of proline (*cis*-4-hydroxy-L-proline, azetidine-Z-carboxylic acid; 3,4-dehydro-L-proline, *cis*-4-bromo-L-proline, and *cis*-4-fluoro-L-proline) have been tested with the thought that they would be incorporated into proteins and yet could not serve as substrates for prolyl hydroxylase and thus might decrease extracellular deposition of collagen (Salvador *et al.*, 1985). Preliminary studies in animal models of pulmonary fibrosis show that proline analogs reduce the amount of collagen in tissues without causing toxic side effects (Riley *et al.*, 1981). Further research is warranted in order to apply these compounds to human pulmonary fibrosis.

7. Vascular Diseases of the Lung

7.1. Pulmonary Edema

7.1.1. *Etiology*

Pulmonary edema can be defined as a pathological state of the lung in which there is an abnormal accumulation of fluid in the pulmonary interstitial tissues or in the alveoli. The causes of this condition can be quite diverse (Table 2). In the majority of cases, however, the edema can be

TABLE 2
PATHOGENIC CAUSES OF PULMONARY EDEMA

Permeability edema (noncardiogenic)
Infectious agents
Environmental intoxicants
Vasoactive substances
Drugs
Adult respiratory distress syndrome
Pulmonary hypertension-associated edema (cardiogenic)
Congestive heart failure
Mitral stenosis
Hypoalbuminemia
Veno-occlusive disease
Lymphatic dysfunction
Unknown mechanisms
High-altitude pulmonary edema
Neurogenic pulmonary edema
Narcotic-induced pulmonary edema

ascribed to factors associated with an altered permeability of the pulmonary capillary endothelial cells or alveolar epithelial cells in the presence of normal pulmonary vascular pressures (noncardiogenic causes) or to factors associated with pulmonary microvascular hypertension (cardiogenic causes). There are also a number of other forms of pulmonary edema that are not classified under cardiogenic or noncardiogenic. These include pulmonary edema produced as a result of lymphatic insufficiency, high-altitude effects, neurogenic causes, and narcotic overdose.

In order to better understand the pathophysiology associated with these various forms of pulmonary edema, a review of the morphology associated with the capillary–alveolar–intestinal interface is useful (Fig. 6). From this review it can be seen that fluid in pulmonary capillaries is separated from the alveolar interstitial tissue by the capillary endothelial cells and the capillary basement membrane (commonly called the endothelial barrier). The alveolar surface is separated from the interstitial space by the alveolar–airway barrier, which consists of the alveolar basement membrane, alveolar epithelium, and a layer of pulmonary surfactant within the alveolus. As described previously, the alveolar interstitial tissue is made up of connective tissue (elastin and collagen), fibronectin, and mucopolysaccharides. The interstitial space also contains the pulmonary lympatic system, which functions to drain proteins, large particulate matter, and excess fluid away from the tissue space and to return them to the blood.

The development of pulmonary edema is normally divided into three stages related to fluid leakage from the pulmonary capillaries to the in-

dynamic, ventilatory, and pulmonary abnormalities are more readily apparent. Thus chest radiographic analysis demonstrates that fluid has accumulated in the acini and that gas exchange is extremely abnormal, with severe decreases in P_aO_2 and increases in P_aCO_2. Alveolar hyperventilation is present and the resultant respiratory alkalosis impairs delivery of oxygen to the tissues. Pulmonary function tests (Fig. 9) show a much decreased functional residual capacity, decreased tidal volume, increased respiratory rate, and increased closing volume (Fig. 14). As fluid accumulates in the lung, interstitial pressure becomes more positive, increasing flow resistance in the pulmonary microvasculature and resulting in a rise in pulmonary artery pressure, which ultimately leads to pulmonary hypertension.

In addition to the general symptoms described, the patient with cardiogenic edema also may become extremely anxious, perspire heavily, and expectorate sputum that is frothy and blood tinged. The skin is usually observed to be cold, ashen, and cyanotic as a result of a low cardiac output and increased sympathetic drive (Ingram and Braunwald, 1980).

Protein analysis of the edema fluid has been shown to be of value in distinguishing cardiogenic from noncardiogenic edema. In general, protein concentrations in edema fluid are usually less than half that of blood in cardiogenic pulmonary edema, whereas the ratio is usually greater than 0.7 in noncardiogenic pulmonary edema (Sprung *et al.*, 1981). Identification of the type and potential cause of pulmonary edema is important because this may facilitate the choice of the therapeutic approach to be taken in the treatment of patients with this disease.

7.1.3. *Therapeutic Treatment*

7.1.3.1. *Noncardiogenic (Permeability) Edema.* Since there are no specific treatments that reverse endothelial cell injury in permeability edema, therapy is directed at supporting optimal tissue oxygenation without creating further damage to lungs and systemic organs (Stevens, 1982).

Early mechanical ventilation and positive end-expiratory pressure (PEEP) are the mainstays of therapy. A reasonable objective is to achieve an arterial oxygen pressure of 60 to 65 mm Hg, since higher levels add little to oxygenation and introduce the chance of oxygen toxicity to the lungs (Ingram, 1982). PEEP is used early to prevent alveolar collapse, increase the functional residual capacity of the lung, and reinflate previously closed alveoli. It does not "push" water back into the pulmonary capillary (Sibbald *et al.*, 1979); it only maintains alveolar integrity until the primary process is identified and corrected.

Therapeutic measures designed to limit lung fluid accumulation such as the intravenous administration of albumin or other high-molecular-weight molecules to raise colloid osmotic pressure are only beneficial in patients

and smoke) have been reported to cause pulmonary edema primarily by altering alveolar or capillary permeability (Cordasco, 1974). The pathogenic mechanisms associated with this cytotoxicity have been hypothesized to be due to the chemical-induced generation of free radicals by alveolar macrophages or the chemical-induced release of lysosomal enzymes or vasoactive substances from activated macrophages in the lung. Once released, such substances can readily injure the alveolar epithelial cell layer. In fact, it appears that certain types of chemicals can cause massive pulmonary edema almost immediately after exposure and can produce fatality within a few hours.

Vasoactive substances. As described in previous sections of this chapter, the lung has a great capacity to metabolize, synthesize, and liberate a number of biologically active substances such as histamine, serotonin, bradykinin, prostaglandins, leukotrienes, platelet-activating factor (PAF), and complement. These substances are synthesized and released by a variety of cell types found within the lung tissue, including platelets, leukocytes, mast cells, and alveolar macrophages. Most of these vasoactive substances have been shown to increase vascular permeability directly by perturbing the alveolar capillary membrane and can lead to protein-rich edema formation (Henson *et al.*, 1982; Vaage, 1982). Other mediators may act indirectly by stimulating the production of thromboxane A_2 within the lung (Heffner *et al.*, 1983), stimulating the production of oxygen radicals, or stimulating the release of proteolytic enzymes from alveolar macrophages. The mechanism by which O_2 radicals cause lung injury and capillary permeability changes is speculative; however, they are hypothesized to affect these processes by interacting with key cellular components in the alveolar epithelium or capillary endothelial cells such as proteins, membrane lipids, and/or nucleic acids (Tate *et al.*, 1982).

Drug-induced edema. Pulmonary edema in humans can be produced as a result of idiosyncratic reactions to a number of drugs, including hydrochlorothiazide, ethclorvynol, salicylates, nifedipine, nitrofurantoin, and sulfonamides. In fact, noncardiogenic pulmonary edema is a frequent complication associated with the ingestion of salicylates. Although the biochemical mechanisms associated with salicylate-induced increases in vascular permeability are unknown, three hypotheses have been suggested: (1) salicylates might inhibit prostaglandin synthesis, thereby eliminating the modulatory effect of prostacyclin on the vascular endothelial cells and shunting arachidonic acid from the cyclooxygenase pathway into the lipoxygenase pathway, thus promoting the formation of leukotrienes with capillary permeability-altering capabilities, (2) salicylates might directly impair platelet function, which is important for maintaining vascular integrity, or (3) salicylates might have a direct toxic effect on pulmonary microvasculature

(Heffner and Sahn, 1981). In the case of salicylate-induced edema, drug levels can be lowered with forced alkaline diuresis and then pulmonary edema clears quickly.

Adult respiratory syndrome. Adult respiratory distress syndrome (ARDS) is the descriptive term given to an extreme form of noncardiogenic pulmonary edema in which both the alveolar and capillary membranes are damaged and "leaky," leading to interstitial and alveolar edema and alveolar collapse. When the ARDS pulmonary edema is associated with shock or trauma, it is termed "shock lung." A number of serious disorders can cause ARDS, but the final common pathway is alveolar–capillary injury that alters permeability. One of the most popular current hypotheses concerning the pathogenesis of ARDS is that complement activation of polymorphonuclear leukocytes (PMN) in the lung leads to the pulmonary endothelial injury that causes the edema (Sprung *et al.*, 1983). Support for this theory is provided by the following clinical and experimental observations: (1) many clinical disorders associated with ARDS (i.e., bacteremia, traumatic shock, pancreatitis) can activate the complement system; (2) an early pathological hallmark of ARDS is leukocyte aggregation and plugging of the pulmonary microvasculature; (3) intravenous infusion of activated complement in animals produces luekocyte activation and aggregation similar to that seen with ARDS; (4) animals depleted of either PMNs or activated C5a are relatively resistant to the development of shock lung; and (5) there is a significant correlation between the presence of PMN-aggregating activity in the plasma and the development of ARDS. Indeed, determination of plasma C5a seems to be a useful predictor of ARDS (Hammerschmidt *et al.*, 1980).

The symptomology associated with ARDS can develop quickly (within 3 to 4 hours) and the prognosis for patients with ARDS is usually poor. Death can occur within 6 to 8 hours, and the mortality rate is greater than 50%. This mortality rate, however, represents an improvement over the 100% mortality rate experienced just a few years ago (Sprung *et al.*, 1983).

7.1.1.2. *Causes of Cardiogenic (Pressure) Edema.* Cardiogenic pulmonary edema is thought to be mediated by pulmonary microvascular hypertension, which results in the damming up of blood in the lungs. The most common cause of cardiogenic pulmonary edema is left ventricular heart failure (congestive heart failure), although mitral stenosis, pulmonary veno-occlusive disease, or hypoalbuminemia can also be the underlying cause. Fortunately, before serious pulmonary edema will develop, the pulmonary capillary pressure must be elevated to an extremely high degree. In human beings, the pulmonary capillary pressure is normally 7 mm Hg and this pressure must rise above 30 mm Hg (greater than the colloid osmotic pressure, which is about 28 mm Hg) before pulmonary edema ensues (Guyton,

1971a). Because cardiogenic edema is the result of cardiovascular disease, it can take years to develop, in contrast to many forms of noncardiogenic edema, which develop in only a few hours.

Surprisingly, pulmonary edema does not occur in patients with severe pulmonary hypertension (primary pulmonary hypertension), even though their pulmonary artery pressure may be chronically elevated as high as 45 mm Hg above the normal value of 13 mm Hg. The reason for this is that the arterial bed is severely narrowed in pulmonary hypertension; thus the alveolar capillaries are not exposed to the increased pressures and there is no engorgement of blood in the pulmonary vasculature (Ingram and Braunwald, 1980).

7.1.1.3. *Edema Caused by Lymphatic Dysfunction.* Pulmonary lymphatic insufficiency resulting from lymphatic carcinoma, silicosis, anesthesia, or sedative drug overdose can cause a decreased clearance of fluid from the lung via the lymphatics. Because of the absence of this important mechanism for fluid removal from the lung, lymphatic insufficiency can rapidly lead to an accumulation of fluid in the lung interstitium and ultimately to pulmonary edema.

7.1.1.4. *Pulmonary Edema of Unknown Origin.* As indicated in Table 2, there are also several clinically distinct forms of pulmonary edema in which the etiology is unknown. These are discussed in some detail below.

High-altitude pulmonary edema. Pulmonary edema develops in certain people who rapidly ascend to altitudes greater than 9000 ft. Symptoms (cough, dyspnea, weakness, chest tightness, tachycardia, bilateral rales, and cyanosis) begin 12–60 hours after ascent to high altitudes and reversal is rapid by returning to a lower altitude (Shanies, 1977). Presently, no single mechanism satisfactorily explains the pathogenesis of this syndrome, but proposed causes include (1) direct injury to the alveolar capillary membrane due to alveolar hypoxia at the high altitudes, (2) transient intravascular coagulation secondary to hypoxic sequestration of platelets and plugging of capillaries in the pulmonary circulation, (3) nonuniform, arteriolar hypoxia-induced vasoconstriction in some areas of the lung resulting in excess blood flow into other areas of the lung, with local pulmonary hypertension and interstitial and alveolar edema, and (4) high-altitude stresses that induce the release from leukocytes or platelets of chemical mediators that increase alveolar capillary membrane permeability (Kleiner and Nelson, 1975). The prognosis for recovery from this type of edema is excellent if the patient is given rest and oxygen and taken to a lower altitude.

Neurogenic pulmonary edema. Neurogenic pulmonary edema develops rapidly (within minutes) after a severe injury to the brain or spinal cord such

as head trauma, stroke, grand mal seizure, hemorrhage, or increased cerebrospinal fluid pressure. Theodore and Robin (1975) were the first to ascribe the pathogenesis of neurogenic pulmonary edema to a rapid outpouring of sympathetic neural impulses from the injured brain. This massive, centrally mediated sympathetic discharge causes profound vasoconstriction and results in a rapid shift of blood from the systemic circulation to the more compliant pulmonary circulation, with the resultant outpouring of fluid into the lung. The systemic and pulmonary hypertension are transient, but the damaged endothelial permeability persists in survivors for a long time. While rapid treatment with antagonists of sympathetic mediators can prevent the development of neurogenic pulmonary edema, these drugs are not beneficial in the reversal of this disorder because pulmonary capillary pressures are usually normalized by the time the edema is recognized.

Narcotic-induced pulmonary edema. Pulmonary edema is a common sequel to narcotic overdose (morphine, heroin, methadone, and propoxyphene) and is associated with a significant mortality rate. The exact mechanism has not been delineated, but two reasonable hypotheses have been suggested. First, narcotic-induced pulmonary edema may be a form of neurogenic pulmonary edema because both syndromes have complications of cerebral edema and hypothalamic dysfunction (Jaffe, 1970). Second, narcotics are known to release histamine, which may alter alveolar capillary membrane permeability (Brashear *et al.*, 1974). Early treatment with narcotic antagonists produces immediate reversal of respiratory depression and miosis, while the pulmonary edema resolves more slowly.

7.1.2. *Diagnostic Procedures*

Because the symptoms of pulmonary edema usually occur after the disease has reached stage two, this disease may be well advanced before patients experience the respiratory distress, decreased lung volumes, and decreased dynamic compliance characteristic of pulmonary edema. The earliest measurable lung dysfunctions are noted in small airway pulmonary function tests (increased closing volume and decreased compliance) and in blood gas analysis (decreased P_aO_2). As the disease progresses, the lungs become stiffer and patients experience increased dyspnea and tachypnea. Premature closure of the small airways, especially at the bases of the lungs, can cause the characteristic fine rale sounds over the lower lobes of the lung.

The best noninvasive method for detection of early pulmonary edema appears to be the chest radiograph. With the use of serial films that allow for sequential comparisons, interstitial edema is characterized by loss of normally sharp radiographic definition of pulmonary vascular markings, haziness, and thickening of interlobar spaces.

Once pulmonary edema has advanced to stage three, a number of hemo-

dynamic, ventilatory, and pulmonary abnormalities are more readily apparent. Thus chest radiographic analysis demonstrates that fluid has accumulated in the acini and that gas exchange is extremely abnormal, with severe decreases in P_aO_2 and increases in P_aCO_2. Alveolar hyperventilation is present and the resultant respiratory alkalosis impairs delivery of oxygen to the tissues. Pulmonary function tests (Fig. 9) show a much decreased functional residual capacity, decreased tidal volume, increased respiratory rate, and increased closing volume (Fig. 14). As fluid accumulates in the lung, interstitial pressure becomes more positive, increasing flow resistance in the pulmonary microvasculature and resulting in a rise in pulmonary artery pressure, which ultimately leads to pulmonary hypertension.

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7.1.3. *Therapeutic Treatment*

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Therapeutic measures designed to limit lung fluid accumulation such as the intravenous administration of albumin or other high-molecular-weight molecules to raise colloid osmotic pressure are only beneficial in patients

with a reduced plasma protein concentration (Ingram and Braunwald, 1980). Indeed, the use of albumin and other colloids may be detrimental in patients with permeability edema because the colloids might leak extravascularly and draw fluid with them (Noble, 1980).

Additional supportive therapeutic measures include (1) administration of nutrients, (2) administration of bronchodilators if there is evidence of bronchospasm, and (3) administration of one or two large doses of corticosteroids. The use of corticosteroids is controversial but gaining support since they have been shown to inhibit PMN aggregation *in vitro* and *in vivo* (Hammerschmidt *et al.*, 1980).

Finally, the investigational use of new pharmacotherapeutics such as thromboxane synthase inhibitors, vasodilator prostaglandin infusions, antioxidants, and β-receptor agonists in the treatment of microvascular permeability edema is well covered in several reviews (Demling, 1982; McMillan and Boyd, 1982; Persson *et al.*, 1982).

7.1.3.2. *Cardiogenic (Pressure) Edema.* Because cardiogenic pulmonary edema is a life-threatening emergency that demands immediate attention, therapeutic measures are instituted before the precipitating cause of heart failure is determined. Treatments are directed at reducing pulmonary microvascular hydrostatic pressure without compromising cardiac output and systemic delivery of oxygen. To reduce venous return to the heart, the patient should sit with legs dangling along the side of the bed and rotating tourniquets should be applied to the extremities.

Pharmacotherapy consists of morphine sulfate, diuretics, vasodilators, inotropic agents, and theophylline. Although morphine sulfate is a valuable drug in the treatment of cardiogenic pulmonary edema, the mechanism by which it decreases venous return is not known. The most likely hypothesis suggests that morphine reflexly decreases sympathetically induced venous and arteriolar constriction by allaying the patient's anxiety and decreasing the rate and depth of breathing (Ingram and Braunwald, 1980). But because of morphine's respiratory depressant effects, it is contraindicated in patients whose pulmonary edema is associated with chronic obstructive lung disease or intracranial bleeding.

Intravenous diuretics, particularly furosemide and ethacrynic acid, effectively mobilize liquid from the lungs. The dual mode of action (i.e., initial venodilation and subsequent diuresis) results in effective reduction of venous return; however, caution must be exercised in patients with low cardiac output because a further reduction may precipitate shock even after the pulmonary edema has subsided.

Administration of vasodilators such as sodium nitroprusside (intravenous) and nitroglycerin (sublingual) promptly relieves the symptoms of acute pul-

monary edema. Indeed, parenteral vasodilators are the treatment of choice in acute pulmonary edema because of their dual actions of venodilation (thereby reducing pulmonary capillary pressure) and reduction of systemic vascular resistance (thereby increasing cardiac output).

Depending on the cause of cardiogenic pulmonary edema, positive inotropic agents such as cardiac glycosides may be beneficial because they directly increase the contractility of the myocardium. Microvascular hydrostatic pressure is reduced because a given cardiac output can be generated at a lower left ventricular filling pressure. Because theophylline is both an airway smooth-muscle relaxant and a cardiac smooth-muscle stimulant, it is often useful in treating pulmonary edema, especially if the edema is associated with chronic obstructive airway disease or is due to cardiac asthma.

7.2. Pulmonary Hypertension

7.2.1. *Etiology*

Usually the cause of pulmonary hypertension can be assigned to related parenchymal lung disease, heart disease, thromboembolism, or pulmonary vascular disease. Pulmonary hypertension is termed idiopathic (or primary), however, when it occurs in patients in the absence of associated cardiopulmonary disease and when no other apparent cause for the disease is discernible. Primary pulmonary hypertension is a very rare disease that occurs predominantly in young female patients between the ages of 20 to 40 years (Wood, 1956). It is usually progressive and fatal, with the average survival time from the onset of symptoms being 2 to 3 years (Bourdillon and Oakley, 1976).

Pulmonary hypertension is characterized by a chronically elevated pulmonary artery pressure. As described in previous sections of this chapter, under normal conditions, the pulmonary artery pressure has a systolic value of 18 to 25 mm Hg, a diastolic value of 6 to 10 mm Hg, and a mean value ranging from 12 to 16 mm Hg. Pulmonary hypertension exists when the pulmonary artery systolic and mean pressures exceed 30 and 20 mm Hg, respectively. In the disease state, the pressure in the pulmonary artery may fluctuate widely and is often so high that it equals the blood pressure in the systemic arterial bed. As would be expected, pulmonary vascular resistance is also extremely high in patients with pulmonary hypertension. In addition, patients with this disease exhibit an enlarged right ventricle and an enlargement of the main pulmonary artery and its branches. Systemic hemodynamic parameters, however, such as cardiac output, cardiac index, systemic artery pressure, and pulmonary artery wedge pressure are usually not elevated.

A number of pathogenic mechanisms have been postulated as mediating primary pulmonary hypertension; however, none of these has been conclusively implicated in the development of the disease. In fact, current knowledge of the cause and treatment of primary pulmonary hypertension has been hampered by the absence of a simple, noninvasive early detection test in man and also by the absence of an acute animal model of this disease, which would allow further research into the pathogenesis of this disease. Several of the current working hypotheses for the causes of primary pulmonary hypertension are described here.

7.2.1.1. *Autoimmune "Collagen" Disease.* About 30% of the cases of primary pulmonary hypertension are associated with autoimmune diseases frequently categorized as "collagen" diseases. These include Raynaud's phenomenon, scleroderma, and rheumatoid arthritis. This has led to speculation that primary pulmonary hypertension is a form of systemic disorder, perhaps a form of collagen vascular disease (Ross, 1983). Interestingly, the pathologic changes in pulmonary vessels in patients with collagen vascular diseases resemble those seen in patients with primary pulmonary hypertension, suggesting a common underlying cause of vasoconstriction in both diseases (Alpert and Braunwald, 1980).

Primary pulmonary hypertension differs from typical autoimmune diseases, however, in that there is no evidence of immune complexes either circulating or locally deposited in the pulmonary vascular bed (Voelkel and Reeves, 1979). In addition, the treatment of primary pulmonary hypertension with immunosuppressive agents (Immuran) has not been successful.

7.2.1.2. *Drug-Induced Pulmonary Hypertension.* Over the past 30 years, many chemicals and drugs taken orally have been shown to selectively affect the pulmonary circulation and cause pulmonary hypertension (Heath and Smith, 1977). The most striking case was an epidemic of pulmonary hypertension in Switzerland, Austria, and West Germany between 1966 and 1968 (Follath *et al.*, 1971) following the introduction of an appetite suppressant, aminorex (2-amino-5-phenyloxazoline, menocil). In controlled clinical studies, it was subsequently shown that prolonged treatment with aminorex produced clinical symptoms of pulmonary hypertension in 2% of the patients taking the drug (Douglas *et al.*, 1981). Moreover, at autopsy, the pulmonary vascular lesions were similar to those seen in primary pulmonary hypertension. Since aminorex chemically resembles epinephrine and amphetamine, it was hypothesized that the underlying mechanism of action of aminorex was release of endogenous catecholamines or serotonin in the pulmonary vasculature. Douglas *et al.* (1981) reported that another anorexic drug, fenfluramine, induced pulmonary hypertension in humans. Fenfluramine is

chemically related to aminorex and is also thought to produce pulmonary hypertension by a mechanism involving the release of serotonin from the lung.

Usage of oral contraceptives in young women has been reported to produce vascular intimal proliferation within the pulmonary artery branches, leading to severe pulmonary hypertension (Kleiger *et al.*, 1976). This knowledge combined with the increased occurrence of primary pulmonary hypertension in young females has prompted the suggestion that recurrent pulmonary thromboemboli are responsible for the disease. Unfortunately, attempts to reverse or ameliorate pulmonary hypertension with anticoagulant therapy have not been successful.

7.2.1.3. *Hypoxia.* Hypoxia is undoubtedly a cause of pulmonary hypertension in patients with chronic bronchitis and emphysema or in people residing at high altitudes (Grossman and Braunwald, 1980). However, the role for hypoxia as a determinant of primary pulmonary hypertension is less clear. Support for a hypoxia-linked mechanism in primary pulmonary hypertension is the fact that this stimulus is the most effective and consistent inducer of pulmonary hypertension in all species. Hypoxia exerts its greatest effect by constricting the arterioles and precapillaries through a mechanism independent of autonomic innervation. In addition, hypoxia can cause hypertrophy and hyperplasia of the pulmonary arterial smooth muscle (Naeye, 1965).

7.2.1.4. *Endogenous Vasoconstrictor Agents.* Pulmonary hypertension can be regarded as a situation in which vasomotor tone in the pulmonary circulation has been heightened, leading to the development of intimal lesions in lung arteries. In recent years, the search for an endogenous vasoconstrictive mediator that might be responsible for the heightened vasomotor tone has resulted in extensive literature associating pulmonary vasoconstriction with a number of vasoactive agents such as catecholamines, angiotensin II, histamine, prostaglandins, thromboxane A_2, leukotrienes, and platelet-activating factor. In this coverage of the etiology of pulmonary hypertension, the information that exists about each vasoconstrictor agent is briefly considered.

Catecholamines. Experimental and clinical observations suggest that elevated levels of neuronally released or circulating catecholamines (primarily norephinephrine) can produce an increase in pulmonary vascular resistance and ultimately pulmonary hypertension (Kadowitz and Hyman, 1973). It has been suggested that elevated norepinephrine levels may be present in the lung if there is a defect in the normal uptake mechanism for these substances in the lung (Fishman, 1974). This could occur when there is some type of

vascular endothelial injury. Animal studies performed in isolated perfused lungs have shown that damage to endothelial cells can impair the metabolic functions of the lungs and result in dramatic reductions in the uptake and inactivation of norepinephrine. Additional support for this theory was provided in studies reported by Sole *et al.* (1979), who showed that the net uptake or extraction of norepinephrine was impaired in patients with primary pulmonary hypertension compared to controls.

Angiotensin II. A number of observations suggest that the renin–angiotensin system may be involved in the genesis of primary pulmonary hypertension. These include the reports that angiotensin II can raise pulmonary artery pressure independent of action on the systemic arterial circulation in animals and man and that angiotensin II potentiates the vasoconstrictor effect of hypoxia on the pulmonary vasculature. Furthermore, it has been reported that angiotensin converting enzyme inhibitors such as saralasin or teprotide decrease pulmonary artery pressure (Niarchos *et al.*, 1982) and reduce hypoxic pulmonary hypertension (Weir and Chesler, 1978). The encouraging but scant data available from animal studies with angiotensin converting enzyme inhibitors warranted a trial of this drug therapy in patients with pulmonary hypertension. Two studies (Schmengler *et al.*, 1982; Ikram *et al.*, 1982) documented a decline in pulmonary artery pressure in parallel with plasma angiotensin II levels after the angiotensin converting enzyme inhibitor captopril was administered to patients with primary pulmonary hypertension. Rich and Rosen (1982) failed to show a fall in pulmonary artery pressure in four cases treated for 48 hours with captopril. More studies with effective, specific, long-lasting, competitive inhibitors of angiotensin converting enzyme are necessary to conclusively prove that a humoral mechanism involving angiotensin II participates in the cause of primary pulmonary hypertension.

Histamine. In considering the mechanisms that might be associated with the development of pulmonary hypertension, Bergofsky (1974) suggested an involvement of histamine, which when released from mast cells in the vicinity of pulmonary arteries could cause the vessels to constrict and could lead to pulmonary hypertension. Indeed, although histamine is a vasodilator in the systemic circulation, it is a potent vasoconstrictor in the pulmonary vascular bed. Pharmacological evaluation of the role of histamine as a humoral mediator of pulmonary hypertension would appear to be a straightforward matter because effective antihistamines (diphenhydramine, tolaxoline) and mediator release inhibitors (disodium cromoglycate) are clinically available. The results with antihistamines, however, have been neither consistent nor conclusive. Disodium cromoglycate was able to attenuate hypoxic pulmonary vasoconstriction in dogs (Rengo *et al.*, 1979); however, no studies involving patients with primary pulmonary hypertension have been con-

ducted. Thus the potential role of histamine in pulmonary hypertension must still be explored.

Prostaglandins. The role of cyclooxygenase products (prostaglandins and thromboxanes) in the pathogenesis of primary pulmonary hypertension is presently uncertain; however, an accumulating amount of evidence suggests that an imbalance in arachidonic acid metabolism may be a major contributing factor to the disease. As discussed in previous sections of this chapter, the lung has an enormous capacity to metabolize, synthesize, and release a number of biologically active substances. Under normal conditions the lung metabolizes arachidonic acid to produce mainly prostaglandins of the F, E, and D series and lesser amounts of thromboxane A (TXA_2) and prostacyclin (PGI_2) (Hyman *et al.*, 1978). It is believed that a balance is maintained between vasodilator–platelet aggregation-preventing substances such as PGE_1 and PGI_2 and vasoconstrictor–platelet aggregators such as $PGF_{2\alpha}$ and TXA_2. Under pathological conditions such as hypoxia (or anaphylaxis), however, the metabolism of arachidonic acid may be altered and the balance shifted to produce greater amounts of $PGF_{2\alpha}$ and TXA_2. Regardless of whether the imbalance is a consequence of increased synthesis or decreased inactivation of prostaglandins, pulmonary hypertension can be one of the ultimate outcomes.

Leukotrienes. Since the structural characterization of SRS-A (now known to consist of leukotriene C_4, D_4, and E_4) in 1979 (Murphy *et al.*, 1979), interest in the physiologic and pharmacologic properties of these arachiodonic acid metabolites has increased exponentially. As described in Section 5.1.1. of this article, the leukotrienes have a wide variety of pharmacological actions; this suggests that they may play a role in mediating allergic asthma. In addition, as our knowledge of the effects of leukotrienes on the pulmonary circulation increases, it becomes clearer that these substances may also have a role in the development of pulmonary hypertension. Evidence in support of this hypothesis includes the observations (1) that leukotrienes constrict isolated pulmonary artery smooth muscle obtained from guinea pigs (Hand *et al.*, 1981) and humans (Hanna *et al.*, 1981), (2) that the leukotrienes have been shown to increase pulmonary artery pressure and pulmonary vascular resistance in animal studies (Berkowitz *et al.*, 1982), and (3) that pulmonary artery endothelial cells are an abundant cellular source of leukotrienes (Piper *et al.*, 1983). Confirmation of a role for leukotrienes in pulmonary hypertension, however, must await further investigation with compounds that act as specific leukotriene receptor antagonists or that have been shown to be potent inhibitors of leukotriene biosynthesis.

Platelet-activating factor—thromboxane A_2. Evidence gathered from numerous experimental approaches has suggested a role for the circulating platelet in pulmonary hypertension. Platelets have been shown to aggregate

in the lung and release vasoactive substances such as serotonin, histamine, and thromboxane, which increase pulmonary vascular resistance and pulmonary hypertension (Miczoch *et al.*, 1977). Moreover, removal of platelets by administration of platelet antiserum or prevention of platelet aggregation with drugs such as sulfinpyrazone lessen but do not completely abolish the hypertensive response (Mlczoch *et al.*, 1978). Further interest in the role of platelets in the pathogenesis of primary pulmonary hypertension was stimulated by the structural elucidation of platelet-activating factor (PAF) (Heffner *et al.*, 1983). This naturally occurring phospholipid is synthesized and released from a number of human cells, including platelets (Snyder, 1983).

The *in vivo* physiological actions associated with PAF include systemic hypotension, pulmonary hypertension, neutropenia, thrombocytopenia, and bronchoconstriction (Vargaftig *et al.*, 1981). On the cellular level, PAF promotes aggregation of platelets and stimulates chemotaxis of neutrophils. Because the chemical structure of PAF was not firmly established until 4 years ago and since only limited quantities of the synthetic material exist, few animal studies and no human data are available concerning the pathogenesis of primary pulmonary hypertension through a mechanism involving PAF-stimulated platelet activation. Support for this theory, however, has been afforded by Heffner *et al.* (1983), who showed that pulmonary hypertension and edema occurred in isolated rabbit lungs perfused with platelets and PAF, but not with platelets alone or PAF alone. Furthermore, it was demonstrated through the use of thromboxane synthase inhibitors and thromboxane receptor antagonists that PAF does not produce pulmonary hypertension directly but rather via release of TXA_2 from stimulated platelets. These observations thus suggest a role for PAF or TXA_2 in pulmonary hypertension. As is the case with other mediators, however, this role cannot be confirmed in human beings until therapeutics are developed that specifically regulate the actions of these substances.

7.2.2. *Diagnostic Measurements for Pulmonary Hypertension*

The majority of patients with pulmonary hypertension are largely asymptomatic until marked vascular alterations have developed. When blood flow through the pulmonary artery is obstructed over a long period of time, however, the clinical picture is predictable and markedly uniform. In general, the patients exhibit normal pulmonary function measurements, a low carbon monoxide diffusion capacity (D_LCO), and marked hyperventilation that leads to hypocapnia and decreased serum bicarbonate concentrations. Additional symptoms include weakness, fatigue, exertional dyspnea, and chest pains upon exertion due to low cardiac output and hypoxemia. Occasionally, hoarseness, hemoptysis, and cyanosis occur.

Tools such as chest radiographs, cardiac catheterization, EKG, and echo-

cardiograms are useful in the objective diagnosis of primary pulmonary hypertension. Histopathological changes such as intimal fibrosing, arterial muscular hypertrophy, necrotizing arteritis, and plexiform lesions are also characteristic of the disease (Alpert and Braunwald, 1980). Indeed, intimal plexiform lesions (which are dilated, thin-walled side branches of muscular pulmonary arteries probably resulting from endothelial cell proliferation) are considered to be the morphologic manifestation of irreversible pulmonary hypertension (Edwards and Edwards, 1977).

Unfortunately, once diagnosis is confirmed, the prognosis of primary pulmonary hypertension is dismal. The disease is fatal in most instances, with the patient ultimately dying of heart failure.

7.2.3. *Therapeutic Treatment*

Currently there exists no singularly effective treatment of primary pulmonary hypertension. Therapeutics that have been tried based upon the proposed pathogenic mechanisms of this disorder, as described earlier, include anticoagulants (heparin, sulfinpyrazone), α-adrenergic receptor antagonists (phentolamine, phenoxybenzamine), antihistamines (diphenhydramine, tolaxoline), mediator release inhibitors (disodium cromoglycate), angiotensin converting enzyme inhibitors (captopril), prostaglandins (PGE_1, PGI_2), and-immunosuppressives (Immuran). Three additional categories of drugs have been found useful in small groups of patients. These include direct vascular smooth-muscle relaxants, β_2 agonists and calcium antagonists.

Direct vascular smooth-muscle relaxants evaluated in primary pulmonary hypertension include hydralazine, isosorbide dinitrate, and diazoxide. In general, the hemodynamic effects of these drugs include modest reduction in mean pulmonary artery pressure, which parallels a significant reduction in systemic arterial pressure, decreased pulmonary vascular resistance, and increased cardiac output.

Hydralazine is the first systemic vasodilator drug advocated for initial treatment in patients with primary pulmonary hypertension. Rubin and Peter (1980) reported that short-term and long-term administration of hydralazine (200–300 mg/day) improved hemodynamics during rest and exercise in patients with primary pulmonary hypertension. The use of hydralazine, however, is not without hazard. In one study with 13 patients (Danahy *et al.*, 1979), hydralazine produced only modest decreases in pulmonary arteriolar resistance and serious adverse effects that included hypotension (resulting in one death), renal insufficiency, and systemic arterial hypoxemia.

Oral isosorbide dinitrate has been added to the list of vasodilator agents used for the treatment of primary pulmonary hypertension. In one study

with 18 patients (Rubino and Schroeder, 1979), pulmonary hypertension was reduced with acute administration of this nitrate, although it is uncertain whether chronic use would lead to nitrate tolerance.

Diazoxide is a potent vasodilator chemically resembling the thiazides, but it is not a diuretic. Diazoxide can be administered both parenterally and orally. Caution has been stressed when diazoxide is administered as an intravenous bolus into the pulmonary artery because the solution is highly alkaline (pH 11.6) and irritating to vascular tissue (Cotter and Honey, 1980). In one study with chronic oral diazoxide administration (300–600 mg/day), five of seven patients had important side effects including diabetes mellitus, fluid retention, nausea, vomiting, and postural hypertension that required discontinuation of the drug (Wise, 1983).

The β agonist isoproterenol effectively decreases pulmonary artery pressure when administered intravenously in acute studies. Long-term studies with sublingual isoproterenol appear to show beneficial effects, but only in a limited number of patients. Since isoproterenol is not a selective pulmonary vasodilator, however, cardiovascular side effects are common.

Within the past few years, reports of the clinical use of calcium antagonists in the treatment of primary pulmonary hypertension have increased. Calcium antagonists produce vasodilation by acting directly on smooth-muscle cells to inhibit excitation–contraction coupling. Treatment with the calcium antagonists, nifedipine, verapamil, and diltiazem have resulted in prompt, prolonged beneficial effects in patients with primary pulmonary hypertension at rest and with exercise (Wood *et al.*, 1982). Hemodynamically, a substantial fall in pulmonary and systemic vascular resistance, an increase in cardiac output, and modest decreases in pulmonary artery pressure are usually observed. Although calcium antagonists have a wide range of safety, undesirable effects such as tolerance and negative inotropic effects may outweigh their vasodilator activity (Farber *et al.*, 1983). One death (Berkenboom *et al.*, 1982) and one heart failure (Rozkovec *et al.*, 1982) following nifedipine treatment for primary pulmonary hypertension suggest that calcium antagonists should be used cautiously to treat severe pulmonary hypertension.

Due to the adverse effects associated with the therapies described, many investigators advocate testing for pulmonary vascular responsiveness. The potential for reversal of pulmonary vascular resistance is assessed either by a selective pulmonary vasodilator such as acetylcholine or by a short-acting, easily titratable, nonselective vasodilator such as prostacyclin. These agents appear to be more predictive of pulmonary vascular responsiveness than administration of 100% oxygen or intravenous phenotolamine. Rozkovec *et al.* (1982) suggest that patients who have a greater than 20% fall in total

pulmonary resistance with PGI_2 (8 ng, intravenously) are more likely to respond favorably to long-term vasodilator therapy than those who do not have this acute response.

In summary, the current therapeutic approach for the treatment of pulmonary hypertension is largely experimental, and the search continues for the ideal therapeutic to produce long-lasting normalization of pulmonary artery pressure by reversing the pathologic process in primary pulmonary hypertension.

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