

Advances in
CLINICAL CHEMISTRY
VOLUME 29

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VOLUME 29

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Advances in
**CLINICAL
CHEMISTRY**

Edited by

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VOLUME 29

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PREFACE

The Editors are continuing their policy of presenting reviews on selected topics, broadly covering the leading edge of science and technology. Through the efforts of experts and an international Board of Editors, this series of volumes attempts to provide a more comprehensive global view of the current and future directions of this expanding discipline.

Clinical chemistry now encompasses aspects of analytical chemistry, biochemistry, clinical laboratory science, toxicology, immunology, and molecular biology. Our intended readership includes the clinical laboratory scientists, practitioners of the medical arts, medical technologists, and associated disciplines.

Volume 29, with chapters devoted mainly to topics related to the kidney, cancer, and applications of the polymerase chain reaction, was an exciting learning experience to review. We are happy to share the philosophy and knowledge of our contributors with you.

This volume is dedicated to my friend and colleague, Alvin Dubin. Al was a continuing source of counsel, knowledge, inspiration, and vision. His energy, enthusiasm, and integrity will be missed. He was a champion for this profession and its members. It was my privilege to have known him and to work closely with him for many years. Professor Dubin was a scholar, a gentleman, and an international ambassador of goodwill for Clinical Biochemistry. The community of clinical biochemists will miss him.

I wish to acknowledge the help and continuous contributions of the Board of Editors. Many of them have become close friends as well as collaborators.

Finally, I would like to thank my wife Joanne for her support and genuine congruence, with all my efforts and aspirations.

HERBERT E. SPIEGEL

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CLINICAL SIGNIFICANCES OF SUPEROXIDE DISMUTASES: CHANGES IN AGING, DIABETES, ISCHEMIA, AND CANCER

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1. Introduction

Since the discovery of superoxide dismutase (SOD) by McCord and Fridovich (M19), increasing numbers of papers have been published concerning the structural and functional aspects of this enzyme. Historically, a variety of SODs were reported as copper-containing proteins: hemocuprein from bovine blood (M2), hepatocuprein from horse liver (M28), cerebrocuprein from human brain (P10), and erythrocuprein from human and beef erythrocytes (M9). Carrico and Deutsch (C2) gave the name "cytocuprein" to these proteins because the cupreins from various organs were found to be essentially identical. Cytocuprein was found to

contain two atoms of zinc per mole in addition to copper. This discovery stimulated further research on SOD, which had been studied only as a cuproprotein.

Excellent reviews on SOD and problems related to its study have been published (B3,F2,F4–F7,H2,M19–M21,O1,S17,T8). In this review an attempt will be made to summarize recent data on the clinical and pathological significance of Cu,Zn-SOD and Mn-SOD isozymes in relation to aging, diabetes, cancer, and ischemia. These data have been mainly obtained by enzyme-linked immunosorbent assays specific for Cu,Zn-SOD or Mn-SOD. These techniques are more convenient for clinical and laboratory work than are the various enzymatic assays in terms of reproducibility, ease of handling, and time consumption. This review will not attempt to cover the enzymatic activity data on SODs, because several excellent reviews on activity changes in various diseases already exist (B3) and, moreover, the results of different studies on SOD activities in various physiological stages are sometimes conflicting and are difficult to interpret.

1.1. GENERAL PROPERTIES OF SOD

Most aerobic cells have an enzyme system to eliminate active oxygen species, because some of these active species are toxic. SOD, catalase, and glutathione peroxidase comprise a major defense system against oxygen toxicity. SOD catalyzes the dismutation of superoxide anion (O_2^-) to produce hydrogen peroxide (H_2O_2) and O_2 . O_2^- is one of the reduced oxygen species generated in cellular metabolism.

There are four kinds of SODs. In mammalian systems three isozymes are known. Table 1 indicates the general properties of the mammalian isozymes. The fourth enzyme, Fe-SOD, is structurally homologous to Mn-SOD (P3,P4,S14) and is only found in bacteria. Cu,Zn-SOD and extracellular (EC) SOD are located in the cytosol and extracellular fluid, respectively, whereas Mn-SOD is mainly located in the matrix of mitochondria (M5,S13,T9,W5,W6,W9). Cu,Zn-SOD and EC SOD contain Cu and Zn in their molecules, whereas Mn-SOD contains the Mn atom. The Cu,Zn-SOD is sensitive to cyanide and H_2O_2 , whereas Mn-SOD is resistant to these reagents (F4,W5). The difference in the cyanide sensitivity of the two isozymes makes it possible to distinguish the enzymatic activities of the SODs.

When conventional enzymatic assays are used to determine SOD activities in tissues, however, nonspecific reactions due to other proteins or low-molecular-weight compounds possessing SOD-like activities in the tissues can cause erroneous results. For example, serum ceruloplasmin reacts stoichiometrically with O_2^- , though the reaction is noncatalytic under physiological conditions (B2,G10,H1,V1). Immunochemical assays for SODs are more reliable because the molecular weights and subunit structures of Cu,Zn-SOD and Mn-SOD are quite different from each other, and immunochemically these proteins do not cross-react.

TABLE 1
SOME PROPERTIES OF HUMAN SOD ISOZYMES

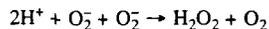
Property	Cu,Zn-SOD	Mn-SOD	Extracellular SOD
Distribution	Cytosol	Mitochondrial matrix	Extracellular
Molecular weight	32,000	88,000	135,000
Subunits	Dimer	Tetramer	Tetramer
(molecular weight)	(16,000)	(22,000)	(35,000)
Metal/monomer	1 Cu, 1 Zn	1 Mn	1 Cu, 1 Zn
Inhibition by CN ⁻	+	-	++
Inhibition by H ₂ O ₂	+	-	+
Inhibition by diethyldithiocarbamate	+	-	++
Inhibition by 2% sodium dodecyl sulfate	-	+	++
Inhibition by chloroform/ethanol	-	+	-
Rate constant for reaction with O ₂ ⁻	0.62 × 10 ⁹	1.2 × 10 ⁹	0.72-1.0 × 10 ⁹
Heterogeneity	Glycation, sulfhydryl	Sulfhydryl	Heparin binding

Recently we have developed polyclonal and monoclonal antibodies to Cu,Zn-SOD and Mn-SOD and have used these in an enzyme-linked immunosorbent assay (ELISA) for the SODs. We found that the level of immunoreactive SOD in tissues or fluids gives valuable information for biochemical and clinical studies (K5). The biochemical properties of EC SOD have been extensively studied by Marklund and his group (H13,K1,M5-M8), but its clinical significance requires further studies.

In this review we will emphasize the importance of this technique and focus on the clinical significance of immunoreactive Cu,Zn-SOD and Mn-SOD isozymes, especially in relation to diabetes, aging, cancer, and ischemia.

1.2. ASSAY OF SOD ACTIVITY BY ENZYMATIC METHODS

SOD catalyzes the dismutation of O₂⁻ to H₂O₂ and O₂ as follows:



Because the enzyme substrate and the products are unstable, it is difficult to measure the disappearance of substrate or the formation of products as is usual in enzymatic assays. Routine assays for SOD usually employ an indirect assay in which one unit of enzyme activity is defined as the amount of enzyme that inhibits the reaction of O₂⁻ with the indicator by 50%. The most frequently used method for measuring SOD activity employs the xanthine/xanthine oxidase reaction for

O₂ generation and reduction of cytochrome *c* or nitroblue tetrazolium for O₂ detection.

In the xanthine/xanthine oxidase–cytochrome *c* method originally developed by McCord and Fridovich (M19), a typical assay mixture consists of oxidized cytochrome *c*, xanthine, sufficient xanthine oxidase, and phosphate buffer at pH 7.8 containing EDTA in a total volume of 3 ml. The rate of reaction is followed at 550 nm. One unit of SOD activity is defined as the amount that causes 50% inhibition of the rate of reduction of cytochrome *c*.

Autooxidation reactions have also been used as O₂-generating systems. Substances that have been utilized as autooxidizing systems include adrenalin (M23,S19), sulfite, pyrogallol (M3), and 6-hydroxydopamine (H11). Direct addition of K[•]O₂ as an O₂ generator has also been reported (M4). Illuminated riboflavin (B11) is another source of O₂.

Systems used to detect O₂ include cytochrome *c* reduction (M4,M11,S4), nitroblue tetrazolium reduction (B11,S3), adrenalin (M24,S19), hydroxyamine (M3), and a hydroxyamine derivative (E2,K14,K15). NADH/lactate dehydrogenase (B15), 6-hydroxydopamine (H10), sulfite (F8,G13), pyrogallol (M3), tetranitromethane (M19), and dianisidine (G13,M24) are frequently employed. NAD(P)H oxidation has also been employed to generate O₂. In this system, NAD(P)H is the O₂ detector (P2). SOD activity has also been estimated using hematoxylin autooxidation and chemiluminescence of a luciferin analog (K8,N6). In the case of illuminated flavin, nitroblue tetrazolium was used for the O₂ detection (S3).

A method utilizing riboflavin as the O₂ generator is widely used to detect SOD after polyacrylamide gel electrophoresis of crude tissue extracts. Gels are soaked in a solution of 2.45×10^{-3} M nitroblue tetrazolium for 30 minutes followed by immersion for another 30 minutes in a solution containing 0.028 M *N,N,N,N'*-tetramethylethylenediamine, 2.8×10^{-5} M riboflavin, and 0.036 M phosphate buffer, pH 7.8. The gels are then removed from the solution and illuminated for 30 minutes. During the illumination procedure, the wells become uniformly blue, except where there are gels loaded with known concentrations of purified enzyme, allowing the concentration to be determined from the peak height. This method is an indirect negative assay. A more convenient indirect positive assay applicable to polyacrylamide gel electrophoresis has been developed (M24,M25). In this assay the oxidation of dianisidine is followed by the absorbance change at 460 nm. The reaction is initiated by the photooxidation of riboflavin. The electronically excited stage of riboflavin oxidizes the dianisidine, yielding the flavin semiquinone and a dianisidine radical which, in the absence of a competing reaction, will dismutate to yield the divalently oxidized dianisidine. SOD scavenges the O₂ formed and therefore inhibits the reduction of the dianisidine radical, which can then dismutate to yield the divalently oxidized dianisidine. This method is also applicable to the staining of SOD in crude enzyme extracts. Beyer and Fridovich (B13) have compared the sensitivities of the above assay methods.

Direct assays for SOD have relied on pulse radiolysis (K12,M15), stopped-flow spectroscopy (M4,M17), rapid-flow electron paramagnetic resonance (EPR) (B1, O5, S1), polarographic techniques (R4), and nuclear magnetic resonance (NMR) spectroscopy (R3).

1.3. IMMUNOCHEMICAL ASSAY FOR SODs

Compared to enzymatic methods, immunochemical assay methods for SODs are more reliable and reproducible, because the determinations are specific to the protein moiety and immunogenicity will not change in the presence of inhibitors or activators of SOD in the tissues (G3,I2). In some cases, both enzymatic and immunochemical assays are requisite for the assessment of tissue SOD levels. In the serum, however, the activity assay is generally too insensitive to detect SOD, and immunochemical assay is much more reliable and convenient.

For the immunochemical assay of SOD, polyclonal or monoclonal antibodies are used. SOD protein levels can be determined by single radial immunodiffusion (H6), radioimmunoassay (A9,A10,B4,D5,H8), or enzyme-linked immunosorbent assay (A1,A2,I2,I4,K5,N8,O3,P9). Making polyclonal antibodies to Cu,Zn-SOD or Mn-SOD is not always simple, because the sequence homology between SODs of various animal species is quite high. Higher titer antibodies to human Cu,Zn-SOD were raised in goats rather than in rabbits. Antibodies to Mn-SOD with high titer are very difficult to obtain in rabbits. In our experience, one or two out of eight rabbits gave antibodies with a high titer to human Mn-SOD or rat Mn-SOD. Usually we employed a lymph node immunization technique (S10) to make the antibodies.

Similar difficulties were encountered in making monoclonal antibodies to human Mn-SOD. Sequence homology between human, rat, and mouse is again very high. Fortunately we obtained several monoclonal antibodies whose antigen-binding epitopes are located on the C-terminus of the Mn-SOD, where sequence differences between the species exist (K4).

In general, a sandwich-type ELISA is the most convenient method of immunochemical assay. Monoclonal antibodies are useful for this purpose. Monoclonal antibodies raised against human Cu,Zn-SOD have been employed in estimating Cu,Zn-SOD levels by a sandwich-type immunoassay (O3).

We have developed ELISA systems using a polyclonal (I2) and a monoclonal antibody (K4) to Mn-SOD. The Mn-SOD is composed of four identical subunits; therefore, one can use a single monoclonal antibody to bind and detect the Mn-SOD. Namely, a monoclonal antibody binds one molecule of the tetrameric enzyme and then the same type of monoclonal antibody linked with peroxidase binds to another monomer of the enzyme. A schematic representation of the ELISA is shown in Fig. 1.

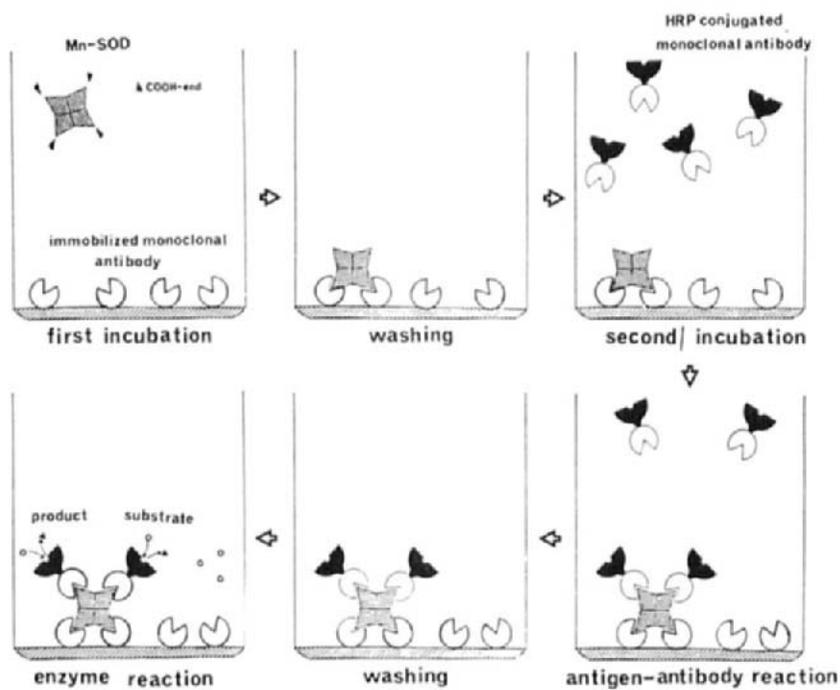


FIG. 1. A schematic drawing of a sandwich ELISA procedure for Mn-SOD using a monoclonal antibody.

2. Cu,Zn-SOD

2.1. OCCURRENCE

Cu,Zn-SOD is widely distributed in the cytosol of tissues. Although the enzyme is localized mainly in the cytosol, it also exists in the mitochondrial intermembrane space (P1,P7,T9) and in lysosomes (G7). A correlation between life span and the Cu,Zn-SOD activity has been suggested (T7).

When assessing the levels of Cu,Zn-SOD in tissues, one must be aware that the enzyme is released from erythrocytes by hemolysis. Particular attention must be paid to this problem when Cu,Zn-SOD is measured in human serum. Human erythrocytes contain approximately 0.5–0.75 μg Cu,Zn-SOD per gram of hemoglobin. Even trace hemolysis can therefore lead to misinterpretation. Because many of the previous studies on tissue and serum Cu,Zn-SOD did not take into account the contribution from hemolysis, many of the data so far reported should be reinvestigated. Hartz and Deutsch carefully determined the content of Cu,Zn-SOD in various human tissues using a single radial immunodiffusion technique

(H6). They obtained the values by subtracting the Cu,Zn-SOD contributed by erythrocytes using hemoglobin as an internal standard. Liver, cerebral gray matter, cardiac muscle, and testis were found to be relatively abundant in Cu,Zn-SOD. In a recent study using ELISA for Cu,Zn-SOD, human liver samples from which blood had been removed were found to contain approximately 0.65–1.5 mg Cu,Zn-SOD per gram of liver protein, a value approximately equal to 130–300 mg Cu,Zn-SOD per kilogram (wet weight) of human liver tissue (D7).

2.2. GENERAL PROPERTIES

Cu,Zn-SOD is composed of two identical subunits of molecular weight 32,000. Each subunit contains one Cu and one Zn molecule, noncovalently linked. The complete amino acid sequence has been determined for various Cu,Zn-SODs (B5,B6,J1,S16,S17). The N-terminus of the enzymes from higher vertebrate species is acetylated. Cyanide is a reversible inhibitor of the Cu,Zn-SOD (C5,R5). The enzyme is also sensitive to diethyldithiocarbamate (H10) and H_2O_2 (B16,F9,R6,S12). The diethyldithiocarbamate binds copper at the active site and removes the metal from the enzyme. The copper ion appears to function in the enzymatic reaction, whereas the zinc ion does not function in the catalytic activity but stabilizes the enzyme.

The Cu,Zn-SOD was purified from erythrocytes by relatively simple procedures, including ion-exchange chromatography (A8,B2,M18), tri-phase partition methods (P8), metal chelate affinity chromatography (M27,W8), and immunoaffinity chromatography (A5). The enzyme contains four cysteine residues and one of them is highly reactive. Alkylation of this cysteine residue results in a marked increase in stability and this form may be easily crystallized (J1).

The three-dimensional structure of bovine Cu,Zn-SOD has been studied by X-ray diffraction analysis at 2 Å resolution (R2,T1). Each subunit is composed of eight antiparallel strands of β -pleated sheet, which form a flattened cylinder, plus three external loops. Cu(II) and Zn(II) are bridged by His-61. Cu(II) is also coordinated to His-44, His-46, and His-118 (bovine sequence) in a square-planar geometry. Zn(II) is bridged to His-61, His-69, His-78, and Asp-81, and the geometry of the ligands is tetrahedral.

Thus, His-61 interacts with both metals and may play an important role in supplying the protons necessary for the dismutation reaction. X-Ray analysis of bovine Cu,Zn-SOD also indicated the existence of a channel "track" with positively charged amino acid residues. Lys-122 and Lys-134 at the top and Arg-143 are located inside the channel, positioned at 13, 12, and 5 Å, respectively, from the active-site copper ion. Most of the surface of Cu,Zn-SOD is negatively charged to repel O_2^- . However, the positively charged channel "track" provides effective long- and short-range electrostatic guidance to the active site for the substrate O_2^- (K14,T1). Chemical modification of the positively charged amino acid side

chains markedly decreased the activity of the enzyme (B17,B18). As described below, Lys-122 and Lys-128 in human Cu,Zn-SOD are sensitive to nonenzymatic glycosylation (glycation), and the SOD loses its activity after ketoamine adduct formation by glucose.

The role of Arg-143 in bovine and human Cu,Zn-SODs has been extensively studied (B12,B17,B18,M1,O6,T2). Horton *et al.* (H17) reported that substitution of Arg-143 with Lys or Ile in site-specific mutants of human Cu,Zn-SOD expressed in yeast brought about the loss of enzymatic activity. This indicates that the residue plays an important, if not crucial, role in the binding of O₂, (H16). X-Ray crystallographic study of the human enzyme has not yet been reported. Recently Kitagawa *et al.* (K10) reported that X-ray crystallography of spinach Cu,Zn-SOD indicated high structural homology to the bovine enzyme. This suggests that the bovine and human enzymes are very similar.

2.3. Cu,Zn-SOD GENE

Cu,Zn-SOD is encoded by human chromosome 21. A cDNA clone was obtained for examining the expression in various tissues (G14,S7,S8). The nucleotide sequence of one clone was found to contain 459 nucleotides followed by a single stop codon (TAA) and by a noncoding sequence of 95 nucleotides (S8).

Northern hybridization of the clone to mRNA from human cells revealed the presence of two mRNA species of 0.7 and 0.5 kb. The two mRNAs were found to code for the same polypeptide chain and were transcribed from the same gene. The major 0.7-kb mRNA was much more abundant than the 0.5-kb one. The larger mRNA contained 222 additional nucleotides at the 3'-polyadenylated terminus, and both species had multiple 5' ends.

The gene locus is at chromosomal region 21q22, the distal portion of the long arm of chromosome 21. The gene was found to be approximately 11 kb in length and to have five exons and four introns. The first intron contains an unusual variant dinucleotide, 5'-GC, rather than the highly conserved 5'-GT. Four processed pseudogenes were isolated in addition to the functional gene (D1). Overexpression of the Cu,Zn-SOD gene in Down's syndrome, which is usually caused by the presence of three copies of chromosome 21, may play an important role in the neurobiological abnormalities of the disease (E1,E3,S11).

2.4. GLYCATION OF Cu,Zn-SOD

2.4.1. Glycated Sites of Cu,Zn-SOD

It is well known that proteins undergo nonenzymatic glycosylation reactions under hyperglycemic conditions. These reactions are referred to as glycation to distinguish them from enzymatic glycosylation catalyzed by glycosyltransferase.

Glycation is a posttranslational modification that occurs *in vivo* by direct chemical reaction between glucose and the primary amino groups of proteins. The initial product is a labile Schiff base adduct, which undergoes a slow Amadori rearrangement to a stable ketoamine derivative of the protein (Fig. 2).

Glycation is considered to be the first step in a complex series of browning, or Maillard, reactions that occur in the presence of reducing sugar. Increased glycation and the subsequent Maillard reaction are thought to be involved in the structural and functional changes in body proteins that occur during normal aging

General Scheme of the Maillard Reaction

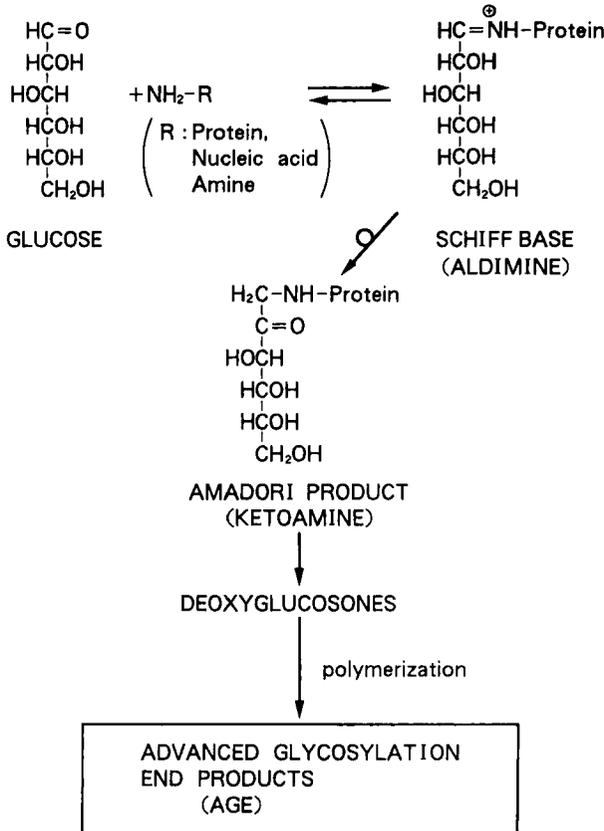


FIG. 2. Reaction mechanism for glycation of proteins.

and at accelerated rates in diabetes (B10,C3,M29). The reaction was found in the browning reaction of food in the nineteenth century. Glycation has also been seen in various proteins such as hemoglobin (G12), albumin (D4,I1), and lens crystallin (C4,S18), and in several enzymes such as ribonuclease (W4), carbonic anhydrase (K13), Cu,Zn-SOD (A5), and Na⁺,K⁺-ATPase (G6).

2.4.2. Glycation of Cu,Zn-SOD and Diabetes

Erythrocytes are subjected to a continuous flux of O₂ and H₂O₂ due to hemoglobin autooxidation (C3,H9,M26,W3) and also undergo oxidative stress from environmental agents (C6). Cu,Zn-SOD in the erythrocytes may have some physiologically important role in combating these processes.

Human erythrocytes contain glycated and nonglycated Cu,Zn-SOD, which can be separated by boronate affinity chromatography (A6,T5). The boronate affinity column binds to protein-bound glucose, which has a *cis*-diol structure. When the chloroform/ethanol extracts from normal human erythrocytes were subjected to boronate affinity chromatography, approximately 10% of the SOD activity was recovered in the binding fractions, eluted by sorbitol. On the other hand, the extracts from diabetic patients gave approximately 20% bound activity, as shown in Fig. 3. However, the percentage of activity bound probably did not accurately reflect the percentage of SOD protein bound, because the bound fraction was glycated SOD and glycation often results in inactivation. ELISA assay for Cu,Zn-SOD overcomes this problem. In fact, when we used ELISA for the measurement of glycated Cu,Zn-SOD, 50% or more of the erythrocyte Cu,Zn-SOD from diabetic patients was found to be glycated, as shown in Fig. 4.

Because such a high percentage of the Cu,Zn-SOD in the erythrocytes of diabetic patients was glycated, the total Cu,Zn-SOD activity did not differ much between the diabetic patients and the controls. Nonetheless, the specific activity of Cu,Zn-SOD in the erythrocytes of patients with diabetes was always lower than in nondiabetics. These facts indicate that the Cu,Zn-SOD is inactivated under hyperglycemic conditions. Indeed, when purified Cu,Zn-SOD was incubated with glucose under sterile conditions, the SOD activity showed a time- and dose-dependent decrease and the amount of ketoamine adduct increased simultaneously (Fig. 5.).

The mechanism by which Cu,Zn-SOD undergoes glycation and inactivation has been studied (A7,T5,T6). The N-terminus of human erythrocyte Cu,Zn-SOD is an acetylated alanine; therefore, lysine residues are the only likely candidates for glycation. The Cu,Zn-SOD is specifically glycated at Lys-122, Lys-128, and several other lysine residues. Considering the positions of the glycated site in Cu,Zn-SOD, it is interesting that three sites lie in Lys-Gly: Lys-9, Lys-36, and Lys-128. In particular, Lys-128 is located in a Gly-Lys-Gly sequence. The glycation of albumin is explained as due to local acid-base catalysis of the Amadori rearrangement, and the glycated sites are likely to be adjacent to posi-

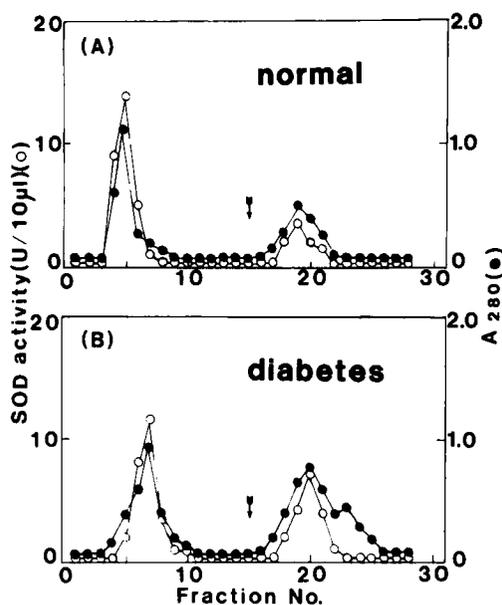


FIG. 3. Separation of glycated and nonglycated human erythrocyte SOD on a boronate affinity column. The arrows indicate addition of a sorbitol solution to elute the glycated SOD. (A) An extract from normal erythrocytes; (B) an extract from erythrocytes from a diabetic patient. The SOD activity was determined by enzymatic methods. The percentage of the glycated SOD was higher in the erythrocytes from the diabetic patient. However, as described in the text, the glycated SOD fraction had lost activity, and therefore the true amount of glycated SOD was much higher. A determination of the amount by ELISA is shown in Fig. 4.

tively charged amino acid residues (11). On the other hand, in hemoglobin and RNase, the nearness to carboxylic acid residues in the primary or three-dimensional structure of the protein is regarded as one of the important factors in the glycation reaction (S6,W5). In the case of Cu,Zn-SOD, however, in the primary sequences, Lys-Glu (Lys-23), Lys-Asp (Lys-75), and Lys-Asp (Lys-91) are not glycated. As described above, the Cu,Zn-SOD has a positively charged channel "track." The positive charges of Lys-122 and Lys-128 play an important role in the entrance of negatively charged O_2^- into the enzyme molecule, and enzyme inactivation is highly associated with a relative decrease in the positive charge of these lysine residues.

The computer image of spinach Cu,Zn-SOD is shown in Fig. 6. As indicated, Lys-122 and Lys-128 are located on the surface of the enzyme molecule and appear to be easily attacked by glucose.

Several groups independently reported that glycated proteins produce O_2^- in the

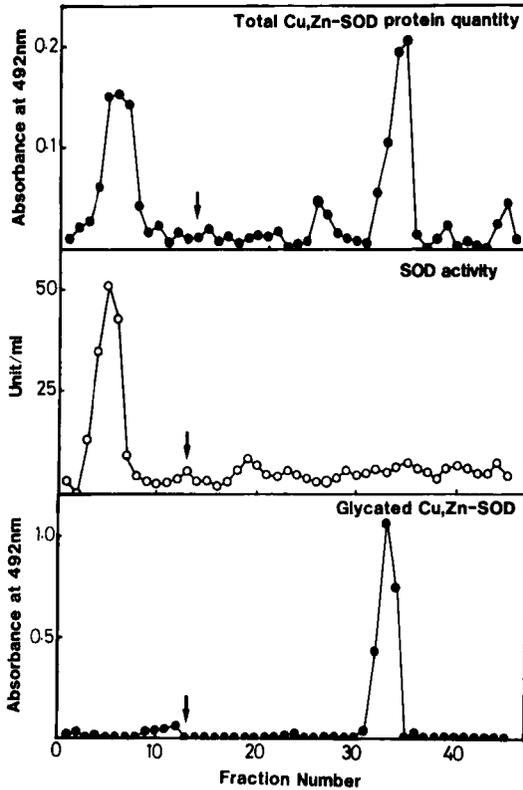


FIG. 4. Specific detection of glycated Cu,Zn-SOD by sandwich immunoassay using anti-Cu,Zn-SOD and antiglycitol lysine antibodies. Upper panel, immunoreactive Cu,Zn-SOD was measured by ELISA as described in the text; middle panel, SOD activity was measured by the xanthine/xanthine oxidase method; lower panel, glycated Cu,Zn-SOD was specifically measured by the sandwich immunoassay.

presence of transition metal ions (H18,M30,S2,S3). Furthermore, it is well known that Cu,Zn-SOD is sensitive to H_2O_2 (B5,B16,F9,H8). Inactivation by H_2O_2 is due to oxidation of Arg-143 by the reagent. In addition to the inactivation, proteolytic fragmentation of Cu,Zn-SOD, as one of the antioxidant defense mechanisms, has been observed following H_2O_2 exposure (D3,S4). The proteolysis was inhibited by metal chelators and serine protease inhibitors but not SH reagents, which indicates that the fragmentation of Cu,Zn-SOD *in vivo*, especially in red cells, occurs by metal protease. This protease was named “macroxyprotease” and has a molecular weight of 90,000.

Thus, the glycated proteins produce O_2^- on one hand, and the Cu,Zn-SOD

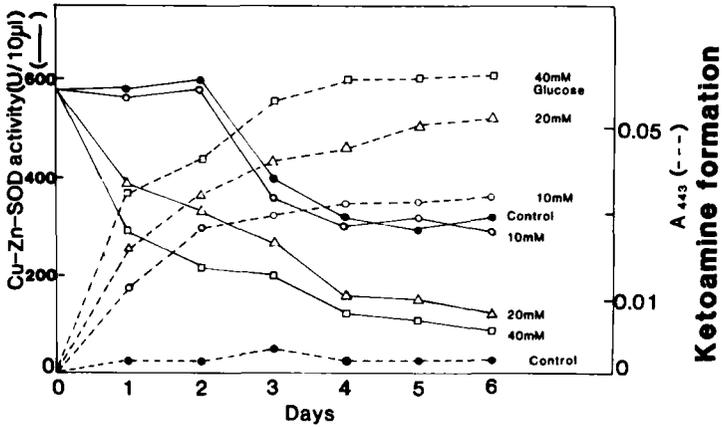


FIG. 5. Inactivation of Cu,Zn-SOD by glycation reaction *in vitro*. Purified Cu,Zn-SOD from human erythrocytes was incubated with 10–20 mM glucose at 4°C under sterile conditions. At appropriate time intervals the SOD activity and thiobarbituric acid-reactive materials were measured.

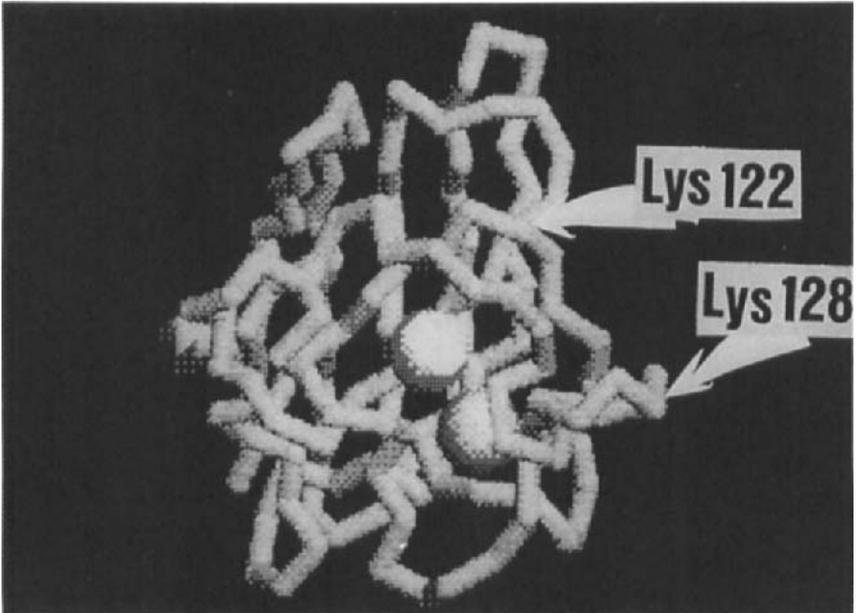


FIG. 6. Computer image of Cu,Zn-SOD (courtesy of Dr. Kitagawa). Lys-122 and Lys-128 are located on the surface of the molecule.

undergoes glycation and inactivation on the other hand. These events may enhance the accumulation of O_2^- in the microenvironment of the tissues and, as a result, the accumulation of H_2O_2 and $OH\cdot$ (see Fig. 7). This may further inhibit Cu,Zn-SOD activity through oxidation of Arg-143 by H_2O_2 .

2.4.3. *The Glycation of Cu,Zn-SOD and Werner's Syndrome*

Werner's syndrome is an autosomal recessive condition and is sometimes referred to as adult progeria. The disease is clinically characterized by accelerated aging and increased frequency of malignant tumors and diabetes (F1). At the cellular and molecular levels, cultured fibroblasts from patients with Werner's syndrome have a markedly decreased replicative life span (G11). In addition, increased proportions of several enzymes in the fibroblasts have been reported to be heat labile (G11,H16), as found in old fibroblasts. The etiology of the disease is still unknown, but an involvement of the free-radical scavenging system has been suggested (N9). An age-related reduction in Cu,Zn-SOD has been reported (G9,R1). In patients with Werner's syndrome, erythrocyte Cu,Zn-SOD undergoes nonenzymatic glycosylation at multiple lysine residues, irrespective of the glycaemic state. The enzyme purified from a patient was found to be unstable and had a very low specific activity due to nonenzymatic glycosylation. As described above, several groups recently reported that glycated protein produces O_2^- in the presence of metal ions such as Cu^{2+} or Fe^{2+} . Accelerated glycation reactions will bring about the production of O_2^- . Inactivation of Cu,Zn-SOD through glycation

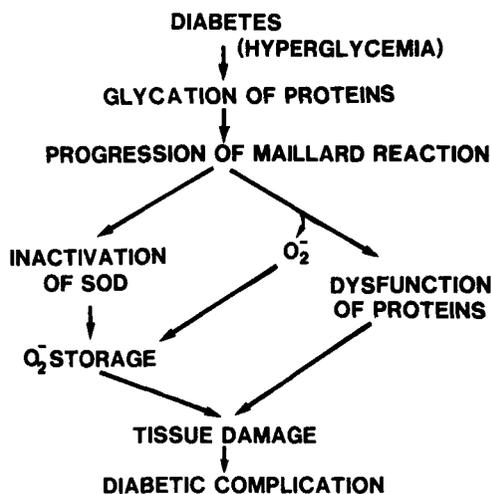


FIG. 7. A possible mechanism for accumulation of O_2^- in a microenvironment due to inactivation of SOD.

reactions also promotes the accumulation of O_2 in the tissues. The enhanced accumulation of O_2 will then lead to the production of H_2O_2 and $OH\cdot$.

2.4.4. Increase of Glycated Cu,Zn-SOD in Diabetic Retinopathy and Cataracts

The glycation of proteins may play an important role in diabetic complications (C3). Cu,Zn-SOD is located in the lens epithelium (B14,S5), as are most of the drug-metabolizing enzymes and antioxidant enzymes (H12). Figure 8 shows the localization of Cu,Zn-SOD in rat lens epithelium using antirat, Cu,Zn-SOD. Glycation of Cu,Zn-SOD in the lens may play an important role in cataractogenesis. In general, glycated Cu,Zn-SOD levels seem to correlate with the level of HbA_{1C} . In patients with diabetic cataracts, however, the correlation is not apparent, and the level of glycated Cu,Zn-SOD is rather high compared to levels in diabetic patients with no complications. Aged persons with senile cataracts also have relatively higher levels of glycated Cu,Zn-SOD in their erythrocytes. It is unclear, however, whether the increased amount of glycated Cu,Zn-SOD is really related to the senile cataracts or due to other minor atherosclerotic changes in aged persons. If one separates younger and older populations of normal erythrocytes by centrifugation and compares the activities of those populations, the activity of normal younger erythrocytes is higher than that of aged erythrocytes, as shown in Fig. 9 (T4).

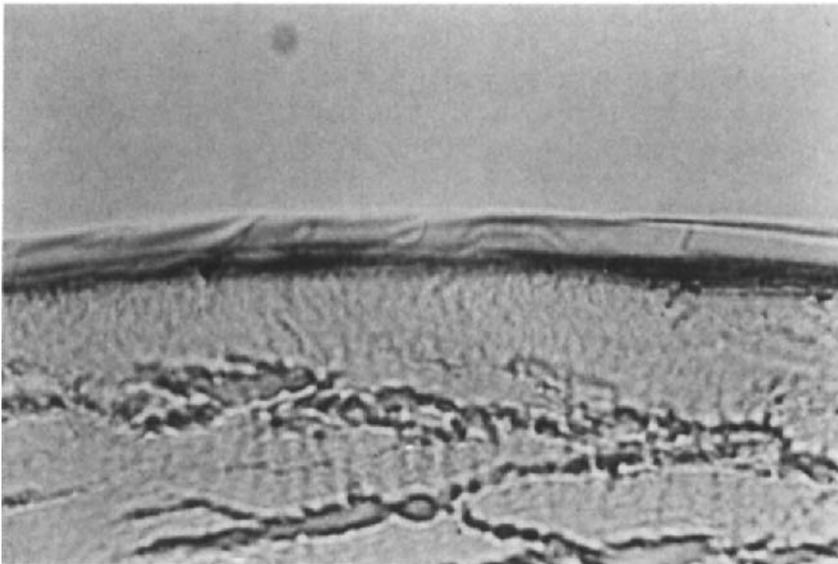


FIG. 8. Cu,Zn-SOD localization in rat lens. Immunostaining using antirat Cu,Zn-SOD IgG detected Cu,Zn-SOD in the epithelial cells of the rat lens.

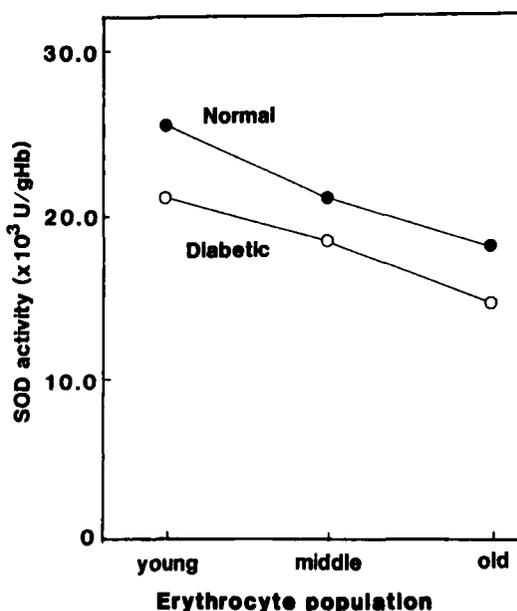


Fig. 9. Cu,Zn-SOD activities of aged and younger populations of erythrocytes. Pooled erythrocytes from normal and from diabetic patients were separated into younger and older populations, and the specific activity of Cu,Zn-SOD was measured. The Cu,Zn-SOD specific activity of older erythrocyte populations was always lower than that of younger ones, and the activities of both aged and younger erythrocytes from diabetic patients were lower than the corresponding normal controls.

Stevens *et al.* (S18) proposed that glycation might have a role in the browning and aging of lens crystallins associated with the development of senile and diabetic cataracts. Several investigators subsequently reported age-related increases in glycation of normal human (G4,G5,O2,V2), bovine (C3), and rat lens (S21). They suggested that increased glycation of proteins with age could cause an age-related acceleration of glucose-dependent damage to protein. However, Patrick *et al.* (P6) reported that glycation of human lens protein is essentially constant with age in normal lens.

Streptozotocin, a nitrosourea compound produced by *Streptomyces achromogenes*, has been used to induce experimental diabetes. The drug also induces DNA strand breakage in islet cells. Streptozotocin injection into rats has been observed to decrease the Cu,Zn-SOD activity of retina cells, erythrocytes, and islet cells (C11,G3,G15). However, the mechanism by which the Cu,Zn-SOD activity decreases in experimental diabetes is not yet known.

Streptozotocin-induced diabetic rats also had high levels of glycated Cu,Zn-SOD in the erythrocytes (K9) and lens, as judged by affinity chromatography on a boronate column (see Fig. 10). As described above, much of the glycated

Cu,Zn-SOD is inactive. This is one of the reasons that Cu,Zn-SOD activity is decreased in rats with streptozotocin-induced diabetes. Even in normal rat lens, approximately 40% of the Cu,Zn-SOD undergoes glycation. Under diabetic conditions, over 80% of the enzyme was found to have undergone glycation (N. Kawamura *et al.*, unpublished data). As described above, the level of the glycated Cu,Zn-SOD is slightly higher than in the normal adult, but Jacques *et al.* (J2) reported that no significant change was observed in Cu,Zn-SOD activity in persons with and without senile cataracts. Whether age-related changes occur in Cu,Zn-SOD in normal rat lens is now under study in our laboratory.

3. Mn-SOD

3.1. OCCURRENCE

3.1.1. *Mn-SOD Protein*

Mn-SOD is localized in the mitochondrial matrix and is considered to play an important role in the dismutation of O_2^- in tissues. Due to the limited availability of human tissues, we have determined Mn-SOD levels in rat tissues. As shown in Table 2, heart, small intestine, kidney, and liver contain relatively high amounts. Interestingly, immunocytochemical distribution of Mn-SOD in rat brain hippocampus indicated that CA1 pyramidal cells were weakly stained, whereas CA3 pyramidal cells were strongly reactive, as shown in Fig. 11 (A3). The CA1 subfield is one of the most sensitive areas in the hippocampus with respect to ischemia. This suggests that Mn-SOD may play an important role in brain ischemia. In rat lung, the Mn-SOD level is not so high, but the lung contains many different cell types, and assay of Mn-SOD levels in the homogenates of whole lungs may underestimate the large changes taking place in only a few cell types (H2). Moreover, the lung tissues respond to oxidant and hyperthermic stresses (H7,H8).

Recently Deutsch *et al.* (D7) examined six human liver samples obtained at autopsy and determined the enzymatic as well as immunoreactive Cu,Zn-SOD and Mn-SOD contents. Human liver contains approximately 1.5–9.2 mg Mn-SOD per gram of liver protein, which is equal to 300–840 mg per kilogram (wet weight) of liver. Interestingly, even though the activities of Cu,Zn-SOD and Mn-SOD varied, the total SOD activity in each liver sample remained in the range of 130–200 U per milligram of protein. These results suggested that in the human the total SOD activity in the liver is rather constant, even though the Cu,Zn and Mn isozymes are located in different compartments. When activity of one of the SOD isozymes decreases, activity of the other increases. Such compensation would serve to protect against O_2^- generated as the result of oxidative stress.

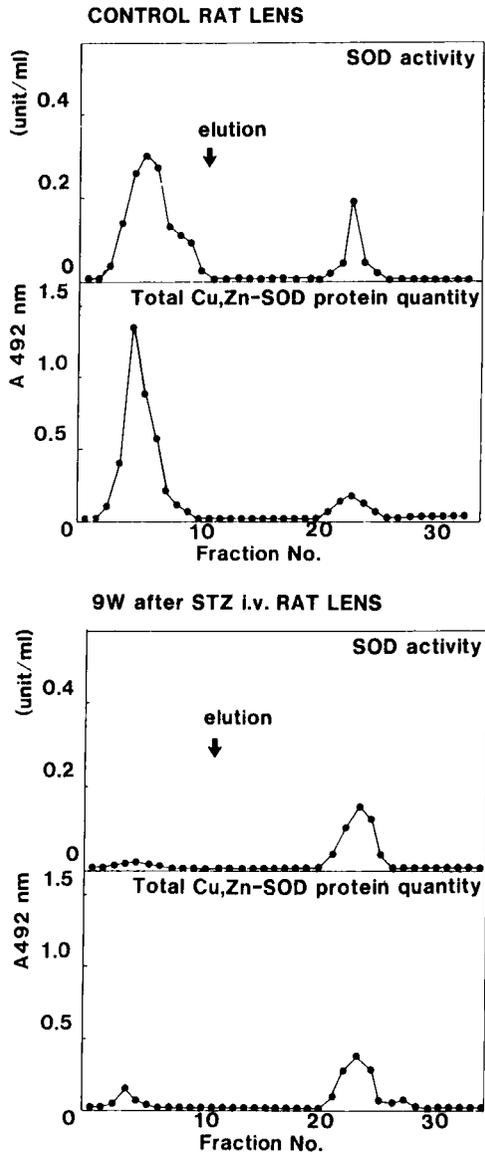


FIG. 10. Glycated Cu,Zn-SOD in streptozotocin-induced diabetic rat lens. Pooled lenses from normal and streptozotocin-induced diabetic rats [9 weeks after intravenous injection of streptozotocin (STZ)] were homogenized, and Cu,Zn-SOD was extracted by chloroform/ethanol and subjected to a boronate affinity column. Upper panel, normal rat lens; lower panel, diabetic rat lens. The SOD activity and immunoreactive Cu,Zn-SOD were measured using the xanthine/xanthine oxidase method and ELISA, respectively.

TABLE 2
Mn-SOD CONTENTS IN VARIOUS RAT TISSUES

Tissue (no. of samples)	Mn-SOD content ($\mu\text{g}/\text{mg}$ protein, mean \pm SD)
Brain (5)	0.236 \pm 0.047
Lung (5)	0.081 \pm 0.023
Heart (5)	1.203 \times 0.244
Stomach (5)	0.054 \pm 0.042
Intestine (5)	0.194 \pm 0.065
Liver (5)	0.772 \pm 0.113
Spleen (5)	0.102 \pm 0.032
Kidney (5)	1.149 \pm 0.292
Testis (5)	0.141 \pm 0.037
Ovary (5)	0.664 \pm 0.066

3.2. GENERAL PROPERTIES

Mn-SOD was first isolated from *Escherichia coli* and was found to be quite different from Cu,Zn-SOD. The enzyme is not inhibited by cyanide or diethyldithiocarbamate, but is destroyed by chloroform plus ethanol (K7). The molecular weight is approximately 40,000 or 80,000. The enzyme has a pink color and contains Mn at its active site. Under native conditions, human liver Mn-SOD contains Mn(III) (M13). The amino acid sequences of the Mn-SODs from various species, including rat, mouse, and human, as well as from bacteria, are very similar (H14). The Fe-SODs from various bacteria also show high homology to Mn-SOD. Most mammalian Mn-SODs are tetrameric enzymes with molecular weights of approximately 80,000, whereas bacterial enzymes are dimeric enzymes of approximately 40,000 Da.

The three-dimensional structure of Mn-SOD has been reported for bacteria (P5,S15) and human recombinant Mn-SOD (W2). X-Ray analysis of *Bacillus stearothermophilis* Mn-SOD indicated that Mn binds His-26, His-81, His-167, and Asp-163 (P5). Human recombinant Mn-SOD has been crystallized. The crystal has no orthorhombic space group $p2_12_12_1$, and the unit cell parameters have been reported (W1,W2). Human liver Mn-SOD has also been crystallized and has hexagonal space group $p6_122$ or $p6_522$, with cell dimensions different than those of recombinant Mn-SOD (D7).

In mammalian Mn-SODs, His-26, His-74, His-163, and Asp-159 are highly conserved. Tyr-34 is also highly conserved and may play an important role in the catalytic activity. The catalytic sites are rich in aromatic amino acids. The whole sequence of Mn-SOD was determined by a chemical technique (B5) as well as by deduction from the cDNA sequence (H14). Human Mn-SOD is composed of four

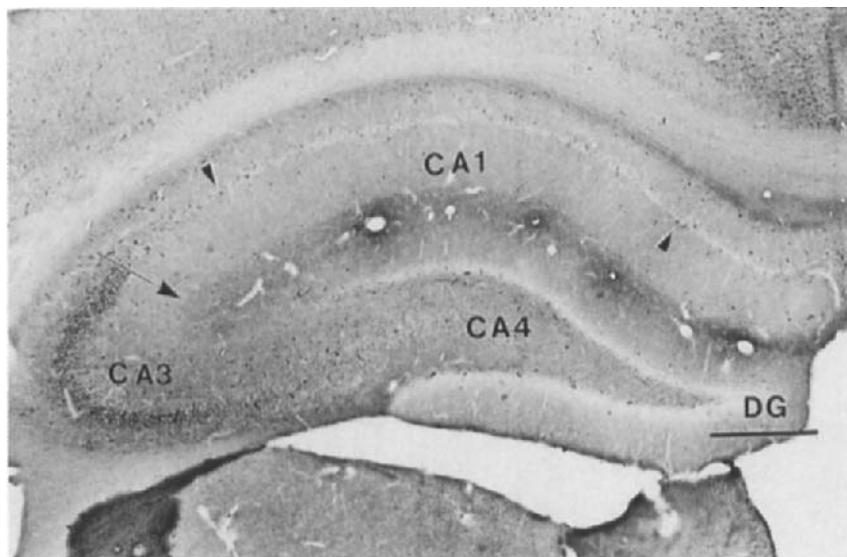


FIG. 11. Light micrographs of the rat hippocampus stained for Mn-SOD (courtesy of Dr. Akai). The arrow indicates the end of CA1 where pyramidal cell bodies are almost devoid of immunoreactivity, in contrast to possible interneurons with strong reactivity (arrowheads). Bar = 50 μ m.

identical subunits. Each monomer molecular weight is 22,000, as judged by the low-laser-angle light-scattering method (M13). Under denaturing conditions the enzyme is easily converted to monomers, which form intradisulfide bonds. Mn-SOD has two cysteine residues. Cys-196, located in the C-terminus, is highly reactive to sulfhydryl reagents (M13). The reaction of Mn-SOD with glutathione *in vitro* brings about mixed disulfide formation and creates an acidic component (M14). Reduction with dithiothreitol converted the acidic variant form to the basic form, indicating that the reaction with glutathione is reversible. The increase of one net negative charge due to addition of a carboxyl group after the reaction of the basic form with glutathione also indicates reversibility (B19,D6,K12, N4,S9,T3). Binding of glutathione to the reactive sulfhydryl group did not affect activity, but may change the conformation of the Mn-SOD molecule. The presence of the reactive sulfhydryl group Cys-196 also suggests that mixed disulfides are formed *in vivo*.

Even though intraperitoneal injection of glucose suppressed Mn-SOD activity in the rat brain and heart (K2), unlike Cu,Zn-SOD, Mn-SOD did not undergo inactivation reaction due to glycation reactions.

3.3. Mn-SOD GENE AND cDNA

The Mn-SOD gene is located on chromosome 6 (M16,X1). Hartman (H5) cloned human genomic Mn-SOD. The gene comprises 15 kb and contains six exons (the sequence of the Mn-SOD gene that is expressed as mRNA) and five introns (the sequence of the Mn-SOD gene that is transcribed but excised before translation). At the exon-intron boundaries the sequences of the donor and acceptor splice junctions are rather unique. The first intron contains either an unusual donor sequence—GG instead of the highly conserved GT—or an unusual acceptor sequence—GG instead of Ag. The promoter region lacks both TATA and CAT boxes. The region is rich in GC and contains eight repeats of the consensus hexanucleotide core for binding transcription factor Sp1 (GGGCGG).

Ho and Crapo (H14) cloned full-length Mn-SOD cDNAs from libraries of human liver and U937 cells and obtained a full primary sequence. The amino acid sequence predicted from the cDNA was almost the same as that obtained from the protein by chemical analysis (B5), except that one Gly and one Tyr were added and Glu replaced Gln residues at positions 42, 88, 109, and 131. The cDNA for human Mn-SOD encoded 222 amino acids. The first 24 amino acids were found to be a leader peptide essential for translocation into mitochondria. The homology between human and rat Mn-SOD was found to be 93% and the homology between human and mouse Mn-SOD was 94%. Moreover, the homology between rat and mouse enzymes was 96%.

3.4. CLINICAL SIGNIFICANCE OF Mn-SOD

It is widely believed that Mn-SOD plays some role in the aging process as well as in carcinogenic processes (D9,O1). However, no direct evidence has been presented linking Mn-SOD to these two processes. Several laboratories found that in cancer tissues or transformed cells, as well as in aged tissues, the activity of SOD decreased or disappeared as compared to that in uninvolved or younger tissues (D2,D9,M10,N1,N2,N3,O1,Y1). In a previous study (I2) we found that the immunoreactive Mn-SOD levels in human lung cancer tissues were increased relative to those in uninvolved tissues in the same patients, whereas the level of the active enzyme did not increase. Thus it seems that the level of immunoreactive enzyme may provide information for the monitoring of cancer tissues.

Very recently we raised three monoclonal antibodies against human liver Mn-SOD. The epitope of one of these antibodies was found to be a COOH-terminal peptide, as judged by competitive inhibition assay using synthetic peptides (K4). Using this antibody we developed an ELISA method and found that the enzyme is also present in human serum. Measurement of the serum immunoreactive Mn-SOD protein levels in various diseases revealed that the enzyme levels are

increased in certain pathological conditions, such as acute myocardial infarction, primary biliary cirrhosis, primary hepatoma, gastric cancer, and acute myeloid leukemia. Mn-SOD levels were also increased in the sera of patients with epithelial-type ovarian cancer.

3.4.1. Purification of Mn-SOD and Its Monoclonal Antibody

Mn-SOD was purified from human liver according to the method described previously (I2). A typical purification procedure for human liver Mn-SOD is shown in Table 3 (M13). A monoclonal antibody, PG 11, was raised in mice against the human liver Mn-SOD. The competition experiments using synthetic peptides revealed that the antibody-binding epitope was localized in the COOH-terminal peptides as described previously (K4).

3.4.2. Enzyme-Linked Immunosorbent Assay for Mn-SOD

An enzyme-linked immunosorbent assay has been developed for human Mn-SOD using the monoclonal antibody PG-11 (K5). As described above, human Mn-SOD is a tetramer composed of identical subunits. Therefore, the same monoclonal antibody could be used in the sandwich immunoassay as both captor and detector; in the case of monomeric enzymes, two different antibodies should be used.

The ELISA offers a specific, sensitive, and convenient means of measuring immunoreactive Mn-SOD in human sera. Under optimum conditions, the sensitivity of the assay permits the detection of 2–200 ng of purified Mn-SOD from human liver, as described below.

3.4.2.1. *ELISA Procedures.* The ELISA was carried out as follows using the monoclonal antibody raised against human liver Mn-SOD. An IgG fraction was obtained from ascitic fluid through 50% ammonium saturation and DEAE–Sephacryl chromatography. The IgG fraction was added to the wells of flat-bottom

TABLE 3
A TYPICAL PROCEDURE FOR PURIFICATION OF Mn-SOD FROM HUMAN LIVER

Step	Volume (ml)	Total protein (mg)	Total activity (U × 10 ⁻⁶)	Specific activity (U/mg)	Fold
Supernatant	9000	289,000	27.2	—	—
DEAE–cellulose	20,000	67,700	8.93	—	—
Hydroxyapatite	210	5230	1.91	369	1
Sephacryl S-300	240	3290	2.49	757	2.1
Chromatofocusing	165	1280	2.10	1640	4.5
Second hydroxyapatite	182	454	2.33	5133	14.0
Red Sepharose	6.6	320	1.96	6100	16.6

polystyrene microtiter plates at a concentration of 1 $\mu\text{g}/100 \mu\text{l}$ of phosphate-buffered saline (PBS), followed by incubation at 4°C overnight. The wells were then washed twice with PBS, filled with 0.1% (w/v) bovine serum albumin in PBS, and then kept at room temperature for 30 minutes. Unbound protein was removed by washing with PBS. Human sera were diluted 10- or 11-fold with PBS containing 0.1% bovine serum albumin, and then 100- μl aliquots were added to the antibody-coated wells. After incubation for 1 hour at room temperature, unbound antigen was removed by washing three times with PBS containing 0.05% Tween 20, and then 100 μl of PG-11 conjugated with horseradish peroxidase (at an IgG concentration of approximately 500 ng/ml) was added to each well. The enzyme was conjugated by sodium metaperiodate (O7). After 1 hour at room temperature, the wells were washed four times with PBS containing 0.05% Tween 20. The substrate for horseradish peroxidase was then added to the wells (100 μl of 0.003% H_2O_2 in 0.1 M sodium citrate buffer, pH 5.0, containing 0.6 mg of *o*-phenylenediamine/ml). The enzymatic reaction was stopped after 15 minutes at room temperature by the addition of 50 μl of 2 N sulfuric acid. The absorbance of the wells was measured at 492 nm with an Immuno-Reader MTP 32 (Corona Electric).

3.4.2.2. *Standard Curve for ELISA of the Mn-SOD in Human Serum.* Mn-SOD purified from human liver was serially diluted and then introduced into the wells of microtiter plates precoated with the Mn-SOD antibody, and then the assay was performed as described above. A typical standard curve for the ELISA using the pure human Mn-SOD and the PG-11 antibody is shown in Fig. 12. The lower limit of detection was 2 ng/ml and the working range was 2–200 ng/ml. Each point represents the mean \pm SD.

The specificity of the ELISA method for Mn-SOD was examined. None of the major proteins found in human sera or erythrocytes (such as human serum albumin; human α -, β -, or γ -globulin; Cu,Zn-SOD; or hemoglobin) showed a reaction with this method. To test the possibility of assaying the enzyme in human sera, three different concentrations of Mn-SOD were added to normal human serum and the effect of dilution on the ELISA was examined. A linear relationship was observed between the immunoreactive Mn-SOD level and the serum dilution in the range of 8- to 128-fold (Fig. 13). The ELISA was performed as described in Section 3.4.2.1, wherein a serum sample is diluted 10- or 11-fold and used for the assay.

3.4.2.3. *Reproducibility and Within-Run Reproducibility of the ELISA.* Repeat assays of a high control sample on six different days showed the following reproducibility. The mean \pm SD was 152 \pm 4.6 ng/ml. The coefficient of variation was 3%. The within-run reproducibility was examined at two different concentrations of Mn-SOD, and the coefficient of variation was found to be less than 5%.

The stability of Mn-SOD in serum was examined. Storage of a serum sample at 4°C for 5 days had no effect on the ELISA value. Freezing and thawing of the serum sample up to five times did not cause any change in the value obtained by

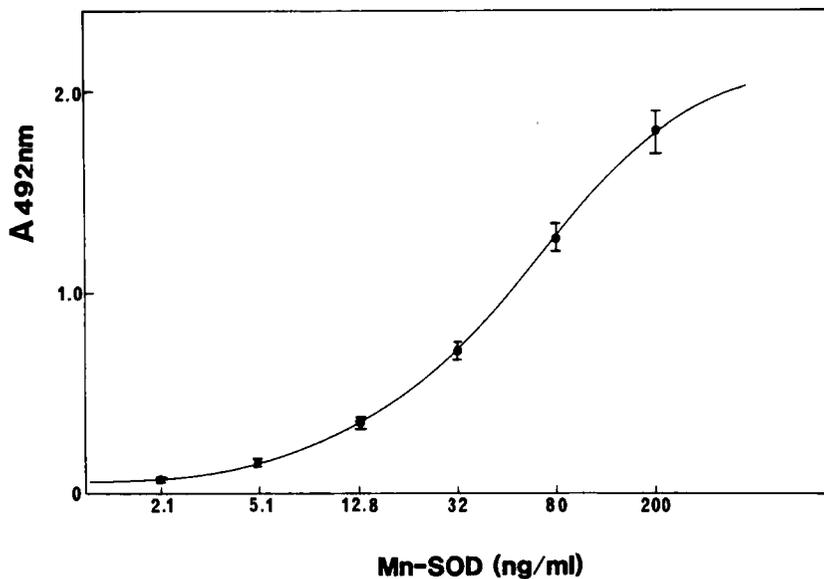


FIG. 12. A typical standard curve obtained with the ELISA for Mn-SOD.

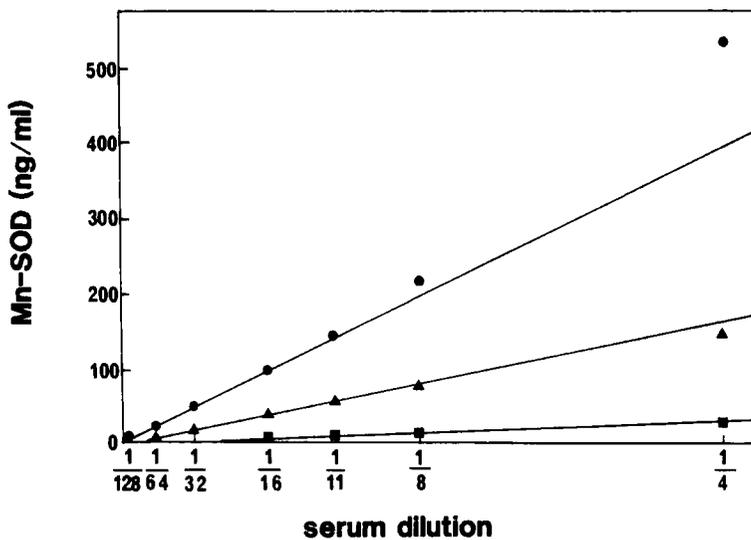


FIG. 13. Effect of serum dilution on the ELISA. The sera from three different individuals were diluted 8- to 128-fold, and aliquots of the dilutions were subjected to ELISA.

ELISA. Incubation of the human serum at room temperature for 1–5 hours also caused no change. Overall, the immunoreactive Mn-SOD in the serum was found to be stable.

The effect of hemolysis on the determination of Mn-SOD levels by ELISA was examined. The lysis of erythrocytes was observed to have no effect. Because erythrocytes do not contain any Mn-SOD, the problems encountered with Cu,Zn-SOD do not occur when serum Mn-SOD levels are measured. The effects of jaundice and lipids were also examined by adding substances such as ascorbic acid, bilirubin, cholesterol, phosphatidylcholine, and triglycerides. None of the above substances affected the detection of Mn-SOD in sera.

3.4.3. *Mn-SOD Levels in Normal, Healthy Controls*

The levels of Mn-SOD in sera from 194 male and 207 female healthy adult individuals were examined by ELISA. As shown in Fig. 14, the frequency distribution of serum Mn-SOD levels for the normal adult male was found to follow a normal distribution pattern. The distribution for the normal female adult was found to be slightly skewed, but the plotting of the cumulative frequency using normal probability paper gave a near-straight line.

The mean level and SD for male and female were $99.8 \text{ ng/ml} \pm 24.8$ and $88.8 \pm 20.8 \text{ ng/ml}$, respectively (Table 4). Assuming the upper limit of the normal male to be 150 ng/ml (equivalent to the mean value for normal male subjects plus 2 SD), the percentage of false positives was 2.1%. Similarly, assuming the upper limit of the normal female to be 130 ng/ml, the percentage of false positives was 1.0%. In children, the serum Mn-SOD levels are slightly lower than in adults and gradually increase in proportion to age. By 10 years of age the Mn-SOD levels are nearly at the adult level, as shown in Fig. 15. In various diseases, including cancer, Mn-SOD levels are relatively high (Fig. 16).

3.4.4. *Serum Mn-SOD Levels in Acute Myocardial Infarction*

A great deal of interest has developed in the role of SOD modifying the toxic effects of O_2^- arising in cardiac tissue during reperfusion following an ischemic episode (F3,F5,H3,M22). Much of the interest has centered on the role of the widely distributed Mn-SOD. The intravenous administration of SOD appears to be effective in reducing the size of experimentally induced infarct (A4,B21,J4,W7). There is still considerable controversy, however, regarding the salvage effects of SOD in the myocardium (G1,U1).

Serum Mn-SOD levels were determined in 29 patients with acute myocardial infarction by an ELISA using a monoclonal antibody (S20). Figure 17 shows typical changes in serum Mn-SOD in two patients following acute myocardial infarction. Case A is an example of a successful reperfusion of the infarcted vessel at an acute stage, and case B is a case without reperfusion. In both instances a biphasic elevation of Mn-SOD is noted—a small early one that is slightly higher

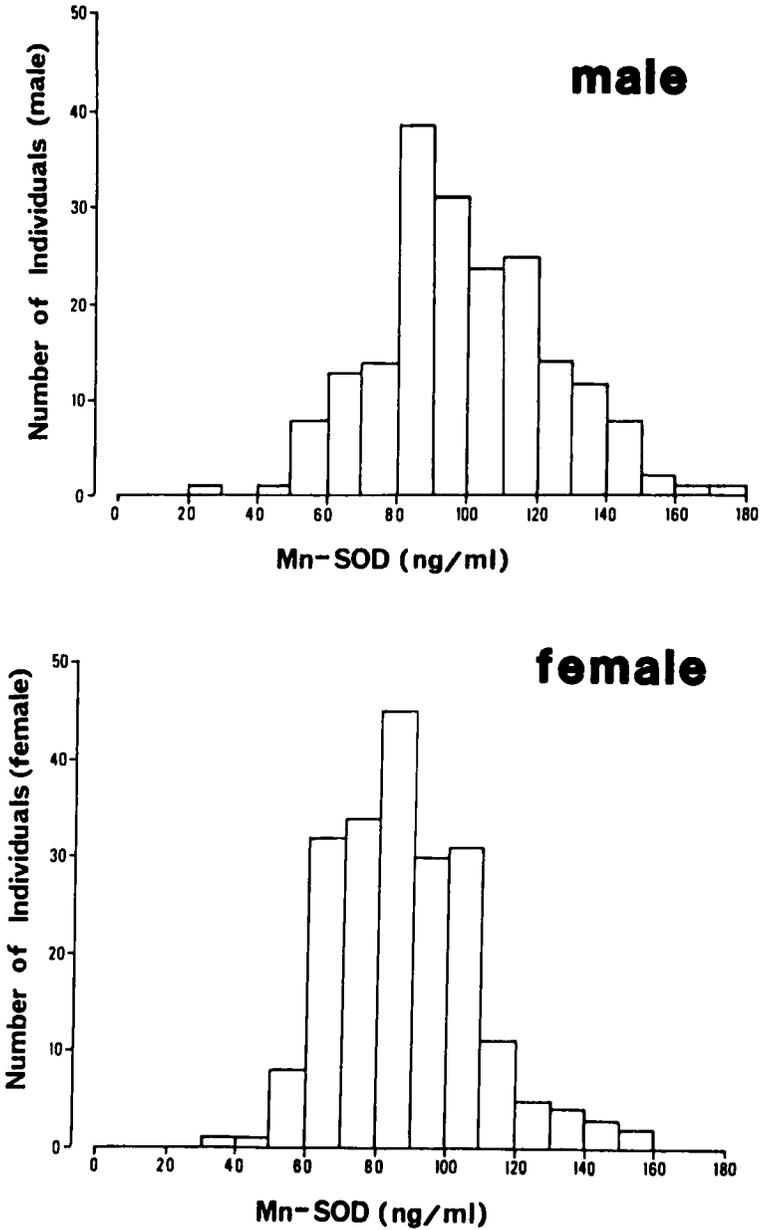


FIG. 14. Distribution of serum Mn-SOD for normal male and female adults, determined by ELISA.

TABLE 4
Mn-SOD VALUES IN HEALTHY CONTROLS

Group	Total no. tested	Mean ± SD (ng/ml)	Range (ng/ml)
Male	195	99.8 ± 24.8	47.2-141.0
Female	207	88.8 ± 20.8	50.1-149.4
Total	402	94.1 ± 23.5	47.2-141.0

than the levels seen in normal, healthy controls and a later phase elevation that is typically much larger. The initial rise follows a pattern similar to that of phosphocreatinine kinase (CK) (W1), whereas the later phase elevation occurs much later than increases in other enzymes.

The results of serial determinations of serum Mn-SOD for the 29 patients are shown in Fig. 18. Figure 18A shows results for 23 reperfused patients, whereas Fig. 18B depicts six cases without reperfusion. In four of the latter patients either intracoronary thrombolysis or percutaneous transluminal coronary angioplasty was unsuccessfully employed. In two cases, reocclusion occurred after reperfusion. This was confirmed later by coronary angiography during the convalescent

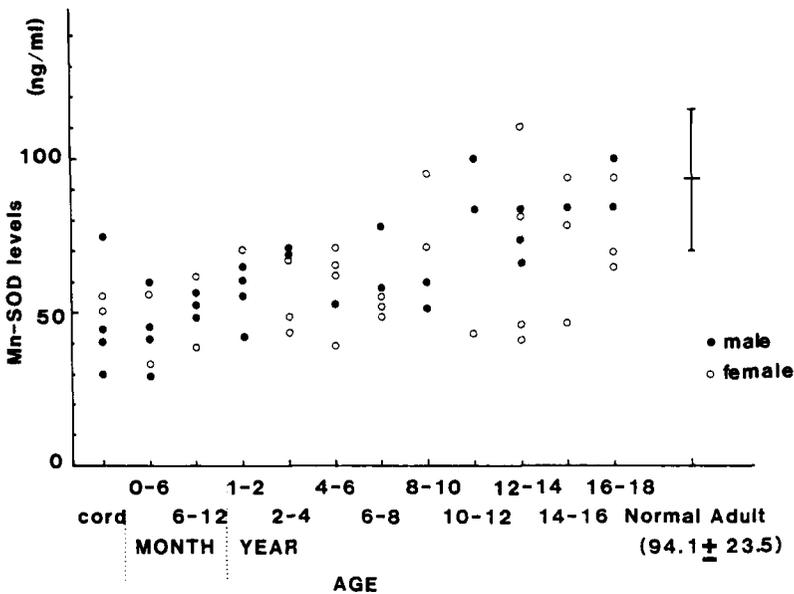


FIG. 15. Developmental changes of human serum Mn-SOD.

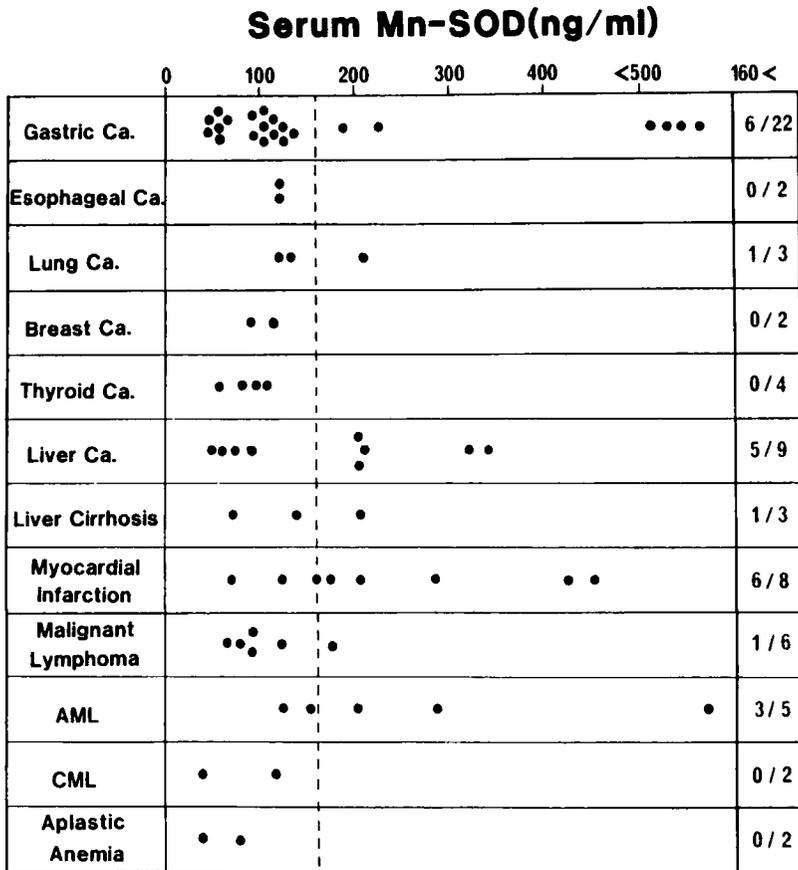


FIG. 16. Immunoreactive Mn-SOD contents of sera from patients with various diseases, determined by the ELISA. The vertical dashed line denotes the normal upper limit for serum immunoreactive Mn-SOD, taken as 150 ng/ml (mean + 2 SD for 194 normal male subjects). Abbreviations: Ca, cancer; AML, acute myeloid leukemia; CML, chronic myeloid leukemia. Each group included both male and female adult subjects.

stage. In most of these cases, irrespective of whether reperfusion was successful, two elevations of Mn-SOD were observed. The maximum levels (\pm SD) of serum Mn-SOD for the early and later stage elevations were 164 ± 84 and 248 ± 103 ng/ml, respectively. The time of appearance of the early elevation was 16.2 ± 7.3 hours, and the later elevation was at 108 ± 20.6 hours. No significant correlation was found between the peak level of CK and the maximum level of the late elevation of Mn-SOD ($r = 0.26$). This indicates that different mechanisms are operating for the release of these two enzymes.

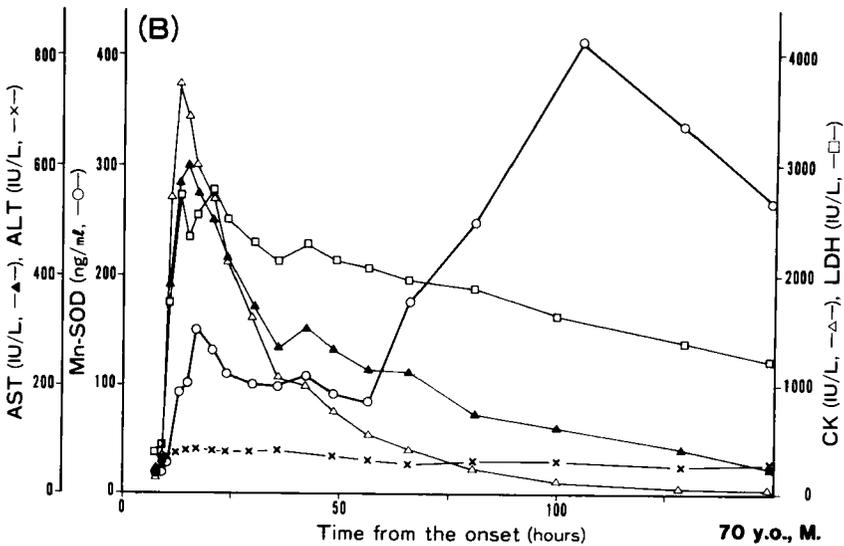
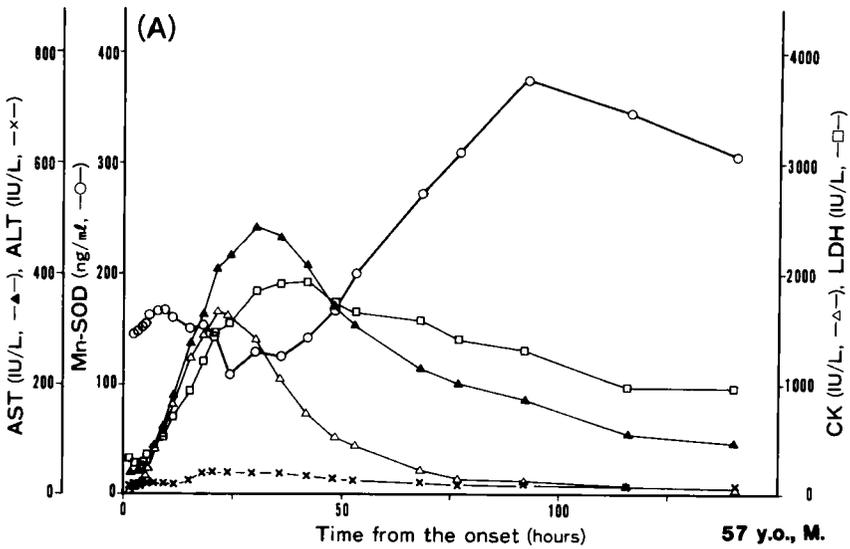


FIG. 17. Typical patterns of Mn-SOD release into serum for two male patients with acute myocardial infarction. (A) A case with an unsuccessful reperfusion; (B) successful case. The mean level for normal male adults is almost 100 ng/ml. Other enzymes: AST, L-aspartate:2-oxoglutarate; ALT, L-alanine:2-oxoglutarate; CK, creatine kinase; LDH, lactate dehydrogenase.

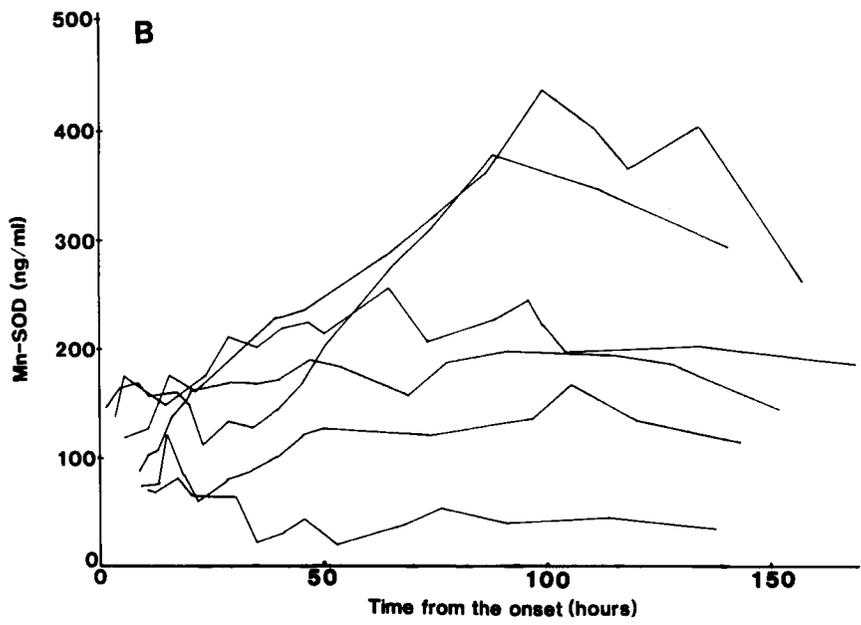
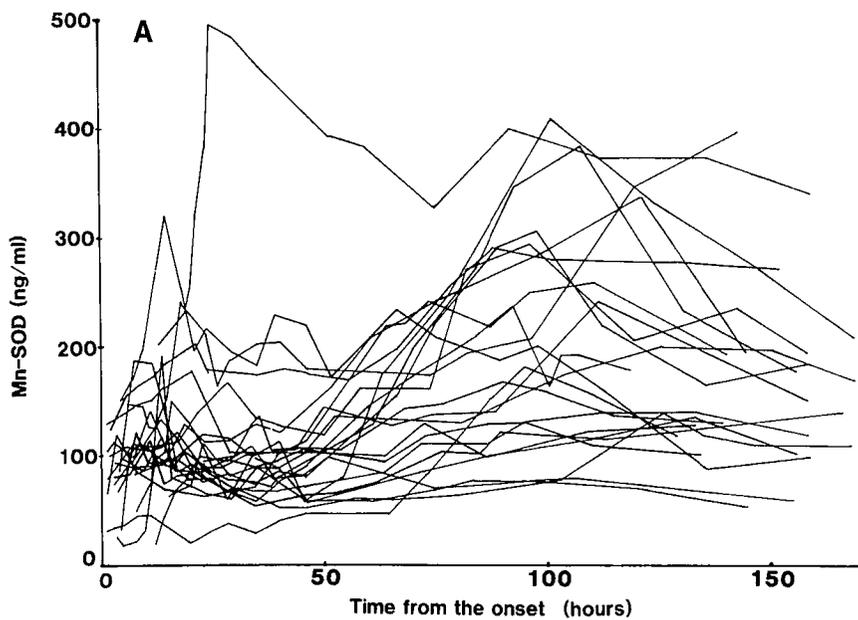


FIG. 18. Serial determinations of serum Mn-SOD in 29 patients with acute myocardial infarction: (A) 23 cases with successful and (B) 6 cases with unsuccessful reperfusion.

Reperfusion did not affect the time required for the late elevation to occur, but the procedure shortened the time for appearance of the early rise. The appearance of the early elevation correlated with the time at which reperfusion was carried out.

3.3.4.1. *The Mechanism of Mn-SOD Release into Serum in Patients with Acute Myocardial Infarction.* Figure 19 shows the difference in serum Mn-SOD levels between samples taken from the ascending portion of the aorta and samples taken from the coronary sinus immediately following reperfusion in four patients with anterior myocardial infarction. In all cases the Mn-SOD levels were higher in the coronary sinus than in the aorta. This suggests that the noted elevations of serum Mn-SOD originated in cardiac tissue.

In order to verify that the Mn-SOD was not released as the result of coronary angiography, serum levels in eight patients with angina pectoris who had undergone coronary angiography were determined. No elevations were found, which indicates that the Mn-SOD release was not due to the angiography.

Immunoelectron microscopy of cardiac muscle revealed that immunogold was deposited on the mitochondria among the myofibrils as well as on the mitochondria beneath the sarcoplasmic membrane (Fig. 20, upper panel). Some immunogold was also seen on the secondary lysosomes and on lipofuscin, which contained heterogeneous substances and showed irregular shapes. In addition, the mitochondria of the endothelial cells of the blood capillaries reacted with this antibody (Fig. 20, lower panel).

Histochemical studies demonstrated that Mn-SOD is localized in the mitochondria of the myocardium, suggesting that it is released from this organelle. However, care must be taken to determine the source of the serum Mn-SOD. The possible role of the liver must be considered, because it is relatively rich in this enzyme (D7,M13). Patients with acute hepatitis that is accompanied by significant necrosis show increased serum levels of Mn-SOD (K6). These elevations, how-

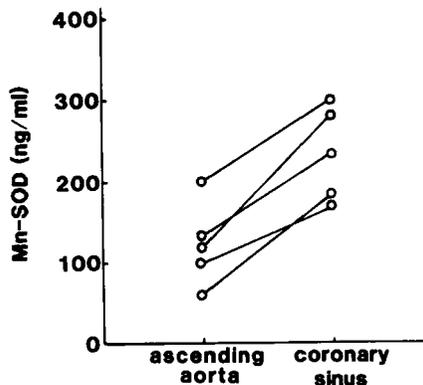


FIG. 19. Difference in serum Mn-SOD levels in aortic and coronary sinus blood in acute anterior myocardial infarction.

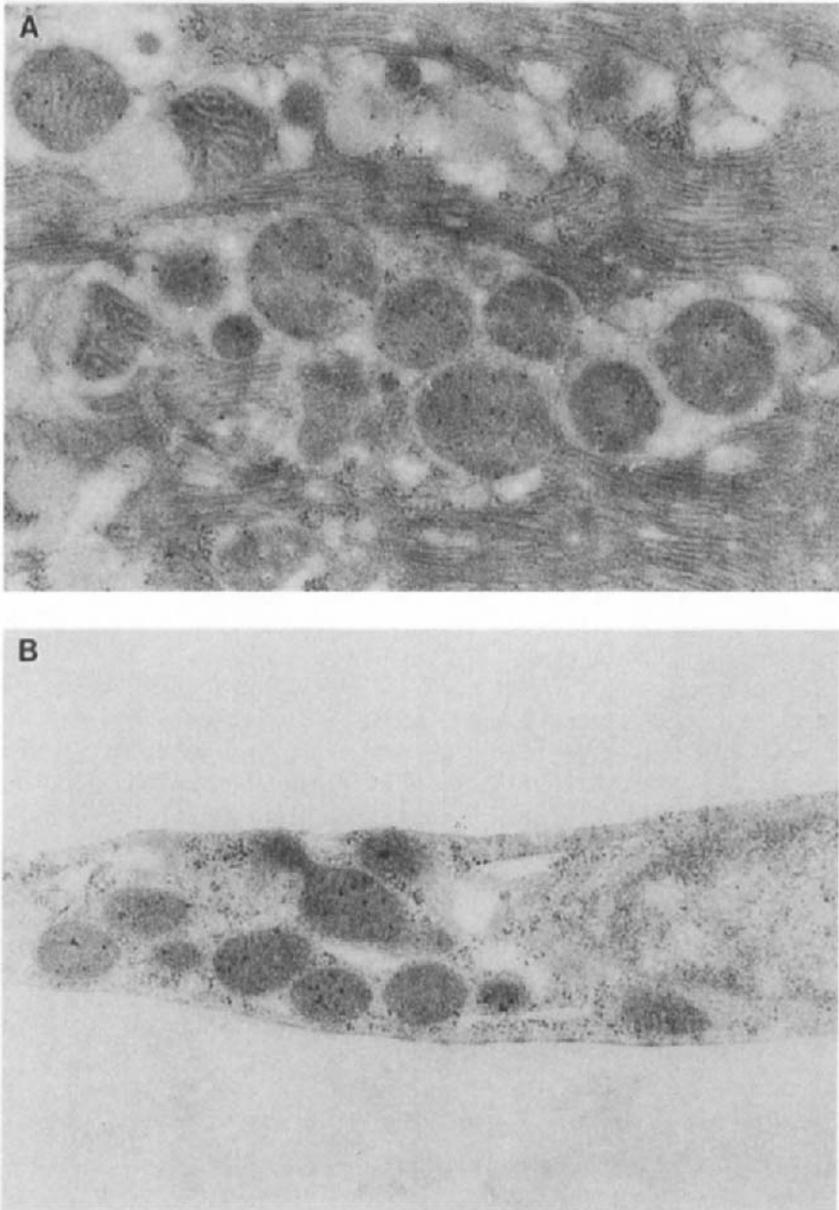


FIG. 20. Upper panel: immunoelectron microscopy of cardiac muscle treated with monoclonal antihuman Mn-SOD IgG. Among the myofibrils are many mitochondria, upon which immunogold is deposited. Lower panel: immunoelectron microscopy of endothelial cells of blood capillaries in cardiac tissue treated with anti-Mn-SOD IgG. Immunogold is seen on the mitochondria.

ever, are in proportion to increases in L-alanine:2-oxoglutarate aminotransferase (ALT) and L-aspartate:2-oxoglutarate aminotransferase (AST). None of the 29 patients with acute myocardial infarction showed increased levels of ALT. Furthermore, serum levels of Mn-SOD in the coronary sinus were higher than those in the ascending aorta. Patients with congestive heart failure showed no increase in serum Mn-SOD. Taken together, these results strongly suggest that Mn-SOD is released from myocardial tissues. In order to determine whether serum Mn-SOD originates in skeletal muscle, we examined the enzyme levels in sera of 21 patients with Duchenne-type muscular dystrophy and found that the mean value was 78 ± 30.5 ng/ml, with a range from 32 to 173 ng/ml. No correlation was observed between serum Mn-SOD and CK values in those patients (H. Ohno *et al.*, unpublished data). This indicates that Mn-SOD, unlike CK, is not likely released from muscle.

No significant difference in the degree of elevation of Mn-SOD was found between groups with successful and unsuccessful reperfusion. However, the early elevation occurred slightly later in the unsuccessful cases, compared to the successful cases. This indicates that the early elevation of Mn-SOD is affected slightly by reperfusion, as is observed for CK. The time of appearance of the late elevation of Mn-SOD in these patients did not change with reperfusion. The secondary elevation of Mn-SOD is, therefore, a more reliable marker for the assessment of ischemic myocardial damage.

As mentioned above, the late elevation of the Mn-SOD level was observed approximately 108 hours after the onset of infarction, which is later than the elevation of other proteins, such as myosin light chain (N1), lactic dehydrogenase isozyme (C7), and mitochondrial AST (I3). It is interesting to note that even though the Mn-SOD is released relatively late, its maximum value correlates with the values of the left ventricular ejection fraction, which reflects the left ventricular function (S20). The mechanism by which the Mn-SOD is released from heart mitochondria is not known. The slight early elevation may originate from endothelial cells and the late one from myocytes. If the second elevation of Mn-SOD were due to mitochondrial breakdown accompanying cell injury, it would seem that other mitochondrial enzymes, such as mitochondrial AST (mAST), which has about the same molecular weight as Mn-SOD, would be released simultaneously. The half-life of serum Mn-SOD has been reported to be only 6 hours (H5). The absence of mAST suggests that the late elevation of Mn-SOD relates to a specific mechanism for its release.

One possibility is that increased synthesis is responsible for the late elevation of Mn-SOD. Recently two groups have independently reported that the mRNA of Mn-SOD is specifically induced by interleukin-1 (IL-1) or tumor necrosis factor (TNF) (M12, W10). In myocardial infarction, neutrophils and macrophages could move to the necrotized tissues following the early elevation and thus release the above cytokines. This could induce Mn-SOD synthesis in mitochondria, from

which the enzyme could be released due to cell damage at the later phase. In such an induction hypothesis, ischemic tissues would be thought of as inflammatory foci.

There are several factors affecting recovery of the infarcted myocardium. The extent of ischemia is related to the time between the occurrence of the infarct and reperfusion, and this will determine the extent of cell injury. The adherence of blood cells to endothelium is a central reaction in the development of inflammatory reactions (H4). In the ischemic myocardium, adhesion of neutrophils to the endothelial cells is observed, which is typical of initiation of an inflammatory process. It is well known that TNF sensitizes neutrophils and macrophages to agents that induce these cells to secrete reactive oxygen species (K11). TNF has also been reported to cause neutrophils to adhere to vascular endothelium (G2). These facts again support the possibility that the late elevation of Mn-SOD reflects the extent of the inflammatory process in the myocardium. It would be interesting to know whether IL-1 or TNF is produced in the ischemia myocardium.

Very recently Visner *et al.* (V3) reported that induction of Mn-SOD by IL-1, TNF, and lipopolysaccharide occurred in pulmonary epithelial cells. Mn-SOD in cultured myocytes was also induced by the addition of TNF. Furthermore, injection of human recombinant TNF into rats resulted in an increase in Mn-SOD in the rat heart (K. Suzuki *et al.*, unpublished data). In this study, immunoreactive Mn-SOD in the rat heart was assayed by ELISA 24 hours after TNF injection. More interestingly, TNF injection led to a decrease in the release of CK both in cultured myocytes and *in vivo* (K. Suzuki *et al.*, unpublished data), which strongly suggests that the TNF induced the synthesis of endogenous Mn-SOD in the cardiac tissues. The localization of Mn-SOD in the mitochondria of both myocytes and endothelium could play a role in protection against O_2^- produced during reperfusion following ischemia. If an induction of Mn-SOD synthesis actually occurs, myocytes could be protected by endogenous Mn-SOD induced by cytokines. Such an approach might be useful in protecting various cells from reactive oxygen radicals not only in ischemia reperfusion injury, but in aging, carcinogenesis, and other disease processes. In fact, hearts isolated from rats treated 36 hours before IL-1 treatment were found to decrease hydrogen peroxide levels and injury after ischemia reperfusion injury (B20).

3.4.5. Mn-SOD Levels in Primary Hepatoma and Primary Biliary Cirrhosis

Approximately 60% of the patients with primary hepatoma gave positive values for Mn-SOD in the ELISA. Because serum Mn-SOD levels were also elevated in various other diseases, including gastric cancer and primary biliary cirrhosis, however, whether the immunoreactive Mn-SOD can be used as a marker for the diagnosis and monitoring of primary hepatoma remains to be clarified.

Interestingly, 30 out of 31 patients with primary biliary cirrhosis had increased levels of serum Mn-SOD. Mn-SOD levels at early stages of the disease were found

to be higher than those at late stages. Primary biliary cirrhosis is an idiopathic liver disease characterized by spontaneous destruction of interlobular bile ducts (J3,R7). The disorder is considered to be the result of an altered autoimmune response mediated by T cells or, although less likely, by disease-specific autoantibodies against mitochondrial enzyme complexes (B9,C8,G8). The mechanism by which Mn-SOD is expressed in the early stage of the disease remains unclear. Cytokines such as IL-1 and TNF may be expressed in this tissue, and these cytokines may stimulate the expression of Mn-SOD. In any case the early elevation of serum Mn-SOD in primary biliary cirrhosis is a very interesting phenomenon.

3.4.6. *Mn-SOD in Leukemias*

We also found that 9 out of 15 patients with acute myeloid leukemia and 4 out of 15 patients with acute lymphocytic leukemia had increased levels of serum Mn-SOD, whereas no increased value was observed in patients with chronic myeloid or chronic lymphocytic leukemias. This suggests that acute leukemia cells, especially myeloid cells, synthesize the Mn-SOD. The mechanism by which the level of serum Mn-SOD increases is currently under investigation in our laboratory. As described above, the mRNA for Mn-SOD is induced by IL-1 or TNF. TNF has been reported to be expressed in human peripheral blood mononuclear cells (K3). Moreover, IL-1 has been reported to act as an autocrine factor in the acute myeloid cells (C10) and hairy cell leukemia (C9). This suggests that the synthesis of IL-1 in acute myeloid cells induces Mn-SOD in the cells, and this is reflected in high levels of serum Mn-SOD in patients with acute myeloid leukemia.

3.4.7. *Serum Mn-SOD in Epithelial Ovarian Cancer and Other Gynecological Malignancies*

A difficult problem often encountered by gynecologists is in differentiating malignant tumors from benign ones in patients with pelvic masses. A monoclonal antibody (OC 125) reactive against an ovarian carcinoma antigen (CA 125) was prepared by Bast *et al.* (B7). The use of the antibody to estimate serum immunoreactive CA 125 levels has become a relatively effective method for evaluation of such malignancies (B7). Approximately two-thirds of patients with adenocarcinoma of the ovary have elevated serum levels of this antigen (B7,C1).

Because of the insidious onset and progression of ovarian cancer, an early diagnosis is very difficult. Moreover, accurate monitoring of tumor status is also difficult because patients are often in clinical remission when subclinical disease is present.

We used ELISA with a monoclonal antibody against human Mn-SOD to investigate the possibility of using this enzyme as a marker for epithelial ovarian carcinoma. Serum samples were taken within 1 week before surgery or radiation therapy from 119 patients with pelvic masses and gynecological malignancies,

which included 21 benign masses, 2 borderline epithelial ovarian tumors, and 33 ovarian carcinomas, and from 63 patients with other gynecological tumors. Fresh blood (1 ml) was collected by venipuncture; samples were randomly numbered and immediately sent to the laboratory for these studies. Clinical diagnoses were not known prior to assay, and patients' diagnoses were not matched with test code numbers until the assay was complete. Diagnoses were confirmed by review of operative and pathology reports. Judgments of disease progression or regression were based on objective intraoperative observation of tumor modules, dimensions of metastases on chest X-ray, or abdominal computed tomography. Acceptance of regression of disease required a greater than 50% reduction in the size of detectable lesions. Disease progression required the appearance of new lesions or a 25% increase in the largest dimension of previously detected tumor nodules.

In our series of 308 patients, 158 proved to have invasive pelvic neoplasms. As described in Section 3,4,3, fewer than 1.4% of healthy adult females have serum Mn-SOD levels above 130 ng/ml. Only 7 of the 39 patients (17.9%) with benign ovarian tumors had Mn-SOD levels exceeding 130 ng/ml. In the nonovarian gynecological malignancies group, 9 out of 40 patients (22.5%) with uterine cervical cancer and 8 of 40 patients (20.0%) with endometrial cancer had Mn-SOD levels above 130 ng/ml (Table 5).

Of 74 patients with malignant ovarian tumors, 34 had epithelial and 12 had nonepithelial carcinomas. When a serum Mn-SOD value greater than 130 ng/ml was utilized as the diagnostic criterion, the positive rate was 59.7% for patients with epithelial ovarian carcinomas and 0% for patients with nonepithelial carci-

TABLE 5
POSITIVITY RATE FOR Mn-SOD IN THE SERUM OF PATIENTS WITH BENIGN
OVARIAN TUMORS AND OTHER GYNECOLOGICAL MALIGNANCIES OF THE
OVARY

Type	Total no. tested	Number (%) of positive cases above 130 ng/ml
Ovarian benign tumors	40	5 (13%)
Endometriosis	68	8 (12%)
Uterine tumors		
Hydantiform mole	2	0
Uterine myoma	54	4 (7%)
Other malignant tumors		
Uterine cervical cancer	42	8 (19%)
Endometrial cancer	41	9 (22%)
Choriocarcinoma	2	0
Vulvar cancer	3	0
Vaginal cancer	1	0

TABLE 6
POSITIVITY RATE FOR SERUM Mn-SOD IN PATIENTS WITH BORDERLINE AND
OVARIAN CARCINOMAS

Type	Total no. tested	Number (%) of positive cases above 150 ng/ml
Epithelial carcinomas	63	39 (62%)
Serous cystadenocarcinoma	33	22 (67%)
Clear cell adenocarcinoma	11	6 (55%)
Endometrioid adenocarcinoma	12	9 (75%)
Mucinous cystadenocarcinoma	7	2 (29%)
Germ-cell tumors	4	0
Sex cord stromal tumors	2	0
Metastatic tumors	4	0
Mucinous tumor of borderline malignancy	2	0

nomas (Table 6). Serum Mn-SOD levels in preoperative patients with ovarian cancer and with borderline malignancy of the ovary are shown in Fig. 21). The mean value of serum Mn-SOD in patients with epithelial carcinomas was 194.8 ng/ml, compared with 92.4 ng/ml in patients with nonepithelial carcinomas. Statistical analysis showed a significant difference ($p = 0.01$) between these two groups (Table 7).

Among the 40 patients with epithelial ovarian carcinomas, the Mn-SOD assay showed a significant difference ($p = 0.05$) between those with stage I (33.3%) and those with other stages of the disease (71.4%) (Table 8).

The correlation between serum Mn-SOD and CA 125 was examined for the 62 patients with epithelial ovarian carcinoma. Serum levels of the Mn-SOD and CA 125 were assayed simultaneously. For CA 125, serum levels over 35 U/ml were taken to be positive. The positive rate of Mn-SOD was 58.5% (20/34) and that of

TABLE 7
CORRELATION BETWEEN SERUM Mn-SOD AND TUMOR TYPE

Type	Total no. tested	Mean value (ng/ml)	p^a
Epithelial	62	194.8	0.01
Nonepithelial	10	92.4	

^aCalculated using Wilcoxon's rank sum test.

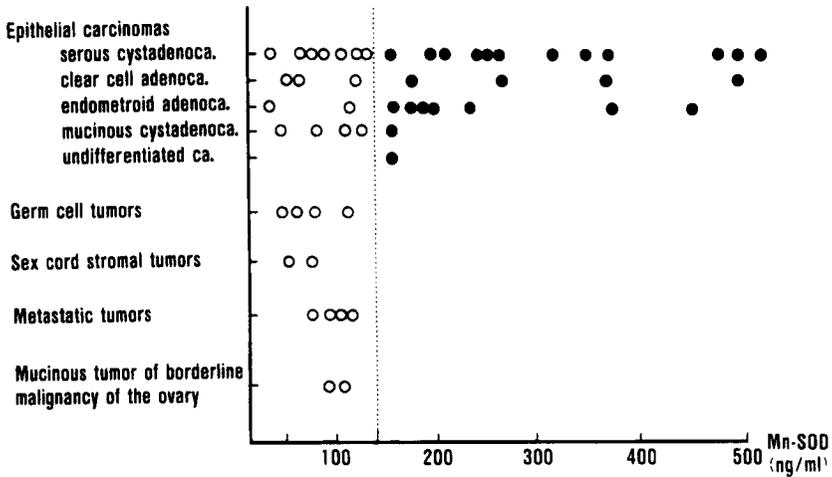


FIG. 21. Preoperative serum Mn-SOD levels in patients with borderline and malignant tumors of the ovary (Ca, carcinoma).

CA 125 was 82.4% (28/34). Positive Mn-SOD values were found in 6 of 18 CA 125-negative epithelial ovarian carcinomas. No significant correlation was noted between serum Mn-SOD and CA 125 levels in epithelial ovarian cancer patients ($r = 0.39$).

Changes in enzyme levels were compared to variations in tumor mass (Fig. 22). Serum specimens from 21 patients with epithelial ovarian carcinomas, taken from 2 to 18 times over 2 to 36 months, showed Mn-SOD levels from 49 to 1925 ng/ml. During tumor regression, Mn-SOD values exceeded 150 ng/ml in one patient and fell below 150 ng/ml in 8 patients. A rising Mn-SOD level or maintenance of a level over 150 ng/ml was associated with disease progression in 8 of 10 cases.

TABLE 8
CORRELATION BETWEEN SERUM Mn-SOD LEVELS AND
CLINICAL STAGE OF EPITHELIAL OVARIAN CARCINOMAS

Stage	Total no. tested	Number above 150 ng/ml
I	16	3 (38%)
II	4	3 (75%)
III	23	16 (70%)
IV	10	8 (80%)
Recurrence	10	6 (60%)

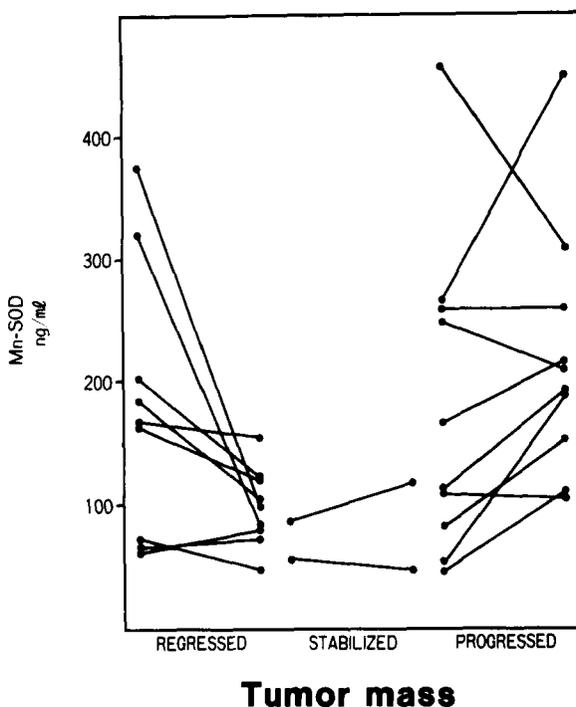


FIG. 22. Mn-SOD levels before and after treatment in patients whose diseases regressed or progressed. Regression or progression of disease was judged according to standard criteria outlined in the text.

In one patient with serious cystadenocarcinoma, stage IIb, it was possible to monitor Mn-SOD levels on nine occasions over 30 months (Fig. 23). After surgical cytoreduction and chemotherapy with a combination of cyclophosphamide, adriamycin, and cisplatin, i.e., CAP, Mn-SOD levels decreased from 266 to 66 ng/ml. Laparotomy failed to reveal residual tumor, and treatment was then continued with cisplatin. After 2 years the Mn-SOD level rose to 166.5 ng/ml, with CA 125 rising to 150 U/ml. At this time, abdominal computerized tomography (CT) revealed a small pelvic mass and ascites. Thus, increases in Mn-SOD and CA 125 were observed upon recurrence of disease.

Immunohistochemical studies of Mn-SOD were done on ovarian cancer tissues. Of four ovarian serous cystadenocarcinoma tissues tested, two stained positively with the antibody used for the ELISA experiments. Antibody localization of Mn-SOD in the tissue is illustrated in Fig. 24. Control sections incubated with a monoclonal antihuman IgG and then stained with fluorescein-conjugated horse antimouse immunoglobulin failed to show uptake of antibody. No reactivity with

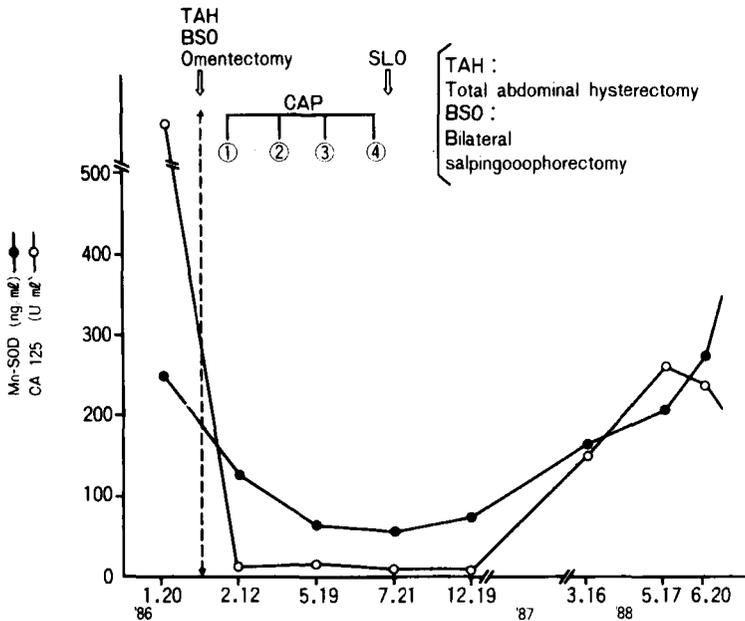


Fig. 23. Serum Mn-SOD levels during the course of treatment of one patient with stage IIb serous cystadenocarcinoma (CAP, cyclophosphamide, adriamycin, and cisplatin treatments; SLO, second-look operation).

the anti-Mn-SOD antibody could be detected in any of the normal tissues tested, including ovary and uterus, nor did any of 12 other gynecological carcinomas react. In rats, experimental serous cystadenocarcinomas induced by a carcinogen, dimethylbenz[*a*]anthracene (N7), were also found to have increased Mn-SOD (T. Nakata *et al.*, unpublished data).

3.4.7.1. *Prospects for Use of Mn-SOD as a Marker for Epithelial Ovarian Cancer.* Bast *et al.* first suggested that serum CA 125 values greater than 35 U/ml should be defined as positive criteria for epithelial ovarian cancer (B7,B8). Based on this criterion, CA 125 positivity rates for this malignancy ranged from 73 to 92.5%, whereas in the present study the rate of CA 125 positivity was 65%. However, positive values for serum CA 125 have also been reported in patients with various benign pelvic masses (D8,N5). The positivity rates in these cases were 10% in patients with benign epithelial ovarian tumors, 9.5% in cases with pelvic inflammatory masses, 44% for leiomyomas, and 37.5% for ovarian endometriosis. In this respect, serum Mn-SOD measurements will be of clinical use because the level of the enzyme is low in gynecological malignancies other than epithelial ovarian cancer, and because benign ovarian tumors do not show significant amounts.

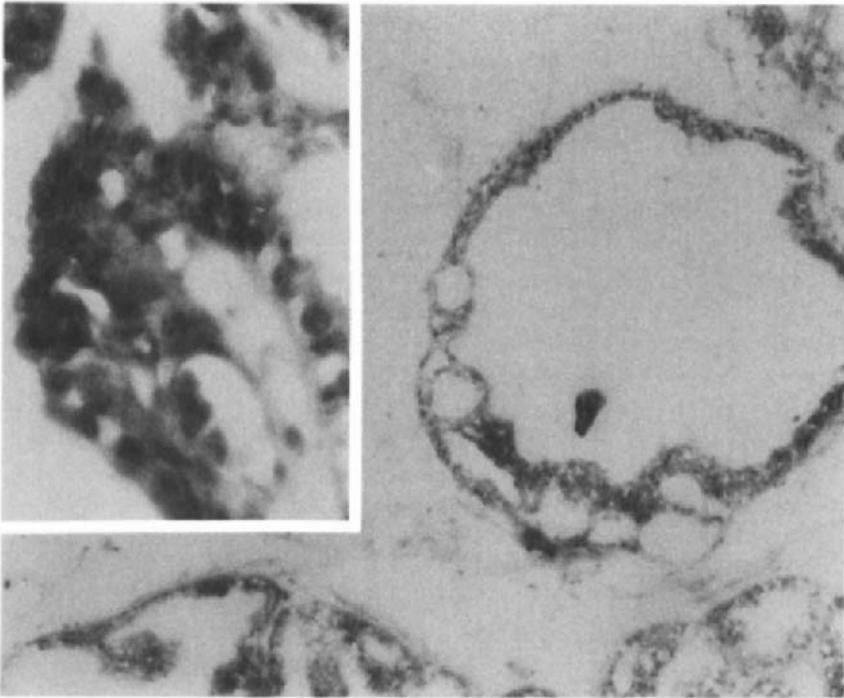


FIG. 24. A section of serous cystadenocarcinoma, stained with monoclonal antibody to Mn-SOD. Bright staining is seen in the cytoplasm of the carcinoma cells. Left panel, $\times 1000$ magnification; right panel, $\times 100$ magnification.

In patients with epithelial ovarian carcinomas, serum Mn-SOD levels increased in accordance with the clinical progression of disease. At the early stage, however, the incidence of cases with elevated serum levels was low. Therefore measurements of serum Mn-SOD may not always be useful for the early diagnosis of epithelial ovarian carcinoma. The decline in serum Mn-SOD levels following affective therapy seems to reflect the disappearance of lesions. Decreases occurred after therapy and increases occurred with recurrences. Thus it appears that the measurement of this enzyme in serum can provide useful data for monitoring epithelial ovarian cancer following therapy, and for the early diagnosis of recurrences of the disease.

3.5. EXPRESSION OF Mn-SOD *in Vitro* IN RESPONSE TO TNF AND TPA TREATMENTS

As described above, TNF or IL-1 specifically induces mRNA for Mn-SOD and this effect is blocked by actinomycin D but not cyclohexamide, indicating that the increase in Mn-SOD mRNA results from an increase in transcription of the

Mn-SOD gene (M12,M15,W10). Lipopolysaccharide also induces Mn-SOD mRNA in pulmonary epithelial cells by a similar mechanism (V3). We showed that phorbol 12-myristate 13-acetate (TPA), a potent tumor promoter and protein kinase C activator, also induced Mn-SOD expression only in TNF-resistant cell lines and presented two hypothetical signal transducing pathways in this gene expression (F10). The importance of Mn-SOD for cellular resistance to TNF cytotoxicity has been reported (A11,I5,W11). However no data were available regarding the levels of Mn-SOD protein after TNF treatments.

The development of a monoclonal antibody and the ELISA technique made it possible to determine quantitatively Mn-SOD protein levels. The ELISA was used to study changes in the immunoreactive Mn-SOD levels in TNF-resistant and -sensitive human cell lines in response to TNF.

3.5.1. *Effect of TNF on the Levels of Mn-SOD and Cu,Zn-SOD in TNF-Resistant Cells*

The effect of TNF on the expression of Mn-SOD protein in TNF-resistant cells was examined. In the case of WI-38 (fetal lung cells), Mn-SOD protein levels increased dramatically, approximately 80-fold, after TNF treatment (Fig. 25). On the other hand, TNF treatment did not cause any changes in Cu,Zn-SOD expression in most of the TNF-sensitive cells (K6).

As described above, Mn-SOD is highly expressed in epithelial-type ovarian cancer (I4) and is one of the best marker proteins for this cancer. For this reason, we investigated the effect of TNF on the induction of the Mn-SOD level prior to the effect of TNF on the induction of Mn-SOD in a cell line, Kuramochi, derived from adenocarcinoma of the ovary. In the case of Kuramochi cells, the Mn-SOD level prior to treatment with TNF was 110 ng/mg protein. Addition of TNF to achieve concentrations of 10^{-6} , 10^{-5} , and 10^{-4} mg/ml resulted in dose-dependent increases in Mn-SOD levels to 180, 270, and 360 ng/mg protein, respectively (Fig. 26). On the other hand, even at 10^{-4} mg/ml, TNF did not induce Cu,Zn-SOD protein in the Kuramochi cells.

These results again indicate that TNF specifically induces Mn-SOD protein but not Cu,Zn-SOD protein. Induction of Mn-SOD was also seen in A549 cells, a human adenocarcinoma cell line.

3.5.2. *Effect of TNF on the Level of Mn-SOD and Cu,Zn-SOD in TNF-Sensitive Cells*

In ME-180, a human cervical epidermoid carcinoma cell line, and KYM-1, a human myosarcoma cell line, which are TNF sensitive, Mn-SOD levels are one order of magnitude lower than in TNF-resistant cells. However, ZR-75-1, a human breast cancer cell line that is also TNF sensitive, contains a relatively high level of Mn-SOD, even though its level is not as high as those of TNF-resistant cells. The basal Mn-SOD protein levels in KYM-1 and ME-180 cells are very low, and

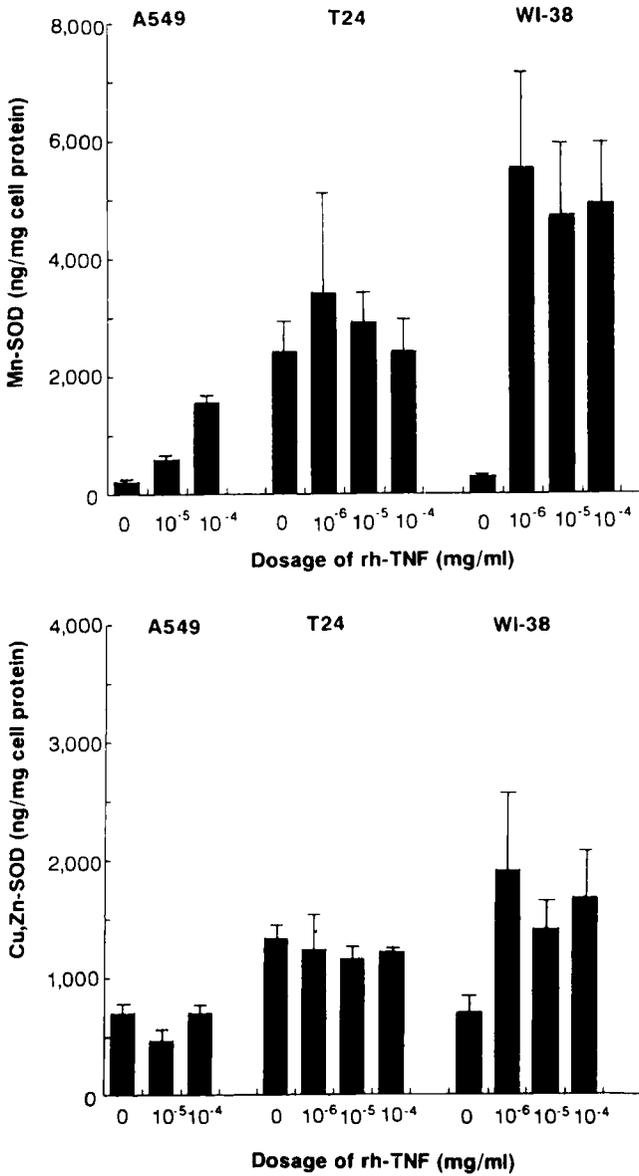


FIG. 25. Effect of TNF on Mn-SOD and Cu,Zn-SOD levels in TNF-resistant cells. Each cell line was treated with the indicated concentrations of TNF for 24 hours before Mn-SOD and Cu,Zn-SOD protein levels were determined. Each value was obtained from triplicate samples as described in the text. Bars indicate the standard deviations.

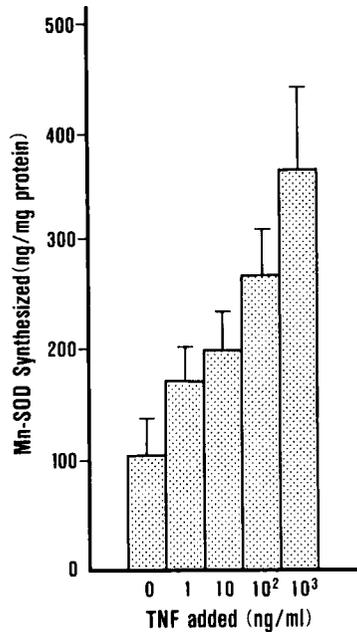


FIG. 26. Effect of TNF on Mn-SOD levels in ovarian carcinoma cells, Kuramochi strain.

even after treatment with TNF, no increase in Mn-SOD was observed. In ZR-75-1 cells, however, a tendency toward increased Mn-SOD levels was observed after TNF treatment. Therefore, in this cell line it seems a different mechanism may control Mn-SOD expression.

3.5.3. *The Mechanism of Mn-SOD Induction by TNF or TPA*

Although several stimulators such as TNF, IL-1, and LPS have been reported to enhance Mn-SOD expression in some cell lines, the pathway that transduces a signal from corresponding receptors to Mn-SOD gene is not clearly understood.

Recently, we showed that TPA also induces Mn-SOD in various cell lines which are all resistant to TNF (Table 9). This gives us clue to investigate the intracellular signal transduction pathway. Since TPA enhanced Mn-SOD mRNA expression in TNF-resistant cell lines in which other stimulators also induced expression of the gene but did not affect TNF-sensitive cells, it is conceivable that protein kinase C is involved in this gene expression by TNF through phosphorylation of certain substrates. One likely candidate for this substrate is nuclear factor κ B (NF- κ B). NF- κ B can be activated by releasing inhibitory protein I κ B after protein kinase C-dependent phosphorylation. This activated NF- κ B may enhance Mn-SOD gene expression like other TNF-responsive genes. Yet another possi-

TABLE 9
RELATIVE STIMULATION OF Mn-SOD mRNA EXPRESSION BY TPA, TNF,
IL-1, AND LPS IN VARIOUS CELL LINES^a

Cell line	Control	TPA (10 ng/ml)	TNF _α (100 ng/ml)	IL -1 _β (1000 U/ml)	LPS (10 μg/ml)
HeLa ^b	1.0 ± 0.1	18.7 ± 1.0	7.8 ± 2.5	8.1 ± 1.2	3.0 ± 0.7
A549 ^b	1.0 ± 0.2	9.0 ± 1.2	18.5 ± 1.4	25.3 ± 3.8	1.0 ± 0.1
Kuramochi ^b	1.0 ± 0.1	6.7 ± 0.1	2.7 ± 0.5	19.8 ± 2.7	1.4 ± 0.1
MCAS ^b	1.0 ± 0.1	11.2 ± 1.3	1.4 ± 0.8	6.0 ± 1.4	16.5 ± 0.7
ME180 ^c	1.0 ± 0.3	1.2 ± 0.1	0.8 ± 0.4	1.3 ± 0.2	0.4 ± 0.2
HL60 ^c	1.0 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.4
K562 ^c	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.3	1.3 ± 0.1	1.0 ± 0.1

^aTotal RNA was prepared from various cells treated with 10 ng/ml TPA, 100 ng/ml TNF, 1000 units/ml IL-1, or 10 μg/ml LPS for 4 hrs. The amount of Mn-SOD mRNA was evaluated by scanning X-ray film exposed to Northern blot membrane filters. The mRNA levels relative to the control are presented as the means ± SD for three experiments.

^bTNF-resistant cell.

^cTNF-sensitive cell.

bility is that AP-1 is responsible for this gene expression. Ho *et al.* (H15) found the consensus sequence for AP-1 enhancer binding protein in the 5'-flanking region of the rat Mn-SOD gene. TPA acts to both activate AP-1 protein and to enhance protooncogene Jun/AP-1 expression.

However, there would be more than one pathway because TNF could induce Mn-SOD in the cells that were desensitized to TPA by TPA-pretreatment. Thus, at least two pathways participate in Mn-SOD expression. One is triggered by protein kinase C activation itself in the absence of new protein synthesis, and the other can be activated by TNF without protein kinase C activation.

4. Conclusion

Strong evidence has been presented indicating that many of the cell alterations seen in normal aging and in various diseases, including cancer, are due to oxidative damage from active oxygen species. SODs function in the cellular defense against the active species, O₂. Study of these enzymes is therefore of potential clinical interest.

Cu,Zn-SOD is subject to nonenzymatic glycosylation (glycation). The reaction often causes inactivation of the protein through glycation of lysine residues 122 and 128, which play a role in the entrance of the substrate O₂ into the enzyme.

Study of erythrocyte Cu,Zn-SOD revealed that the percentage of glycated enzyme increased in diabetes. Presumably the increased glycation was due to the

hyperglycemic conditions existing in the diabetic erythrocytes. Interestingly, in erythrocytes of patients with Werner's syndrome, glycation of Cu,Zn-SOD was found to occur irrespective of the glycemic stage. Werner's syndrome is characterized by accelerated aging and increased frequency of malignant tumors and diabetes. Increased glycation resulted in a reduction of Cu,Zn-SOD activity in this disease. A progressive decrease in Cu,Zn-SOD activity is also associated with normal aging.

One age-related process in which glycation of Cu,Zn-SOD may play a role is cataractogenesis. Relatively high levels of the glycated enzyme are found in erythrocytes and lens tissues of aged persons with senile cataracts. In addition, diabetic patients with cataracts have higher levels of glycated Cu,Zn-SOD than do patients without complications. Increased glycation of lens epithelial Cu,Zn-SOD has also been seen in rats with streptozotocin-induced diabetes. These findings suggest that glycation of Cu,Zn-SOD in the lens epithelium can be used as a marker for cataractogenesis.

Involvement of the Mn isozyme of superoxide dismutase in aging and disease has also been implicated. High levels of Mn-SOD are present in sera of patients with certain malignant conditions, including gastric cancer, primary hepatoma, epithelial ovarian cancer, and acute myeloid leukemia. Furthermore, Mn-SOD protein levels were found to be higher in human lung cancer tissue than in uninvolved tissues of the same patients. In the case of epithelial ovarian cancer, the serum level of Mn-SOD correlated with progression of the disease. In general, progression was accompanied by an increase of the enzyme level and tumor regression was accompanied by a decrease. Recurrence of the cancer following surgery and chemotherapy also coincided with rising levels of Mn-SOD. In contrast, high levels of serum Mn-SOD were not seen with other gynecological malignancies or with benign ovarian tumors. The specificity of the increased Mn-SOD to epithelial ovarian cancer would therefore appear to be greater than that of the currently used marker for the disease, CA 125. Assay of serum Mn-SOD protein levels will likely provide a useful additional technique for monitoring treatment of epithelial ovarian cancer and for early detection of tumor recurrence.

Expression of Mn-SOD can be induced by IL-1 or TNF as well as by TPA. Inducibility of the enzyme by TNF is abolished by actinomycin D, which indicates that the effect is due to *de novo* biosynthesis of Mn-SOD. TNF-resistant cell lines respond to TNF treatment with increases in Mn-SOD. A similar increase was seen after TNF treatment of a cell line derived from adenocarcinoma of the ovary. On the other hand, most TNF-sensitive cell lines do not show any changes in Mn-SOD levels following exposure to TNF. The level of Cu,Zn-SOD is essentially unchanged by TNF addition.

One possible explanation for the elevation of Mn-SOD seen in certain cancers is that TNF or IL-1 produced by cancer cells or tumor-associated macrophages (E4) has been found to act as an autocrine factor in acute myeloid leukemia cells

and is likely involved in the specific increase in serum Mn-SOD associated with this type of leukemia.

Interestingly, a biphasic increase in serum Mn-SOD has been seen in patients with acute myocardial infarction. The later, much larger increase is not affected by reperfusion and may be due to *de novo* synthesis of the enzyme by myocytes. The possible involvement of TNF in this process has been described.

Given the wide tissue distribution of Cu,Zn-SOD and Mn-SOD and the important role of these enzymes in the cellular defense against O₂, it is not surprising that changes in the SODs are associated with such a variety of pathological conditions. The recent development of enzyme-linked immunoassays for Cu,Zn-SOD and Mn-SOD has facilitated accurate and reproducible determination of the protein levels of these enzymes in serum and other tissues. The ELISA is preferable to enzymatic assays for SOD because SOD-like activities in tissues can interfere with the quantitation of SOD activity. Also, modifications of SOD such as glycation can result in inactivation, thereby precluding accurate determination of enzyme levels by activity assay. Measurement of serum immunoreactive SOD levels shows promise as a diagnostic tool, and assessment of changes in tissue levels of the enzymes will likely provide insight into the biochemical processes involved in a number of diseases.

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BIOCHEMISTRY OF THE UREMIC SYNDROME

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1. Introduction

In recent years our ability to treat uremia has outstripped our understanding of its pathogenesis. At the present time more than 300,000 people throughout the world are being maintained by dialysis; active renal transplant programs have been set up in most developed countries, and under optimal conditions a successful transplant recipient, or a 20- to 40-year-old dialysis patient free of systemic disease, has an over 90% chance of being alive 10 years later. A precise understanding of the pathogenesis of the uremic syndrome, however, continues to elude us. Numerous substances have been isolated from the blood of uremic subjects and have been studied *in vivo* and *in vitro*. The subject of uremia has been reviewed many times and the literature is enormous (B18). Yet almost 200 years after the first investigation into the nature of uremia, no single retention compound has qualified for the title of a true uremic toxin (R12,R13).

Many investigators, indeed, have objected to the very concept of a specific toxin, arguing that the uremic syndrome represents the sum total of all the perturbations caused by renal failure. Increasingly clinicians have come to appreciate

the role of acidosis, hyponatremia and water intoxication, anemia, hyperkalemia, hypocalcemia, hyperphosphatemia, hypertension, and heart failure. The recent therapeutic use of erythropoietin to correct anemia may well lead to a reassessment of what symptoms can be attributed to uremia *per se*.

Clinicians rely mainly on blood urea nitrogen (BUN) and serum creatinine measurements to evaluate patients with renal failure. Yet the correlation between symptoms and blood levels is at best approximate. In acute renal failure the underlying disease and its associated complications often dominate the clinical picture and determine the prognosis, and it is unclear at what level of nitrogen retention symptoms may be attributed to uremia. Clinicians generally institute dialysis when the BUN exceeds 100 mg/dl or the serum creatinine exceeds 10 mg/dl, but sometimes earlier or later, and early dialysis has not been shown to confer distinct benefits. In chronic renal failure, patients may be quite asymptomatic despite very high BUN and serum creatinine levels. Many so called uremic symptoms may be more properly attributed to anemia, heart failure, nephrotic edema and hypoproteinemia, hypertension, malnutrition, or uncontrolled diabetes or its complications, such as gastroparesis, diarrhea, and neuropathy.

Yet the clinician usually makes the diagnosis of uremia when patients with high BUN (≥ 100 mg/dl) and serum creatinine (≥ 10 mg/dl) levels develop symptoms of anorexia, nausea, vomiting, abnormal taste in the mouth, uremic fetor, insomnia, loss of energy and libido, dyspnea from pulmonary congestion, or chest pain from pericarditis. Such patients may also exhibit normocytic normochromic anemia, metabolic acidosis, hyperkalemia, hyperphosphatemia, hypocalcemia, or hypoalbuminemia.

Even when these characteristic uremic symptoms appear, they are not necessarily caused by urea retention, as Osler observed in 1892 (O6). Neither does the retention of other products fully explain the uremic symptom complex, for progressive nephron loss initiates complex adaptive processes. The resulting clinical picture will depend on the magnitude and rate of development of renal failure as well as on these countering mechanisms.

The metabolic changes observed in uremia result from the decline in renal excretory function, hence the retention of a legion of substances; the loss of vital renal hormones (e.g., erythropoietin) and enzymes (e.g., 1α -hydroxylase); the effect of the uremic environment on organ function, intermediary metabolism, and transport processes; dialysis-related problems; and exogenous toxins.

2. Retention of Inorganic Substances

Electrolyte and acid–base disorders in chronic renal failure are well known and will therefore be presented briefly. A more detailed discussion may be found in standard textbooks of nephrology (M25).

2.1. HYPERKALEMIA

Hyperkalemia poses an immediate threat to the life of the uremic patient. Although potassium excretion decreases with increasing nephron loss, hyperkalemia occurs infrequently in stable chronic renal failure when the glomerular filtration rate exceeds 10 ml/minute. Serum potassium, however, may rise sharply if renal function deteriorates suddenly or if an excessive potassium load enters the extracellular fluid space. The latter event may result from dietary indiscretion; extracellular shift of potassium by acidemia; potassium release by hemolysis, rhabdomyolysis, or tumor lysis, or administration of potassium-containing drugs.

Acute hyperkalemia causes a hypopolarization of the cardiac muscle cell membrane, resulting in characteristic electrocardiographic changes followed by serious and often fatal arrhythmias; in most cases there are no warning symptoms. Immediate treatment is needed and consists of giving sodium bicarbonate, glucose, and insulin intravenously to shift K^+ into the cells; calcium intravenously to minimize the cardiotoxicity of hyperkalemia; and polysterene sodium (a Na/K exchange resin) rectally or orally to remove potassium from the body; if all fails, the performance of dialysis may be required (S18).

2.2. SODIUM AND WATER

Retention of sodium and water in progressive renal failure results in hypertension, edema, pleural and pericardial effusions, and pulmonary edema, the last being a life-threatening event requiring immediate dialysis. Hypertension may be severe, leading to heart failure or encephalopathy. Hyponatremia occurs if water is retained in excess of sodium. If severe, it may cause muscular twitching and convulsions (water intoxication). Dialysis is the treatment of choice (R14).

2.3. HYDROGEN ION

Hydrogen ion is generated from protein metabolism to the order of 1 mmol H^+ per kilogram of body weight per day, mainly as sulfuric and phosphoric acids. The kidneys dispose of this acid load by excreting ammonia and titratable acid (NaH_2PO_4). With the loss of renal mass, each remaining nephron increases its excretion of titratable acid and ammonia, so that total acid output does not decrease until renal failure is advanced. Metabolic acidosis with a high anion gap usually occurs when the glomerular filtration rate has fallen to 10–15 ml/minute.

The increased H^+ concentration in the blood stimulates the chemoreceptors, causing hyperventilation and a fall in alveolar and hence arterial pCO_2 . The resulting decrease in the ratio of pCO_2 to serum HCO_3^- tends to restore H^+ concentration toward normal. This pulmonary compensation for metabolic acidemia, though effective, is usually not complete (C15). In the long run the excess H^+ ions are buffered by skeletal tissues, thus causing a loss of calcium and contributing to the secondary hyperparathyroidism of uremia.

Severe acidemia impairs cardiac contractility and predisposes to arrhythmias. Treatment with sodium bicarbonate or Shohl's solution (sodium citrate and citric acid) is in order if the blood pH falls below 7.2 (N1). Dialysis should be started when uremia and acidosis are more advanced.

2.4. MAGNESIUM

Retention of magnesium also occurs, but significant hypermagnesemia is unusual in the absence of an exogenous magnesium load. Because magnesium is excreted by the kidneys, magnesium-containing antacids and laxatives should be avoided in uremic patients. Magnesium intoxication may cause respiratory paralysis by blocking the neuromuscular junction.

2.5. TRACE ELEMENTS

Although trace element abnormalities occur in chronic renal failure, few symptoms have been attributed to them in nondialyzed patients. In dialysis patients these disturbances appear to be qualitatively similar but more severe (T7). They have been extensively reviewed by Alfrey (A5). Total body zinc (except in erythrocytes), strontium, aluminum, and tin are generally increased, whereas total body rubidium is decreased. Iron stores tend to be increased in the spleen and liver in dialyzed patients, especially after ferrous sulfate therapy. Copper is increased in lung tissue and decreased in heart tissue and erythrocytes. Molybdenum and cadmium are decreased in renal tissue but increased in liver tissue of dialyzed and nondialyzed patients. Total body zinc content is significantly increased (A5), but hypozincemia, frequently observed in dialysis patients, has been blamed for taste impairment and impotence and there is conflicting evidence on whether zinc repletion corrects these abnormalities (K4, M12). Nickel is also increased in the serum of uremic patients, but this does not appear to be associated with a corresponding increase in tissues (S5). It cannot be concluded that trace element retention in renal failure is of no clinical importance, as shown by the problem of aluminum intoxication, to be discussed later. In addition, trace elements such as rubidium and bromine, which are rapidly depleted in uremic patients on maintenance dialysis (A5), may prove to be essential in normal metabolism. Thus the clinical importance of these element alterations remains unclear.

3. Retention of Organic Substances

3.1. UREA

The end product of protein catabolism, urea, has been studied for almost 200 years and the literature on this subject is vast (B18, F9, K11, R12). In 1921 Leiter observed that infusing urea into normal dogs produced hypothermia, a frequent

finding in uremia (L5). However, in 1927 Bollman and Mann showed that when the ureters of dogs were transplanted into the ileum, toxic symptoms did not occur despite urea concentrations of 800 mg/dl (B25). Merrill *et al.*, in 1953, noted that patients with acute renal failure in whom urea concentrations were maintained by adding urea to the dialysate improved even though the blood urea did not decrease (M34). At this time urea infusions were being widely used to relieve intraocular pressure in acute glaucoma and increased intracranial pressure in neurosurgical cases. Although the concentrations of urea rarely approached the higher levels seen in uremia, no major toxic effects were observed except for headache and mild gastrointestinal symptoms.

In 1959 Grollman and Grollman added urea to the dialysate in animals and showed that weakness and anorexia at blood urea concentrations of 370–480 mg/dl appeared within 4 days of bilateral nephrectomy, followed by vomiting, hemorrhagic diarrhea, hypothermia, stupor, and coma. The urea at death or termination of the experiment reached concentrations of 740–1690 mg/dl, concentrations greatly exceeding those found clinically (G17). Other investigators have shown that urea administered to man or experimental animals caused dehydration, headaches, asthenia, vomiting, hypothermia (H10), glucose intolerance (B8, H10, P8), and a bleeding tendency (E3) at blood urea concentrations of 200–300 mg/dl. Numerous *in vitro* experiments have been performed implicating urea as a potential uremic toxin. Urea reduced myocardial contractility in concentrations of 60–600 mg/dl (K6), retarded platelet aggregation in concentrations of 100–300 mg/dl (D2), and inhibited several enzyme systems, but only at very high concentrations, exceeding those found in uremic patients (R4).

During the 1970s several urea-loading studies were conducted in patients with chronic renal failure. Thus Johnson and co-workers demonstrated that blood urea concentrations of 300 mg/dl were well tolerated (J2, J3). Despite prolonged severe azotemia (predialysis blood urea concentrations of 500–600 mg/dl), uremic fetor, stomatitis, myoclonus, and pericarditis were not observed, nor were gastrointestinal, cardiovascular, or neurological disturbances. In animal studies chronic subcutaneous urea administration (200–700 mg/dl) in partially nephrectomized dogs produced none of the symptoms normally associated with uremia (B9).

High blood urea concentrations, however, have been implicated in the pathogenesis of the dialysis dysequilibrium, or reverse urea syndrome. This is characterized by headache, nausea, vomiting, blurred vision, disorientation, muscle twitching, and sometimes convulsions, coma, and death (K3, M14). It was proposed that during dialysis the decrease in cerebrospinal fluid urea lagged behind the plasma, causing water movement from plasma to brain with resulting cerebral edema. Urea may also play a role in the induction of generalized myoclonus by blocking glycinergic neurotransmission (C9). It may also act as an enzyme inhibitor (G8). Yet it is the consensus of most clinicians that urea is not a major toxin in renal failure, though at very high concentrations (BUN >300 mg/dl) it may cause headache, vomiting, and fatigue.

3.2. CREATININE/CREATINE

Another consequence of renal failure is an increase in plasma creatinine. Creatinine, the anhydride of creatine, is synthesized in muscle by irreversible and nonenzymatic removal of water from creatine and therefore is an index of skeletal muscle mass. In normal subjects the amount of creatinine excreted is primarily influenced by lean body mass and diet. Although the serum creatinine increases in chronic renal failure, the increase is less than predicted because creatinine is also removed by extrarenal mechanisms. In general, creatinine and creatine play a minor role as uremic toxins. Although creatinine at very high concentrations might affect glucose tolerance and erythrocyte survival (B8), it and its degradation products have not been shown to be toxic at the levels found in chronic renal failure (D17, J4, J5, O8). Predialysis values for creatinine and BUN do not predict adequate dialysis, clinical rehabilitation, or longevity (M38), and may be quite elevated in totally asymptomatic patients.

3.3. URIC ACID

In renal failure uric acid is also increased, its levels correlating poorly with creatinine (G16). Apparently there is an increase in the extrarenal uric acid elimination by uricolysis in the intestinal tract (B14, S29, S30). This becomes progressively important as plasma uric acid concentration rises (S30). Although hyperuricemia has been implicated in the precipitation of uremic pericarditis (C10), most investigators believe that uric acid is innocuous, though clearly it may precipitate gout.

3.4. OXALATE

Retention of oxalic acid and consequent crystal deposition in both myocardium (Z1) and renal tissue (B16, F3) are recognized features in chronic renal failure. Crystal deposition in these tissues may be of importance in the genesis of some of the clinical features of the uremic syndrome. It has been suggested that the serum oxalic acid concentration may rise following dialysis against high glucose baths. Oxalic acid in concentrations comparable to those in uremic plasma inhibits lactic dehydrogenase (E4) and conceivably other enzymes. Nevertheless, the role of oxalate in the pathogenesis of the uremic syndrome has yet to be defined.

3.5. CYANATE

Some investigators suggested that the toxic effects observed during urea infusion experiments were due to products formed from urea (G17) (Fig. 1). Dirnhuber and Schultz had shown earlier that cyanate, formed in urea solutions, could cause drowsiness and hyperglycemia (D16, S15). Gilboe and Javid had likewise con-

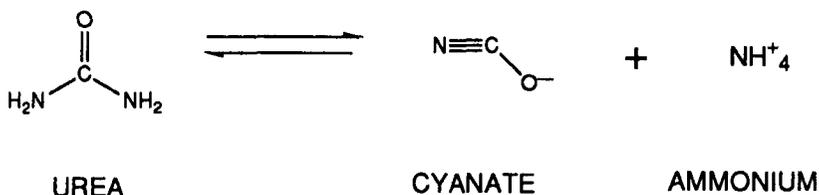


FIG. 1. Spontaneous degradation of urea.

cluded that cyanate formed from urea, rather than urea itself, caused the toxic effects (G7). Urea is known to break down spontaneously to cyanate, which is liposoluble and can diffuse through cellular membranes, reacting irreversibly with the N-terminal groups of many amino acids, peptides, or proteins to form carbamyl derivatives (B21, S32) (Fig. 2). Thus the long-term administration of cyanate may be neurotoxic because it is incorporated into brain protein as carbamyl derivatives (F2). Cyanate given to rats and mice in moderate doses decreases motor activity; in higher doses it induces drowsiness (C24). Yet inactivation of hormones and enzymes by carbamylation does not necessarily occur *in vivo* (G15, V1); long-term administration of cyanate in low doses does not seem to produce ill effects in mice, dogs, or monkeys (C4). Furthermore, it has not been proved that cyanate accumulates in uremic patients in sufficient amounts to produce toxicity.

3.6. MYOINOSITOL/POLYOLS

Myoinositol and other polyols, such as scylloinositol and neoinositol, are normal constituents of a class of phospholipids known as the phosphoinositides. These compounds, closely related to nervous tissue and neuronal function, are retained in uremia and have been considered a possible cause of peripheral neuropathy (D7, H8, N10). Rats receiving large amounts of myoinositol show a decrease in nerve conduction velocity (C12). Adding myoinositol to root ganglion cells *in vitro* in concentrations known to occur in uremic plasma produces cytotoxic changes (L14).

In uremic patients an inverse correlation has been observed between nerve conduction velocity and the blood level of myoinositol (R11). Uremic nerve tissue has been shown to contain increased levels of myoinositol (N6). In hemodialyzed patients, however, there is no correlation between nerve conduction times, the degree of clinical neuropathy or electroencephalographic changes, and the levels of plasma or cerebrospinal fluid myoinositol (B24, R11). Thus, there is little convincing evidence of an etiological role of myoinositol in the development of neuropathy.

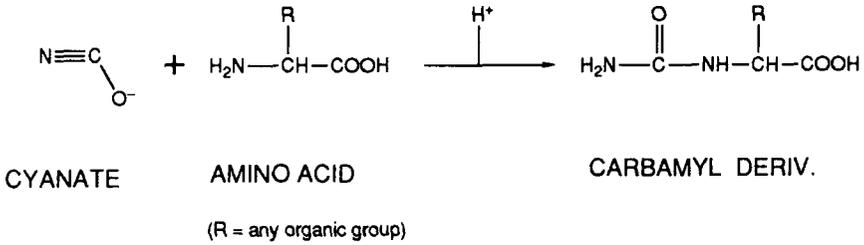


FIG. 2. Carbamylation.

3.7. GUANIDINES

Guanidines are strong organic bases formed from nitrogen in the urea cycle. They accumulate in renal failure because of a reduced clearance and increased production, presumably due to increased conversion of creatinine to methylguanidine (G9, O4). It has also been suggested that urea may be converted to creatine through a guanidine cycle (Fig. 3), urea being oxidized to hydroxyurea and creatinine being formed from canavanine after a series of condensation reactions, with guanidinosuccinic acid formed as a by-product (N3). Guanidinosuccinic acid may also be produced by inhibition of glycine amidinotransferase activity, thus reducing guanidinoacetic acid synthesis. Under these conditions the amidino group of arginine is transferred to aspartate instead of glycine, with the concomitant formation of guanidinosuccinic acid (P7). Recent metabolic studies have confirmed that urea is a source of guanidinosuccinate (C16, G9), increased concentrations of which support the aforementioned proposed mechanism (N2).

Guanidines and related compounds, i.e., methylguanidine, guanidinosuccinate, and guanidinoacetate, are increased in the plasma and tissues in renal failure and have long been implicated as uremic toxins (D4, D5, G9, G12, K7). In animals large doses of guanidines, especially methylguanidine, produce many of the characteristics of uremia, i.e., hemolytic anemia, reduced erythropoiesis, gastric ulceration, and peripheral neuropathy (G10). To obtain changes in the nervous system, however, higher serum concentrations of methylguanidine are needed than those found in uremic patients (B3). Also, rabbits given sufficient guanidinosuccinic acid to raise their serum levels to those found in uremic patients do not appear to develop signs of toxicity (M29).

Nevertheless, some investigators have suggested that guanidines could be toxic. *In vitro* methylguanidine inhibits oxidative phosphorylation (G9), and guanidinosuccinate interferes with platelet aggregation and DNA synthesis in lymphocytes (A12, H13). On the other hand, high concentrations of methylguanidine do not inhibit oxygen uptake in tissue respiration studies of slices of rat liver (F6). The concentrations of methylguanidine used in many studies were much higher than

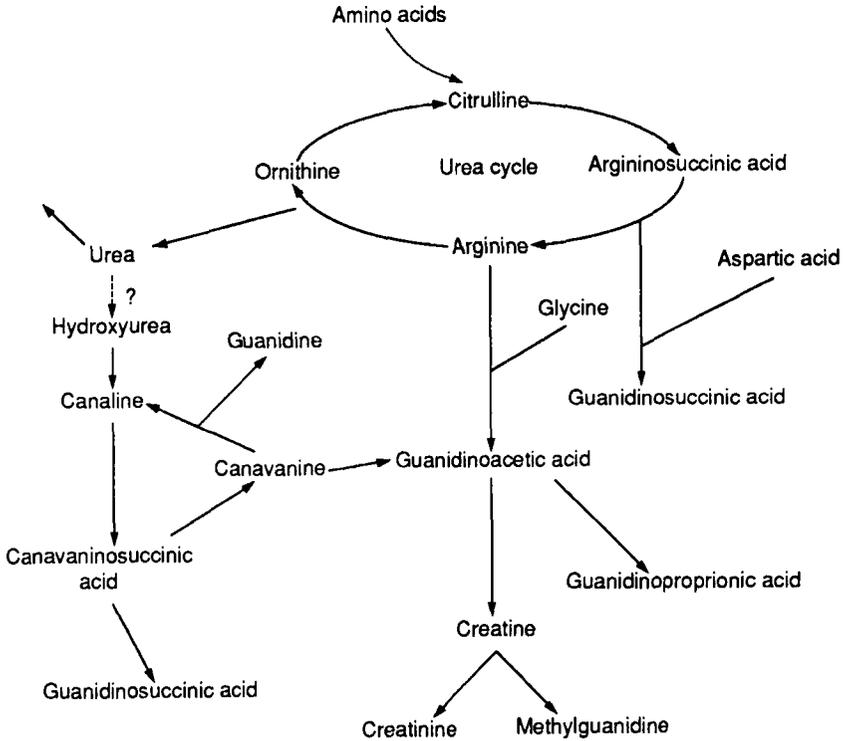


FIG. 3. Proposed relationships between urea metabolism and the production of guanidino compounds.

those found in renal failure. Moreover, the *in vitro* addition of guanidinoacetic acid to the blood of normal volunteers had no apparent effect of the osmotic fragility of erythrocytes, even at concentrations higher than those of uremic patients (G12).

In addition to methylguanidine, guanidinosuccinate, and guanidinoacetate, other guanidino compounds accumulate in uremia; i.e., guanidine, guanidinobutyric acid, guanidinopropionic acid, and taurocyamine. However, there is little evidence to implicate these compounds in the pathogenesis of uremic neuropathy or toxicity (D18, R7).

3.8. POLYAMINES: ALIPHATIC AND HETEROCYCLIC AMINES

These basic compounds are derived from various metabolic pathways, from endogenous tissue sources, from a deranged biochemical pathway involving normal intestinal flora, or from exogenous sources. Their retention in renal failure makes them potential uremic toxins.

Polyamines are derived from ornithine and methionine. Putrescine is formed from ornithine via ornithine decarboxylase. Through additional enzymatic steps, methionine contributes aminopropyl moieties to putrescine to form spermidine and spermine (Fig. 4). Polyamines participate in stimulating RNA and DNA synthesis and facilitate gene transcription and translation (H9). Intracellular polyamine concentrations are restricted to narrow limits to ensure efficient protein synthesis and high fidelity of amino acid sequences (A3). These compounds also regulate enzyme activities (O2) and promote cell growth (R3). Their biological breakdown products are toxic, hence the interest in their accumulation in uremia (C1, S1, S36). Experimentally, polyamines inhibit many enzymes (A17, Q1), interfere with erythroid colony formation in human bone marrow culture (R2), and reduce erythrocyte deformability at concentrations found in uremic serum (B10). A basic peptide containing spermidine, isolated from the plasma and peritoneal fluid of uremic patients, will readily complex with insulin and may contribute to glucose intolerance and hypertriglyceridemia (L17).

The bowel, one of the largest and most metabolically active organs, contains bacteria that may change the chemical composition of the human body. In renal failure the altered bacterial flora cause the accumulation of aliphatic amines in the gut (O9, S25). Bacteria transform part of the choline in the gut to trimethylamine, which is reabsorbed and then either oxidized or demethylated to dimethylamine in the liver (S24). Dimethylamine enters the circulation and is excreted in the bile and urine. The trimethylamine and dimethylamine in the exhaled air of uremic patients may contribute to the classic "fishy breath," which can be improved by hemodialysis or by gut sterilization with nonabsorbable antibiotics (S23, S25). The overall role of these compounds as uremic toxins, however, remains to be defined.

Heterocyclic amines could prove to be toxic after enzymatic conversion to their *N*-hydroxylamine derivatives (Y1). Several of these "mutagenic" amines have been found in cooked foods (S34) and can be absorbed by the gut (Y2). They are largely excreted in the bile, but also by the kidneys (M21), and conceivably could accumulate in uremia. Indeed, Japanese investigators have reported a marked increase in the concentration of two heterocyclic amines, 2-amino-6-methylpyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1) and 2-aminodipyridol[1,2-*a*:3',2'-*d*]imidazole (Glu-P-2) (Table 1), in the dialysate and serum of patients with chronic renal failure (M21, M22, Y2). Although these heterocyclic amines are partially removed by dialysis, significant amounts of these potentially carcinogenic substances can still be detected even after 1 month of hemodialysis (M21). Further clinical studies are needed to confirm these findings and determine their significance.

3.9. PHENOLS/POLYPHENOLS/AROMATIC ACIDS

First shown to accumulate in uremia in 1925 (B13), phenols are derived from protein and bacterial metabolism. Highly toxic, they cause central and peripheral

TABLE 1
 Glu-P-1 AND Glu-P-2 LEVELS IN PLASMA OF NORMAL SUBJECTS AND
 PATIENTS WITH CHRONIC RENAL FAILURE^a

Normal (N = 7) ^b		Chronic renal failure (N = 5)	
Glu-P-1 (pmol/ml)	Glu-P-2 (pmol/ml)	Glu-P-1 (pmol/ml)	Glu-P-2 (pmol/ml)
2.50	3.10	17.83	19.38
ND	2.20	13.46	20.15
ND	ND	11.24	10.10
ND	ND	7.76	15.50
ND	ND	12.80	8.90
ND	ND	ND	ND

^aCompiled from data of Manabe *et al.* (M21).

^bND, Not detectable (for Glu-P-1, levels <1.00 pmol/ml; for Glu-P-2, levels 1.67 pmol/ml).

nerve damage because they are liposoluble and can easily cross the cell membrane (H11, W1, W2). Phenols are poorly removed by hemodialysis, their serum concentration rebounding to 80% of the predialysis level within a few hours after dialysis (J6). Phenols are usually rendered water soluble by esterification to glucuronic acid or sulfate, which greatly reduces their toxicity. An exception is phenylglucuronide, which, though water soluble, definitely inhibits the enzyme Na⁺,K⁺-ATPase. Recently Niwa *et al.* demonstrated an increase in several free polyphenols in the blood of hemodialysis patients (N7) and suggested that these were potential uremic toxins. Nevertheless, the role of phenols in uremia needs further clarification.

The aryl acids or aromatic acids are a heterogeneous group of substances that include the hippurates, benzoic acid, and phenolic acids. Some of these could be toxic by causing enzyme inhibition (interestingly, hippurates are almost as soluble in lipids as in water). Many aromatic acids, especially those with an unsaturated side chain, depress enzyme reaction rates.

Phenolic acids affect cerebral metabolism, as measured by the rate of respiration and anaerobic glycolysis of guinea pig brain slices, and they also inhibit the activity of some selected enzymes, i.e., Na⁺,K⁺-ATPase (W2), the carboxylases of 3,4-dihydroxyphenylalanine, 5-hydroxytryptophan, glutamic acid, amine oxidase, and lactate dehydrogenase (H11). Special note must be made of *o*-hydroxyphenolic acid, which is increased markedly in uremic sera (N8) and inhibits peripheral glucose utilization (T8). In general, the mono- and dihydroxybenzoic acids are poorly conjugated with glucuronide. However, Monti *et al.* have isolated

an endogenous compound from uremic plasma and determined it to be a glucuronide conjugate of *o*-hydroxybenzoic acid (M37). It has been observed that the mono- and dihydroxybenzoic acids are more readily conjugated with glycine; however, conjugation does not appear to prevent enzyme inhibition (R16). Conjugation of benzoic acid to glycine forms hippuric acid, which accumulates in renal failure (F4) and may displace several albumin-bound drugs (T3). Abnormal binding of drugs and endogenous compounds has been reported in uremic patients (G18, R6); the inhibitors of protein binding appear to be aromatic carboxylic acids (D9, D10).

Lichtenwalner *et al.* have suggested that *o*-hydroxyhippurate may be an important binding inhibitor in azotemic plasma (L8). However, Gulyassy *et al.* found that the concentration of *o*-hydroxyhippurate in uremic plasma are undetectable or minimally elevated, except among patients with elevated plasma salicylate (G19). They also reported increased hippurate concentrations in these patients, but found no correlation between these levels and inhibition of protein binding of drugs. Their studies indicated that other ligands remain to be identified as the major determinants of impaired albumin binding.

Recently Gallice *et al.* used high-performance liquid chromatography (HPLC) and nuclear magnetic resonance spectroscopy (NMR) to identify a double conjugate of glucuronidate-*o*-hydroxyhippuric acid in ultrafiltrates of uremic plasma (G6). This compound had been previously described by Zimmerman *et al.* as a fraction of the so-called uremic middle molecules (Z7, Z8). So far no data have been reported on the toxicity of this compound.

3.10. UROFURANIC ACIDS

Of the endogenous organic acids retained in chronic renal failure, some have been proposed as inhibitors of drug binding (S12). These include hippuric acid (T3), indoxyl sulfate (N9), 2-hydroxyhippuric acid (2-hydroxybenzoylglycine) (F8), 4-hydroxyphenylacetic acid (N8), and indole-3-acetic acid (B27). Recently Mabuchi and Nakahashi profiled the endogenous ligands in uremic serum by HPLC (M5). They proposed that an unidentified compound corresponding to "peak P" in the chromatogram was a major inhibitor of drug binding in uremic patients.

Using HPLC and gas chromatography-mass spectrometry (GC-MS), Mabuchi and Nakahashi (M6) and Takeda *et al.* (T2) have identified the compound as 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) (Fig. 5). This substance, first detected in normal urine (S31) and later in uremic sera (L9, M3), belongs to a new class of endogenous metabolites called urofuranic acids (Fig. 6). Although furanoid fatty acids with similar chemical structures have been found in lipids in the liver and testes of several fish species, the source and metabolism of

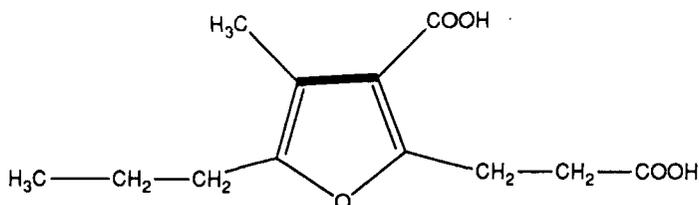


FIG. 5. Chemical structure of 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid.

CMPF in humans remain unknown (G11). Furan fatty acids have yet to be found in human lipids. However, urofuranic acids are present in the urine of babies and adults who have not consumed fish products for months (S3). Consequently, ingestion of fish does not appear to be the source of these furanoid fatty acids. Although the metabolic role of these compounds remains to be elucidated, substituted furan substances may be involved in the metabolism of uronic acid and carbohydrates (M40).

CMPF accumulates in uremia and is poorly removed by hemodialysis, apparently because of its strong affinity for serum albumin (M4). It impairs albumin binding of several drugs, e.g., phenytoin and salicylate, and many endogenous substances such as tryptophan and bilirubin (M2), and in that capacity has been proposed as a potential uremic toxin. It is unique in being one of the few albumin-associated "fluorescent" substances known to be increased in uremic patients (M1).

3.11. MIDDLE MOLECULES

During the early years of dialysis, peripheral neuropathy often occurred because of delayed or inadequate dialysis. One could not, however, correlate the symptoms to urea or creatinine blood levels. In 1965 Tenckhoff *et al.* reported that chronic peritoneal dialysis patients had fewer signs of neuropathy, although their blood levels of urea and creatinine were just as high (T4). Scribner proposed in that same year that the "leakiness" of the peritoneal membrane enhanced the removal of substances with a higher molecular weight than urea and creatinine (S14). In 1971 Babb *et al.* formulated the square-meter-hour hypothesis, which stated that larger molecules (2000–5000 Da) were well removed, despite reduced blood and dialysate flow by dialyzers with a larger surface area (B2). Because peritoneal dialysis is considered a slow-flow system with a large surface area, neuropathy seemed to improve with this treatment, it was proposed that larger molecules were responsible for some of the uremic toxicity. In 1972 Babb *et al.* changed the square-meter-hour hypothesis to the middle molecule hypothesis after the molecular

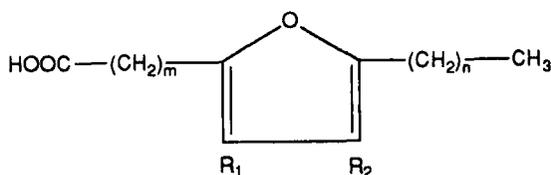


FIG. 6. General structure of urofuranic acid.

weight range was reevaluated and presumed to be closer to 500–2000 (B1). Since then most investigators have regarded middle molecules as being substances of 300–2000 Da (F1, G4, S13).

Because the middle molecule hypothesis was based on the size of the supposed solutes, Dall'Aglio *et al.* introduced size-exclusion techniques, such as membrane filtration or gel chromatography, to isolate these substances (D1). In subsequent studies they used Sephadex (G-75 and G-15), ultrafiltration and diafiltration through selective membranes, and obtained chromatograms showing an accumulation of middle molecules in the sera of uremic patients. Other investigators confirmed these findings using gel chromatography (Fig. 7) in conjunction with other techniques such as ultrafiltration, electrophoresis, isotachopheresis, and ion-exchange chromatography (D21, G1, M20, M35, P9, S19). Subsequently attempts were made to identify middle molecular weight substances and perform *in vitro* toxicity tests with these fractions.

Furst *et al.* reported on the separation of middle molecules that they contended were peptides (F12). Using high-speed gel filtration and ultraviolet absorption at 254 and 206 nm, they separated normal and uremic plasma into 10 and 11 peaks. One of these peaks, peak 7, was present in uremia but not in normal serum. Using ion-exchange chromatography, Furst and Bergström further separated peak 7 into seven or eight subpeaks (7a, 7b, 7c, etc.); peak 7 was prominent in uremic patients with symptoms such as malnutrition and infection (F13). Amino acid analysis indicated that peak 7c consisted of a small peptide chain with 8–10 amino acids (B17).

Chapman *et al.* later improved the resolution of Furst and Bergström's techniques and separated peak 7c further into peaks 7c₁ and 7c₂ (C7). They also demonstrated that various drugs and salts and hemodialysis could artificially increase the subpeaks of peak 7, and they stated that many of the subpeaks of fraction 7 could be found in normal plasma. The most interesting study of peak 7c was by Zimmerman *et al.* in 1981 (Z8). Using gas chromatography and mass spectrometry, they found that the main component of this middle molecule peak was not a peptide substance but a glucuronide of *o*-hydroxyhippuric acid (GOHH) (see Section 3.9) of 371 Da. Some correlations were found between the accumulation of these middle molecule fractions, especially 7c, and uremic "sickness" (A16).

Man *et al.* and Cueille *et al.* subsequently used a similar technique and separated a middle molecular substance that they designated b_{4-2} , which was found to be neurotoxic to frog sural nerve (C25, M19). It was subsequently shown that this b_{4-2} fraction was heterogeneous but contained a glucuronide of around 568 Da (C25, L6).

Other groups also used gel filtration exchange to measure middle molecules and perform *in vitro* toxicity tests (C7, D21). However, many of the middle molecular weight substances isolated by these techniques proved to be much smaller than anticipated. This discrepancy was due to the intrinsic inadequacies of the standard gel filtration techniques for the isolation of middle molecules, as pointed out by Furst *et al.* (F13) and later by Schoots *et al.* (S13). These investigators used analytical techniques to demonstrate that middle molecular fractions obtained by gel filtration comprised many low-molecular-weight solutes, such as carbohydrates, amino acids, polyols, aromatic substances, and other UV-absorbing solutes, and also sodium chloride, acetate, phosphate, and sulfate (S10). Thus these fractions do not exclusively represent middle molecules.

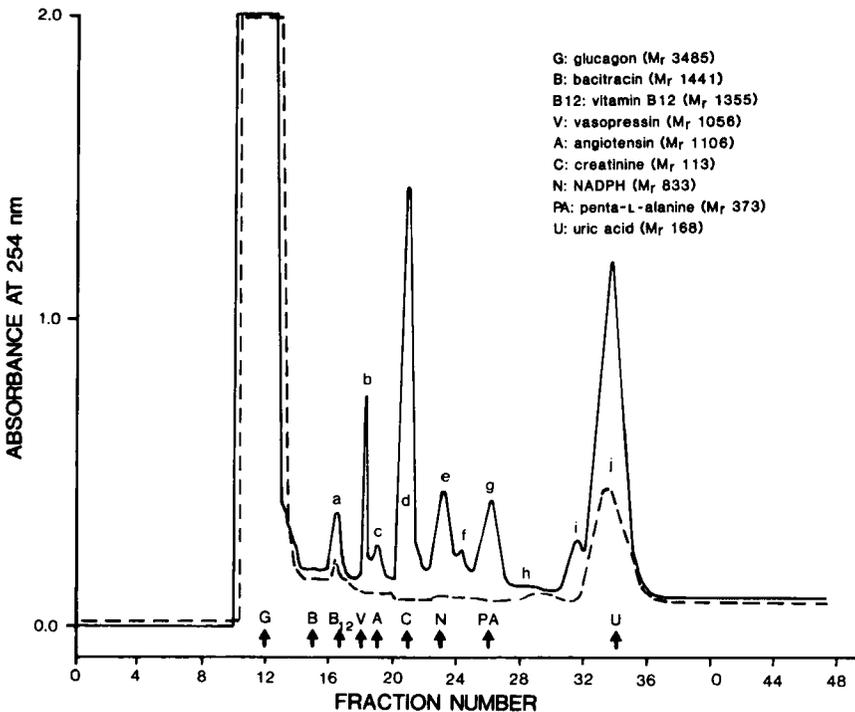


FIG. 7. Characteristic Sephadex G-15 gel chromatogram of uremic (solid line) and normal (dashed line) serum, showing elution position of various molecular mass markers. From Shaykh *et al.* (S19).

Although gel filtration is commonly used as a preliminary purification step, interpretation of molecular size/weight using calibrated columns should be approached with caution. This also applies to ultrafiltration membranes, another size-exclusion technique used to isolate middle molecules (B31, B33, C18). Although these membranes ostensibly have an exclusion limit, variable adsorption phenomena, ionic interactions, and steric hindrance occurring with the membrane affect the retention of complex biological mixtures during ultrafiltration (B33, C18). For example, Buzio *et al.* (B33) observed that membranes with a theoretical cutoff of 400 Da were permeable to molecules between 300 and 1000 Da; only molecules above 1100 Da were efficiently retained. Contreras *et al.*, using a similar membrane, noted that many amino acids were retained despite their molecular weights being much lower than the reported exclusion limit (C18). Consequently, such aberrations in ultrafiltration prevent accurate prediction of the molecular size of compounds eluted through these membranes.

Another approach to middle molecule analysis has been to search for specific solutes. Lutz (L18) isolated several basic peptides using gel filtration, ion exchange, and paper chromatography of peritoneal dialysis fluid that contained nonamino acid ninhydrin-positive groups such as spermidine, amino sugars, and guanidine. Three peptides of different amino acid sequence were isolated and identified by Abiko *et al.* (A1, A2) following an elaborate procedure of ultrafiltration on membranes with different molecular weight cutoffs, and chromatography using different Sephadex size-exclusion and ion-exchange gels. These workers identified a pentapeptide, possibly representing a fragment of fibrinogen β chain, and a heptapeptide corresponding to positions 13–19 of β_2 -microglobulin. Both peptides inhibited T lymphocyte E-rosette formation *in vitro* (A1, A2).

Other authors also described ninhydrin-positive compounds and peptidic material in uremic serum by combinations of bag dialysis, ion-exchange, paper chromatography, and thin-layer electrophoresis (E2, K8, M33). Subsequently Mabuchi and Nakahashi used HPLC to analyze uremic serum and ultrafiltrates for peptides using precolumn fluorecamine derivatization and fluorescence detection (M8, M9). Several investigators also used HPLC in conjunction with other separation methods and demonstrated heterogeneity of substances beneath peptidic fractions during analysis of uremic biological fluids (B31, G4, M10, S10, S11). The main factor hindering the quantitative and qualitative assessment of middle molecular fractions was the abundance of low-molecular-weight solutes present in the fractions obtained for analysis.

In only a few cases have pure substances been isolated and identified (Fig. 8). *In vitro* and *in vivo* toxicity tests have been performed using middle molecular fraction of varying purity to demonstrate biological activity (B32, N5). Various fractions have decreased ^{59}Fe incorporation into heme, inhibited lymphocyte proliferation, impaired glucose utilization, decreased motor nerve conduction velocity

in rats, induced cardiotoxic effects and hypotension in rats, and inhibited platelet aggregation (B26, D22, G5, G20, N4, P13). However, firm proof of a causative relationship between these toxic effects in the test systems and clinical signs has not been established. In only a few instances were pure substances tested, and many of the fractions studied were later determined to be heterogeneous. Contamination with electrolytes and lower molecular-weight substances would suggest that some of the effects were nonspecific, and moreover the experimental conditions were often not standardized, different flow rates and buffers being used for gel filtration elution. All this indicates that at present the chemical nature of most middle molecules is undetermined and their role in producing uremic symptoms remains unclear.

3.12. NUCLEOTIDES/PURINES/PYRIMIDINES

Plasma levels of the nucleotides ATP, ADP, AMP, cyclic GMP, and cyclic AMP are elevated in renal failure (H6, M39, S9, U1). Cyclic AMP (cAMP) levels

PEPTIDES

Dipeptide

H-Asp-Gly-OH
(Position 73-74 β -chain of hemoglobin -Asp-Gly-)

Hexapeptide

H-Ala-Phe-Phe-Gly-Gly-Glu-OH

Pentapeptide

H-Asp-Leu-Trp-Gln-Lys-OH
(position 123-127 of β -chain of fibrinogen)

Basic tripeptide

H-His-Gly-Lys-OH

Heptapeptide

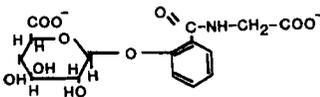
H-His-Pro-Ala-Glu-Asn-Gly-Lys-OH
(Position 13-19 of β_2 -microglobulin)

Acidic tripeptide

H-Glu-Asp-Gly-OH

GLUCURONIDE CONJUGATES

Glucuronidated o-hydroxybenzoylglycine



Glucuronide b4-2

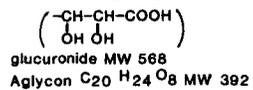


FIG. 8. Structures of the peptides and glucuronide conjugates isolated from uremic patients (see Refs. A1, A2, C25, and Z1).

are increased twofold and correlate with plasma creatinine concentrations (H6). This could be explained by either a decrease in the metabolic clearance rate or an increase in the production of cAMP (S26). Although it has been suggested that parathyroid hormone could stimulate cAMP production, plasma cAMP levels are equal in uremic patients with and without parathyroidectomy (H6). Other hormones also increase cAMP production (E5, M27). The hyperphosphatemia of uremia has been invoked to explain increased nucleotide levels (A15), which also rise rapidly during acute transplant rejection (R8). The most persuasive evidence that cAMP is toxic is its ability to inhibit platelet aggregation *in vitro* at concentrations found in the plasma of uremic patients (W3).

3.13. FLUORESCENT SUBSTANCES/XANTHOPTERIN

Several investigators (H2, M7, S17, S19, S35, W6) have reported an increase in endogenous fluorescence in renal failure (Table 2). This was first encountered as a source of error in the electrophoretic analysis of various enzymes in uremic patients (M31, W4). Later, fluorescence was determined to originate not from the enzymes but from other substances (A4, C19). These fluorescent species have been found in the serum, urine, and hemodialysate samples from patients with chronic renal failure (M7, S17, S35). Some are nondialyzable and albumin bound, others are dialyzable and found in dialysate or hemofiltrate (B12). Some of the fluorescent substances may represent drug-binding inhibitors retained in uremic serum (D10, S17). For example, 2-hydroxybenzoylglycine has a fluorescent emission maximum of 425 nm (L8), and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid fluoresces at 410 or 490 nm, depending upon the excitation wavelengths of 350 or 400 nm, respectively (M1).

Schwertner has isolated albumin-associated fluorescent ligands that have an emission maximum of 415 nm (S17). The fluorescent species is very water soluble and can be removed by charcoal (S16). A positive correlation was found between fluorescence and serum creatinine in patients maintained on conservative treatment (D15), but not in patients already on hemodialysis (S16). Interestingly, the serum of patients with acute renal failure does not emit this fluorescence, a fact that has been proposed as a differential criterion between acute and chronic renal failure (V2). Mabuchi *et al.* have used HPLC to demonstrate numerous endogenous fluorescent substances at excitation (Ex) 322 nm/emission (Em) 415 nm in chronic renal failure and concluded that some of these fluorescent peaks probably represented peptidic substances, but did not identify any of them (M7).

Indeed, so far only a few fluorescent compounds have been isolated. Using reverse-phase HPLC with fluorescence detection at Ex/Em 295/405 nm, one group was able to identify indican (indoxyl sulfate), kynurenic acid, tryptophan, and indole-3-acetic acid, all markedly elevated in uremic sera (B12, S35).

TABLE 2
A SUMMARY OF PREVIOUSLY REPORTED FLUORESCENCE CHARACTERISTICS OF UREMIC SERA^a

Wavelengths (nm)			
Excitation	Emission	Other characteristics ^b	Methods of analysis ^c
356	405	Fluorescence in region at anodic band corresponding to albumin	NADH produced by substrate overlay in fluorometric scan of LDH isoenzymes
320–370	400–450	Fluorescent band unique to chronic renal failure	Fluorescent spectra of serum from a patient with chronic renal failure
360	460	Fluorescence ascribed to CK-BB; intensity correlated with the duration of renal failure	CK isoenzyme measurement by fluorogenic substrate
365	455	Fluorescent material different from CK-BB, with electrophoretic mobility similar but somewhat slower than that of CK-BB	Electrophoresis of CK isoenzymes, measuring fluorescence of NADPH
340–375	455	Nonprotein artifact of CK-BB associated with albumin fraction of protein electrophoresis	Serum protein electrophoresis, CK fractionation by ion-exchange chromatography
366	425	Fluorescent band with emission maximum at 425 nm, distinct from albumin and NADH	Fluorescence in whole serum before and after addition of CK assay mixture
340	420	Water-soluble, heat-stable inhibitor of albumin-binding capacity, $M_r < 10,000$; has acidic group with $pK = 4-5$	Acidified serum applied to hydrophobic resin, eluted with ethanol, measured albumin binding of phenytoin and tryptophan
345	415	Fluorescence bound to albumin, extractable with charcoal treatment under acidic conditions, soluble in water and polar alcohols, diminished at pH <3 or >12	Gel chromatography, isoelectric focusing, protein electrophoresis, thin-layer chromatography, extraction with charcoal and alcohol
322	415	Two emission maxima attributed to substances with $M_r < 1000$, ninhydrin negative	Methanol extraction of hemofiltrates, dialysis fluids, and normal urines; gel filtration for estimation of size
342	430	Fluorescence intensity shows a linear correlation with serum creatinine	Fluorescence measurement of serum, hemodialysate, and urine
295	405	Identified possible substances to be indican, kynurenic acid, tryptophan, and 5-hydroxy-3-indoleacetic acid	Sera and hemodialysate was filtered; used reversed-phase HPLC with fluorescence detection, cochromatography, and arylsulfatase-induced peak shift
322	415	Numerous endogenous fluorescent substances are present in uremic sera and urine	HPLC with fluorescence detection of uremic serum and urine passed through Centriflo CF 25 filter; guanidine compound and amino acids removed
295	405	Identified indican, tryptophan, and indoleacetic acid; eight unidentified components	Reversed-phase HPLC with fluorescence detection of uremic hemodialysate; cochromatography and tryptophanase-induced peak shift

^aFrom Shaykh *et al.* (S19).

^bCK-BB is an isoenzyme of creatine kinase.

^cLDH, Lactate dehydrogenase; CK, creatine kinase; HPLC, high-performance liquid chromatography.

Shaykh *et al.*, using gel filtration chromatography, reported in uremic patients a hitherto undescribed endogenous fluorescence with Ex/Em maxima of 380/440 nm and 400/460 nm (S19). These fluorescent spectral properties were unique in that the Ex/Em maxima did not occur in the highly fluorescent far-ultraviolet region, but rather in the near-ultraviolet and visible part of the spectrum (Fig. 9). The fluorescence remained unaltered after serum was passed through ultrafiltration membranes with molecular weight cutoffs from 10,000 to 500 (W6), suggesting that these compounds have a molecular mass of less than 500 Da (Table 3).

Subsequent studies by the same group (W7) have shown that the increased endogenous fluorescence in patients with chronic renal failure is due to the unconjugated pteridine, xanthopterin (2-amino-4,6-pteridinedione, 179 Da; see Table 4 and Figs. 10 and 11) (B23, D11, L15, P11). Unlike the conjugated pteridines (folates), the function of many of the unconjugated pteridines (pterins) has yet to be elucidated (U2, Z3). So far only biopterin has been shown to have a defined role, being a cofactor in the hydroxylation of several aromatic amino acids involved in the formation of neuronal hormones such as catecholamines and serotonin.

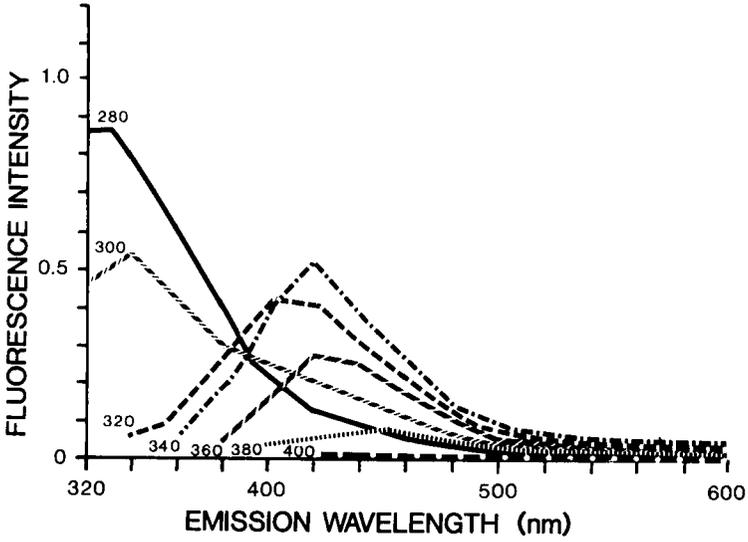
Several studies have shown altered catecholamine metabolism in uremia and have suggested that the unconjugated pteridines may play a role in the causation of some of the neurological symptoms (C21, D8). Dhondt and Vahille have reported an increase of the pteridines neopterin and biopterin in the serum of maintenance dialysis patients (D12). They did not, however, describe an accumulation of xanthopterin, perhaps because this compound, unlike the blue-fluorescing pteridines (excitation maxima = 360 ± 10 nm), has its maximum excitation at 390 nm.

The increase in serum xanthopterin levels may be directly related to renal failure, because the kidney maintains pteridine concentrations within narrow limits (L4, P1, Z5). However, xanthopterin is one of the end products of biopterin and neopterin metabolism, which are elevated in uremia (D12, R9). Thus an increase in their catabolism could be a source of increased xanthopterin production.

Xanthopterin appears to have some metabolic activity. It can induce hematopoiesis and reticulocytosis in anemic animals (T10, T11) and stimulate renal cell mitosis in animal paradigms of uremia (H1). The administration of xanthopterin to animals induces hyperplasia of the kidney (H1). Because the residual nephrons in man also hypertrophy as renal disease progresses, xanthopterin could conceivably play a role in the mechanism of renal hypertrophy.

Because patients with chronic renal failure may have a higher incidence of renal cancer (P14), it is of interest that xanthopterin and other pteridines are increased in the serum or urine of cancer patients (D13, R15, S33). They are likewise elevated in patients with renal allografts (F7). Some of this may be a response to lymphocyte activation (Z6). Several pteridines, i.e., biopterin and neopterin, are increased during lymphocyte proliferation, and the catabolism of these pteridines

FLUORESCENCE OF NORMAL ULTRAFILTRATE



FLUORESCENCE OF CRF ULTRAFILTRATE

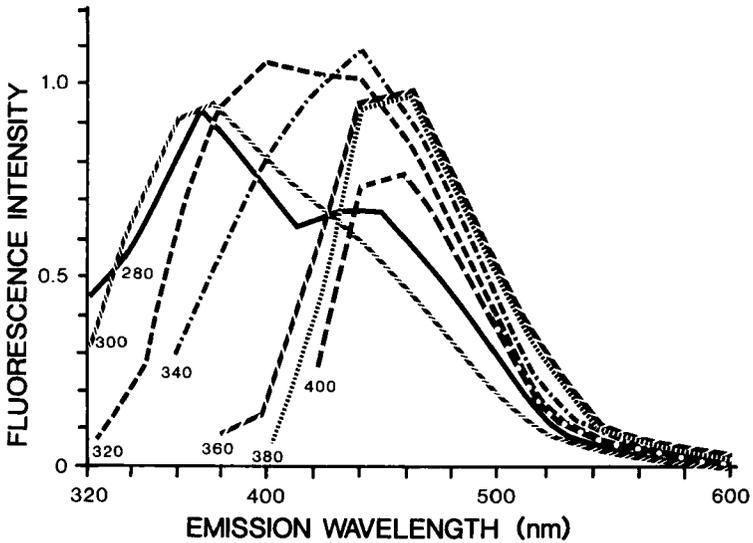


FIG. 9. Fluorescence emission spectra of the PM 10 (10,000-Da cutoff) ultrafiltrates of sera from normal subjects and patients with chronic renal failure (CRF). From Shaykh *et al.* (S19).

TABLE 3
 FLUORESCENCE MEASURED IN THE ULTRAFILTRATES OF UREMIC SERA AT
 Ex/Em 380/440 nm^a

Measure	Uremic ultrafiltrates		
	10,000 Da ^b	1000 Da	500 Da
Mean	9.14 ^c	9.53	8.54
SD	1.44	2.05	1.32

^aFrom Shaykh *et al.* (S19).

^bCutoff value of ultrafiltration membranes.

^cμmol/liter, determined by comparison with (i.e., in terms of) fluorescence of 13.3. μmol/liter quinine standard.

SEPHADEX G-10 GEL CHROMATOGRAM OF PTERIN STANDARDS AND YC05
 ULTRAFILTRATE FROM UREMIC SERUM AND HEMOFILTRATE

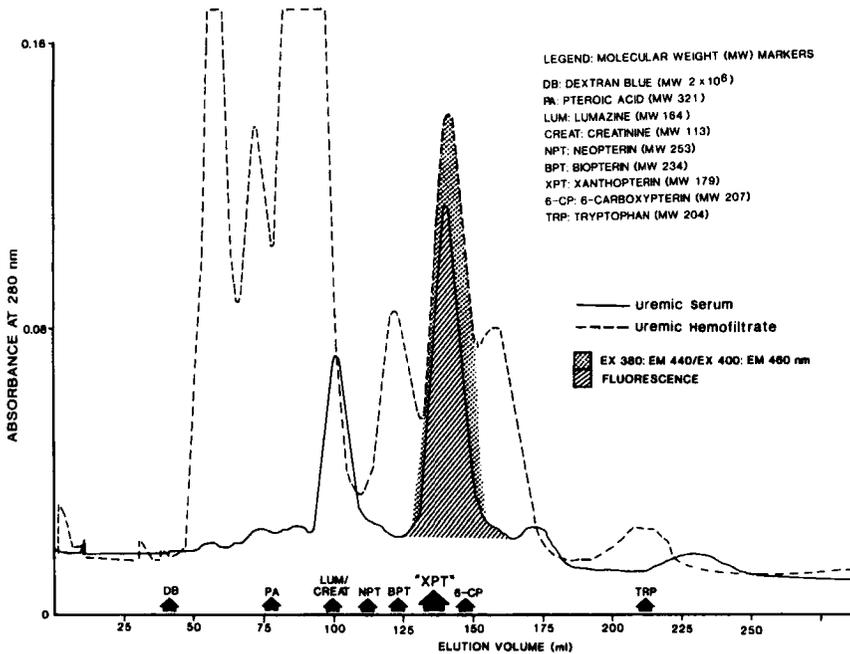


FIG. 10. Sephadex G-10 gel chromatogram of pterin standards and YC05 ultrafiltrate from uremic serum and hemofiltrate. (R. W. Williams *et al.*, unpublished data, 1990.)

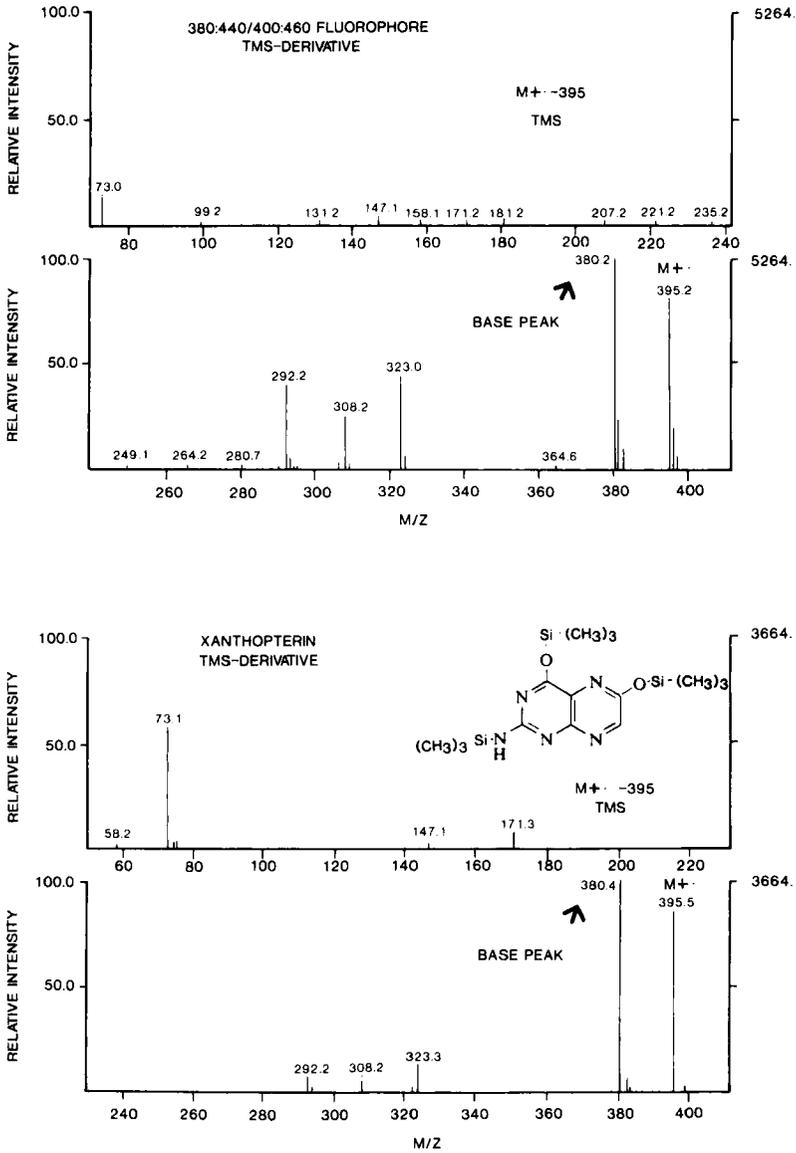


Fig. 11. Comparison of electron impact (EI) mass spectrum of the 380/440–400/460 fluorophore and xanthopterin from gas chromatography using trimethylsilyl (TMS) derivatives; base peak, fragment with greatest intensity; M+, TMS–molecular ion. Arabic numbers represent the mass/charge ratio (m/z). (R. W. Williams *et al.*, unpublished data, 1990.)

TABLE 4
 R_f VALUES (× 100) OF SOME PTERIDINES AND THE 380/440–400/460 FLUOROPHORE FROM UREMIC
 HEMOFILTRATE USING MN-300F CELLULOSE THIN-LAYER CHROMATOGRAPHY PLATES^a

Compound ^b	Solvents ^c					
	NH ₄ Cl	P-A	B-A-W	I-NH ₄ Ac	P-W	E-W
6-Biopterin	73	40	42	51	32	70
6-Hydroxymethylpterin	57	34	32	43	23	61
Isoxanthopterin	40	16	25	29	15	39
Leucopterin	42	3	9	7	0	2
Lumazine	74	40	45	49	1	73
6-Methylpterin	43	45	53	50	35	60
L-Monapterin	68	27	17	37	15	64
D(+)-Neopterin	70	30	19	43	16	67
Pterin	58	42	43	46	26	60
Pterin-6-carboxylic acid	53	14	12	19	10	54
Xanthopterin (dione)	45	23	39	20	6	26
Xanthopterin monohydrate	45	23	39	20	6	26
Pteric acid	8	1	40	7	7	55
Folic acid	34	3	40	13	5	21
380/440–400/460 fluorophore	45	23	39	20	6	26

^aFrom R. W. Williams *et al.* (unpublished data, 1990).

^bConcentration = 100 µg/ml.

^cNH₄Cl = 3% NH₄Cl in distilled water; P-A = *n*-propanol–1% NH₃ (2:1); B-A-W = butanol–acetic acid–water (4:1:1); I-NH₄Ac = isopropanol–2% NH₄ acetate (1:1); P-W = *n*-propanol–water (70:30); E-W = ethanol–water (1:3).

results in increased levels of xanthopterin, which appears to terminate lymphocyte stimulation by inhibiting the synthesis of 45 S pre-rRNA (Z4). This inhibition appears to be specific and may be due to the pterin forming cross-links with double-stranded deoxyribonucleic acid (Z3).

3.14. β₂-MICROGLOBULIN

This 11,800-Da HLA class I light chain is present on all mammalian cells. It is filtered by the glomerulus and reabsorbed by the proximal tubular cells, where it is degraded. It accumulates in renal failure, causing deposition of an amyloid material in the carpal tunnel, synovial membranes, and the ends of long bones. This leads to the carpal tunnel syndrome, a destructive arthropathy, and bone cysts (D14). There is also some evidence that complement activation by bioincompatible dialyzer membranes may cause the generation of β₂-microglobulin by stimulated neutrophils (M30).

4. The Loss of Hormones and Enzymes Produced by the Kidneys

4.1. ERYTHROPOIETIN

Most patients who require dialysis have a normocytic normochronic anemia and a hypoproliferative bone marrow. As erythropoiesis decreases with advancing renal disease, iron shifts from circulating red cells to the reticuloendothelial system, leading to high serum ferritin levels. Repeated blood transfusion is also a common cause of iron overload and hyperferritinemia. Clearly the most important cause of the anemia of chronic renal failure is decreased erythropoietin production by the kidneys; uremic patients have much lower plasma erythropoietin levels than comparably anemic patients with normal renal function (E8). Less important causes are shortened red cell survival, iron or folate deficiency, aluminum intoxication, and osteitis fibrosa cystica (E8). Uremic retention products such as methylguanidine (G10) and spermidine (R2) may also have an adverse effect on erythropoiesis.

Recombinant erythropoietin, recently produced in large quantities by innoculating the erythropoietin gene into the Chinese hamster ovary, has been used to treat the anemia of chronic renal failure with dramatic results (E6, E7, H5, L13, W8). Anemia is corrected in most patients, and there is a greater sense of well being and exercise tolerance (M11). Iron overload from previous blood transfusions improves and serum ferritin levels fall as treatment with erythropoietin is continued.

4.2. 1α -HYDROXYLASE

Vitamin D₃, pivotal in calcium metabolism, is derived from dairy products, from which it is absorbed in the small intestine and carried to the liver by the portal veins. It is also produced from dermal 7-dehydrocholesterol upon exposure to solar ultraviolet radiation, and is then transported to the liver (H12).

Vitamin D₃, whether of dietary or skin origin, is hydroxylated in the liver to 25-hydroxycholecalciferol (25-HCC). This undergoes another hydroxylation in the kidneys to the very active compound 1,25-dihydroxycholecalciferol (1,25-DHCC). This reaction is catalyzed by 1α -hydroxylase, a mitochondrial cytochrome *P*-450 mixed-function oxidase normally found in the kidneys, although some pathological tissues such as sarcoid granulomas may also possess the enzyme (H12).

1,25-DHCC acts like a steroidal hormone. It enters the small intestinal mucosal cell and binds to the nuclear membrane, stimulating the formation of a messenger RNA that diffuses back to the cytosol and directs the formation of several peptides. These are then assembled to form a calcium-binding protein that controls calcium absorption and transport by the small intestine (F5, H12). Besides its role in

calcium absorption, 1,25-DHCC also stimulates intestinal absorption of phosphorus; hypophosphatemia enhances while hyperphosphatemia suppresses the renal production of the hormone. 1,25-DHCC also plays a permissive role in the parathormone-induced calcium resorption from bone (F5).

In progressive renal disease two main events alter calcium and phosphorus metabolism (Fig. 12). The first is a decline in glomerular filtration, leading to phosphate retention. This induces a reciprocal decrease in the plasma ionized calcium, which promptly causes a surge of parathormone secretion. Parathormone raises the plasma calcium concentration by stimulating skeletal resorption and increasing renal phosphate excretion. The resulting decrease in intracellular phosphorus is believed to stimulate renal production of 1,25-DHCC, which will raise the level of plasma calcium by increasing its absorption from the intestines and abetting the parathormone-induced bone resorption. The rebound in the plasma calcium concentration will then shut off further parathormone secretion (F5, H12, L2).

The second event is a progressive loss of the 1α -hydroxylase enzyme, leading to decreased 1,25-DHCC, intestinal malabsorption of calcium, decreased plasma calcium, and increased parathormone secretion. Parathormone will then attempt to restore the plasma calcium level by increasing skeletal resorption. As uremia worsens, however, the bones become increasingly resistant to the calcemic effect of parathormone (G3). Thus the two events of phosphate retention and 1,25-DHCC depletion, by causing hypocalcemia, will initiate the development of secondary hyperparathyroidism. Parathormone will restore plasma calcium and phosphorus toward normal, and a new steady state will be established. With additional nephron loss, this cascade of events will come into play again, until a new steady state has been achieved.

The parathyroid glands initially show hyperplasia of the clear-water cells and a disappearance of fat, but later, actual micronodular hyperplasia and enlargement of the glands occur and the basal secretion of parathormone increases. This is better reflected by measuring the N-terminal or the middle fragment of parathormone rather than the large C-terminal fragment, which is catabolized slowly and is retained in renal failure. The parathyroid glands remain responsive to the plasma calcium level, and when the latter is elevated by the oral administration of 1,25-DHCC, parathyroid hormone (PTH) secretion decreases. However, there is an upward shift of the set point for calcium-regulated PTH secretion, and higher serum calcium levels are required to produce a significant decline in serum PTH (B30). On the other hand administration of 1,25-DHCC intravenously may have the added effect of directly suppressing PTH secretion by inhibiting gene transcription (O3) and synthesis of pre-pro-PTH messenger RNA (S21).

The clinical end product of the pathophysiological processes just described is usually osteitis fibrosa cystica. Skeletal radiography may show characteristic

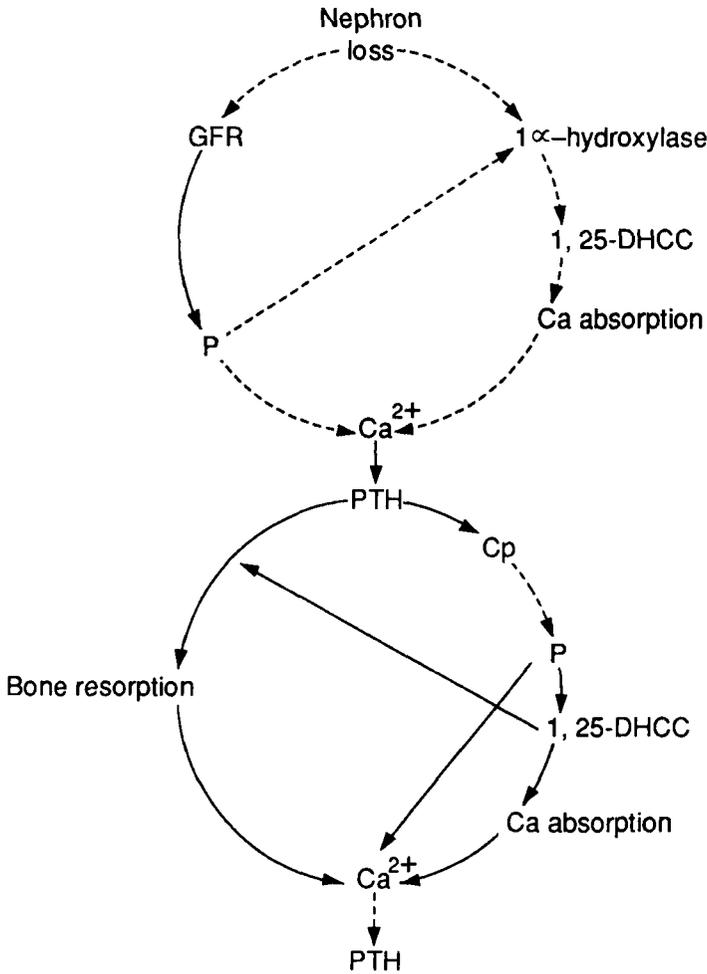


FIG. 12. Scheme of calcium and phosphorus homeostasis in progressive renal disease: —→, increases; - -→, decreases; GFR, glomerular filtration rate; P, serum phosphorus; Ca²⁺, serum ionized calcium; Ca, calcium; 1,25-DHCC, 1,25-dihydroxy cholecalciferol; PTH, serum parathyroid hormone; Cp, phosphate clearance.

changes such as subperiosteal resorption along the radial border of the middle phalanges, resorption of the outer third of the clavicles, and sandy stippling of the skull. Bone turnover, the number and activity of osteoclasts, and the serum alkaline phosphatase level all increase. The treatment of osteitis fibrosa cystica includes the oral administration of phosphate binders, e.g., aluminum hydroxide

or calcium carbonate, which decrease the absorption of dietary phosphorous (C13), the oral or intravenous administration of 1,25-DHCC (A14, M16, M17), and, when all fails, parathyroidectomy (K2).

Other forms of uremic bone disease also occur. They include osteosclerosis (rigger jersey spine), nutritional osteomalacia, aluminum-induced osteodystrophy (low-turnover bone disease, or osteomalacia), and mixed uremic osteodystrophy (hyperparathyroid bone disease and defective mineralization) (M17).

5. The Effects of the Uremic Environment

5.1. ORGAN FUNCTION

5.1.1. Heart Disease

Heart disease is the leading cause of mortality in uremic patients, accounting for half of all deaths. Hypertension and diabetes mellitus, both common causes of chronic renal failure, clearly play an important role in the prevalence of cardiovascular disease. Furthermore, coronary atherosclerosis is common in caucasian uremic patients, many of whom have hypertriglyceridemia and low serum levels of high-density lipoprotein (HDL) cholesterol.

Pericarditis complicating severe uremia was recognized by Richard Bright in 1836 (B28). It presaged imminent death before the era of dialysis, but nowadays it resolves with adequate dialysis in most cases. Often, however, it first appears in dialyzed patients, being possibly related to viral infections, anticoagulation, sepsis, or fluid overload (C17, D19). A putative uremic toxin has not been found, but hyperparathyroidism, a hyperdynamic ventricle, platelet dysfunction, hyperphosphatemia, hyperuricemia, and treatment with the antihypertensive drug minoxidil have all been cited as possible contributing factors (C17, D19).

Congestive heart failure is common and is related to fluid overload, hypertension, or atherosclerosis. Some workers have postulated a uremic cardiomyopathy. The enhancement by parathormone of cellular calcium uptake may contribute to myocardial calcification, degeneration, and fibrosis (M26). There is a higher incidence of calcification of the aortic and mitral valves, as well as of visceral and peripheral arteries in association with uremic hyperparathyroidism (M13). In addition to PTH, middle molecules (B19), phenols (L3), guanidinosuccinic acid (K5), or cobalt (P6) may contribute to the observed cardiotoxicity *in vitro* of uremic serum.

5.1.2. Respiratory System

Pulmonary edema constitutes an immediate threat to life. It usually results from fluid overload or congestive heart failure, but some workers have postulated a pulmonary capillary leak in uremia. However, this abnormality may not be pe-

cular to uremia, for many progressive renal diseases are characterized by the nephrotic syndrome in which a generalized capillary leak of plasma water and albumin may occur.

5.1.3. *Gastrointestinal Symptoms*

Symptoms consisting of anorexia, nausea, vomiting, weight loss, and uremic fetor are characteristic of untreated uremia. Telangiectasia (angiodyplasia) of the gastrointestinal mucosa, especially of the ascending colon, is probably the most common cause of bleeding in dialysis patients (Z9). Some workers have blamed the aluminum-containing phosphate binders for the development of these lesions, observing that skin telangiectasia develops in aluminum factory workers (T5).

In addition to the decreased intestinal absorption of calcium in uremia, calcium ATPase activity is also low (S6). Many gastrointestinal hormones are found in increased concentrations in uremia. These include gastrin (O10), cholecystokinin (O10), pepsinogen I (I1), gastric inhibitory peptide (L1), amylase, and trypsin (R17). There is a decrease in the conjugation of cholic to deoxycholic acid (G13) and in the pancreatic secretion of bicarbonate (O11). Hepatic output of urea is decreased, as is the activity of some urea cycle enzymes; e.g., ornithine transcarbamylase (T9). The clinical significance of these hormonal and enzymatic perturbations remains to be elucidated.

5.1.4. *Bleeding Tendency*

Patients with renal failure have an abnormal bleeding tendency (R10). In fact, uremic bleeding was described as early as 1764 by Morgagni. There is probably a defect in primary hemostasis, reflected by a prolonged bleeding time and impaired platelet adhesion to foreign surfaces. Anemia is probably the most important factor contributing to the prolongation of the bleeding time, which is shortened considerably but not normalized by red blood cell transfusion. Furthermore, platelet aggregation may be defective, an abnormality attributed by some workers to the accumulation in uremic serum of guanidinosuccinic acid. High parathormone levels and abnormalities of von Willebrand factor, thromboxane A₂, and prostacyclin may also contribute to the hemostatic defect (R10).

Clinically the bleeding is usually not severe, occurring mainly as epistaxis, echymoses, and bleeding from venipuncture sites and from the gastrointestinal tract. In the latter instance mucosal ulcerations or more commonly telangiectasia are the underlying lesions. Uremic bleeding may improve with packed red blood cell transfusion, dialysis, cryoprecipitate, intravenous or intranasal desmopressin (a synthetic derivative of vasopressin), or conjugated estrogens (B29, R10).

5.1.5. *Neurologic Abnormalities*

Insomnia, tremor, asterixis, and the restless leg syndrome are probably central in origin, and are usually seen in severe uremia. Peripheral neuropathy involves

sensory and motor nerve fibers. The ankle jerk and the vibration perception over the halluces are the first to disappear. The nerve conduction velocity is decreased but does not correlate with the clinical severity of the neuropathy. The peripheral neuropathy improves partially with dialysis, but usually resolves completely after renal transplantation. Autonomic neuropathy may also occur and may account for the failure of some patients to develop tachycardia during dialysis-induced hypotension (J1).

As discussed earlier peripheral neuropathy has been blamed on uremic products such as myoinositol (C12, D7, H8, L14, N6, R11) and methylguanidine (B3), whereas the latter compound and cyanate are claimed to suppress central nervous function (B3, C24, F2). However, there is no convincing clinical evidence that these compounds are responsible for uremic neuropathy (B24, J1, R11).

5.1.6. Immune System

The immune system is also compromised in uremia. That and the use of dialysis catheters and synthetic grafts, the frequent blood transfusions, the exposure to nosocomial organisms during repeated hospitalization, the presence of anemia and malnutrition, and the immunocompromised state associated with certain renal diseases (nephrotic syndrome, lupus erythematosus) all conspire to make infection a major cause of mortality, accounting for 15–20% of uremic deaths. Pyogenic infections are facilitated by impaired neutrophil adherence and chemotaxis (L7, S27), blunted antibody response (B15), and defective phagocytosis by macrophages due to decreased Fc-receptor activity (R19).

Uremic patients also exhibit impairment of type IV immunity, manifested clinically by delayed rejection of skin and renal allografts, a protracted but mild infection with the hepatitis B virus with a higher rate of asymptomatic carriage, and a “burning out” of lupus nephritis with the onset of advanced renal failure (C20). On the cellular level there is lymphopenia (R5), a decrease in helper T cells (C8), and impaired T cell blastogenic response to mitogens (K1). Lymphocytes from uremic and hemodialyzed patients show decreased *in vitro* production of interferon (an activator of macrophages, B lymphocytes, and cytotoxic T cells) (G2). Likewise, mitogen-stimulated lymphocytes from hemodialyzed patients produce low amounts of interleukin-2, needed to stimulate antibody production by B lymphocytes and differentiation of T cells and natural killer lymphocytes (C8).

5.1.7. Endocrine System

Abnormalities of the endocrine system are also seen (M41). Pregnancy is rare in women whose serum creatinine exceeds 3 mg% and often ends in spontaneous abortion. Amenorrhea and dysfunctional uterine bleeding are common in women on hemodialysis. Ovarian production of estrogen and progesterone is decreased, leading to an increase in follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The secretion of FSH and LH increases after the administration of

LH-releasing hormone, indicating normal gonadotropin production, storage, and release. The clomiphene-stimulated rise in FSH does not increase estrogen levels, further confirming primary ovarian dysfunction (L12). Fertility is restored after renal transplantation, and several hundred women with renal allografts have borne normal babies.

In men decreased libido and impotence are common (M42). There is atrophy of the seminiferous tubules, decreased or absent spermatogenesis, and fibrosis of the interstitial cells of Leydig. Testosterone and dihydrotestosterone secretions are decreased both before and after stimulation by human chorionic gonadotrophin. LH and FSH are increased but they respond normally to gonadotrophin-releasing hormone and clomiphene (T12).

Although there may be a defective thyroidal incorporation of iodide, free thyroxin (T4) levels are normal. Serum triiodothyronine (T3) is decreased owing to decreased peripheral conversion of T4 to T3 (L11). Patients are generally euthyroid and serum thyroid-stimulating hormone (TSH) levels are normal. Most uremic men and women have high serum prolactin levels resulting from increased production and, to a lesser extent, decreased degradation. Prolactin levels are not increased by thyrotropin-releasing hormone, phenothiazines, arginine, or insulin hypoglycemia, and they are not decreased by dopamine and L-dopa (L10, S8). The chronic, but not acute, administration of bromocriptine may decrease prolactin levels (L12, M41).

Serum growth hormone level is usually increased, mainly owing to decreased metabolic clearance (S7). Somatomedin levels are normal by radioimmunoassay (T1), but the activity is decreased on bioassay, perhaps because of the presence of a low-molecular-weight inhibitor in the serum. The decreased somatomedin activity may contribute to the protein catabolic state, is inversely related to the BUN and serum creatinine levels, and may improve during dialysis (P12).

5.1.8. *Skin*

The most common cutaneous problem is pruritis, which occurs in 50–75% of patients. It is very distressing and may cause severe skin excoriations. It may be related to secondary hyperparathyroidism, in which case parathyroidectomy may provide dramatic relief (H7). In some cases the dermal content of phosphorous, calcium, and magnesium is increased. Ultraviolet B radiation, which decreases dermal phosphorous, may relieve the itching, thus suggesting a causative role for skin deposits of calcium or magnesium phosphate (B20). Histamine may play a role because dermal mast cells are increased; ketotifen, which stabilizes these cells, may relieve the itching (M28). Exposure to dialyzer membranes, to the potting compounds used to cap dialyzers, or to plasticizers in the tubing may cause IgE-mediated release of histamine by mast cells (C6, P2). Removing the allergenic material and using antihistaminics may relieve the pruritis, as may oral charcoal (P5) or cholestyramine (S22).

5.2. INTERMEDIARY METABOLISM

5.2.1. *Carbohydrates*

More than half of uremic patients exhibit insulin resistance manifested by fasting euglycemia despite hyperinsulinemia, blunted hypoglycemic response to administered insulin, persistent hyperinsulinemia late into a glucose tolerance test (L16), and decreased glucose uptake by skeletal muscle (D6) and adipose tissue (M18). The increased plasma levels of glucagon (M36,S20) and growth hormone (S7) may play a role in insulin resistance, but a postreceptor defect in insulin action (S28) and uremic toxins such as spermidine (L17) and *o*-hydroxyphenolic acid (T8) may be more important, because hemodialysis reverses glucose intolerance without decreasing glucagon or growth hormone levels (M24).

Fasting plasma insulin is therefore increased owing not only to peripheral resistance to its action but also to decreased renal excretion and degradation by skeletal muscle (R1). Plasma levels of proinsulin and C-peptide, both excreted by the kidney, are also increased. Glucagon (3500 Da) and its larger precursor (9000 Da) are also increased because of reduced degradation (M36). Hyperglucagonemia is corrected by renal transplantation but not by dialysis.

Insulin-dependent diabetic patients with advancing renal failure often develop more frequent episodes of hypoglycemia resulting from decreased renal degradation of insulin. Emaciated nondiabetic patients may also develop spontaneous hypoglycemia caused by low glycogen stores and the decreased contribution by the diseased kidneys to gluconeogenesis (B11, W5).

5.2.2. *Proteins*

Uremic patients are often catabolic and exhibit a negative nitrogen balance that takes a greater protein intake to correct (K9). Moreover, amino acid losses during hemodialysis (K10) and albumin losses from peritoneal dialysis are not insignificant. There is a decrease in the body pool of albumin and other proteins (Z2). Several essential amino acids in the plasma are decreased, while some non-essential ones are increased. Tyrosine:phenylalanine and valine:glycine ratios are decreased. A most common abnormality is an increase in hydroxyproline, citrulline, and 1,3-methylhistidine. These abnormalities are corrected by hemodialysis (F14).

There may also be characteristic changes in the intracellular content of amino acids. Several essential amino acids, which in uremia also include histidine and tyrosine, are decreased. Intracellular depletion of valine may contribute to increased muscle protein catabolism and negative nitrogen balance. These derangements are corrected by dietary manipulation and dialysis, unlike the increased muscle content of urea cycle amino acids (F14).

5.2.3. Lipids

The common lipid abnormalities are hypertriglyceridemia in 30–50% of patients, low serum HDL cholesterol in 50–75% (mostly white patients), and hypercholesterolemia in 20% (C5, H3). Hypertriglyceridemia is usually evident when the BUN has exceeded 50 mg% and is caused by decreased lipoprotein lipase activity. Low enzyme activator concentration (low apoC11:apoC111 ratio), diminished enzyme synthesis due to insulin resistance, a smaller releasable pool of enzyme due to repeated heparinization, and enzyme inhibition by uremic toxins (e.g., spermidine) have all been invoked to explain the decreased lipoprotein lipase activity (C26). Acetate used in the dialysis bath is also claimed to contribute to hypertriglyceridemia through its conversion to acetyl-Co A, but this effect of acetate is probably insignificant (S4).

The hypertriglyceridemia of renal failure resembles the endogenous familial variety, and may likewise coexist with insulin resistance, glucose intolerance, and hyperuricemia. In dialysis patients it is associated with a high rate of coronary artery disease in women and white men under 60 years (C26). The low HDL cholesterol levels, found mainly in white men on hemodialysis, are attributed to decreased activity of the enzyme lecithin cholesterol acyltransferase (L-CAT). Decreased enzyme activator apo-apo A1 and inhibitory uremic toxins have been proposed as causes for the decreased enzyme activity. Low HDL cholesterol levels are associated with an increased risk of atherosclerotic heart disease (C26).

Coronary artery disease is a leading cause of death in dialysis patients, but it has not been proved that its prevalence exceeds that in age- and risk-matched controls. It is clear, however, that white men have greater triglyceridemia and lower HDL cholesterol levels and greater coronary mortality than do black men, despite the fact that the latter exhibit a higher prevalence of hypertension, smoking, and left ventricular hypertrophy (C26).

5.3. TRANSPORT DERANGEMENTS

Decreased activity of the enzyme sodium potassium ATPase increases cellular sodium and decreases potassium, resulting in a decreased potential difference across the cell membrane. Because cellular sodium gain exceeds potassium loss, the cell water content increases. The decreased enzyme activity and the derangement in cell sodium, potassium, and water content are corrected partially by hemodialysis and completely by renal transplantation (C22, P3).

The disturbance in the membrane potential difference is corrected by hemodialysis or protein restriction, suggesting a causative role for dialyzable products of protein metabolism. Phenolic acids (W2), methylguanidine, atriopeptins, and tissue hypothyroidism have been suggested as causes for the decreased sodium potassium ATPase activity (C23).

6. Dialysis-Related Problems

6.1. HYPOTENSION

Hypotension is common during hemodialysis. When it occurs early (within the first half hour), it may be due to overmedication with antihypertensive agents, pericardial effusion, or the "first-use syndrome." Later in dialysis hypotension usually results from excessive ultrafiltration, abnormal left ventricular function, or the peripheral vasodilatory effect of acetate in the dialysis bath. Prevention depends on the cause of hypotension. It includes the administration of saline intravenously, controlling the rate of ultrafiltration, increasing the dialysate sodium concentration to about 140 mEq/liter, or substituting bicarbonate for acetate in the dialysis bath (B22).

6.2. FIRST-USE SYNDROME

This syndrome may occur occasionally with the first use of a dialyzer, usually of the cuprophane type, and is an anaphylactic reaction with hypotension, wheezing, and flushing. Rarely it may be severe, resulting in death from marked bronchospasm and shock. In most cases the symptoms subside upon stopping the dialysis without returning the blood in the extracorporeal circuit to the patient, but a few patients require vigorous treatment for anaphylactic shock.

There may be more than one cause for this syndrome. Complement activation by bioincompatible membranes (H4); hypersensitivity to the ethylene oxide used in dialyzer sterilization, to phthalic anhydride, or to isocyanates (plasticizers) used in potting compounds present in the dialyzer caps; and entry of bacterial endotoxin from the dialysate into the bloodstream may each play a role (B22, H4, I2). Hypoxemia and high serum levels of the C3a and C5a complement fragments would suggest complement activation (C2, H4), and peripheral blood eosinophilia and high plasma IgE levels would point to a hypersensitivity reactions. Fever and chills, on the other hand, would incriminate endotoxemia.

The first-use reaction does not usually occur with subsequent uses of the dialyzer, and may be prevented by thorough rinsing of new dialyzers. It is less common with more biocompatible membranes; e.g., cellulose acetate, polyacrylonitrile, or polymethacrylate (B22, H4, I2).

6.3. MUSCLE CRAMPS

Muscle cramps occur in about 20% of hemodialysis patients. They may be excruciating but are of no serious consequence. They occur mainly in the legs, but may involve the muscles of the abdomen, chest wall, and upper extremities. Cramps occur late in dialysis, may last several minutes, and are usually related to

rapid or excessive extracellular fluid loss, especially with high-flux or large dialyzers. Increasing the bath sodium concentration to 140 mEq/liter, the intravenous administration of a small volume of hypertonic saline or dextrose solution, or the oral administration of quinine sulfate may all have some benefit (B22).

6.4. POSTDIALYSIS SYNDROME

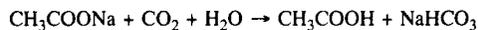
This syndrome is often seen after hemodialysis and may last throughout the following day. It occurs mostly in elderly or debilitated patients who feel weak, "washed out," and are hardly functional after dialysis. It has been suggested that the symptoms are caused by interleukin-1 released from activated monocytes (B22). More likely, however, the syndrome is related to water and electrolyte fluxes and the cardiovascular stress associated with hemodialysis.

6.5. PRURITIS

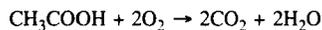
Pruritis may be exacerbated during hemodialysis. Sometimes it is ameliorated by using a different dialyzer, changing from porcine to bovine heparin, the oral administration of an antihistaminic agent, or intravenous administration of lidocaine (B22).

6.6. HYPOXEMIA

Hypoxemia occurs during hemodialysis but is mild and asymptomatic unless there is underlying cardiopulmonary disease. The arterial pO_2 does not drop below 70 mm Hg, hence significant hemoglobin desaturation does not occur. However, there is an extensive literature on dialysis-induced hypoxemia, partly owing to its interesting pathophysiology. Hypoxemia is most likely to occur with the use of a sodium acetate bath and bioincompatible (cuprophane) dialyzer membranes. Acetate is metabolized in the liver to bicarbonate, fixing in the process metabolic CO_2 , thus,



Sodium acetate Acetic acid



It can therefore be seen that 1 mol of acetate will consume 2 mol of oxygen and net 1 mol of CO_2 , thereby decreasing the respiratory quotient (C2).

At the usual rate of 240 mmol/hour of acetate entering the body during dialysis, 240 mmol of CO_2 will be converted to bicarbonate. Furthermore, 12 mmol/hour of gaseous CO_2 will be lost from the blood across the dialyzer, making for a total

of 252 mmol/hour, which is diverted away from the lungs. The resulting decrease in arterial $p\text{CO}_2$ will trigger the chemoreceptors and lead to hypoventilation and hypoxemia. These effects can be reproduced by infusing sodium acetate into patients with chronic renal failure (C2).

If sodium bicarbonate is used instead of acetate, no such fixation of metabolic CO_2 occurs and the gaseous CO_2 loss through the dialyzer is too small to cause significant hypocapnia and reciprocal hypoventilation and hypoxemia. However, a high bath bicarbonate concentration or the use of high-flux dialyzers will cause a large bicarbonate influx and rapid alkalemia, which would lead to alveolar hypoventilation and hypoxemia (C2).

Another cause of dialysis-induced hypoxemia is the use of bioincompatible dialyzer membranes, which activate complement (C3 and C5). This causes the formation of C3a and C5a, which are chemotactic for neutrophils and are anaphylatoxic, thereby causing pulmonary sequestration of neutrophils and platelets (hence neutropenia and thrombocytopenia), with resultant ventilation-perfusion (V/Q) mismatch and hypoxemia. This is evidenced by the simultaneous development 15 minutes after the start of dialysis of hypoxemia, neutropenia, and high circulating levels of C3a and C5a (C2).

Dialyzer membrane bioincompatibility, hence complement activation, is maximal with the first use of cuprophane membranes, is considerably less with cellulose acetate, and is negligible or absent with polyacrylonitrile and polymethacrylate membranes. With dialyzer reuse, complement activation is greatly attenuated (C2). The effects of dialysate and dialyzer on the induction of hypoxemia are additive, so that hypoxemia is greatest during the first use of a cuprophane membrane and acetate bath, but is much improved by the substitution of a bicarbonate bath. The use of a polyacrylonitrile or polymethacrylate membrane with a bicarbonate bath will not cause hypoxemia (D3).

Acetate is also said to contribute to hypoxemia by directly depressing the respiratory muscles and myocardium, the latter effect causing decreased cardiac output with subsequent pulmonary V/Q mismatch and hypoxemia (C2). Although not all workers agree that acetate is a significant myocardial depressant (A10), it may nevertheless cause hypotension by its peripheral vasodilatory effect.

Patients are usually asymptomatic during dialysis-associated hypoxemia, unless they have severe cardiac or pulmonary disease. The hypoxemia can be corrected by using a bicarbonate dialysate, a biocompatible dialyzer, or the administration of O_2 by nasal mask or cannula (C2).

6.7. PRIAPISM

Priapism may also occur in hemodialyzed males. It may be related to heparin, anabolic steroids, or increased blood viscosity resulting from ultrafiltration (B22).

6.8. DIALYSIS DYSEQUILIBRIUM

Dialysis dysequilibrium is not seen often nowadays. It may occur in very uremic patients during the first dialysis, particularly with high-flux dialysis or very rapid blood flow. Its etiology is not clear, but is probably related to increased cerebrospinal fluid pressure and cerebral edema. The possible role of urea has already been discussed (see Section 3.1). Another hypothesis is that the brain cells of uremic patients produce "idiogenic" osmoles to prevent water loss to the hyperosmolar extracellular fluid. When high-flux dialysis rapidly decreases extracellular fluid osmolality, it may cause a water shift into the brain (B22).

Dialysis dysequilibrium may be prevented by avoiding vigorous hemodialysis in the early treatment of patients with severe uremia, infusing osmotic agents (e.g., mannitol) during dialysis, premedication with phenytoin, or by performing the slower and less stressful peritoneal dialysis (B22).

6.9. MECHANICAL FAILURE OR INCORRECT PROCEDURE

Complications resulting from these problems are rare nowadays. They include hyper- and hyponatremia, hyper- and hypokalemia, hypercalcemia, hypermagnesemia, air embolism, overheated dialysate resulting in hemolysis and possibly fatal hyperkalemia, and electrocution (B22).

6.10. SUDDEN DEATH

This is very rare during hemodialysis. It may result from cardiac arrhythmias induced by electrolyte abnormalities or hypoxemia, the first-use syndrome, severe hemorrhage, or mechanical or procedural failure (B22).

7. Drugs and Exogenous Toxins

Uremic patients are at an increased risk from drugs and exogenous toxins. The potential toxicity of drugs is enhanced by decreased renal excretion (digoxin, procainamide, aminoglycoside antibiotics, aluminum compounds), diminished protein binding (phenytoin, warfarin), prolonged serum half-life (penicillins, cephalosporins), and an impaired blood-brain barrier. The dosages of many drugs must be decreased according to the extent of renal failure if serious morbidity is to be avoided.

7.1. ALUMINUM

In the early 1970s a serious encephalopathic syndrome was described in patients receiving hemodialysis. It consisted of stuttering speech or mutism, myoclonic

jerks, ataxia, progressive dementia, convulsions, and death (A6, M15). Aluminum contamination of the dialysis water was later shown to be responsible for that fatal syndrome (A7, A8, D20, M32) and also for a painful fracturing osteomalacia (O7) and microcytic anemia (A9, O1). Epidemic dialysis dementia all but disappeared with the removal of aluminum from the dialysis water.

However, varying degrees of aluminum retention continue to occur in uremic patients from the ingestion of aluminum-containing phosphate binders, usually aluminum hydroxide. Although aluminum is poorly absorbed from the gastrointestinal tract, long-term intake in the face of decreasing renal excretion leads to a significant increase in the body burden of the metal (C11).

Furthermore, certain factors may increase intestinal aluminum absorption. We have previously described severe hyperaluminemia resulting in an acute rapidly fatal encephalopathy in four uremic patients who had been taking aluminum hydroxide and Shohl's solution, an alkalinizing buffer of sodium citrate and citric acid. The encephalopathy resembled the earlier dialysis dementia of the 1970s, but was much more acute, culminating in death within 3 weeks (B4, B5).

This led us to suspect that citrate had enhanced intestinal aluminum absorption in those four patients (B5). We subsequently showed that the concomitant ingestion of citrate and aluminum hydroxide caused high serum aluminum levels in patients with chronic renal failure, and high serum and urinary levels in healthy volunteers (B6, B7). These findings were also confirmed by others (R18, S2). Furthermore, it was recently shown that citrate enhances intestinal aluminum absorption by opening up the tight junctions between the duodenal mucosal cells (F10). Citrate has a great affinity for aluminum binding owing to the small ionic radius and polyvalence of aluminum, which allow it to link with the hydroxyl groups of citrate (P4). Citrate may thus act as a carrier for aluminum, transporting it to the brain (encephalopathy) or to the mineralization front of bones (osteomalacia) (T6).

We have also found that aluminum retention is increased in the older azotemic patient taking aluminum hydroxide (B6). This may be related to increased aluminum absorption owing to the decreased gastric acidity of old age (average pH of 4). Other factors enhancing the intestinal absorption of aluminum are diabetes mellitus (A13), poor food intake, impairment of the gastric mucosal barrier by telangiectasia or inflammation, and H₂-blocking drugs (C14).

To prevent large aluminum loads in uremic patients, aluminum-containing phosphate binders are given nowadays in smaller doses, or other phosphate binders, such as calcium carbonate, are given instead (F11). More emphasis is placed on decreasing dietary phosphorus intake. For the established case of symptomatic aluminum overload, the chelating agent desferroxamine is given intravenously at the end of dialysis sessions. This treatment may have to be continued for many months before it shows any benefits.

7.2. COPPER

Copper used in the wiring of dialysis systems may be leached in an acidic medium. It then diffuses into the blood, causing hemolysis. Symptoms consist of flushing, chills, nausea, vomiting, abdominal cramps, and diarrhea. This problem can be avoided by deionization of the dialysis water. The deionizer should be checked periodically to assure it is not being exhausted (M23).

7.3. ZINC

Zinc may also be leached from new galvanized iron piping. The plasma and red blood cells take up zinc even when its concentration in the dialysate is only one-third that of the plasma, and this may cause a severe hemolytic anemia. Deionization of the dialysis water should prevent this problem (P10).

7.4. SODIUM AZIDE

Sodium azide in glycerine solution is used as a preservative for new ultrafilters used in the dialysis circuit. If the filters are not thoroughly rinsed prior to mounting, sodium azide may gain access to the bloodstream, causing hypotension, nausea, vomiting, headache, blurred vision, cramps, and syncope. The symptoms resolve within minutes after stopping the dialysis. Sodium azide, a stable salt of hydrazoic acid, causes hypotension, even when given by mouth, by relaxing arteriolar smooth muscle, thereby decreasing the systemic vascular resistance (G14).

7.5. CHLORAMINES

Chloramines are formed from the reaction of chlorine and ammonia used in most urban water purification plants. Chloramines are removed by treating the dialysis water with activated charcoal. Failure to do so may allow chloramine to diffuse into the blood, causing severe hemolysis by converting hemoglobin to methemoglobin. It also inhibits the hexose monophosphate shunt pathway in the red blood cells, thereby impairing the generation of NADPH and rendering the cells susceptible to oxidant damage. Neither chlorine alone nor sodium hypochlorite inhibits this shunt pathway (E1).

The use of reverse osmosis to treat dialysis water does not remove chloramine, but the addition of ascorbic acid does. Treating the dialysis water with activated charcoal effectively removes chloramine, but periodic water testing with *o*-toluidine should nevertheless be undertaken. This reagent detects total chloride, i.e., OCl_1 , HOCl_1 , NHC_1_2 , and NC_1_3 . Interestingly, chloramine contamination of natural waters has also caused hemolysis in several species of freshwater fish (E1).

7.6. FORMALDEHYDE

Formaldehyde may be used in water filtration systems (formaldehyde-melamine), to disinfect dialysis machines, or to clean dialyzers before reuse. In all cases it should be thoroughly washed away and its absence confirmed by a negative test with a clintest paper strip. The entry of formaldehyde into the bloodstream will cause hemolysis. This occurs because the red blood cells are rich in the enzyme aldehyde dehydrogenase, which oxidizes formaldehyde to formic acid, using up in the process NADP and generating NADPH. This would inhibit glycolysis at the glyceraldehyde-3-phosphate step, depleting ATP and causing red blood cell fragility and hemolysis (O5).

7.7. NITRATE

Nitrate intoxication has been described in a patient receiving hemodialysis at home. Using well water contaminated with nitrates, the patient developed dyspnea, sweating, and cyanosis. The venous blood acquired a brownish color and the arterial pO_2 decreased and failed to rise with the administration of O_2 . His condition reversed with the intravenous administration of methylene blue. Nitrate must be converted to nitrite before it can convert hemoglobin (ferrous) to methemoglobin (ferric). The authors of this case report postulated that nitrate must have diffused from the blood into the gut, where the resident bacteria reduced it to nitrite, which then diffused back into the blood (C3).

7.8. FLUORIDE

Fluoride intoxication was reported in 1980 in eight patients from a single dialysis unit. The patients developed nausea, vomiting, diarrhea, itching, hypotension, and substernal pain. One patient died. The incident was traced to the spillage of a large amount of hydrofluosilicic acid into the district's water purification plant because of the failure to close a valve. The dialysis unit in question used a water softener only and did not employ reverse osmosis or deionization (A11).

8. Conclusions

The nature of uremic toxicity remains elusive, and laboratory indices of renal functions do not necessarily correlate with uremic symptoms. To the clinician, however, the decision to initiate dialysis treatment for chronic renal failure is based largely on a combination of uremic symptoms and high BUN (≥ 100 mg/dl) and serum creatinine (≥ 10 mg/dl) levels.

Uremia results from a number of processes arising from the progressive loss of

renal functional mass. These include the loss of excretory function, hence the retention of toxic inorganic and organic compounds, and the depletion of crucial renal hormones (erythropoietin) and enzymes (1α -hydroxylase), hence the development of anemia and hyperparathyroidism. These perturbations and the adaptive responses they engender produce a uremic environment which in turn causes abnormalities of organ function, intermediary metabolism, and transport processes.

The retention of inorganic substances causes the well-known complications of hyperkalemia, acidosis, pulmonary edema, and hypertension. However, the clinical effects of the retention of organic substances are less clear, except for β_2 -microglobulin. The role of the so called middle molecules is not well defined, and some of these molecules are in fact quite small. Numerous *in vitro* experiments suggest that a number of these middle and smaller molecular compounds contribute to the neuropathy, anemia, platelet dysfunction, glucose intolerance, and impaired albumin binding seen in uremia. Unconjugated fluorescent pteridines, including their end product xanthopterin, are also retained in uremia, but it is not known if they exert any adverse effects.

As patients live longer with dialysis treatment, they begin to develop yet another set of problems related to dialysis itself, and to the chronic accumulation of endogenous compounds (β_2 -microglobulin) and exogenous toxins (aluminum). β_2 -Microglobulin causes "dialysis amyloidosis" with the carpal tunnel syndrome and destructive arthropathy. Aluminum causes anemia, osteomalacia or adynamic bone disease, and encephalopathy.

The treatment of end-stage renal failure by dialysis and transplantation has made great strides, but much more research is still needed if we are to unravel the mystery of the uremic environment.

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RENAL BIOCHEMISTRY AND PHYSIOLOGY: PATHOPHYSIOLOGY AND ANALYTICAL PERSPECTIVES

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1. Anatomical Features of the Kidney

The nephron is the functional unit of the kidney. Each of the two kidneys contains approximately 1.3 million nephrons. The components of the nephron include the glomerulus and Bowman’s capsule, the proximal and distal tubules,

and the loop of Henle (Fig. 1). The collecting tubules are not considered to be part of the nephron, because embryologically they develop from the ureteric bud. However, because the collecting tubules play a major role in determining the composition of the urine, they are generally discussed in relation to the nephron. The cut surface of a bisected kidney consists of an outer region, the cortex, and an inner region, the medulla. The medulla is further divided into an inner and outer zone. The outer zone is further divided into inner and outer stripes. Nephrons found in the outer cortex are designated as the cortical nephrons. Nephrons found near the corticomedullary boundary are called juxtamedullary nephrons. These juxtamedullary nephrons have long loops of Henle with characteristic descending and ascending thin limb portions that enter the inner medullary zone. In contrast, cortical nephrons have loops of Henle that are short, with thin limb portions sometimes lacking and, when present, they generally do not enter the medullary zone. The short cortical nephrons are seven times more abundant than the long juxtamedullary nephrons (1).

The glomerulus consists of capillary loops that provide a filtration surface of approximately 1 m² that is involved in the formation of an ultrafiltrate of plasma (2). It is seated in the Bowman's capsule. The capillary wall is made up of three layers, beginning with an endothelial layer containing centrally located mesangial

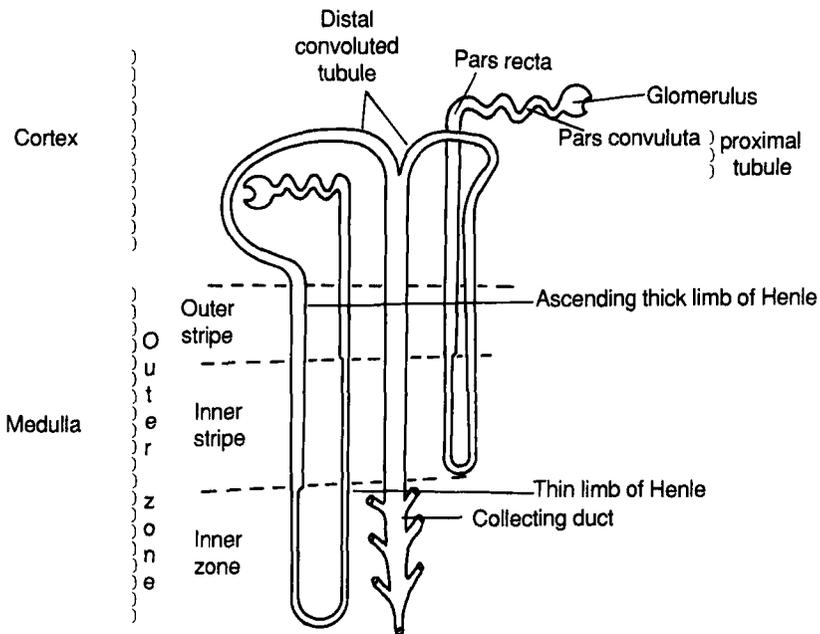


FIG. 1. Components of the nephron

cells, the basement membrane of the Bowman's capsule, and the Bowman's epithelial cell layer, with the foot processes of the epithelial cell or podocyte being fixed on the basement membrane (Fig. 2). The mesangial cells have phagocytic properties and are contractile.

The basement membrane has collagen and glycoproteins as its major constituents. Anionic regions present on the basement membrane influence the filtration of molecules. The juxtaglomerular apparatus located at the hilus of the glomerulus consists of three parts. These are the macula densa, which is a small segment of the distal tubule near the afferent arteriole, the epithelioid cells, which are the site of renin synthesis, and the lacis cells located in the region between the macula densa and the afferent and efferent arterioles (Fig. 3).

The proximal tubule extends from the glomerulus to the descending thin limb of the loop of Henle. The convoluted segment of the proximal tubule is distinguished as the pars convoluta, in contrast to the straight segment of the tubule, which is referred to as the pars recta. The cells of the pars convoluta are extremely complex and contain elongated mitochondria; an endocytic apparatus with its complement of apical vacuoles, vesicles, and dense tubules; and microvilli, constituting the brush border. The brush border microvilli serve to increase the surface area of the cell significantly. The endocytic apparatus enables the cell to uptake and digest both protein and nonprotein constituents.

The cell structure of the pars recta, less complex than that seen in the cells associated with the pars convoluta, is characterized by a decrease in the size and number of mitochondria. As such, the pars recta, in comparison with pars convoluta, is less involved in functions requiring active transport, such as sodium and water reabsorption.

The terminal portion of the proximal tubule leads into the descending thin limb of Henle in the region corresponding to the demarcation of outer and inner stripes of the outer zone of the medulla. The thin limb loops upward to form the ascending thin limb of Henle, which differentiates into the ascending thick limb of Henle at the intersection of the inner zone of medulla and the inner stripe of the outer medullary zone. This is characteristic of juxtamedullary nephrons that have long loops of Henle. In contrast, as noted earlier, cortical nephrons have short loops of

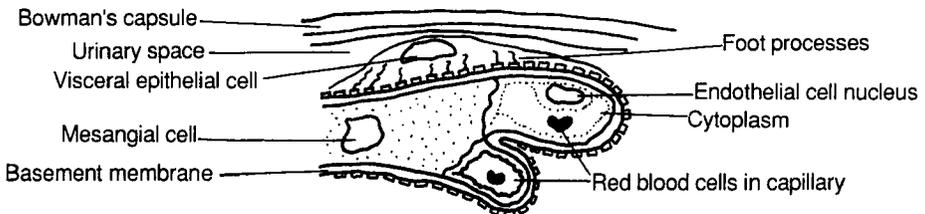


FIG. 2. Schematic representation of the glomerulus.

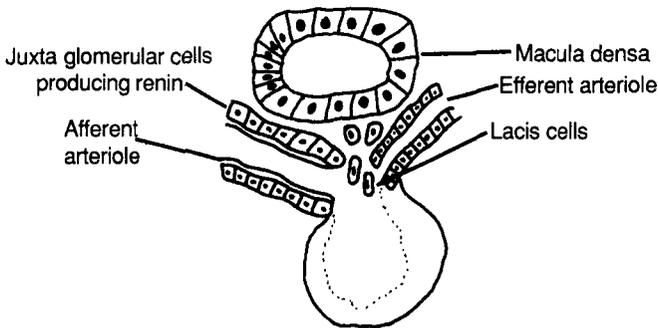


FIG. 3. The juxtaglomerular apparatus

Henle. There is a variation in morphology corresponding to the portions of limb structure of the loop of Henle. In general, the shapes of the thin limb cells are less complex and are characterized by a thick basement membrane and a cytoplasm rich in granulated endoplasmic reticulum. The concentration of urine is effected by the ascending and descending thin limb regions of the loop of Henle. Structurally, the ascending thick limb of the loop of Henle, with its abundance of elongated mitochondria, appears to be well suited for the role of active transport of sodium chloride, whereas the ascending thin limb segment of the loop of Henle is not suited to this role, due to the paucity of mitochondria.

The ascending thick limb or the pars recta of the distal tubule leads into the distal convoluted tubules or the pars convoluta of the distal tubule. The macula densa is a specialized region corresponding to the initial portion of the pars convoluta of the distal tubule. The two segments of the distal tubule (the pars recta and pars convoluta) are similar morphologically and functionally in terms of active chloride transport and permeability to water.

The distal convoluted tubule empties into the collecting duct, which can be differentiated into the cortical collecting segment, the outer medullary, and the inner medullary segments. The collecting duct has a major role in effecting concentration of urine. The collecting duct is under the hormonal influence of antidiuretic hormone. The reabsorption of sodium chloride and secretion of potassium take place in the collecting duct.

2. Renal Physiology

2.1. FUNCTIONS OF THE NEPHRON

A discussion of the functions of the nephron usually begins with the ultrafiltration unit, the glomerulus. Approximately 173 liters of fluid containing

solute is filtered through the glomerulus in 24 hours. The fluid courses through a network of glomerular capillaries that constitute the ultrafiltration unit. The structural configuration of the ultrafiltration unit plays a pivotal role in determining the efficiency of ultrafiltration. Structurally, the ultrafiltration unit consists of a fenestrated endothelium, the glomerular basement membrane, and epithelial foot processes separated by slit diaphragms (Fig. 4). The fenestrated endothelium permits the passage of the fluid containing solute that is continually presented to the glomerular capillary for ultrafiltration. Biochemically, the fenestrated endothelium is characterized by the sialic acid content of its plasmalemma and is believed to contain glycoprotein in the form of heparin sulfate proteoglycan (2-4). The structural features of the glomerular basement membrane have a key role in ultrafiltration. In the electron microscope, glomerular basement membrane can be characterized by a middle electron-dense region called the lamina densa that is surrounded by electron-lucent regions, one on each side, called the lamina rara externa and lamina rara interna. Through the fenestrated endothelium, the lamina rara interna region of the glomerular basement membrane is exposed to the bloodstream. The ultrastructure of the lamina densa region with its tightly packed fibrils provides the glomerular basement membrane with the tensile strength necessary to cope with a broad range of intraglomerular pressure changes that it may be subject to during ultrafiltration. The firm attachment of endothelium and epithelial foot processes to the glomerular basement membrane is perhaps facilitated by the loosely packed fibrils of the lamina rara interna and lamina rara externa. Biochemically, the collagen-rich lamina densa region of the glomerular basement membrane is implicated in the size-selective sieving of the solute present in the fluid-selective nature of heparin sulfate proteoglycan in the glomerular basement membrane, especially in the lamina rara externa and interna regions. These anionic regions characterized by the presence of heparin sulfate proteoglycan ensure the hydration of the glomerular basement membrane surface. This prevents absorption of negatively charged proteins, such as albumin, to the glom-

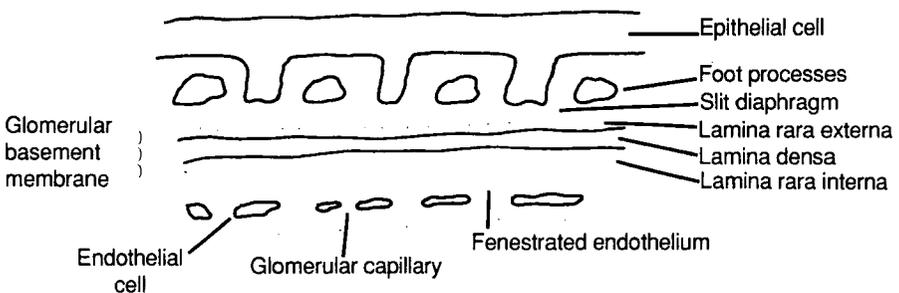


FIG. 4. Schematic representation of the ultrafiltration unit.

erular basement membrane surface during the transit of fluid containing solute across the glomerular capillary.

Due to their sialic acid content, the epithelial foot processes are also negatively charged. This negative charge apparently helps in maintaining the integrity of the foot processes, which are kept separated by the slit diaphragms (5). Fluid is thus able to filter through the slit diaphragm space. According to current knowledge, the glomerular basement membrane is one of the major determinants of the size-selective and charge-selective nature of the ultrafiltration unit. The anionic regions of the glomerular basement membrane, characterized by the presence of heparin sulfate proteoglycan, dictate the movement of solute either by interaction with these negatively charged sites or by electrostatic repulsion. As noted earlier, the heparin sulfate proteoglycan regions ensure the hydration of glomerular basement membrane surface that precludes hydrogen bonding and adsorption of negatively charged proteins such as albumin, and thus ensure continuous filtration of fluid containing solute through the glomerular capillary. These concepts have emerged from studies using tracer molecules. Thus, in one study using ferritins of similar size but increasing isoelectric points, it was demonstrated that increasing the cationic nature of the molecule resulted in the increased permeability of the molecule into the glomerular basement membrane (6).

The molecular-sieving properties of the glomerular ultrafiltration unit are also governed by the flow rate of renal glomerular plasma, and the difference between the hydrostatic and oncotic pressure differential leads to a decrease in the glomerular filtration rate, whereas an increase in these two variables results in an increase in the glomerular filtration rate. The glomerular permeability of molecules can be related to the clearance of a small molecule, such as inulin, that is fully permeable across the glomerular capillary. Thus, the concept of fractional clearance relates the ratio of the clearance of a macromolecule to the ratio of the clearance of inulin. The fractional clearance is inversely related to the glomerular filtration rate. The fractional clearance of a macromolecule is governed both by its size configuration or effective hydrodynamic radius and, within a certain size range, by its charge characteristics. The effective hydrodynamic, or Stokes, radius has an inverse relationship to fractional clearance (7). Thus, molecules with a Stokes radius greater than 4.0 nm are relatively impermeable through the glomerular filter, whereas molecules with a Stokes radius less than 1.8 are readily permeable.

The glomerular filtrate containing virtually very little protein (approximately 30 mg/dl) enters the proximal tubule. Approximately 80% of the water and solute from the glomerular filtrate is reabsorbed in the proximal tubule as an isotonic solution. Solutes such as glucose and proteins are almost totally reabsorbed. Creatinine, in contrast, is not reabsorbed. Approximately 65% of the sodium in the glomerular filtrate is reabsorbed in the proximal tubule by active transport. The rate of reabsorption of sodium in the proximal tubule, however, is under the

control of a variety of factors, such as the glomerular filtration rate, the peritubular oncotic pressure, and, perhaps, also the presence of a "natriuretic hormone."

Bicarbonate reabsorption in the proximal tubule is related to the formation of hydrogen ions in the tubule cells and their excretion in the tubular lumen, which is controlled by the $p\text{CO}_2$ content of the blood traversing the tubule. The reabsorption of approximately 95% of the potassium present in the glomerular filtrate occurs in the proximal tubule and the loop of Henle. The reabsorption of potassium in the proximal tubule is independent of dietary intake. The fluid remaining in the proximal tubule after approximately 70% of the glomerular filtrate is reabsorbed enters the descending thin limb of the loop of Henle, which is permeable to salt, urea, and water. Prior to entering the ascending thin and thick limbs of the loop of Henle, its water content is further reduced. The ascending thin and thick limbs of the loop of Henle, however, are relatively impermeable to water and the ascending thick limb in particular is capable of actively transferring salt in the form of sodium chloride and also urea from the tubular lumen to the interstitial fluid in the medulla. As a consequence, the interstitial fluid in the medulla becomes hypertonic and the tubular fluid that reaches the distal tubule is hypotonic. The ascending thick limb has been identified as the site of active chloride reabsorption. The hairpin loop structure of the loop of Henle permits a progressive increase in hypertonicity as salt in the form of sodium chloride is continually trapped in the medullary interstitium. This countercurrent multiplier effect results in maximum hypertonicity being attained at the tip of the papilla (8). The osmolality of the medullary interstitial fluid, which is always hypertonic, varies, however, with the osmolality of urine. Thus, it can range from 40 mOsmol/kg of water with hypotonic urine to a maximum of 1300 mOsmol/kg of water attained when urine osmolality reaches its maximum hyperosmolality. The hypotonic fluid reaching the distal convoluted tubule has approximately the same pH as the proximal tubular fluid. The first portion of the distal convoluted tubule is relatively impermeable to water. However, the latter part of the distal convoluted tubule and the collecting duct that follows become permeable to water only in the presence of antidiuretic hormone (ADH). While the medullary interstitial fluid is hypertonic, the interstitial fluid of the cortex is isotonic. As such, in the presence of ADH water moves out of the distal tubule and the cortical collecting duct into the interstitium of the cortex. The cortical collecting duct is not permeable to urea. As a result, as water leaves the cortical collecting duct the concentration of urea in the cortical collecting duct increases. Urea, however, is diffusible in the medullary region of the collecting duct. Thus the hypertonic medullary interstitium enriched with salt and urea abstracts water from the medullary collecting duct. The abstracted water is removed by blood vessels called vasa recta. This countercurrent exchange by vasa recta preserves the hypertonicity of the medullary interstitium achieved as a result of the countercurrent multiplier effect of the loop of Henle. The abstraction of water from the collecting duct results in the production of concentrated urine. This situation

only prevails in the presence of ADH when urea and sodium chloride are concentrated in the medullary interstitium, thus drawing out water in proportion to the hypertonicity of the interstitium and producing urine with an osmolality directly related to the urea content of the medullary interstitium. Because of the fact that the metabolism of each gram of protein results in 0.3 g of urea, subjects consuming a high-protein diet are better able to concentrate their urine.

In the absence of ADH, however, the hypotonic fluid reaching the distal convoluted tubule remains hypotonic during its passage through the collecting duct, because the distal convoluted tubule and the collecting duct both remain impermeable to water. This results in a hypotonic urine. In the absence of ADH, instead of urea being concentrated in the medullary interstitium, it is excreted. The production of concentrated urine requires not only the presence of ADH, but sufficient availability of salt and urea, active transport of salt, an adequate number of functionally intact nephrons, and sufficiently optimal blood and tubular fluid flow rates. Concentration of salt becomes limiting if the flow rate is low. In contrast, if the flow rate is high, there is a risk of washout of salts, thus affecting the ability to concentrate urine. Incidentally, the flow of blood through the vasa recta is controlled by the osmolality of the medullary interstitium. An increase in the osmolality of the medullary interstitium has the effect of constricting the vasa recta and thus reducing its blood flow. This ensures the efficiency of the countercurrent exchanger effect of the vasa recta by maintaining the high osmolality of the medullary interstitium, which is a prerequisite for the production of a concentrated urine.

The distal convoluted tubule and the collecting duct contribute to the adjustment of urine pH and osmolality and ensure elimination of waste products. Approximately 97% of sodium entering the distal convoluted tubule from the loop of Henle is reabsorbed. Sodium reabsorption is related to the excretion of potassium ions and also to a certain extent to hydrogen ion excretion. Reabsorption of sodium into the tubule cells sets up a gradient that leads to entry of chloride ions into the tubule cell while potassium and hydrogen ions move out of the cell into the tubular fluid. In the distal tubule, sodium reabsorption is under the control of the hormone aldosterone. Either a diminution of sodium levels in the fluid coursing through the distal convoluted tubule in the macula densa region or a drop in the pressure in the afferent arteriole stimulates the juxtaglomerular cells to release the hormone renin. Once in circulation, renin acts on its substrate angiotensinogen derived from the liver and circulating in the plasma as an α_2 -globulin to produce angiotensin I. A converting enzyme released from the lung cleaves a dipeptide residue from angiotensin I to produce angiotensin II, which is not only a powerful vasoconstrictor but stimulates the adrenal cortex to produce aldosterone. The latter, as noted earlier, acts on the distal tubule to retain sodium and excrete potassium. This effect of aldosterone is counteracted by drugs such as spironolactone and thiazide diuretics.

The distal convoluted tubule regulates the amount of potassium that is secreted and in turn excreted in urine. This is dictated by metabolic needs. The cells of

the cortical collecting duct appear to possess a sodium- and potassium-dependent adenosine triphosphatase ion pump in their basolateral membranes, secreting two potassium ions for every three sodium ions that are reabsorbed. The secreted potassium moves down the tubular lumen. Its excretion in the urine can be prevented by a potassium-absorbing pump that is located in the luminal membrane of the collecting tubule cells. This mechanism ensures that potassium is retained in states of potassium depletion. The secretion of potassium in the distal tubule is related to the electrochemical gradient that is set up as sodium is reabsorbed. Thus, as increased amounts of sodium are delivered to the distal tubule, the intratubular negativity increases, thus resulting in an increased secretion of potassium. As sodium content of the distal tubular fluid is decreased, coupled with a diminution of the distal tubular flow rate, the intratubular negativity correspondingly decreases, resulting in decreased secretion of potassium by the distal tubule. Such a situation may prevail in a salt-depleted state with the resulting hyperkalemia.

The formation and secretion of hydrogen ions from the tubule cells into the tubular lumen is regulated by many variables, including the $p\text{CO}_2$ of the blood traversing the renal tubule, the activity of the enzyme carbonic anhydrase (which is responsible for the hydration of carbon dioxide to form carbonic acid), and the extent of bicarbonate reabsorption. The enzyme carbonic anhydrase, in addition to being present on the basolateral membrane and in the cytoplasm of cells of the proximal tubule, is also present on the brush border of the luminal membrane. As such, the luminal fluid in the proximal tubule lumen comes into contact with this enzyme, which accelerates the conversion of carbon dioxide to carbonic acid. The hydrogen ions secreted into the proximal and distal tubular lumina buffer the filtered bicarbonate to form carbonic acid. This in turn is converted to carbon dioxide and water, a reaction catalyzed by the enzyme carbonic anhydrase in the proximal lumen membrane. Carbon dioxide diffuses out of the lumen into the tubule cell, where again, mediated by the enzyme carbonic anhydrase, carbonic acid is formed and then dissociates into a bicarbonate and a hydrogen ion. The bicarbonate is reabsorbed along with sodium. The hydrogen ion is secreted into the tubule lumen. Carbonic anhydrase is not present on the distal tubule luminal membrane. However, it is present within the distal tubule cell, where it can catalyze the hydration of carbon dioxide. The flow of bicarbonate from the tubular lumen into the cell will also cause secretion of potassium ions into the tubular lumen. The extent of secretion of hydrogen ions and potassium ions into the tubular lumina and ultimate excretion in urine will be dependent on the relative intracellular concentration of these two ions. There are additional variables that effect the secretion of hydrogen ions and the reabsorption of bicarbonate. Increase in plasma calcium levels apparently increases the secretion of hydrogen ions by activating the enzyme carbonic anhydrase. Decrease in plasma chloride levels will limit the amount of chloride that can diffuse back from the tubule lumen into the cell during the reabsorption of bicarbonate and thus will accentuate the potential

difference between the tubule cell and the lumen. This increased potential difference will increase the secretion of hydrogen ions from the tubule cell into the tubule lumen. The presence of hydrogen ions in excess of those required to buffer bicarbonate creates a significant drop in pH in the distal tubular lumen, which can drop as low as pH 4.5. Thus the excess hydrogen ions have to be neutralized, which is accomplished by combination with phosphate and also with ammonia. A drop in tubular fluid pH is restricted to a certain extent by the creation of a hydrogen ion gradient from the tubular lumen to blood.

Phosphate ions are in their dibasic form (HPO_4^{2-}) at the pH of the glomerular filtrate. The hydrogen ions of the distal tubular lumen are neutralized by HPO_4^{2-} . The resulting monobasic form of the phosphate (H_2PO_4^-) is excreted in urine as NaH_2PO_4 , thereby conserving the sodium ions required for the reabsorption of bicarbonate. However, when hydrogen ions need to be conserved to combat alkalosis, phosphate is excreted as Na_2HPO_4 , thus permitting excretion of bicarbonate in urine. Hydrogen ions in combination with phosphate constitute a major portion of titratable acid in urine, which can be determined by titrating urine with sodium hydroxide until the pH of the urine is equal to 7.4, corresponding to the approximate pH of the plasma.

Another mechanism for neutralizing excess hydrogen ions in the distal tubule lumen utilizes ammonia, which is formed by the renal cortical cells from glutamine in the presence of the enzyme glutaminase (9). Ammonia, being lipid soluble, permeates the cells of the distal tubule and then diffuses into the distal tubular lumen. Here it traps hydrogen ion as ammonium ion (NH_4^+), which, being charged, is poorly lipid soluble and is unable to diffuse back in the cell. The trapped ammonia is excreted. Ammonia formation is a mechanism to process the body's acid load, which consists of volatile hydrogen ions formed from carbonic acid, and, in addition, nonvolatile hydrogen ions resulting from the metabolism of phosphoproteins, phospholipids, and sulfur-containing proteins. The formation of ammonia spares precious cations such as sodium, calcium, and potassium, which would otherwise have to be excreted with anions such as sulfate, chloride, and phosphate resulting from strong acids (sulfuric, hydrochloric, and phosphoric acids) generated in the body. Although negligible amounts of hydrogen ions resulting from the production of strong acids can be excreted at the minimum pH (4.5) attainable in the distal tubular lumen fluid, they can, however, be easily eliminated in combination with ammonia as ammonium ion. The activity of glutaminase is stimulated by acidosis and is depressed by the decrease in the secretion of hydrogen ions into the tubular lumen and also by an accumulation of ammonia within the tubule cell. The excretion of ammonium ions in urine is in proportion to its pH, being considerable when the urine pH is low and becoming negligible as urine pH approaches alkalinity.

The utilization of hydrogen ions to conserve bicarbonate and the titration of excess hydrogen ions with phosphate and ammonia is a means by which the

kidney handles its hydrogen ion load. In terms of handling other constituents, mention should be made of the effect of parathyroid hormone (PTH) on the distal tubule. Although calcium is absorbed from the proximal tubule without the aid of PTH, in the distal tubule, however, this hormone increases the reabsorption of calcium and inhibits the tubular reabsorption of phosphate. The hydroxylation of 25-hydroxycalciferol in the 1-position to yield 1,25-dihydroxycholecalciferol, the active form of vitamin D, is mediated by the enzyme 1α -hydroxylase found in the mitochondria of the cells of the proximal tubule. Vitamin D increases tubular reabsorption of calcium, promotes mobilization of calcium from the bone, and reduces tubular reabsorption of phosphate. PTH stimulates the synthesis of vitamin D. The reabsorption of magnesium parallels that of calcium and is dependent on PTH.

The distal tubule secretes 80% of the uric acid content in urine. The reabsorption of most of the uric acid (98%) in the glomerular filtrate takes place in the proximal tubule. This reabsorption can be inhibited by thiazide diuretics, thus increasing uric acid excretion in urine. The chronic use of diuretics, however, by depleting the extracellular fluid volume provides a stimulus for uric acid reabsorption. Drugs that promote uric acid excretion (uricosuric drugs) include probenecid, sulfipyrazole, and salicylates in high doses. In low doses salicylates depress uric acid excretion.

A certain amount of creatinine is secreted by the tubules, augmenting creatinine filtered at the glomerulus, which, as was noted earlier, is not reabsorbed. Thus, the amount of creatinine in urine represents the amount filtered at the glomerulus and the amount secreted by the tubules.

Conservation of amino acids filtered at the glomerulus is made possible by the existence of four main transport systems for specific amino acids that facilitate active reabsorption of these amino acids from the proximal tubule. A lack or deficiency of the transport system responsible for the absorption of valine, alanine, cystine, and tryptophan, and of the transport system for arginine, lysine, cystine, and ornithine, leads to excretion of these specific amino acids in urine, which is characterized as renal aminoaciduria to distinguish it from overflow aminoaciduria. In the latter situation, the production of amino acids far exceeds the proximal tubular reabsorption capacity, thus leading to overflow of amino acids into urine. This can occur due to defective metabolism of amino acids, as is the case when phenylalanine cannot be metabolized due to the deficiency of the enzyme phenylalanine hydroxylase, or to the inability to deaminate amino acids in liver disease.

2.2. BIOCHEMICAL FUNCTIONS OF THE KIDNEY

Gluconeogenesis takes place in the renal cortical cells. The activity of enzymes that favor gluconeogenesis, such as phosphofructokinase, pyruvate carboxylase,

and phosphoenolpyruvate carboxykinase, is stimulated by acidosis. Acidosis also stimulates the enzyme glutaminase by making available more of the coenzyme NAD⁺ for ammonia synthesis to proceed in the cortical cells and in the cells of the outer medulla. The energy requirement of the renal cortex commensurate with its increased blood flow is aerobic, utilizing primarily free fatty acids and to a certain extent glucose as its energy sources (10).

The renal medulla uses glucose as its main source of energy. Anaerobic glycolysis proceeds in the inner medulla and papilla. The outer medulla, however, can utilize glucose both aerobically and anaerobically. The availability of α -ketoglutarate by transamination favors gluconeogenesis.

2.3. CONCEPT OF T_m

The ability of tubules to reabsorb or secrete a substance is variable. The maximum amount of a substance that the tubules can either reabsorb or secrete in 1 minute is called the T_m for that substance. ("T" refers to the tubule and "m" stands for maximal). This parameter can be calculated by infusing the substance of interest intravenously until the capacity of the tubules to reabsorb or secrete that substance is exceeded. The reabsorptive capacity of the tubules (T_m) can be calculated by knowing the concentration of the substance of interest in both urine and plasma (P_s and U_s, respectively), the rate of urine flow per minute (V), and the glomerular filtration rate (GFR). The formula for calculation of reabsorptive T_m for a substance is

$$T_m = GFR \times (P_s - U_s) \times V$$

The capacity of tubules to secrete a substance can be determined by infusing a nonbiological compound, such as *p*-aminohippuric acid (PAH), which is actively secreted by the tubules. In general, a substance is regarded as being actively secreted if the amounts excreted in urine are greater than the amount filtered, although substances such as potassium, which are actively secreted by the tubules, are filtered in amounts greater than that excreted in urine. The formula for calculating the secretion T_m using a compound such as PAH is

$$T_m = \text{urine PAH} \times (V - GFR) \times \text{plasma PAH}$$

The capacity of the tubules to reabsorb or secrete a substance is subject to several variables. Thus, the expansion of extracellular fluid volume inhibits the reabsorption of sodium, bicarbonate, and phosphate and depresses the secretion of PAH. The tubular reabsorption of phosphate is inhibited by increased circulating levels of parathyroid hormone. The reabsorption of phosphate and bicarbonate by the tubules is directly related to the glomerular filtration rate. The reabsorption of bicarbonate by the tubule is also directly related to the *p*CO₂ level.

3. Pathophysiology

3.1. ACID-BASE DISORDERS

Metabolic acidosis can arise due to a variety of conditions. Thus, in diabetic ketoacidosis there is an excess of metabolic acids in the form of acetoacetic acid and β -hydroxybutyric acid. In oxygen-depleted states, there is an excess of hydrogen ions due to their inability to be converted to water by combination with oxygen. Thus, in states of temporary oxygen deficit, such as in strenuous exercise, there is an accumulation of lactic acid due to anaerobic glycolysis. In starvation, there is an increase in hydrogen ions due to ketosis and also due to increased catabolism. In uremia, the retention of waste products, including a variety of metabolic acids, leads to metabolic acidosis. Metabolic acidosis can also result from ingestion of salicylate, ethylene glycol, or methanol.

In the conditions discussed above (diabetic ketoacidosis, lactic acidosis, uremia, and ingestion of salicylate, ethylene glycol, or methanol) metabolic acidosis is associated with an increased anion gap. In the face of excess metabolic acids, bicarbonate is depleted in the process of buffering excess hydrogen ions. Provided that the renal functions is normal, the kidney attempts to compensate by secreting an acid urine and retaining bicarbonate.

Aberrations in kidney function lead to metabolic acidosis. Defects may lie both with the tubules and the glomeruli, such as in generalized renal failure. On the other hand, the defect may be confined either to the tubules or to the glomeruli. A loss or reduction in the number of functioning tubular cells compromises the function of tubules, such as secretion of hydrogen ions in exchange for sodium and regulation of urine pH between 4.5 and 7.8. Excretion of hydrogen ions buffered with ammonium ions may be affected by a reduction in the formation of ammonia from glutamine mediated by the enzyme glutaminase in the tubule cell. Defective hydrogen ion secretion affects reabsorption and regeneration of bicarbonate, thus affecting its regulation.

Acidification of urine is affected in renal tubular acidosis. This condition may be due to an inborn error of metabolism or to an acquired tubular lesion. The defect may be related either to the secretion of hydrogen ions or to the diffusion of hydrogen ions into the blood as a result of increased permeability of the distal tubule cell wall to secreted hydrogen ions. Because renal tubular acidosis is primarily a defect in hydrogen ion secretion, the formation of ammonia by tubule cells is not affected.

Renal tubular acidosis can be classified into two main types, type I and type II, which are hereditary (11). Renal tubular acidosis can also result from accumulation of waste products, including a variety of metabolic acids in uremia. Another type of renal tubular acidosis, type IV, is due to hyporeninemic hypoaldosteronism. Hypoaldosteronism appears to be secondary to the inability of the kidney to

secrete renin. Type IV is usually acquired. The hallmarks of this condition are hyperkalemia and hyperchloremic acidosis.

In type I renal tubular acidosis, or the classic type, there is an impairment in the formation of a hydrogen ion gradient in the distal tubule. Hence, this condition is also called distal tubular acidosis. Normally the tubular epithelium can achieve a maximum pH gradient of 1000 to 1 between the luminal fluid and the blood. In distal tubular acidosis this gradient is 100 to 1 or even less. Hence, in distal tubular acidosis, the urine pH is maintained above 5.5–6.0. The existence of distal tubular acidosis can be confirmed by the administration of 0.1 gram of ammonium chloride per kilogram of body weight and measurement of urine pH between 2 and 8 hours after administration. Whereas in normal subjects the pH of urine falls to 5.3 or below, in patients with distal tubular acidosis the urine pH stays above 5.5–6.0. The increased urine pH in distal tubular acidosis depresses secretion of ammonia into the tubular lumen. In distal tubular acidosis the bicarbonate absorption by the proximal tubule, which accounts for the major portion of bicarbonate that is absorbed, is normal. Because the fraction of bicarbonate normally reabsorbed from the distal tubule is of the order of 10–15%, losses of bicarbonate in type I renal tubular acidosis are not substantial. Only about 70 mEq of hydrogen ions are secreted by the distal tubule in a day, thus acidosis in the classic type I can be corrected by the administration of 70 mEq of bicarbonate. As a consequence of acidosis, ionization of calcium and its desorption from bone are increased. Calcium deposits in renal tubules lead to uremia due to nephrocalcinosis. The depletion of calcium from bone leads to osteomalacia. The breakdown of the calcium phosphate matrix results in phosphaturia.

In type II renal tubular acidosis there is a defect in the secretion of hydrogen ions by the proximal tubule. Because the proximal tubule is the major site of bicarbonate reabsorption (4000 mEq of bicarbonate per day as compared to 70 mEq in the distal tubule), the defect in secretion of hydrogen ions in this condition leads to the flooding of the distal tubule with bicarbonate. The capacity of hydrogen ions secreted by the distal tubule to buffer this massive efflux of bicarbonate is soon overwhelmed and, as a result, large quantities of bicarbonate are excreted in the urine. Much more bicarbonate needs to be administered in this condition to correct the acidosis than is necessary in type I renal tubular acidosis. In general, in renal tubular acidosis the impairment in hydrogen ion secretion leads to excretion of potassium ions in urine.

Metabolic acidosis can also result from loss of bicarbonate, such as in severe diarrhea, especially in infants or due to the depletion of bicarbonate when urine is delivered to the colon after transplantation of ureters into the colon. The administration of carbonic anhydrase inhibitors such as acetazolamide results in excretion of bicarbonate in urine and retention of hydrogen ions, leading to metabolic acidosis. Because of impaired hydrogen ion secretion potassium is

excreted in urine. Acidosis in this case may be accompanied by hypokalemia and hyperchloremia. Administration of ammonium chloride also results in metabolic acidosis. In all these conditions (renal tubular acidosis, severe diarrhea, the administration of acetazolamide or ammonium chloride) metabolic acidosis is present without an accompanying anion gap. In the absence of a glomerular defect serum creatinine and blood urea nitrogen (BUN) levels are generally within the normal range in renal tubular acidosis.

In glomerular disease or in water-depleted states, such as renal circulatory insufficiency, the glomerular filtration rate is low. As a consequence of a low glomerular filtration rate, the amount of sodium in tubular fluid that is available for exchange with hydrogen ions is reduced. The secretion of hydrogen ions by the renal tubular cells and its subsequent elimination is affected, thereby resulting in metabolic acidosis due to the accumulation of hydrogen ions produced during metabolism.

In some cases, such as in pyloric stenosis, which involves obstruction between the stomach and the duodenum with consequent vomiting, leading to loss of hydrochloric acid and volume depletion, a diminished glomerular filtration rate leads to metabolic alkalosis. Because a low glomerular filtration rate affects the secretion of hydrogen ions, the ability to reabsorb bicarbonate is also affected. A low glomerular filtration rate also reduces the quantity of bicarbonate that is filtered. As such, the ability to correct acidosis or alkalosis is affected when glomerular filtration rate is reduced.

Metabolic alkalosis is less common compared to metabolic acidosis because hydrogen ions, not hydroxyl ions, are produced during metabolism. The ingestion of excess bicarbonate as sodium bicarbonate to overcome indigestion, the loss of acid during vomiting associated with pyloric stenosis, and potassium loss due to steroid or diuretic administration are some of the frequent causes of metabolic alkalosis. Potassium depletion as a consequence of steroid or diuretic therapy is more common and results in extracellular alkalosis. The loss of potassium from the cells and the extracellular fluid results in the passage of hydrogen ions from the extracellular fluid into the cells in order to compensate for the intracellular cation deficit. This influx of hydrogen ions into the cells leads to the availability of more hydrogen ions for secretion into urine, thus resulting in an acidic urine. The secretion of hydrogen ions due to potassium depletion causes the reabsorption of bicarbonate, resulting in an increase in the plasma bicarbonate concentration. This situation is aggravated if potassium is lost together with chloride. In the face of chloride deficiency the reabsorption of sodium by the proximal tubule is limited. This increases the quantity of sodium available for exchanging with hydrogen ions, thus leading to a paradoxical aciduria. Increase in hydrogen ion secretion, as noted previously, causes an increase in bicarbonate reabsorption. The metabolic alkalosis resulting from potassium and chloride depletion can be corrected by the administration of chloride.

Some of the rare causes of metabolic alkalosis due to potassium depletion are those found in subjects with either Cushing's syndrome, primary aldosteronism, or Bartter's syndrome. In Cushing's syndrome, the potassium ions and alkalosis are related to increased mineralocorticoid activity resulting from an increase in adrenocorticotrophic hormone (ACTH), cortisol, deoxycorticosterone, and corticosterone. In primary aldosteronism, the effects of increased aldosterone are manifest on the distal tubule of the kidney. In Bartter's syndrome, the basic abnormality appears to be a defect in the reabsorption of chloride in the ascending limb of loop of Henle, leading to loss of potassium (12).

3.2. DIURNAL RHYTHM AND EDEMA

Normally the amount of water and ions such as sodium, potassium, chloride, and bicarbonate in urine is greater during the day than during evening and night. This diurnal rhythm is abolished in edema resulting from a reduced plasma oncotic pressure associated with low albumin levels, such as in severe liver diseases, anorexia nervosa due to protein malnutrition, or nephrotic syndrome. The diurnal rhythm is also abolished in cardiac failure, when the increased intravascular hydrostatic pressure on the venous side of the circulation results in edema. The loop of Henle, the distal convoluted tubule, and the collecting duct retain sodium to expand plasma volume in edema, thus restoring sodium balance in such patients. Correcting measures for edema include use of diuretics, such as thiazide, to treat moderate edema, for example, in nephrotic syndrome. Diuretics such as furosemide and ethacrynic acid have found application not only in treating all forms of edema, but also in acute tubular necrosis to thwart oliguria.

3.3. ACUTE RENAL FAILURE

Nephrotoxins or ischemic disorders can initiate acute renal failure. Shock, hemorrhage, septicemia, or vasodilation due to hypertensive medication can precipitate ischemic acute renal failure. Systemic reactions to certain drugs and nephrotoxins such as aminoglycoside antibiotics and heavy metals lead to acute renal failure. The extent of retention of creatinine and urea in blood is directly related to the severity of acute renal failure. This condition is not readily reversible and, as such, should be distinguished from reversible phenomena such as prerenal or postrenal azotemia, in which there is also an increase in levels of plasma urea and creatinine (13). In volume-depleted states, for example, diarrhea, the kidney is hypoperfused. This results in increased back diffusion of urea into the circulation from the tubular fluid because of the reduced urine flow. In addition to an increase in urea levels in circulation, there is also a slow increase in creatinine levels. Plasma urea and creatinine levels can be restored to normal within 24 hours by appropriate fluid and electrolyte replacement in prerenal azotemia. In condi-

tions where there is bladder-outlet obstruction, such as in prostatic hypertrophy, the reduced renal tubular fluid flow also leads to increases in serum urea and creatinine. This postrenal azotemia can be corrected by relieving the bladder-outlet obstruction with a catheter.

In contrast, acute renal failure is not a readily reversible phenomena, and may require dialysis to relieve uremia, especially if the patient is oliguric with an urine output less than 400 ml per day. In contrast, acute renal failure in nonoliguric patients with urine volumes in excess of 800 ml per day appears to be more benign, and the majority of such patients do not require hemodialysis. Apparently, non-oliguric patients have a relatively large number of functioning nephrons and, as such, a relatively higher glomerular filtration rate compared to oliguric patients.

Serum creatinine level is not a good index for the early diagnosis of acute renal failure. Thus the creatinine level in the serum of patients with acute renal failure may vary from 2 to 16 mg/dl when the glomerular filtration rate drops below 10 ml/minutes, depending on the duration of the reduced GFR. The determination of urinary indices permits the diagnosis of acute renal failure. A urine:plasma creatinine ratio of 60 would suggest that tubular function is intact, and that one is dealing with prerenal azotemia due to either volume depletion or cardiac failure. However, a urine:plasma creatinine ratio of 8 would indicate that the patient has either acute renal failure or postrenal azotemia. It is desirable to determine the urine:plasma creatinine ratio prior to administering diuretics such as furosemide or mannitol, which would otherwise mimic the low urine:plasma creatinine ratios characteristic of acute renal failure or postrenal azotemia. An early diagnosis of acute renal failure provides the opportunity to overcome oliguria by the administration of diuretics such as furosemide or mannitol. Such maneuvers have little chance of success once the serum creatinine level exceeds 8 to 10 mg/dl. Other useful urinary indices for the diagnosis of acute renal failure are the urine:plasma osmolality ratio, the urinary sodium, and the renal failure index (RFI), which is obtained by dividing the urinary sodium value by the urine:plasma creatinine ratio. Another index, the fractional excretion of sodium (FE_{Na}), which is equal to $(\text{urine sodium/plasma sodium}) \div (\text{urine creatinine/plasma creatinine}) \times 100$, is also useful. Patients with acute renal failure or urinary tract obstruction lack the ability to concentrate their urine. Thus a urine:plasma osmolality ratio in excess of 1.5 would indicate prerenal azotemia, where the urine osmolality is usually greater than 500 mOsmol/kg. However, prerenal azotemia may coexist with an inability to concentrate the urine due to either malnutrition, old age, or hypokalemia, in which case the urine:plasma osmolality ratio may not exceed unity. In acute renal failure or urinary tract obstruction, the urine osmolality is generally less than 400 mOsmol/kg.

Patients with prerenal azotemia have urinary sodium levels of less than 20 mEq/liter, consistent with normal tubular function. In contrast, patients with either acute renal failure or postrenal azotemia have urinary sodium levels greater than

40 mEq/liter. Urinary sodium levels between 20 and 40 mEq/liter would indicate that the patient is in the process of progressing from prerenal azotemia to acute renal failure.

A renal index of less than 1 is suggestive of prerenal azotemia, whereas values greater than 1 indicate acute renal failure. A low fractional excretion of sodium, less than 1%, is found in prerenal azotemia and in hemoglobin- and myoglobin-induced acute renal failure (14). An urine uric acid (UUA) and urine creatinine (UC) concentration ratio greater than 1 has been found in patients with acute renal failure due to uric acid nephropathy. Jaundice and overproduction of uric acid due to increased catabolism are believed to be responsible for the increased UUA:UC ratio (15). Because urinary indices cannot distinguish between acute renal failure or postrenal azotemia due to urinary tract obstruction, the latter can be ruled out by diagnostic maneuvers such as bladder catheterization to detect bladder-outlet obstruction and ultrasound to detect urinary tract obstruction above the bladder, which may be due to renal calculi. In spite of its limitations, retrograde pyelography is still used to rule out urinary tract obstruction.

Mitochondrial dysfunction is believed to play a vital role in the pathogenesis of acute renal failure. In the face of inadequate production of mitochondrial ATP, sodium and calcium efflux from the cell, which requires ATP, is curtailed. This leads to the swelling of the cell and activation of the calcium-calmodulin complex. The latter may activate phospholipases, which in turn can damage the cell membrane and cause swelling of the cell, leading to its death. The cell debris serves as a substrate for tubular obstruction and supports the maintenance phase of acute renal failure. Complications of casts solidifying in the tubular lumen can be avoided by early measures to prevent cell death.

In the initiation phase of acute renal failure, mitochondrial oxygen uptake is affected by ischemia. The resulting vasoconstriction in the kidney activates the renin-angiotensin system. The intracellular calcium level in the mitochondria increases. The administration of a calcium transport blocker such as Verapamil can reverse or prevent renal vasoconstriction and also restore the mitochondrial oxygen uptake and thus prevent cell necrosis and the development of the maintenance phase of acute renal failure.

3.4. CHRONIC RENAL FAILURE

The progressive loss of functioning nephrons leads to chronic renal failure (16). The urea level in blood is increased as the rate of urea excretion falls. In the initial stages, there may be polyuria due to the osmotic diuresis resulting from an increase in the urea level in the nephron filtrate. Adequate water intake by such persons can maintain them in a stable condition for several years, although with a moderately increased blood urea level. It is only when the great majority of nephrons are destroyed that terminal stage of renal failure is reached, marked by oliguria and

a very high blood urea level. The creatinine clearance, a reflection of the glomerular filtration rate, provides the best estimate of the stages of renal disease. A creatinine clearance greater than 50 ml/minute is an indication of mild renal disease. A clearance in the range of 15–50 ml/minute reflects moderate renal dysfunction. A clearance between 5 and 15 ml/minute signifies severe renal disease. Patients with creatinine clearance below 5 ml/minute may require kidney transplantation or dialysis to prevent death.

In addition to an increase in serum urea and creatinine levels, uric acid and inorganic phosphate levels also increase in chronic renal failure. The increase in serum inorganic phosphate leads to deposition of calcium phosphate in bones, causing hypocalcemia. In the early stages of chronic renal failure, calcium levels are restored by the stimulation of parathyroid hormone. However, as the renal disease progresses, the ability of the kidney to hydroxylate vitamin D and thus convert it to the active form decreases, thereby affecting the uptake of calcium by the gut and thus perpetuating hypocalcemia. Serum alkaline phosphatase levels increase due to disordered bone metabolism. Loss of bicarbonate is seen in some patients with increased parathyroid hormone activity.

The ability to form ammonia is impaired with loss of functioning nephrons. This reduces the excretion of hydrogen ions, thus causing acidosis. Initially the mild acidosis does stimulate the production of ammonia by the remaining functional nephrons. However, the net result is continuing acidosis in the face of chronic renal failure. The most common causes of chronic renal failure are glomerulonephritis, pyelonephritis, obstructive nephropathy, and vascular nephropathy in severe hypertension.

Acute glomerulonephritis is associated with the sudden onset of proteinuria, hematuria, and the appearance of red cell casts in urine. Demonstration of the presence of red cell casts in the urinary sediment establishes the presence of active glomerular inflammation. Proteinuria is a characteristic finding in glomerulonephritis, with levels ranging from 0.5 to 3.0 g per day. Proteinuria in excess of 2.5 g per day characteristic of nephrotic syndrome are found in some cases of glomerulonephritis.

Glomerulonephritis can be classified into three main forms based on histological findings: these are the proliferative form, the minimal-lesion form, and the membranous form. Proliferative glomerulonephritis is characterized by the presence of a large number of inflammatory and glomerular cells. Changes in minimal-lesion glomerulonephritis involve fusion of foot processes of the epithelial cell and a thickening of the glomerular basement membrane that is discernible with electron microscopy. On the other hand, a light microscope is sufficient to observe the characteristic changes in membranous glomerulonephritis, such as the thickening of the glomerular basement membranes and the presence of electron-dense material in them. Membranoproliferative glomerulonephritis shares the histological features of both the minimal-lesion and membranous glomerulonephritis.

Acute glomerulonephritis may develop as a result of streptococcal infection (17). Patients with poststreptococcal glomerulonephritis usually present with proteinuria and hematuria with red cell casts. The distinction between acute poststreptococcal glomerulonephritis and membranoproliferative glomerulonephritis is difficult, especially because the latter condition may also develop after a streptococcal infection. Even so, the analysis of the complement cascade might help in distinguishing between acute poststreptococcal glomerulonephritis and membranoproliferative glomerulonephritis. Antibodies directed against complement components such as C3 nephritic factor are found in the serum of patients with membranoproliferative glomerulonephritis.

Serum complement levels are useful to distinguish between various forms of acute nephritis that may be due to either a primary renal disorder or a systemic disease. Glomerular diseases associated with decreased complement levels include systemic diseases such as systemic lupus erythematosus, subacute bacterial endocarditis, and cryoglobulinemia, and renal diseases such as acute poststreptococcal glomerulonephritis. Glomerular diseases in which serum complement levels are normal include systemic diseases such as Goodpasture's syndrome and renal diseases such as IgG-IgA nephropathy, antiglomerular basement membrane disease, and immune-complex disease.

From 70 to 80% of patients with glomerulonephritis may have deposits of immune complexes in the glomerular basement membrane. In a rare condition such as Goodpasture's syndrome, in which antibasement membrane antibodies attack the lung and the kidney, linear deposits of immunoglobulin are present on the glomerular basement membrane. Linear deposits of immunoglobulin are also seen in the glomerular basement membrane of patients with antiglomerular basement membrane nephritis. Immune-complex glomerulonephritis may result from exposure to certain drugs, bacteria such as streptococci and *Salmonella*, viruses, and endogenous antigens such as thyroglobulin and DNA. Even so, rapidly progressive glomerulonephritis with no immune-complex deposits is more common. For instance, there is little evidence of immune-complex-initiated or antiglomerular basement membrane-initiated disease in approximately one-third of reported cases of glomerulonephritis, which include nephrotic syndrome in children and proteinuria due to minimal-lesion disease. The measurement of serum antibodies against antiglomerular basement membrane is useful in patients with idiopathic rapidly progressive glomerulonephritis.

Nephrotic syndrome characteristic of excretion of protein in urine in excess of 2.5 g per day is not present in most patients with glomerulonephritis. These patients may present with a reduced glomerular filtration rate and varying degrees of proteinuria with or without hematuria, a condition referred to as the nephritic syndrome. Patients with nephritic syndrome may recover or worsen with chronic renal failure.

Pyelonephritis develops as a result of bacterial infection or from retrograde infection through the ureter. In the acute stage, bacterial infection is accompanied with infiltration with inflammatory cells and the development of edema. Obstructive nephropathy develops in chronic stages of bacterial infection.

Vascular nephropathy in prolonged hypertension is associated with a degeneration of the arterial walls. The resulting nephrosclerosis is due to the loss of functional glomeruli, leading to diminished renal function.

Renal hypertension can be due to either renal vascular hypertension or renal parenchymal hypertension (18). Renal vascular hypertension results from the activation of the renin-angiotensin system as a consequence of the decreased perfusion of renal tissue due to stenosis of a branch or main renal artery. The role of angiotensin II (which activates the adrenal secretion of aldosterone, which in turn leads to sodium retention and expansion of extracellular fluid volume) in renal hypertension has been elucidated by the use of the angiotensin II antagonist Saralasin. Renal parenchymal hypertension is also due to the activation of the renin-angiotensin system. In renal parenchymal hypertension, the decreased perfusion of renal tissue, however, is due to fibrotic and inflammatory changes in multiple small infrarenal vessels. Additional mechanisms may be operative in renal parenchymal hypertension, including the inability to dispose of sodium. As such, hypertension can be controlled in a majority of patients with renal parenchymal disease by removal of sodium with the administration of diuretics. Excess secretion of renin by nephroblastomas or juxtaglomerular cell tumors is also a cause of renal hypertension.

Characteristic features suggestive of renal ischemia can be gleaned from the intravenous pyelogram, which is used as a standard screening test for renal vascular hypertension. The reduction in blood pressure following the administration of the angiotensin II antagonist Saralasin is also used as a screening test for renal vascular hypertension. A combination of renal angiogram and renal vein renin determinations is useful in deciding whether the renal disease is surgically correctable. The presence of a renal arterial lesion and whether the lesion is due to atherosclerosis or fibrotic changes can be established with a renal arteriogram. The plasma renin level from the vein serving the ischemic kidney is in excess of 1.5 times or greater of the plasma renin activity in the vein from the unaffected kidney.

3.5. DIABETIC KIDNEY DISEASE

Patients with diabetes mellitus have a wide range of glomerular and tubular lesions. Glomerular lesions are more common. Increased glomerular filtration rates in diabetics correlate with increased glomerular and kidney size, and these changes are ascribed to increased levels of growth hormone. The most common

lesion found in diabetics is diffuse glomerulosclerosis and is characterized by the thickening of the mesangial matrix and an increase in the width of the glomerular basement membrane. Hyalin atherosclerosis of both the afferent and efferent arterioles may be involved. The efferent arteriole is rarely affected in nondiabetics.

Nodular glomerulosclerosis, first described by Kimmelstiel and Wilson in 1936, appears to be specific for juvenile onset or islet cell antibody-positive diabetes mellitus. The nodular lesion is found in the intercapillary tissue or mesangium (19).

Basement membrane thickening plays a key role in the pathogenesis of diabetic nephropathy. Extra renal sites such as the retina, peripheral nerves, and skeletal muscle capillaries may be involved. The normal basement membrane is made up of collagen-like glycoproteins with the carbohydrate subunits linked to hydroxylysine and asparagine residues. The increased blood glucose in diabetics perhaps results in increased enzymatic incorporation of carbohydrates into the basement membrane. A reduction in cross-linked cystine residues may render these basement membranes leakier than normal. It is known that diabetic capillaries leak plasma proteins excessively and the size of the leak is dependent on both the duration and success of diabetic control.

Glomerulosclerosis with basement membrane thickening is characteristic of diabetic microangiopathy involving small blood vessels in the kidney and other organs, such as the skin, muscles, and retina. The reduction of the width of the capillary basement membrane in controlled diabetics is evidence that basement membrane thickening is related to hyperglycemia.

Proteinuria is characteristic of diabetic nephropathy (20). Patients with diabetes mellitus may have massive proteinuria and the nephrotic syndrome. Some of these patients never develop glomerulosclerosis. Hypertension develops but is rarely malignant. Plasma renin activity is normal or decreased. More commonly, hyporeninemic hypoaldosteronism with hyperkalemia and mild hyperchloremic metabolic acidosis is found.

Diabetics are at an increased risk of urinary tract infections and there is an increased incidence of chronic pyelonephritis. By meticulous diabetic control one may be able to reverse the early defects at the onset of diabetes evidenced by hyperfunction of kidneys and mild proteinuria.

3.6. NEPHROGENIC DIABETES INSIPIDUS

In this condition the renal tubules are unresponsive to antidiuretic hormone and, as such, the subject has polyuria. The condition may be congenital or acquired. Acquired nephrogenic diabetes insipidus can result from several causes, such as chronic renal disease, potassium deficiency including primary aldosteronism, drugs such as lithium, systemic diseases such as multiple myeloma, and chronic hypercalcemias, including hyperparathyroidism. The damage to the renal tubules

by hypercalcemia prevents their responsiveness to ADH. Nephrogenic diabetes insipidus is diagnosed by failure of the subject to concentrate urine after administration of exogenous ADH.

3.7. RENAL STONES

These are generally made up of metabolic products present in the glomerular filtrate at levels close to their maximum solubility limit. Variation of the pH and composition of the urine or obstruction to urine flow may result in precipitation of substances in the kidney and contribute to calculi formation. Urinary calculi may be composed of calcium-containing stones such as calcium oxalate or calcium phosphate, both with and without magnesium ammonium phosphate, and stones containing either cystine, xanthine, or uric acid (21). The mucoprotein core around which the mineral salts precipitate account for 60% of the composition of the stone.

Calcium stones account for 70% or more of all renal stones. The most common cause of calcium stone formation is hereditary and is called idiopathic hypercalciuria. Thus, healthy subjects may absorb more calcium than normal subjects and have absorption hypercalciuria, leading to calcium stone formation. Increased calcium absorption due to excess vitamin D intake, or milk alkali syndrome and increased bone resorption, as in hyperparathyroidism, contribute to calcium stone formation. Thiazide diuretics are effective in preventing formation of calcium-containing stones. Alkaline pH favors calcium precipitation. Although calcium oxalate stones can form at any urinary pH, calcium phosphate formation is facilitated at high urine pH. Calcium phosphate stones are common in renal infections with organisms that convert urea to ammonia, such as *Proteus vulgaris*. Calcium and magnesium ammonium phosphate stones form staghorn calculi in the renal pelvis.

Uric acid stones account for approximately 10% of all renal calculi. Formation of uric acid stones is favored by acid urinary pH. Measures to prevent uric acid stone formation include a low-purine diet or treatment with allopurinol, which lowers levels of uric acid by inhibiting the enzyme xanthine oxidase. A high rate of urine flow, on the order of 3 liters per 24 hours, especially at night, or administration of bicarbonate to maintain urine pH alkaline facilitates dissolution of uric acid stones and prevents their recurrence after surgery.

Cystine stones are rare except in cases of an inborn error of metabolism (cystinuria). Cystine, like uric acid, is more soluble in alkaline urine than in acidic urine. Xanthine stones are very rare except in cases of an inborn error of metabolism (xanthinuria).

Hyperoxaluria due to overabsorption of dietary oxalate in fat malabsorption can contribute to formation of oxalate stones. Hyperoxaluria can also be due to a hereditary enzymatic defect. Cholestyramine, a resin that binds oxalate, is useful

to correct fat malabsorption, and low-fat diets are measures to correct hyperoxaluria secondary to intestinal absorption. Calcium lactate administration can be useful in precipitating oxalate in the gut lumen. Urinary levels of magnesium pyrophosphate and some polypeptides are believed to prevent stone formation.

Renal failure can result from a host of pathophysiological changes, all of which would be too numerous to cover, given the space limitations of this article. However, mention should be made of renal failure in lymphoma (22). The lymphomas can either obstruct, infiltrate, or rupture portions of the urinary tract. Retroperitoneal obstruction of both ureters is thus one of the most common causes of renal failure in lymphoma. Lymphomas may obstruct the renal artery and cause hypertension; they may also occlude the renal vein. Hypercalcemia in lymphoma can cause nephrocalcinosis and renal failure. Patients with malignant lymphoma, similar to patients with multiple myeloma, can have casts of monoclonal paraprotein or light chains in the distal tubules and collecting ducts. These casts can damage the adjacent tubular epithelial cells and interstitium, the severity of which correlates with the concentration of light chains (Bence-Jones proteins) in urine. The demonstration of light chains in urine requires using either the heat and acetic acid procedure or quantitative techniques, such as the sulfosalicylic acid procedure, as light chains will be missed in the urine dipstick procedure, which relies on a pH change of the indicator dye that the light chains are unable to effect. Deposition of immune complexes containing tumor-related antigens can cause glomerular injury in lymphoma. Some patients with lymphoma, particularly those with Hodgkin's disease, may have amyloid infiltration of the kidney, resulting in renal failure. Finally, tumor lysis as a result of therapy can precipitate renal failure because of uric acid nephropathy and deposition of phosphate in the renal tubules (23).

3.8. METHODOLOGY AND ANALYTICAL CONSIDERATIONS

Methods for the measurement of BUN and uric acid are well defined. Both colorimetric and enzymatic methods have been utilized for the measurement of these two constituents.

Because the measurement of BUN by nesslerization subsequent to hydrolysis by urease is cumbersome, the colorimetric method most widely used for the measurement of BUN is the diacetyl monoxime procedure (24). In this procedure diacetyl released from diacetyl monoxime under acidic conditions is reacted with urea to yield a yellow diazine derivative that is measured spectrophotometrically at 520 or 550 nm. The color intensity of the reaction is increased by use of either pentavalent arsenic or other polyvalent ions, such as ferric ions, or by the use of thiosemicarbazide. Although compounds such as citrulline, allantoin, and alloxan interfere, in practice the level of these constituents in serum is so low as to be of no significance. Even so, the hazard posed by the reagents used in this reaction has

encouraged development of specific enzymatic procedures. The commonly used procedure for the measurement of BUN is a coupled-enzyme procedure utilizing urease and glutamate dehydrogenase (25). In this procedure the ammonium ion resulting from the conversion of urea by urease to ammonium carbonate is coupled to glutamate dehydrogenase in the presence of α -ketoglutarate and NADH. The resulting decrease in absorbance at 340 nm as NADH is converted to NAD is directly related to the concentration of BUN. This coupled-enzyme reaction can be used for the measurement of BUN in serum, plasma, or urine. Urine samples need to be diluted 20–50 times because of the high concentration of urea in urine. A correction for ammonia may also be necessary because urine contains 1000 times more ammonia than serum. The kinetic assay using the urease–glutamate dehydrogenase coupled-enzyme reaction is suited to the measurement of urine urea in the presence of normal amounts of endogenous ammonia (26). The endogenous ammonia is readily consumed in the initial few seconds of the reaction, and any subsequent decrease in absorbance at 340 nm is related to formation of ammonia from urea. Ammonia may be present in the reagents and may be a potential source of interference. Other endogenous dehydrogenases may compete for the oxidation of NADH, causing potential interference. The specificity of the urease reaction for the measurement of urea is improved by following the change of conductivity with time as urea is hydrolyzed to form ammonium ions and carbon dioxide (27). The conductimetric approach has been adapted for automation. In the kinetic mode the conductimetric reaction, with corrections made for endogenous conductivity, is readily suited for the measurement of urine, serum, or plasma samples. Blood specimens containing fluoride as the glycolytic inhibitor should not be used to measure BUN by the urease procedure because the enzyme urease is inhibited by fluoride. Fluoride does not interfere, however, with the diacetyl monoxime procedure. The glycolytic inhibitor iodoacetate is satisfactory for the collection of blood specimens intended for the measurement of BUN by either the urease or the diacetyl monoxime procedure. Heparin is the desired anticoagulant for plasma BUN analysis. However, ammonium heparin should be avoided because of the obvious interference of ammonium ions in the urease assay. Because of the possibilities of bacterial degradation of BUN, specimens that cannot be analyzed the same day should be stored at 4°C. Maintaining urine pH below pH 4 preserves BUN. The optimum procedure for the measurement of BUN is the kinetic assay using either the urease–glutamate dehydrogenase coupled-enzyme reaction or the urease conductimetric measurement. Either of the two methods can be adapted to automated analysis and possess speed, precision, and specificity.

Enzymatic methods generally offer greater specificity over conventional non-enzymatic colorimetric methods for the measurement of uric acid. Even so, methods that depend upon the use of oxidizing agents—such as phosphotungstic acid to oxidize uric acid to allantoin and carbon dioxide, and the resulting reduction of

phosphotungstic acid to tungsten blue, with its characteristic absorbance between 660 and 710 nm—are still used widely in spite of their nonspecificity. Enzymatic procedures utilizing uricase, however, constitute the most widely used procedures for the measurement of uric acid. Uric acid has a characteristic absorption between 290 and 293 nm at pH ≥ 7 , and at 283 nm at pH < 7 . The measurements of the decrease in absorbance in this region as uric acid is converted to allantoin, hydrogen peroxide, and carbon dioxide qualify the procedure as a reference method for the measurement of uric acid (28). This method is highly specific; xanthine, however interferes significantly. Blood specimens collected either in sodium fluoride or EDTA also interfere, although not significantly. The uricase reaction can also be measured polarographically with an oxygen electrode because oxygen is consumed in the reaction (29). Because many clinical laboratories do not have the capability to measure UV absorbance in the 283- to 293-nm region, the hydrogen peroxide produced in the uricase reaction has been coupled to the catalyzed oxidation of ethanol by catalase to acetaldehyde. The latter is coupled to aldehyde dehydrogenase and NAD to yield acetate and NADH. The increase in absorbance of NADH at 340 nm is directly related to the concentration of uric acid (30). Alternatively, hydrogen peroxide liberated in the uricase reaction can be coupled to a chromogen in presence of the enzyme peroxidase. For example, hydrogen peroxide can be coupled to peroxidase in presence of 3-methyl-2-benzothiazolinone and *N,N*-dimethylaniline to yield a blue indamine dye that can be measured at 600 nm (31). Procedures dependent on coupling of hydrogen peroxide with peroxidase suffer from the fact that other reducing substances, such as vitamin C, compete for the hydrogen peroxide. However, the incorporation of ascorbate oxidase in the reaction mixture can overcome the interference by vitamin C.

Procedures using high-performance liquid chromatography on an ion-exchange material and subsequent electrochemical detection on reversed-phase high-performance liquid chromatography with spectrophotometric detection at 280 or 235 nm offer increased specificity and are candidates for reference methods (32,33).

Uric acid is stable for 3 to 5 days at 4°C. The collection of blood specimens in either EDTA or sodium fluoride should be avoided as these additives may cause positive interference. Collection of 24-hour urine specimens intended for uric acid measurements should be in a container that has 10 ml of 12.5 *N* sodium hydroxide to prevent precipitation of uric acid.

The measurement of creatinine to assess renal function is fraught with technical problems. The reaction described by Jaffé in 1886, and which bears his name, is still widely used for the measurement of creatinine. The Jaffé reaction, however, despite its remarkable longevity, is notoriously nonspecific. The reaction involves the development of a red-colored complex between creatinine and picric acid in alkaline solution. Although the absorbance maximum for the creatinine–picrate

complex is at 485 nm, in practice absorbance measurements are made in the wavelength range of 520–520 nm to overcome steep increases in the absorbance of the alkaline picrate blank at wavelengths below 510 nm. Included in the long list of compounds that interfere positively with the measurement of creatinine by the Jaffé reaction are glucose, acetone, acetoacetic acid, fructose, pyruvate, ascorbic acid, protein, uric acid, glycoxyamidine, indole, and certain cephalosporin antibiotics. Methodological considerations in creatinine analysis have been reviewed (34). Attempts to improve the specificity of the Jaffé reaction by using an aluminum silicate clay, or Lloyd's reagent, have not been totally satisfactory because this absorbent, in addition to retaining creatinine, also retains many constituents that interfere with the Jaffé reaction. Some of the limitations of the Jaffé reaction can be minimized by appropriate choice of time interval of the measurement in a kinetic assay. Performing the kinetic assay during a 10-second interval between 15.6 and 25.6 seconds ensures that color due to fast-reacting interferents (such as acetoacetate) fades before the initial measurements, whereas the slow-reacting constituents will not have reacted prior to 25.6 seconds (35). The kinetic assay does not require deproteinization and is readily adaptable to automation. Bilirubin causes a negative interference in some kinetic assays because some of the reagents are able to oxidize bilirubin to colorless compounds thus decreasing the blank absorbance to reflect an apparently low measured creatinine level. Because urine contains negligible Jaffé-positive interferents, the Jaffé reaction has been shown to be nearly specific for the measurement of creatinine in urine (36).

Currently, enzymatic procedures and procedures utilizing high-performance liquid chromatography (HPLC) offer an alternative to the traditional Jaffé reaction in terms of increased specificity. Whereas HPLC procedures may have a limitation in terms of the speed of analysis required in routine clinical laboratory procedures, the enzymatic procedures are amenable to automation and offer the best alternative to the Jaffé reaction. One such enzymatic procedure uses the enzyme creatinine amidohydrolase (EC 3.5.2.10) to hydrolyze creatinine to creatine. Creatine is then coupled to the enzyme creatine amidinohydrolase (EC 3.5.3.3) to yield urea and sarcosine. The latter is coupled to the enzyme sarcosine oxidase (EC 1.5.3.1) to produce hydrogen peroxide, glycine, and formaldehyde. Finally, hydrogen peroxide is coupled to the enzyme peroxidase in the presence of 4-aminophenazone and 3,5-dichloro-2-hydroxybenzenesulfonic acid to form a quinone–monimine dye, which is measured spectrophotometrically at 510 nm (37). This enzymatic procedure is reported to be free from many of the interferences associated with the traditional Jaffé reaction, including cephalothin antibiotics. Interference by bilirubin in this procedure is minimized by incorporating potassium ferrocyanide in the assay mixture. The addition of the enzyme ascorbate oxidase to the assay mixture prevents competition by ascorbic acid for the hydrogen peroxide generated in this coupled-enzyme reaction for the measure-

ment of creatinine. This enzymatic procedure can also be used with urine samples diluted 1:100.

HPLC procedures for the measurement of creatinine provide increased specificity. Cation-exchange chromatographic procedures have permitted selective adsorption and subsequent elution of creatinine by varying the pH of the mobile buffer phase (38,39). The eluted creatinine may either be measured by the Jaffé reaction (38) or by its absorbance in the UV region at 234 nm (39). Reversed-phase HPLC procedures have served as definitive procedures for creatinine measurements. However, some of these procedures require tedious sample treatment (40,41) and are plagued with technical problems such as column instability (40) and a need for a prolonged column preconditioning (42). However, the current availability of simple, specific, and relatively rapid HPLC separation methods using reversed-phase chromatography and UV spectrophotometric measurement of creatinine (43) should permit one to assay samples, especially when a result is suspect based on correlation with the results of other tests. The Jaffé reaction, because of its low costs and simplicity, is still widely used, thus laboratories unable to afford routinely enzymatic and HPLC procedures may yet resort to either of these two procedures for verification when a result is in doubt.

Blood specimens collected in tubes containing either fluoride or heparin are satisfactory for the measurement of creatinine. Ammonium heparin, however, should not be used to collect blood specimens, if the assay is to be performed with a coupled-enzyme procedure that measures ammonia. To minimize *in vitro* ammonia formation, serum or plasma should be promptly separated from the cells for creatinine assay procedures that depend on ammonia generation. Creatinine is fairly stable in serum stored at 4°C for at least 1 week.

It should be noted that the determination of creatinine in urine is impractical in terms of assessing the completeness of 24-hour urine collections. This is so because of the considerable variation in the amount of creatinine excreted in a 24-hour period (44). Variations within the same subject occur from time to time. In one study, creatinine excretion by the same individual averaged 10% variation, whereas, among individuals, it was 29% (45).

In discussing methodology for assessing renal disease, a few comments on routine urine analysis are in order, including microscopic examination of the urinary sediment. Currently, there is controversy as to whether a microscopic examination of the urinary sediment should be performed routinely (46,47). The availability of reagent dipsticks that can measure pH, protein, glucose, ketones, bilirubin, hemoglobin, specific gravity, and urobilinogen and that detect asymptomatic bacteria (as indicated by reduction of nitrate to nitrite) and leukocytes (both lysed and intact, by the leukocyte esterase test) has made it possible to reserve microscopic examination of urinary sediment only to those urinary specimens that are abnormal by the dipstick method, particularly with respect to the leukocyte esterase and nitrite tests. The criteria for determining that a urinary

sediment is abnormal includes finding one or more red blood cells per high-power field (HpF), more than three leukocytes per HpF, one or more hyaline casts per low-power field, yeast cells with pseudohyphae, any waxy or fatty casts, more than occasional tubular epithelial cell casts or granular casts, occasional leukocyte casts, and any red blood cell casts.

Sensitive radioimmunoassays for assay of β_2 -microglobulin (β_2 -M) allow evaluation of various aspects of renal function (48). Because of its low molecular weight (11,800) β_2 -M is freely filtered through the glomerulus (49). Of this β_2 -M, 99.9% is reabsorbed by and catabolized by the proximal tubular cells. As a result, with normal renal function, only very small quantities of β_2 -M, approximately in the range of 40–360 μg per day, are excreted in urine. Thus, increased levels of β_2 -M excretion in urine would reflect impairment of proximal tubular function. The serum levels of β_2 -M would, however, be normal in proximal tubular dysfunction as long as the glomerular filtration is normal. Increased urinary levels of β_2 -M are also found in cadmium poisoning, Fanconi's syndrome, aminoglycoside nephrotoxicity, and acute renal failure (50–53). The measurement of β_2 -M in urine is inaccurate when urine pH drops below 5.5. At acid pH, β_2 -M undergoes changes in its tertiary structure and is subject to rapid proteolytic degradation. A preservative such as sodium azide (0.1 g/liter) should be used in 24-hour urine samples. Urine samples may be collected over a short period of time (such as 1 hour), after a meal, or after consumption of 150 ml of water containing 10 g/liter sodium bicarbonate. Urine samples that cannot be analyzed promptly may be stored frozen at -20°C .

Increased levels of serum β_2 -M reflect either increased synthesis or reduced glomerular filtration rate. If the glomerular filtration rate is greater than 80 ml/minute/1.73 m^2 body surface area, increased serum β_2 -M levels signify increased synthesis (54). An increase in serum β_2 -M levels with a reduction in glomerular filtration rate is an early indicator of kidney transplant rejection (55). An increase in urinary β_2 -M in kidney transplant patients reflects proximal tubular damage caused by immunologic attack of the transplant by the host immune system. Serial determinations of β_2 -M provide important information about the viability of a graft. Either serum or heparin- or EDTA-anticoagulated plasma is suitable for the assay of β_2 -M by radioimmunoassay. In summary, the combined use of serum creatinine and serum β_2 -M in patients with renal disease ought to provide more information than either measurement alone.

4. Summation of Current Concepts

To obtain a total perspective on glomerular disease, first it is important to recognize that progressive glomerular disease is accompanied by persistent proteinuria. Renal function begins to deteriorate as glomerulosclerosis develops.

However, one of the hallmarks of glomerular diseases that lead to the development of glomerulosclerosis is the proliferation of mesangial cells at an early stage of the development of glomerular disease (56). Examples in this category are membranoproliferative glomerulosclerosis, diabetes mellitus, light chain systemic disease, and IgA disease. In contrast, glomerular disease wherein mesangial proliferation is accompanied by an acute inflammatory episode rarely leads to development of glomerulosclerosis. This would imply that in poststreptococcal glomerulonephritis and in mixed cryoglobulinemia, even when faced with acute injury, glomerular cell number is, by and large, normally strictly regulated. While discussing diabetic kidney disease we noted earlier that the most common lesion found in diabetics is diffuse glomerulosclerosis, which is characterized by thickening of the mesangial matrix and an increase in the width of the glomerular basement membrane. Additionally there is a gradual obliteration of the glomerular capillaries with the resultant loss of surface area for filtration. Thus in the development of diabetic nephropathy, the increase in the volume of the mesangial matrix can be correlated with the onset of proteinuria, azotemia, and hypertension.

4.1. ROLE OF GROWTH FACTORS AND CYTOKINES IN RENAL DISEASE

From our current knowledge on the role of transforming growth factor- β (TGF- β), it is recognized that it is a multifunctional regulator of cellular activity (57). It has dual effects in that it can both stimulate or inhibit cell growth and proliferation. However, in its regulatory role, its predominant effect is to inhibit cell growth. Its importance can be recognized by the fact that most cells produce TGF- β , with the greatest concentration found in platelets and bone. As a master growth factor, TGF- β regulates actions of growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), interleukin-1 (IL-1), and interleukin-2 (IL-2). TGF- β has a strong regulatory influence on the extracellular matrix, and thus plays a vital role in response of cells to stress or injury. In this context, the response of glomerular cells to injury can be pictured as resulting from increased release of substances with multiple effects. Thus the release of IL-1, leukotrienes with inflammatory properties, platelet-activating factor, thromboxane A_2 and PDGF with procoagulant activity, and EGF and PDGF with mitogenic activity, leading to matrix proliferation, can be viewed as a response of glomerular cells to injury.

The release of some of these mediators attracts inflammatory cells such as macrophages, neutrophils, and T cells to the renal parenchyma, in addition to platelets. The consequence of infiltration of the renal parenchyma by these inflammatory cells and platelets is the activation of glomerular cells to produce mitogenic or growth factors.

There are other causes of glomerular injury. They range from deposition of compounds with biological activity, such as immunoglobulins, cryoglobulins,

amyloid proteins, complement, lipoproteins, components of cell walls or bacteria, and toxins, to other factors that involve damage to epithelial cells, such as intra- or extra-capillary coagulation and hypertension. Conditions such as hyperlipidemia, hypertension, and proteinuria can modify the progression of glomerulosclerosis. This can be due either to a direct effect of these conditions, or to the abolition of release of certain factors or by stimulating release of factors with mitogenic or inflammatory effects.

Glomerular injury brings in its wake a host of consequences. There is a decreased production of factors important in maintaining the normal biology and function of glomerular cells. These include plasminogen activator by endothelial cells, prostacyclin, neutral proteinases elaborated by the mesangial cells, and heparin-like proteoglycans produced by the epithelial cells. Thus functions such as regulation of the extracellular matrix by neutral proteinases and control of glomerular cell proliferation by heparin-like proteoglycans are compromised.

4.2. CURRENT INTEREST IN THE MEASUREMENT OF GLOMERULAR FILTRATION RATE

The measurement of the GFR is of current interest in view of the fact that recent studies have questioned the accuracy of creatinine clearance and serum creatinine level for assessing kidney function and the rate of progression of renal disease (58). To recapitulate the value of the GFR as an index of the severity of renal disease, it should be pointed out that the GFR provides, in a sense, a direct measure of renal function and is reduced relatively early in the course of renal disease.

The severity of structural and functional renal impairment can be assessed with the GFR, which correlates with the intensity of tubular and interstitial damage. Furthermore, signs and symptoms of uremia arise when the GFR decreases to less than 5–10 ml/minute. However, the GFR has its limitations in terms of its ability to assess the severity of renal disease. To begin with, the GFR offers a measure of renal function, and, as such, is also affected by other factors, such as circulatory disorders, state of hydration, and drugs, to mention a few. Critics of the use of the GFR point out that it represents a balance of transglomerular filtration pressure and ultrafiltration coefficient. As such, even in the face of considerable destruction of glomerular structure, the GFR in single nephrons could remain normal or even increased. Despite this observation, which raises doubts on the sensitivity and specificity of the GFR in assessing the seriousness of renal disease, current interest is in the use of filtration markers to assess the GFR. Compared to the classical but inconvenient inulin clearance procedure for the estimation of the GFR, the use of labeled filtration markers using bolus infusion and spontaneous bladder emptying provides a convenient alternative for the precise and accurate estimation of the GFR.

Radio-labeled filtration markers that have been used to assess the GFR include chromium-51-labeled EDTA and iodine-125-labeled sodium iothalamate. Either

of these markers is satisfactory. Plasma clearance of ^{51}Cr -labeled EDTA approximates renal inulin clearance and offers excellent precision. In contrast to ^{51}Cr -labeled EDTA, which has a half-life of 28 days, ^{125}I -labeled sodium iothalamate has a longer half-life of 60 days. Although the renal clearance of the latter slightly exceeds renal inulin clearance, for routine clinical practice it is convenient and has good precision.

Recently, minute doses of nonradioactive filtration markers such as iothalamate and diatrizoate meglumine (or Hypaque) have been used for assessing GFR. The development of high-performance liquid chromatography procedures for measurement of these nonradioactive filtration markers in plasma and urine provides a practical alternative to the laboratorian wary of the hazards associated with a radiolabel.

4.3. MICROALBUMINURIA

In recent years microalbuminuria has received considerable attention due to the fact that it is a risk factor for the development of diabetic nephropathy and increased cardiovascular mortality in insulin-dependent diabetes mellitus (IDDM), and cardiovascular death in hypertension and noninsulin-dependent diabetes mellitus (NIDDM).

It has been clearly recognized that one of the clinical hallmarks of diabetic nephropathy is proteinuria in excess of 500 mg/24 hours; this can be easily detected by urine dipstick testing, which has a detection limit of 150–200 mg/liter of albumin. Such clinical proteinuria portends a progressive decline in kidney function leading to end-stage renal failure over an average 7-year period. This clinical proteinuria is not sudden but is preceded by years of microalbuminuria. Thus microalbuminuria represents an early manifestation of diabetic kidney disease. In fact, 6–20% of patients with IDDM present with microalbuminuria. Left uncontrolled, albumin excretion in microalbuminuric subjects can increase at the rate of 7–18.6% per year.

IDDM subjects who have achieved glycemic control through insulin therapy have been known to retard or even reverse the progression of microalbuminuria. Even so, control studies demonstrating the reduction in either patient mortality or the development of end-stage renal disease, with the cessation of microalbuminuria in either IDDM or NIDDM subjects, are lacking. Whereas albumin excretion in the urine of a healthy individual is approximately less than 20 $\mu\text{g}/\text{minute}$, urine albumin excretion greater than 30 $\mu\text{g}/\text{minute}$, usually seen in the early stages of diabetes, is classified as microalbuminuria (59).

There has been considerable discussion as to what constitutes an appropriate urine sample for the detection of microalbuminuria. It is the prevailing view that the albumin excretion rate (AER) and albumin concentration in spot or random

samples are of limited value for initial screening, and frequently 24-hour urine or timed overnight samples are required for long-term monitoring of the diabetic subject (60). To obtain a precise urinary albumin excretion rate (AER) range for microalbuminuria, two out of three timed overnight or 24-hour urine collections done within 6 months should be in the range of 30–300 mg/24 hours, or 3–30 mg/mmol of creatinine. This represents an AER range of 20–200 $\mu\text{g}/\text{minute}$ (61).

Timed overnight urine samples, or the so-called first-morning specimen, collected after overnight sleep have been helpful in predicting microalbuminuria. In such samples, an albumin concentration greater than 17 mg/liter or an albumin:creatinine ratio greater than 3 mg/mmol has been shown to predict the overnight albumin excretion rate greater than 30 $\mu\text{g}/\text{minute}$ with 97% sensitivity and 90% specificity. An albumin:creatinine ratio of 3.5 in a first-morning urine sample is a sensitive predictor of an AER equal to or greater than 30 $\mu\text{g}/\text{minute}$. It is recommended that all patients with an albumin:creatinine ratio greater than 3.5 should have timed overnight urine samples collected for evaluation of microalbuminuria. Use of the albumin:creatinine ratio determined on a random urine sample is of questionable value (62).

How stable is albumin in urine specimens collected for detecting microalbuminuria? There has been a debate on the effect of storage temperature on the stability of albumin in urine. Albumin levels have been reported to be stable in urine samples stored at 4°C and assayed within 2 weeks of collection (63). A loss in albumin has been reported during storage at -20°C, due to the formation of a precipitate. Thus storage at -20°C for 2 months resulted in lowering of albumin levels of 2.4 mg/liter, and storage at the same temperature for 6 months resulted in lowering of albumin by 3.0 mg/liter (63). Adjustment of pH or urine sample to neutrality has been reported to prevent precipitation of albumin during storage at deep-freeze temperatures (64). Other investigators have avoided loss of albumin during deep-freeze storage by vortexing and disrupting the precipitate in the thawed specimen prior to analysis (65). As for the methodology for establishing microalbuminuria, the urine dipstick procedure is clearly insensitive because it does not even detect albumin excretion greater than 20 times the normal limit.

Colorimetric methods such as pyragallol red–molybdate IV or coomassie blue dye binding methods are inadequate because they measure other proteins in addition to albumin. Qualitative screening tests based on turbidimetric or latex immunoagglutination principles are also inadequate due to their poor analytical sensitivity. The choice is then between procedures such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), radial immunodiffusion (RID), polyethylene glycol-accelerated immunoturbidimetry and immunonephelometry, and antibody-coated latex particle-enhanced nephelometry (66). Some of the above immunological methods provide an analytical sensitivity of 5 $\mu\text{g}/\text{liter}$, thus making them suitable for establishing and monitoring microalbuminuria.

Microalbuminuria, in a sense, portends complications that lie ahead and provides an opportunity to arrest disease progression by ensuring proper glycemic control.

4.4. PLASMA PRORENIN ACTIVITY

The juxtaglomerular cells of the kidneys produce prorenin, which is the precursor of renin that is secreted into the blood. Plasma prorenin and renin activities increase when there is a sodium deficit or when diuretics such as furosemide are administered. Several studies have reported that adult normotensive diabetics in whom microvascular complications have developed have higher plasma prorenin activity than those patients without microvascular complications. What makes plasma prorenin activity measurement so valuable is that the prorenin plasma level in adolescents with diabetes can reach above the upper limit of the reference range, as much as 36 months prior to the detection of a complication (67). The mean plasma prorenin activity was significantly higher 18 months prior to the development of retinopathy or overt albuminuria than in those subjects who did not subsequently develop such complications (67). Because children with diabetes develop microvascular complications as they approach adolescence, and the duration of diabetes lengthens, plasma prorenin activity measurements would be very useful to screen such children and initiate treatment toward glycemic control.

Just why is plasma prorenin activity elevated in diabetics? An explanation can be offered in the finding that in adult diabetics administered furosemide, large increases in plasma prorenin activity are seen, especially in those subjects with albuminuria, and plasma renin activity is below the normal range. Thus, it would appear that the efficiency of the conversion of prorenin to renin is affected in diabetics, with the effect being more severe in diabetics with hyporeninemic hypoaldosteronism (67).

The measurement of prorenin activity should be on blood specimens collected in EDTA; the plasma must be promptly processed, frozen, and stored at -20°C . In performing the analysis, active renin activity in plasma is first determined by measuring generation of angiotensin I by radioimmunoassay, during incubation of plasma at 37°C , pH 7.4, with sheep angiotensinogen. To determine total renin activity, an aliquot of plasma is dialyzed first to pH 3.3 and then to pH 7.4, to activate prorenin before incubation with sheep angiotensinogen and measuring angiotensin I generation. Plasma prorenin activity is calculated by subtracting active renin activity from total renin activity. Because an increase in plasma prorenin value precedes by several months the development of diabetic complications, its measurement would be useful to screen for children with diabetes who are at high risk for developing diabetic nephropathy or retinopathy.

4.5. TREATMENT MODALITIES TO REDUCE GLOMERULAR FILTRATION RATE AND KIDNEY SIZE IN INSULIN-DEPENDENT DIABETES MELLITUS

Although it is not uniformly accepted, it has been proposed that glomerular hyperfiltration is an early marker of diabetic nephropathy in IDDM patients. It has been pointed out that this increased glomerular filtration rate appears to be closely correlated with increased glomerular hypertrophy. Hence kidney size has been postulated to have a prognostic implication in IDDM.

Treatment with the somatostatin analog Octreotide has been reported to reduce increased glomerular filtration rate and kidney size in IDDM subjects (68). The mechanisms by which Octreotide reduces renal filtration rate and volume are not well understood. However, the fact that plasma levels of insulin-like growth factor-I (IGF-I) were reduced significantly at 3 and 12 weeks of Octreotide treatment would indicate that reduction of the glomerular filtration rate and glomerular hypertrophy by Octreotide is due to the inhibition of IGF-I. This explanation is plausible, because in the study with Octreotide it was noted that a correlation existed between plasma glomerular filtration rate and the plasma level of IGF-I. Additional evidence is that in normal subjects exogenous IGF-I has been shown to have an enhancing effect on the glomerular filtration rate. Octreotide, by acting directly on the liver, could inhibit formation of IGF-I. Current interest in such treatment modalities is encouraging.

4.6. ANALYTICAL STRATEGIES

In the search for absolute specificity of measurement of renal analytes such as creatinine, newer or modified methods, especially those utilizing the resolving power of HPLC, are being proposed year after year. It is almost impossible to keep pace with all of the literature on key (but interference-ridden) methodologies for the measurement of creatinine. However, a few of the recently proposed procedures deserve mention. One is a rapid HPLC ion-pair procedure for measurement of creatinine in serum and urine by measuring the absorbance of creatinine in the ultraviolet region at 236 nm (69). Another HPLC procedure was proposed as a candidate reference method (70). This method requires the precipitation of protein prior to separation on an ion-exchange column. Creatinine was eluted with one solvent (isocratic elution) and measured in the ultraviolet region at 234 nm. The accuracy of this reference method was proved by accuracy studies done in comparison with isotope-dilution mass spectrometry, a definitive method.

With the multiplicity of drugs that are administered, one must constantly be aware of interferences in methodology, not only by the parent drug but also its metabolites. Even very specific methods are not exempt; for example, it was shown that *N*-ethylglycine, the metabolite of lidocaine, was an interferent in the sarcosine oxidase coupled-enzymatic reaction for the measurement of creatinine

(71,72). The debate does continue as to what constitutes a very specific laboratory marker for renal disease, and, as in every debate, there are vociferous proponents, as well as those who look at the total perspective and argue that currently used routine analytes such as BUN and creatinine are adequate (in spite of the notoriety of the classical Jaffé reaction for its share of interferences). But mention should be made of a few other classical markers, especially in urine. Quantitative assays of retinol-binding protein or α_1 -microglobulin are sensitive indicators of proximal tubular damage. Both *N*-acetyl- β -D-glucosaminidase (NAG), a lysosomal enzyme, and alanine aminopeptidase (AAP), a brush border enzyme, are highly sensitive markers of renal tubular damage (73). The activity of both NAG and AAP in urine increases prior to changes seen in traditional renal function tests. The two enzymes have been used to predict renal allograft rejection as well as renal involvement in diabetes mellitus and hypertension. They have also been used to monitor the nephrotoxic effects of drugs such as cyclosporin A, among others, and to screen for renal damage in subjects exposed to environmentally toxic metals. The measurement of NAG and AAP in urine thus permits early detection of renal tubular damage (73).

Research in the coming years ought to further validate additional sensitive laboratory tests for the assessment of renal disease.

5. Perspectives for the Future

Developments in ultrasound equipment and scanners hold promise for further understanding and characterizing different types of renal disease. Delineation of a specific ultrasonic pattern for glomerulonephritis would dispense with the need for many renal biopsies. Advances in computers and mechanical scanners that can produce a picture in 2 seconds should enhance the usefulness of ultrasound, which is currently the logical choice for uremic patients who are unable to excrete isotopes and contrast media.

Kidney transplantation is now the recognized treatment for patients in end-stage renal failure unless medical or immunological reasons contraindicate it, in which case the patient's life is sustained by dialysis. The relationship between blood transfusions and the suppression of the rejection response is being recognized. Our further understanding of the HLA system and factors controlling the immune response may permit us to effect and maintain successful transplants and thus treat effectively patients with end-stage renal failure.

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THE POLYMERASE CHAIN REACTION: CLINICAL APPLICATIONS

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1. Introduction

In the 6 years that have elapsed since the polymerase chain reaction (PCR) technique was published it has had a major impact on medical research (E6, M8, M9, S1). Previous reviews (E3, G1, P2, W7, W9) have focused on its clinical applications in diagnosing viral and genetic diseases, and several books (E4, E5, I1) provide detailed protocols appropriate for the research laboratory.

This article will cover the principle and practice of PCR in the clinical laboratory and applications for diagnosing viral, bacterial, fungal, and parasitic diseases. Other target amplification strategies, such as the ligase amplification system and transcription-based systems derived from PCR, have been reviewed elsewhere (P2). Applications of the method in the diagnosis of cancer (G1, K3) and genetic disease (R1) were reviewed recently. This article contains an outline of the PCR method and its power and limitations, the key issues involved in using it in the clinical reference laboratory, the challenges ahead in producing diagnostic kits, and an overview of its application in specific infectious disease diagnosis.

One objective of this article is to identify those diseases for which PCR offers an advantage over conventional diagnostic methods. For example, it may be the preferred method for detecting pathogens that are difficult, slow, or impossible to culture. Another objective is to address the issues and technical challenges that must be solved before the method will be widely available in the form of FDA-approved standardized kits suitable for the clinical laboratory. Examples of these challenges include procedures for (1) simplifying specimen preparation; (2) eliminating false positives; and (3) colorimetric detection. Questions that we address include whether PCR is too sensitive, whether more than one gene target is necessary, and how positive results can be confirmed if the method is more sensitive than culture. Finally, although we have not cited all of the published articles on clinical applications of PCR, we have provided summary tables for each of the main areas of disease.

2. Principle of PCR

2.1. THE TECHNIQUE

The polymerase chain reaction, developed at the Cetus Corporation in Emeryville, California, employs the enzymatic amplification of DNA *in vitro* (M8, M9, S1). By synthesizing many copies of a selected DNA sequence, PCR is capable of substantially increasing the quantity of this target DNA segment in a sample. This results in a corresponding reduction in the complexity of the nucleic acid sequences in the sample, i.e., the ratio of target to extraneous sequences is vastly increased. Amplification is performed in discrete cycles, and each cycle can, in principle, double the amount of target DNA. The target is therefore exponentially amplified, such that after n cycles there is $(1 + x)^n$ times as much target as was present initially, where x is the mean efficiency of each cycle.

The principle of the method is shown in Fig. 1 (A1). A target DNA sequence to be amplified is chosen first. The nucleotide sequence of the target DNA may be unknown, but sequences of short stretches of DNA on either side of the target must be known. These sequences are used to design two oligonucleotide primers, which

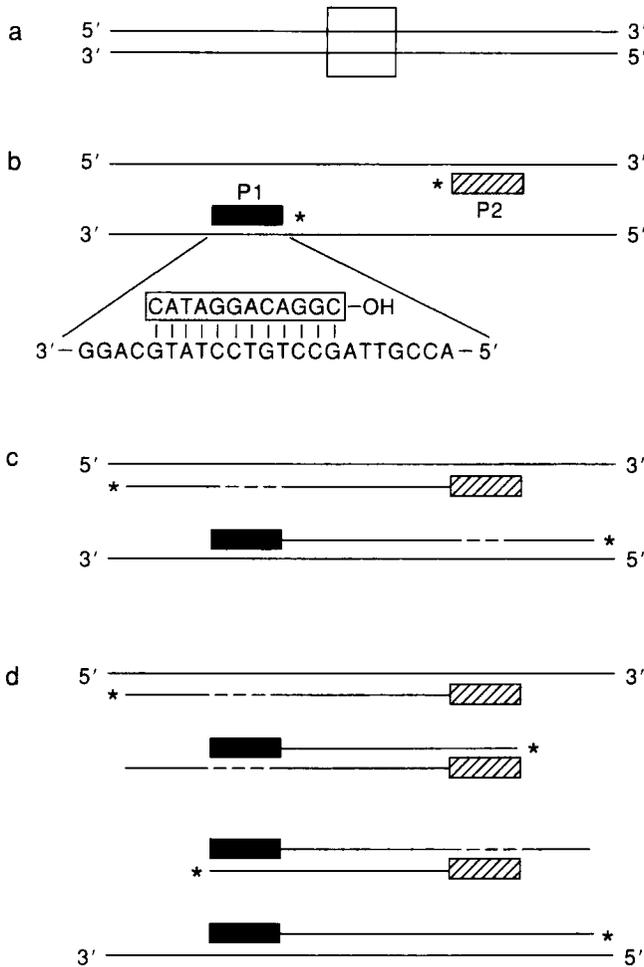


FIG. 1. Principle of the polymerase chain reaction. (a) The target sequence within a double-stranded DNA molecule is indicated with a box. (b) After the DNA has been denatured, the two PCR primers, P1 and P2, anneal to the sequences flanking the target. The 3' end of the primer undergoing elongation by DNA polymerase is denoted by an asterisk. Below primer P1 are the details of the base pairing between the primer (boxed) and the DNA strand. (c) DNA polymerase extends the two primers. The region of the extension product complementary to the other primer is shown by a broken line. (d) In the second cycle of PCR, each of the four DNA strands shown in c anneals to a primer, which is subsequently extended. Note that, at the completion of the second cycle, there are four double-stranded copies of the target, which was originally present as one double-stranded molecule in a. Note also that two of the eight single-stranded products are equal in length to the two primers and the intervening target. Products of this size accumulate exponentially during additional cycles. Reprinted with permission of Arnheim *et al.* (A1) and *BioSciences*.

are single strands of DNA, each approximately 20 nucleotides long. The primers are made by an automated DNA synthesizer. Specific primers can be ordered from commercial suppliers.

Each pair of primers is designed so that the nucleotide sequence of one primer is complementary to sequences flanking one end of the target DNA, whereas the other primer is complementary to the other flanking sequence. After the double-stranded target DNA is denatured into single strands, the primers hybridize to their complementary sequences flanking the target gene. The primers are oriented so that when they bind to the flanking sequences, their 3'-hydroxyl ends face the target sequence. Next, a thermostable DNA polymerase (S2) is added, and, because DNA polymerases extend DNA chains by adding deoxyribonucleoside monophosphates to the 3' end of each chain, the polymerase extends the primers, thereby making copies of the target. The extension products of each primer must be long enough so that they include the sequences complementary to the other primer.

This series of steps—DNA denaturation, primer hybridization, and DNA polymerase extension—represents a PCR cycle. Each of the three steps must be carried out at an appropriate temperature. Because the products of one cycle can serve as templates for the next, if the first cycle is followed by additional ones, more copies of the target sequence will be made. The main product of the procedure is a double-stranded DNA fragment equal in length to the sum of the lengths of the two primers and the intervening DNA. Single-stranded DNA can also serve as a template, as can RNA after a complementary DNA strand has been synthesized with a reverse transcriptase. Because the quantity of target DNA theoretically doubles with each cycle, as few as 20 cycles generates approximately a million times the amount of target sequence present initially. If only 90% of the targets are extended in each cycle, 20 cycles would yield a 375,000-fold amplification. Nontarget sequences that anneal to one primer and become extended could at most increase 20-fold in concentration during 20 cycles because the product of the first primer extension is not likely to contain the sequence region complementary to the other primer. In some cases, nonspecific binding of the other primer to the extension product of the first primer may result in exponential amplification of nontarget sequences.

Although the theory of PCR is straightforward, a major problem might be expected. In a human genome containing in excess of 10^9 nucleotides, a 1000-base pair target would represent only 10^{-6} – 10^{-7} of the available DNA. The annealing of the primer to the many nontarget sequences in such complex templates, even if it occurred infrequently, would lower the purity of the target in the final product. The extent to which imperfect annealing and extension can occur depends on the temperatures during the primer-annealing and polymerase extension steps, because the specificity of primer annealing is greater at higher temperatures. The use of a heat-resistant DNA polymerase allows annealing to

be carried out at an elevated temperature, thus reducing annealing to nontarget sequences. This added selectivity now allows the experimenter to produce large amounts of virtually pure target DNA for characterization. Parameters of the reaction that affect its efficiency include the concentration of enzyme, magnesium ion, and primers. Optimization of the reaction by comprehensive titration of the components is essential for the development of a highly sensitive, reproducible, and robust assay.

2.2. TARGET SELECTION

For detecting an infectious disease organism one can choose among several strategies in selecting the genetic target to be amplified. Genes that provide essential functions and contain both conserved (invariant) and variable sequence regions can be targeted. Specificity can be obtained either at the amplification (primer) or detection (probe) stage. Alternatively, the target might be a virulence gene that is uniquely responsible for distinguishing pathogenic from closely related nonpathogenic strains, types, or species. Pathogen-associated targets, such as cryptic plasmid genes, surface membrane protein genes, or randomly cloned sequences, can also be used as long as specificity can be demonstrated. General guidelines for the design of primers have been described (I1).

To illustrate the first strategy for detecting the bacterial pathogen that causes Lyme disease, one could target a segment of the essential, multicopy, small-subunit ribosomal RNA (rRNA) gene and design primers based on sequences that are invariant among all *Borrelia* species, but different from other bacteria outside this genus (P4). The intervening region within the amplified segment can be used to distinguish DNA amplified from *Borrelia burgdorferi* from other *Borrelia* species with a species-specific probe. Variations on this approach can be used at any level of the molecular taxonomic hierarchy, i.e., for bacterial meningitis one could use primers based on rRNA gene sequences that are identical among all eubacteria and identify specific pathogens with probes for *Haemophilus influenzae*, *Neisseria meningitidis*, etc. (L1). Alternatively, species specificity can be conferred at the amplification level by using primer-target mismatches to prevent primer extension of sequences from the nontarget organisms (K14, N1, W15). This general approach can be used for viral diseases by targeting regions of essential genes that are either conserved or pathogen specific among, for example, herpesviruses or retroviruses.

Examples of the second approach to target selection are provided by the work of Frankel and co-workers (F2) and Persing *et al* (P3). In the former study, toxin genes and an invasion-associated locus were used to distinguish pathogenic *Escherichia coli* and *Shigella* from normal gut flora. Persing and co-workers (P3) targeted a plasmid-encoded outer-surface protein gene to identify the Lyme disease pathogen. A key factor to consider when selecting sequences for primers and

probes is that most published gene sequences have been obtained from single isolates of a particular bacterial species or virus. Therefore, regardless of the target selected, one must ensure that the primer sequences are conserved in all isolates of the target microorganism by empirically testing many strains and closely related nontarget organisms from different geographic regions.

In developing a PCR assay for a microorganism it is generally advisable to evaluate initially multiple primer pair systems, ideally for different genes, particularly if the primary gene target is nonessential for viability. Multiple primer sets can be used to clarify any negative results that may be due to plasmid target loss, sequence deletion, or unanticipated natural variation. If the data from multiple isolates demonstrate perfect concordance between the primary and confirmatory targets and high clinical sensitivity with a single primer pair/probe system, then the confirmatory sets can subsequently be dropped. Discordance among primer sets may be due to factors other than sequence variability, such as (1) sample bias due to low numbers of target organisms, (2) different analytical sensitivities for different primer sets, and (3) the length of the primer. In this regard, standardized reagents and procedures in the form of approved kits may go a long way toward determining whether different clinical sensitivities reported from various laboratories were due to the absence of a standardized procedure and reagents or to target gene variability. In the largest study reported to date (S9), five laboratories analyzed 200 coded blood samples for HIV-1. One lab had 100% sensitivity and specificity for all samples; in this lab the concordance between two primer pair sets for different regions of the HIV-1 *gag* gene was 100%. The lack of concordance between primer systems in other labs was clearly due to variations in the reagents and procedure rather than to an inherent inability to amplify some isolates with either primer pair. These results, if confirmed with additional HIV specimens, will have important implications for the design, speed, and complexity of PCR tests in the clinical laboratory. Degenerate primers (which are composed of a mixture of oligonucleotides containing a mixture of bases at some positions) offer an additional approach to extending the range of the amplification system to identify isolates with heterogeneous target sequences (C6, K7, M2, N3, S4).

In some cases, intraspecies sequence variability, or "microheterogeneity," may be highly useful for designing probes that can both identify and distinguish pathogenic isolates for epidemiologic studies. For example, PCR confirmed that a patient had become infected with the same strain of HIV-1 that was isolated from her dentist (W11). In another study, Rosa *et al.* (R5) identified sequence variability in a randomly cloned gene from North American and European isolates of *B. burgdorferi*. This variation may prove useful in following the spread of this disease in different geographic areas. Kwok *et al.* (K10, K11) identified variable regions of HTLV that are flanked by conserved regions. Their primers amplify all isolates of HTLV-I and -II while specific probes to the variable sequences are conserved within a type, but can distinguish type I from II.

2.3. SAMPLE PREPARATION

One advantage that PCR has over many other DNA probe diagnostic methods is that small, degraded, damaged, and unpurified DNA can still serve as a template for the first cycle of amplification. Because subsequent cycles mainly use the newly synthesized product of previous cycles as template, poor-quality targets are irrelevant once amplification is underway.

Simple dilution of crude extracts of clinical specimens often eliminates inhibitors of the amplification reaction while still providing enough initial template for it to proceed. Thus, microorganism DNA or RNA has been amplified directly from crude lysates of human peripheral blood mononuclear cells, bacteria, insect vectors, cervical swabs, urine, hair, sputum, and preserved tissues (W9). Nonetheless, most current procedures for blood-borne viruses such as HIV and HTLV still require a time-consuming isolation of leukocytes prior to the cell lysis step. What is still needed in order to move these relatively cumbersome specimen handling methods out of the skilled reference lab is more rapid, simple, and preferably automated procedures. Because hematin in blood (H6) is the primary inhibitor of the DNA polymerase in the PCR, perhaps a rapid method will be discovered for inactivating it (L2). Then it might be possible to consider detecting a single bacterium (which contains 15,000 copies of rRNA) in 10 ml of blood from a sepsis patient, because a 50- μ l aliquot of a completely lysed and homogeneous sample would contain 75 copies of the target.

Another promising approach to sample preparation is capture probe technology. In this method the target DNA is hybridized to a probe attached to a magnetic bead. Nontarget sequences are removed by washing prior to amplification. This procedure was developed in conjunction with the Q β signal amplification system (G2, H10). Target capture may prove to be a necessary step for concentrating small numbers of target molecules from a biological fluid. For efficient recovery of *Listeria monocytogenes*, 10 ml of cerebrospinal fluid is required. Likewise, increasing the volume of blood culture inoculum from 5 to 10 ml substantially improves bacterial recovery. Such large volumes are cumbersome for DNA extraction; thus a magnetic target capture system following chaotropic lysis may improve assay sensitivity and reduce sampling error. This source of error becomes important when testing small amounts of specimens that may have minute amounts of target (i.e., 1–10 molecules), because variation can occur simply from nonuniform distribution of the target throughout the sample. Thus far, the capture probe approach has not been reported to detect fewer than several thousand copies of target, though this limitation might be a feature of the Q β system due to the inability to remove nonspecifically bound, but still amplifiable, detection probe. However, in combination with PCR, the capture probe sample preparation technology might be capable of detecting 10 or fewer molecules of target.

One implication of the high sensitivity of PCR is that it may alter patient sampling requirements so that they can be convenient and less invasive. Buccal epithelial cells derived from a mouthwash have been used successfully to identify carrier status for a cystic fibrosis mutation. Hair samples from a patient, relatives at geographically distant locations, and unrelated donors can be rapidly analyzed for histocompatibility leukocyte antigen (HLA) genotype in order to identify compatible donors for bone marrow transplantation (Table 1). Urinary sediments might be used in place of urethral swabs in the diagnosis of infectious urethritis, peripheral blood might be used instead of bone marrow or liver biopsy in the diagnosis of atypical mycobacterial infections, and peripheral blood mononuclear cells (PBMCs) may be used instead of bone marrow in the detection of recurrent leukemias or lymphomas. The ability to HLA type a patient's specimen also offers the potential for resolving instances of sample mix-up, i.e., a particular specimen can be unequivocally associated with a patient of the same type(s).

2.4. DETECTION OF AMPLIFIED DNA

The first detection methods used with PCR were radioactively labeled probes to identify specific amplified sequences (M8, S1). With improvements in amplification specificity it became possible to visualize amplified DNA of the predicted size directly by its fluorescence on an agarose or polyacrylamide gel (M9) following staining with ethidium bromide. Probe-based methods remain a key feature of current detection systems primarily because of the additional information and sequence specificity they provide. Probes have been converted to nonisotopic colorimetric systems (B6) by labeling them with an enzyme such as

TABLE 1
HLA GENOTYPING AT THE DQA, DPB, AND DRB LOCI USING DNA AMPLIFIED FROM SINGLE HAIRS
OF RELATIVES OF A BONE MARROW TRANSPLANT CANDIDATE

Individual	DQA type	DPB type	DRB type
Patient	4,4	1,1	3,5 (DRB1:0301/1102)
Parent	4,4	4.1,1	NT ^b
Sibling	4,4	2,1,1	5,5 (DRB1:1101/1102)
Sibling ^a	4,4	1,1	3,5 (DRB1:0301/1102)
Son	3,4	4.1,1	NT
Daughter	3,4	4.1,1	NT
Cousin	1.1,4	2.1,14	NT

^aThis sibling inherited the same chromosomes 6 and was a candidate donor for bone marrow transplantation. Subsequent analysis of the class I loci confirmed their genetic identity at the relevant transplantation loci.

^bNT, Not tested.

horseradish peroxidase. In another approach, the probe is “reversed” or bound to a membrane, where it “captures” a specific allele or sequence variant if it is present in the amplified DNA (S3). This reverse dot-blot format is currently available as a kit for HLA-DQA genotyping and offers the conceptual and practical advantage of simultaneously allowing the detection of multiple alleles (or different pathogens) in a single hybridization reaction (Fig. 2). Probes have also been bound to the wells of microtiter plates (H5). This format (Fig. 3) has certain advantages for automation with equipment already present in clinical labs such as liquid-transfer devices, plate washers, and readers.

Another probe-based method of detection that looks promising for automation is the colorimetric oligonucleotide ligation assay (OLA), shown in Fig. 4 (N2). This format employs two adjacent oligonucleotides, a 5'-biotinylated probe (with its 3' end at the nucleotide to be assayed) and a 3' reporter probe that is labeled with an enzyme. The probes are hybridized to the amplified target DNA, and if there is perfect complementarity, DNA ligase covalently joins the two probes. Conversely, if there is a mismatch at the junction, ligation is prevented. Capture of the biotinylated, ligated probes on immobilized streptavidin and colorimetric detection of the reporter have been automated.

A luminescent probe detection system called the hybridization protection assay, or HPA, makes use of an acridinium ester-derivatized oligonucleotide that is hybridized to the amplified DNA (O2). Unhybridized probe is preferentially

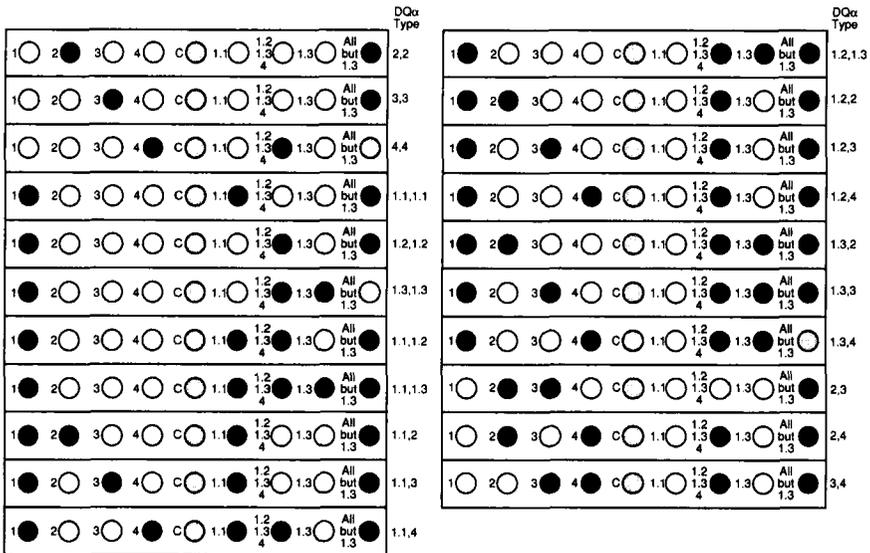


FIG. 2. Reverse dot-blot colorimetric detection format for analysis of HLA-DQA genotypes (S3). Reprinted with the permission of Cetus Corporation.

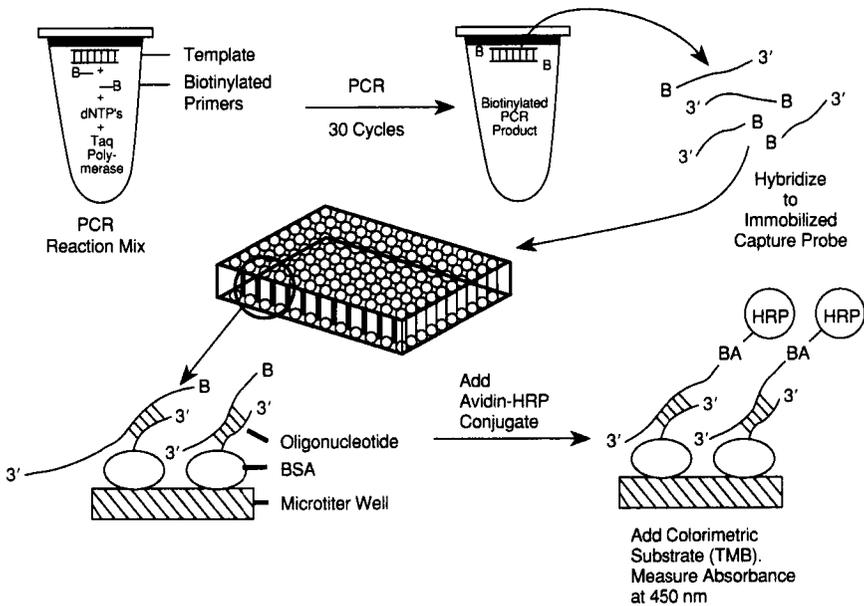


FIG. 3. Microtiter plate colorimetric assay format. Specific probe is bound to the wells of the plate and hybridizes to the amplified, biotinylated DNA target. Unbound primers are removed by washing and the DNA is detected with avidin-horseradish peroxidase and a chromogenic substrate. Reprinted with the permission of Herman *et al.* (H5) and The American Association of Clinical Chemistry.

converted to a form that does not emit light. This format is convenient, rapid, and capable of detecting single nucleotide substitutions, but presently has a limited throughput because each sample must be individually read in a luminometer. An instrument that lends itself to batch processing would enable this detection technology to find wider use in clinical laboratories.

An alternative to probe-based detection systems uses labels on the primers and strives for perfect target specificity in the amplification reaction. This is straightforward if the target gene differs from unintended targets due to the presence of a deletion or gene rearrangement. However, it may require special conditions such as sequence-specific PCR (N1, W15) or "nested" PCR (two sets of primers, one set internal to the first set, in two sequential reactions) (M8) to discriminate between target sequences that differ by only one or a small number of nucleotides. Chehab and Kan (C2) used this approach by derivatizing primers with different fluorescent dyes and scoring the fluorescence relative to the amplified mutant or normal allele in a fluorimeter (Fig. 5). This method has been automated using the Duchenne muscular dystrophy gene deletions as a model. It should prove highly useful in forensic investigations for rapid analysis of amplified targets that differ in length, such as variable-number tandem repeat (VNTR) loci.

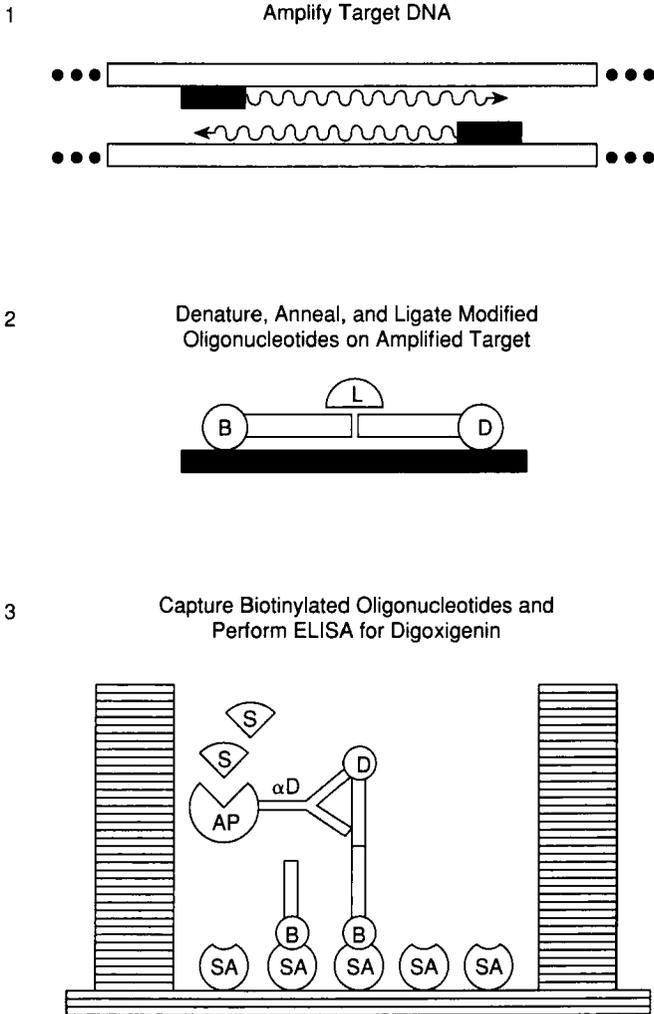


FIG. 4. Schematic diagram of the steps in the automated PCR/OLA procedure performed with a robotic workstation. The assay contains three steps: (1) DNA target amplification; (2) analysis of target nucleotide sequences with biotin (B)-labeled and digoxigenin (D)-labeled oligonucleotide probes and T4 DNA ligase (L); and (3) capture of the biotin-labeled probes on streptavidin (SA)-coated microtiter wells and analysis for covalently linked digoxigenin by using an ELISA procedure with alkaline phosphatase (AP)-conjugated antidigoxigenin (α D) antibodies and a substrate (S). Reprinted with the permission of Nickerson *et al.* (N2) and the *Proc. Natl. Acad. Sci. (U.S.A.)*.

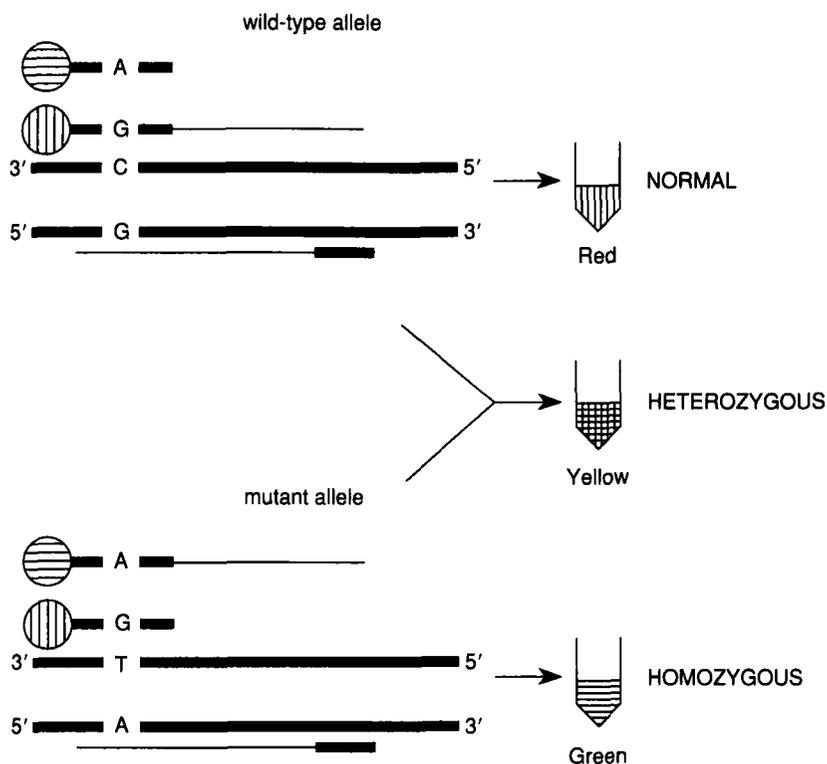


FIG. 5. Strategy for color complementation assay detection of point mutations. A cytidine-to-thymidine mutation is illustrated in the example. Two allele-specific primers corresponding to this region are labeled with red dye (corresponding to the wild-type allele) or green dye (mutant allele). The primer amplifying the opposite strand is unlabeled. After PCR and removal of unincorporated primers, the amplified products for normal, heterozygous, and homozygous DNA are red, yellow, and green, respectively. Reprinted with the permission of Chehab and Kan (C2) and the *Proc. Natl. Acad. Sci. (U.S.A.)*.

Kemp and co-workers (K6) developed a colorimetric detection system that incorporates biotin into one (nested) primer and the sequence for a DNA-binding protein (e.g., the *GCN4* gene from *Saccharomyces cerevisiae*) into the other primer. Amplified DNA is captured on an immobilized affinity reagent and the biotinylated product is detected with avidin-horseradish peroxidase and a chromogenic substrate.

2.5. INACTIVATION OF AMPLIFIED DNA

There are three types of sample contamination that can result in false positives with DNA amplification methods. Two types are familiar to clinical chemists. For

example, sample-to-sample contamination occurs when a positive specimen contaminates a negative one during sample preparation or during the procedure. Another possible source of sample contamination is from nonviable organisms previously grown or prepared in the sample preparation area. Because some PCR tests are optimized in sensitivity to the level of being able to detect 1–10 molecules of template, it is important with such tests to use positive-displacement pipets when aliquoting clinical specimens or when isolating nucleic acids from them. For less sensitive tests, i.e., genetic disease analyses that may start with several hundred thousand molecules of target (e.g., in a 1 μg of total DNA), such precautions are important but less critical. Pipets that have been used on amplified DNA must never be used for isolation or aliquoting of sample DNA.

The second type of false-positive result can occur at the detection stage, e.g., when the liquid-transfer device pipets a strong positive sample followed by a negative sample. Because a typical PCR reaction can produce 10^{11} – 10^{12} molecules of amplified DNA in a 100- μl reaction and a radioactive probe can detect about 10^7 molecules, it is necessary to limit liquid carryover to less than 0.001–0.01 μl (Table 2). Such levels are possible with available automated pipeting devices, but manual pipeting should employ positive-displacement pipets or tips that prevent aerosols from contaminating the device.

TABLE 2
RELATIONSHIP BETWEEN VOLUME OF PCR REACTION
CARRYOVER, NUMBER OF MOLECULES PRESENT, AND
DETECTABILITY BY HYBRIDIZATION TO A RADIOACTIVE
PROBE OF MOLECULES CARRIED OVER^a

Volumes of carryover (μl)	Molecules of carryover	Direct hybridization detection (cpm)
100	10^{12}	11×10^6
10	10^{11}	1.1×10^6
1	10^{10}	1.1×10^5
0.1	10^9	11,000
0.01	10^8	1,100
10^{-3}	10^7	110
10^{-4}	10^6	—
10^{-5}	10^5	—
10^{-6}	10^4	—
10^{-7}	10^3	—
10^{-8}	10^2	—
10^{-9}	10	—
10^{-10}	1	—
10^{-11}	<1	—

^aReprinted with the permission of Cimino *et al.* (C4) and *Nucleic Acids Research*.

A third type of contamination is unique to PCR and other amplification methods, such as the ligase chain reaction. It involves the inadvertent contamination of a new reaction with the aerosolized products of a previous reaction. As shown in Table 2, as little as 10^{-7} μl of a tube of amplified DNA can contain 10^3 molecules of target (C4). Recommended precautions (K13) involve the use of positive-displacement pipets and the physical separation of areas where PCR reactions are analyzed from those where new reactions are setup. In laboratories that use these precautions, contamination is infrequent, and, when it does occur, is usually at the 1- to 100-molecule level. However, in addition to these procedural measures, it would be useful to have chemical or enzymatic methods of selectively inactivating amplified DNA—similar to the sterilization procedures used to inactivate large numbers of cultured viruses or bacteria.

Procedurally, there are two points in the assay where inactivation of amplified DNA can be implemented: during setup of a new reaction or at the end of the reaction prior to the detection step. Exploiting principles of the restriction modification and excision repair systems of cells, two groups independently developed a pre-PCR procedure (Fig. 6) that leads to the specific degradation of polynucleotide products from previous reactions but does not affect nucleic acid templates from the clinical specimen (L3, J. Sninsky, unpublished data). Deoxy-UTP (dUTP) is substituted for dTTP and is incorporated into the amplified DNA. In setting up a new reaction, the reagent mixture in the tube contains the enzyme uracil *N*-glycosylase (UNG), which catalyzes the excision of uracil from single- and double-stranded DNA (but not RNA) prior to initiating the temperature cycling process. The resulting abasic polynucleotides are refractory to amplification. This is due to the stalling of the DNA polymerase and/or strand scission because of the alkaline lability of the aglycosidic linkage at the elevated temperature of the first denaturation step. The high denaturation temperature also inactivates the UNG. Conditions have been identified in which every molecule of greater than 10^6 dUTP-containing templates added to a new reaction can be inactivated. This solution to the carryover of PCR products into reactions about to undergo amplification has several attractive features: (1) both single- and double-stranded DNA contaminants from previous PCR reactions are inactivated, (2) the similarity of the A:U and A:T base pairs in the amplified DNA function in a manner equivalent to hybridization targets, and (3) uracil-containing DNA can be readily cloned and sequenced. Deoxy-UTP incorporation and UNG treatment promise to dramatically reduce false positives in all applications of PCR and thereby lead to its even broader use (J. Sninsky, unpublished data).

Another pre-PCR sterilization process utilizes short-wavelength ultraviolet irradiation of the reaction mixture prior to amplification (S6). Although this procedure can inactivate long DNA fragments, or small numbers of shorter fragments (S7), it is ineffective for more than 10^3 molecules and requires that both the DNA polymerase and the target nucleic acid be absent from the reaction mixture during

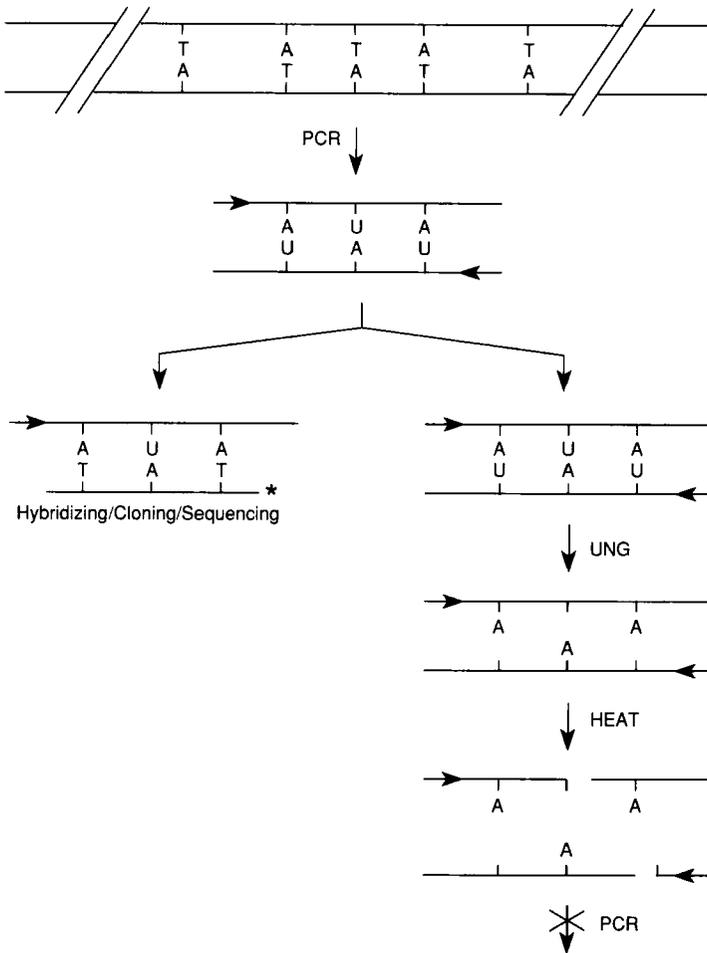


FIG. 6. Schematic representation of the dUTP/uracil-*N*-glycosylase "sterilization" procedure. Reprinted with the permission of Sninsky *et al.* (S11).

irradiation (C3). Because the PCR tube must be opened to add these components following irradiation, contamination can still occur.

Cimino and co-workers (C4, I2) developed a post-PCR photochemical procedure for the inactivation of polynucleotides. The procedure is based upon the blockage of *Taq* DNA polymerase when it encounters a photochemically modified base in a polynucleotide strand. Isopsoralen reagents that are added to a reaction mixture prior to amplification tolerate the thermal cycles, are photoactivated after amplification, and form cyclobutane adducts with pyrimidine bases in the DNA.

If the damaged strand is carried over into a new reaction vessel, it is prevented from functioning as a template for amplification. Effective sterilization requires the use of these reagents at concentrations that are tailored to the length and sequence of the target and the level of amplification (Table 3).

2.6. FUTURE IMPROVEMENTS IN TECHNOLOGY

In addition to predictable improvements in speed, simplicity, and automation of PCR diagnostic tests, there are several improvements in the procedure that will find use in the reference laboratory prior to their incorporation into kits. First, it has been shown that considerable improvement in target specificity and concomitant improvements in specific product yield and test sensitivity can be achieved by adding or "activating" the thermostable DNA polymerase before the first cycle at a temperature at or above the primer-annealing temperature (F1). This activation can be accomplished in several ways: (1) by adding the enzyme after the reaction has reached an elevated temperature and (2) by sequestering the primers by

TABLE 3
EXPECTED NUMBER OF NONSTERILIZED PCR MOLECULES AS A
FUNCTION OF PCR PRODUCT LENGTH^a

Length of PCR product	Average effective adducts/strand	Nonsterilized molecules per 6×10^{11} molecules
Case A (1 adduct per 5 bases)		
100	10	2.7×10^7
150	20	1.2×10^3
200	30	<1
250	40	<<1
300	50	<<1
Case B (1 adduct per 15 bases)		
250	13.3	9.7×10^5
300	16.6	3.4×10^4
350	20	1.2×10^3
400	23.3	4.4
450	26.6	1.5

^aPhotochemical sterilization with isopsoralens is a statistical process characterized by measuring the average number a of adducts per strand of amplified DNA assuming a 50% A:T content and a symmetrical distribution of Ts. If the addition reaction is governed by Poisson statistics, the fraction of molecules with no modifications in a large population of amplified DNA molecules that has an average of a modifications is given by $f_0(0) = e^{-a}$. Reprinted with the permission of Cimino *et al.* (C4) and *Nucleic Acids Research*.

binding them with a single-stranded DNA-binding protein (H9) that is inactivated at high temperature. These approaches avoid any nonspecific priming and synthesis that occur during the reaction setup and during the first rise in temperature to denature the template.

Another improvement for tests when the initial template is RNA rather than DNA is to have a single thermostable enzyme that can function both as a reverse transcriptase and as a DNA polymerase (M12). This would greatly simplify tests for RNA viruses, for bacterial targets that utilize ribosomal RNA targets, and for cancer tests such as for the hybrid mRNA associated with the *BCR-ABL* chromosomal translocation in chronic myeloid leukemia.

Nested PCR reactions can offer significant advantages in sensitivity and specificity yet are prone to carryover contamination of the second reaction that uses the internal primer set. Such contamination cannot be prevented by the sterilization methods described above. Future nested systems may incorporate both the external and internal primers in the same reaction, wherein intentional differences in annealing and denaturation temperatures of the respective primers or target sequences would only permit the intended target to function in primer annealing, denaturation, and synthesis (E6, Y2).

Finally, a major improvement would occur if quantitative procedures (G3, K4, S8, W3) could be simplified and especially if potentially homogeneous methods could be devised (H7). This would greatly extend the utility of diagnostic tests for measuring response to therapy or recurrent disease, and would help address the relationship of pathogen load to clinically significant disease. Like culture, PCR can be optimized to detect single microorganisms and quantitative experiments might offer the opportunity to distinguish septicemia from transient bacteremia (H4) or acute from chronic or latent viral infections. Once the baseline numbers are obtained for a particular microorganism/disease, either (1) the desired level of sensitivity of a qualitative PCR test can be fixed by adjusting parameters such as cycle number or (2) appropriate standards can be run to determine absolute or relative infection levels. Although the potential for detecting nonviable microorganisms following antibiotic therapy is a possible drawback to PCR, a recent study suggests otherwise. Claas and co-workers (C5) found complete agreement between the results of a *Chlamydia* culture technique and PCR in a follow-up study of patients treated with doxycycline. No chlamydial DNA was detected in the patients' samples after treatment. Thus, in our view, the "PCR is too sensitive" critique will become as obsolete as when the same argument was applied to bacterial or viral culture.

A final area for improvement is test turnaround time. Although many current procedures involve 1–2 hours of sample preparation, 2–3 hours of thermocycling, and 1–12 hours for detection, there is nothing sacred about these times. Some whole-blood sample preparation procedures can be done in 1–15 minutes and several nonisotopic detection methods for amplified DNA have been described

that can be completed in 15 minutes. Thermocycling times have to date been limited by the slowness of temperature changes using available instruments, but a recent paper demonstrated that it is possible to reduce each complete cycle of denaturation, annealing, and synthesis to as few as 20 seconds (W12). Because a single molecule of template can be amplified to the point of being visualized by fluorescence on a gel after 42–45 cycles at 85% efficiency, it is possible to consider reducing test thermocycling time to approximately 30 minutes. A specimen-to-result turnaround time of 1 hour is theoretically possible with appropriate instrumentation. It is more likely that times of 2–6 hours will be the practice for several more years.

3. PCR in the Clinical Laboratory: Practical Considerations

Clinical reference laboratories are currently performing PCR test services for infectious diseases, genetic diseases, and oncology. These clinical research formats are being streamlined and developed for FDA approval and subsequent availability as kits to all clinical laboratories. Reference laboratories have become an important element in learning about the reliability of this technology. Though the results of tests in the current research format are not treated as clinically diagnostic, the tests are being performed under the rigors of the clinical laboratory. The complexity of these assays has required the technical and theoretical education of the staff performing them, and the cumulative expertise of these personnel has led to specific recommendations in the areas of quality assurance, sample preparation, amplification, detection, and interpretation of results. The typical protocols currently being used in reference laboratories are reviewed below.

3.1. INTRODUCTION

In this section of the protocol, the clinical and epidemiologic significance of the PCR test is described, with special attention to the contribution of PCR results to a possible diagnosis. The theory and methodology of the specific assay are also described, including the primers and probes that are used, whether the system employs simultaneous amplification of several targets, the species or allelic differences that are detected, and the detection system that is employed. An assay flow chart (Fig. 7) can be helpful in orienting the technicians to the relationships among the steps of a complex procedure.

3.2. QUALITY ASSURANCE OF REAGENTS AND EQUIPMENT

Reference laboratories performing PCR assays in the research format make, test, and cross-over many of their own reagents. Even reagents that are purchased

Sample Preparation

Whole blood
Ficoll-Hypaque to isolate lymphocytes
Digest lymphocytes in detergent/proteinase K

PCR: HLA Gel Check for lysate competence

50 μ l lysate is amplified
Primers: GH26 and GH27
25 cycles of amplification

Detection

3% NuSieve/1% agarose in TBE gel check

PCR: HIV**Sterilization and Amplification**

Note: To ensure consistency of results, amplify each sample in duplicate

25 μ l or 50 μ l (depending on HLA gel-check results) lysate is amplified
Primers: SK431 and SK145
UNG (Uracil N Glycosidase)
Light mineral overlay of reactions

37°C 10-minute pretreatment
95°C 10-minute preincubation
30 cycles of amplification

Detection

Oligomer hybridization with γ -³²P labeled SK102 probe

Run on 10% acrylamide gel
Autoradiograph of gel

FIG. 7. Assay flow chart.

(e.g., oligonucleotides and enzymes) are tested in-house with recommended quality assurance procedures. Future licensed test kits will not require extensive preparation of reagents, as they will be provided by the manufacturer.

Detailed recommendations pertaining to equipment and supplies should also be given in the protocols. Positive-displacement pipets are crucial for successful PCR operations; temperature-cycling instruments must meet certain minimum stan-

dards. Specifications for detection format equipment (e.g., electrophoresis or transfer apparatus, membranes, probe-labeling supplies) are necessary. Compliance with these requirements contributes to the level of standardization among reference laboratories performing PCR assays.

3.3. SAMPLE PREPARATION

Sample preparation methods in PCR diagnostics may be different from those that the clinical laboratory has customarily encountered, including the use of unconventional specimens such as hair, chorionic villi, or synovial fluids. In essence, the target DNA or RNA within a clinical specimen must be released and stabilized. The predicted presence of the target nucleic acid defines the sample to be used. In some cases this will entail isolating a group of cells and lysing them, or pelleting samples and treating the pellets, or digesting tissue. An example of a sample preparation method is the one used for the detection of HIV-1 in clinical samples (K12). For this assay, peripheral blood mononuclear cells are isolated from whole blood by density-gradient centrifugation using Ficoll-Hypaque. These cells are then lysed by proteinase K and detergents that release the proviral DNA for amplification.

3.4. SETUP AND AMPLIFICATION

Specific instructions must be given to combine the sample DNA with the buffers, dNTPs, specific primers, and DNA polymerase used in the amplification reaction. Each of the components of the reaction has previously been carefully optimized to amplify the intended target efficiently and to prevent extraneous amplification of nonspecific targets. The times and temperatures of each step of an amplification cycle are also optimized specifically for each assay. The information gained during optimization experiments should be described in a robustness table that delineates the tolerance ranges for the particular system. Table 4 is an example generated for a viral pathogen/ β -globin coamplification system. In this test, in which the presence of an infectious virus is being examined, the β -globin gene amplification serves as a positive control for sample preparation and for whether the reaction mixture will allow a target to be amplified if it is indeed present. The β -globin gene is present in the cellular material used as the specimen in this assay. A negative viral result without assurance of amplification by a positive β -globin gene result might be a false negative. Controls for successful sample preparation and amplification such as the β -globin and HLA-DQA gene targets are common in infectious disease PCR assays. For genetic and cancer targets, the normal gene, an invariant section of the target gene, or transcribed RNA is a common control (F6).

TABLE 4
ROBUSTNESS OF A TYPICAL AMPLIFICATION SYSTEM

Parameter	Optimum ^a	Tolerance range
[Mg ²⁺]	4 mM	3–8 mM; background increases at 8 mM
[dNTPs]	200 μM each	200–400 μM; viral gene yield decreases below 200 μM
<i>Taq</i> DNA polymerase	2.5 U/reaction	2–5 U/reaction; background increases with 5 U; β-globin yield decreases with 5 U
Pathogen primers	50 pmol each/reaction	25–100 pmol each/reaction
Cellular control gene primer	5 pmol each/reaction	2.5–10 pmol each/reaction
Denaturing temperature	95°C	92–98°C; viral gene yield decreases above 98°C
Annealing temperature	55°C	50–55°C; viral gene decreases at 60°C
[NaCl]	—	0–30 mM; cannot exceed 20 μl 0.9% saline/reaction
PBS	0	Cannot exceed 10 μl PBS/reaction
[EDTA]	—	0–500 μM
SDS	0	0–0.01%; 0–0.03% with 1% Laureth-12

^aParameters optimized by simultaneous β-globin/virus amplification.

The setup and amplification section of a protocol also contains specific recommendations for the prevention of carryover of aerosolized DNA into the new reaction. Dedicated hoods or dead-air boxes are recommended in this step of the procedure. All pipets should be of the positive-displacement type. They should be kept in a dedicated setup hood and should never have previously been used to pipet amplified target. No amplified DNA should ever be brought into this area. During the reaction setup, either dUTP and UNG or isopsoralens may be added.

3.5. DETECTION/DNA HYBRIDIZATION

There are currently several methods for analysis of the amplified target DNA. For HIV-1, liquid hybridization with radioactively labeled probes is used (K12). Tests for HLA genes and sickle cell anemia utilize the reverse dot-blot format with a nylon membrane (S3). Each clinical research format has a well-characterized detection method defining the optimum probe concentration, the hybridization times and temperatures, as well as the concentrations of indicator reagents. Table 5 describes the optima and tolerances of a nonradioactive dot-blot assay that uses biotinylated probes and detection by a chemiluminescent substrate and a streptavidin–HRP conjugate.

TABLE 5
OPTIMA AND TOLERANCES IN OLIGONUCLEOTIDE PROBING

Probe	Optimum	Tolerances
Hybridization	Probe concentration, 0.5 pmol/ml final	$\geq 1-4$ pmol/ml results in higher nonspecific background and occasional nonspecific cross-reactivity to high concentration of target.
	Time, 1-2 hours	≤ 1 hour results in lower sensitivity; at ≥ 2 hours there is no appreciable increase in sensitivity or nonspecific background
	Wash time, 10 minutes, twice 2 \times SSPE/0.1% SDS	SSC can substitute for SSPE
	Wash temperature, 56°C	At 54-56°C there is no loss of specificity or sensitivity; at ≤ 54 °C cross-reactivity may occur; at ≥ 57 °C loss of specific signal and decreased sensitivity occurs
Streptavidin horseradish peroxidase (SAHRP) binding	SAHRP concentration, 40 ng/ml final	30-40 ng/ml; ≥ 40 ng/ml results in higher nonspecific background; ≤ 30 ng/ml results in concomitant loss of signal and assay sensitivity

3.6. INTERPRETATION

The research formats presently used in clinical reference laboratories employ complex interpretation schemes, though they have been streamlined to be as decisive as is practical. Compatible with traditional interpretation, the first decision to be made is whether the test is valid through examination of the control results. PCR tests may include controls to test for sample preparation, amplification, and the detection of the amplified DNA. Additionally, for each patient specimen, an internal amplification control (e.g., for normal and mutant genes) may be included. Table 6 lists one interpretation scheme for an HIV assay wherein one primer pair and probe are used in the test and the decision is based on replicate testing.

When these research assays have been developed into licensed diagnostic kits, many of the reagent preparation steps, quality assurance provisions, and interpretation schemes may be invisible to the user. However, generating FDA-approved diagnostic kits involves concerns in addition to those we have just examined. Developers must consider the range of skills of clients likely to use the kits for diagnostic purposes and design formats such that all users can run the tests with confidence. Procedures for the licensed clinical kits must be as concise as

TABLE 6
INTERPRETATION SCHEME FOR AN HIV TEST PROCEDURE

Duplicates:	A	B	Action taken
	0	0	No repeats (no HIV DNA detected)
	+	+	No repeats (HIV DNA detected)
	0	+	Re-PCR and oligomer hybridization
	+	0	Re-PCR and oligomer hybridization

practical while maintaining reproducibility and accuracy. Kits are run thousands of times before tests are marketed and each step of a test is studied for efficiency and effectiveness.

To monitor each test run, developers must give careful consideration to controls. Manufacturers must assure the quality of specimen preparation reagents, enzyme amplification systems, detection systems, oligonucleotide probes, and DNA controls in the test kit. This means that functional enzymes, DNA, substrates, and other chemicals critically optimized to each other must remain so throughout shipping, storage, and use, and the clinical laboratory must be able to evaluate this stability. PCR assay controls are useful for both the amplification and detection systems, because one or both of these components could be at fault if the test does not work. Controls for testing amplification might include one to test the operation of the thermal cycler, one for DNA polymerase function, and a control to test the amplification competence of each specimen. Traditional controls to verify detection system reagents are employed specifically for each type of system (e.g., a color development control for a colorimetric format). A particular challenge to manufacturers is to develop robust kits and to select the appropriate controls to test each component of the system without overwhelming each run with controls.

In addition to assuring the stability and performance of the reagents within a kit, manufacturers must establish the sensitivity, specificity, reproducibility, and accuracy of the entire procedure. Reproducibility and accuracy must be defined for a test system in which logarithmic signal differences can theoretically occur because of differences in amplification efficiency or initial target numbers. The data gathered by the routine use of PCR in reference laboratories will help manufacturers understand the limitations and attributes of each test when confronted with clinical specimens of variable quality. So far, the optimized research formats are performing as predicted and the sensitivity and reproducibility of the methods should be well established when clinical diagnostic kits are available.

DNA testing is relatively new in diagnostics, and amplification technology is as yet untried in a licensed format. Proficiency panels have been made and used in the reference laboratories for the research formats, and researchers have been constructing and sharing their panels for over 2 years. However, these panels, as

well as their subscribers, vary and they do not approach the rigor present in established clinical proficiency testing. PCR diagnostics will not be fully integrated into the clinical laboratory until there is a way to assure the standardization of the results across all laboratories by independent means. Many of the targets described below are unique and approaches to making and supporting proficiency panels may be complex. It would be judicious to plan for the launch of this technology in the clinical setting by providing laboratories with a means to evaluate their proficiency.

4. Specific Applications

4.1. DIAGNOSIS OF VIRAL INFECTIONS

The first medical applications of the polymerase chain reactions were for the diagnosis of genetic disease (e.g., sickle cell anemia), because the mutations could be directly studied, and for the detection of the virus known to cause AIDS, because of the advantage of speed compared to culture and of greater sensitivity relative to viral antigen tests (K12, S1). For viruses, PCR also offers the advantages of the ability to detect dormant viruses and noncultivable viruses such as human papillomaviruses and some enteroviruses, and the direct detection of the pathogen instead of the host's serological response to an infection. The clinical applications of PCR for diagnosis of viral infections have been recently reviewed (W9) and include detection of neonatal infection, early infection, resolution of indeterminate serologies, viral typing, differentiation of indigenous viruses and vaccine strains, and identification of new agents. Table 7 lists some of the viral diseases that can be diagnosed with PCR.

TABLE 7
REPRESENTATIVE VIRUSES AND ASSOCIATED DISEASES THAT HAVE
BEEN DETECTED WITH PCR^a

Family	Virus	Disease
Herpesviridae	HSV-1, -2	Encephalitis
	CMV	Deafness
Papovaviridae (papillomaviruses)	HPV	Cervical cancer
Flaviviridae	HCV	Hepatitis
	Enteroviruses	Encephalitis
Retroviridae	HIV-1, 2	AIDS
	HTLV-I, II	ATL/TSP

^aReprinted with permission from Williams and Kwok (W9) and Marcel Dekker.

4.2. BACTERIAL INFECTIONS

Because culture is the “gold standard” diagnostic method for most bacterial infections, the greatest potential opportunity for PCR to contribute to clinical medicine is in detecting pathogens that are slow, fastidious, or dangerous to grow, or where other DNA probe methods lack sensitivity. Thus, the first reports addressed pathogens such as mycobacteria, *Chlamydia trachomatis*, *Legionella pneumophila*, and the Lyme disease pathogen *B. burgdorferi* (B5, P5, R4, S11). Recently, many more papers have appeared on mycobacterial PCR tests. These have targeted a diversity of genes—some tests amplify all mycobacteria but detect *Mycobacterium tuberculosis* via a species-specific probe, and some are specific to *M. tuberculosis* at the level of amplification (E2, R3, S10). A similar range of targets has been used for the Lyme pathogen encompassing both universal, plasmid-borne, and randomly cloned chromosomal genes (P3, P4, R5). For this pathogen, much work remains to be done in defining how often the organism can be detected in various clinical specimens at each stage of the disease. Although PCR can detect the organism in synovial fluid, urine, and cerebrospinal fluid from Lyme disease patients with advanced disease, it is not yet clear what the clinical sensitivity would be from a blood or urine specimen in early disease or in patients with ambiguous neurological symptoms and serology.

Table 8 summarizes PCR applications in detecting bacterial pathogens. Most of these studies concern simple detection of the organism rather than diagnosis and await standardized procedures, simplified sample preparation methods, and colorimetric detection formats before they will be practical for clinical laboratories.

Future opportunities include rapid detection and identification of bacterial causes of sepsis and meningitis, with the ability in the latter to differentiate viral and fungal etiologies. Universal primers have been described that are conserved in all eubacteria tested and a similar situation exists for fungi and enteroviruses (B3, R6, W8). A complication test that combines primers for the three types of agents could prove medically useful in selecting therapy if the result can be obtained in 2 hours.

Another opportunity for rapid PCR diagnosis involves antibiotic susceptibility testing (C7). Ounissi and co-workers (O3) have demonstrated concordance between the presence of various genes for antibiotic-modifying enzymes and bacterial sensitivity *in vitro*. By judicious selection of conserved or enzyme-specific gene sequences as primer sites, it is possible to detect, for example, all β -lactamases or only those associated with penicillinase-producing *Neisseria gonorrhoea* (M1, S5). Such tests would have to be used on specimens from fluids that are usually sterile (blood or CSF). Preferably they should be directed toward those pathogens for which the spectrum of resistance mechanisms is restricted and the importance of predicting drug resistance is great, e.g., methicillin-resistant *Staphylococcus aureus* infections (H1, M10), or toward the DNA gyrase A mutation that confers resistance to quinolones.

TABLE 8
 DEMONSTRATED AND POTENTIAL CLINICAL UTILITY OF BACTERIAL DIAGNOSIS BY PCR

Pathogen	Associated disease	Clinical utility	References
<i>Mycobacterium tuberculosis</i>	Tuberculosis	More rapid than culture, possibly more sensitive? less hazardous in lab	B5, H2, S10
<i>Mycobacterium leprae</i>	Leprosy	More rapid than culture	H3
<i>Mycobacterium avium</i>	Atypical mycobacterial infections, AIDS	More convenient specimens? definitive species identification	F5
<i>Chlamydia trachomatis</i>	Venereal disease	More rapid than culture, improved sensitivity	B2, C5, D1, D3, F5, G4, O1, W6, W13
<i>Borrelia burgdorferi</i>	Lyme disease	Easier, faster than culture, higher specificity than serology	M3, P3, P4, R4, R5
<i>Mycoplasma pneumoniae</i>	Atypical pneumonia	More sensitive than culture	B2
<i>Mycoplasma genitalium</i>	Urethritis	Study role in disease	J1
Enterotoxigenic <i>Escherichia coli</i> , <i>vibrio</i> and <i>Shigella</i>	Acute diarrhea	Detect multiple pathogens	F4, K8, O1, V1
<i>Salmonella typhi</i>	Typhoid fever	Monitor carriers	F3
<i>Bordetella pertussis</i>	Whooping cough	Diagnosis	H8
<i>Escherichia coli</i> Shiga-toxin		More rapid than conventional methods	K1
<i>Legionella pneumophila</i>	Pneumonia	More rapid than culture	S11
<i>Rickettsia rickettsii</i>	Rocky Mountain spotted fever	Early detection	C1, T2, W10
<i>Rickettsia conorii</i>	Boutonneuse fever	Choice of therapy	T2
<i>Rickettsia tsutsugamushi</i>	Scrub typhus	Rapid, differential diagnosis	K5
<i>Rickettsia typhi</i>	Murine typhus	Prevalence; vector control	W5
<i>Clostridium difficile</i>	Diarrhea	Prophylactic therapy? distinguish toxigenic strains	K2, W14
<i>Treponema pallidum</i>	Syphilis	Improved sensitivity	B3

With further simplifications of the technology it may be feasible to consider patient-specific microorganism diagnoses, in the sense that the immunoglobulin V-D-J rearrangement that is unique to a B cell leukemia patient can be characterized with respect to its sequence (Y1). This information could be used to monitor response to therapy and to detect residual or recurrent disease. Using similar molecular epidemiological information might someday prove useful in selecting therapy or predicting responsiveness, particularly for those pathogens for which different phylogenetic lines are associated with distinctive clinical symptoms (M11).

4.3. FUNGAL AND PARASITIC INFECTIONS

The advantages of speed, simplicity, and laboratory safety that PCR offers compared to culture of some bacterial pathogens extend to the diagnosis of many fungal and parasitic infections as well (D2). The rapid and definitive diagnosis of congenital *Toxoplasma gondii* infection from amniotic fluid samples may reduce unnecessary abortions and promote earlier treatment of infected fetuses (B7). An important use is to distinguish pathogenic and nonpathogenic isolates of parasites such as amoeba (M4, T1). Rapid detection and differentiation of the mucocutaneous and sylvatic forms of *Leishmania brasiliensis* permit rapid treatment of the former prior to extensive tissue destruction (L4). Lopez *et al.* (L4) also demonstrated that PCR can be performed in the field and can provide highly useful medical information on diseases common in less developed countries.

PCR is just beginning to have an impact on identification and diagnosis of fungal pathogens such as *Cryptococcus neoformans* (V2) and *Pneumocystis carinii* (W1, W2). Universal fungal primers (M5, W8) and pathogen-specific probes (B4) promise to allow rapid and sensitive diagnosis of fungal sepsis and pulmonary disease. Table 9 summarizes the published applications for diagnosis of fungal and parasitic infections.

5. Conclusion

Several predictions can be made regarding the impact of PCR on the clinical laboratory. First, the diagnostic repertoire of the clinical laboratory will expand, primarily because the applications of PCR in human genetics, cancer, and infectious disease are increasing rapidly. In the clinical microbiology laboratory, it will facilitate the detection of pathogens whose identification has previously been limited by the lack of a practical culture system. This will add to the list of organisms that can be detected, increasing both the services and responsibility of the clinical laboratory. In addition, our list of previously unrecognized or unidentified pathogens will grow (R2). Second, the technological nature of these methods will create a demand for laboratory professionals with training in this technology. Currently, very few pathology residency programs, fellowship training, or medical technology programs offer formal instruction in molecular techniques. Given the potential for widespread applications of these methods in the clinical laboratory, consideration must now be given to the adequate training of these future laboratory directors and staff, knowing that those entering programs now will be directly confronted with this technology when they complete their training. Continuing-education programs will have to be developed that are tailored to the needs of this group of professionals to provide them with an understanding of both the power and limitations of these methods. Third, there will be a need to carefully evaluate the data derived from these new methods in light of

TABLE 9
 DEMONSTRATED AND POTENTIAL CLINICAL UTILITY OF FUNGAL AND PARASITIC DIAGNOSIS BY PCR

Pathogen	Associated disease	Clinical utility	References
1. <i>Cryptococcus neoformans</i>	Meningitis	Faster than culture, more sensitive than antigen test	V2, M5
2. <i>Histoplasma capsulatum</i> <i>Blastomyces dermatitidis</i>	Pulmonary disease	Faster, safer than culture Faster, safer than culture	B4
3. <i>Coccidioides immitis</i>			B4
4. <i>Pneumocystis carinii</i>	AIDS	More sensitive and specific	
5. <i>Toxoplasma gondii</i>	Toxoplasmosis	Fetal infection; more sensitive than fetal IgM, faster than tissue culture	B7, G5
6. <i>Trypanosoma brucei</i> <i>Trypanosoma congolense</i>	Sleeping sickness	Rapid and sensitive diagnosis	M6
7. <i>Trypanosoma cruzi</i>	Chagas disease	Sensitivity; replaces xenodiagnosis	E1, M7
8. <i>Plasmodium falciparum</i>	Malaria	Higher sensitivity; detection of pyrimethamine resistance	W4, Z1
9. <i>Leishmania braziliensis</i>	Leishmaniasis	Differentiation of cutaneous and sylvatic types	L4
10. <i>Naegleria fowleri</i>	Amebic meningoencephalitis	Early diagnosis from CSF?	M4
11. <i>Entamoeba histolytica</i>	Hemorrhagic colitis	Differentiation of pathogenic strains	T1

the clinical picture, especially if the performance of PCR-based tests significantly exceeds the "gold standards" currently in place. One can envision that PCR could indeed prove more sensitive than standard methods for the diagnosis of infectious disease, because it is able to detect nonviable organisms. Cooperation of clinicians, researchers, developers, and laboratory professionals will be required to integrate PCR results with the clinical presentation, patient history, supporting laboratory data, and treatment records. Only then will the true clinical significance of PCR results be known. Finally, the introduction of molecular diagnostic techniques will create a concurrent demand for proficiency testing and laboratory site visits administered through independent agencies such as the College of American Pathologists (CAP). Though many tests may be offered on an experimental basis, it would be prudent to begin developing CAP proficiency panels in order to achieve rigorous laboratory standards for both experimental and FDA-approved tests.

Nucleic acid amplification techniques will undoubtedly have a substantial future impact on the practice of laboratory medicine. Ultimately, the spread and acceptance of these techniques will be limited by cost and other considerations.

Although amplification methods are now the standard for most genetic disease tests and are becoming important for many infectious agents, conventional culture for most bacterial pathogens is often rapid, inexpensive, as sensitive, and allows detection of multiple organisms from a single procedure. Culture also allows determination of phenotypic characteristics such as antibiotic resistance, virulence factors, and strain differences, which may be difficult or impossible to determine by amplification alone. For infectious diseases, modern serological techniques, especially latex agglutination and solid-phase antibody methods, are also rapid and less labor intensive. The decision to use an amplification method is thus likely to be dictated by the sensitivity and specificity of the PCR procedure versus the low-cost, time-proven conventional method, factoring in the turnaround time and the clinical need for definitive results. With the evolution of this technology, however, will come increased sophistication and automation of many of the steps involved in PCR technology, resulting in lower per-test costs. This will likely result in increased use and may cause us to consider applying it to new areas. Inevitably, the acceptance and competent application of this technology will lead to great improvements in our diagnostic capabilities and to better clinical understanding.

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ONCOGENES IN CANCER

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1. Carcinogenesis

1.1. NEOPLASIA

Neoplasia is cellular proliferation that exceeds and is uncoordinated with normal growth and persists at the expense of the host. It is a stable, irreversible phenotypic change in the cells and is classified into two subgroups: benign and malignant neoplasias. Benign neoplasms do not invade or metastasize, but grow slowly and are well differentiated and frequently encapsulated. Malignant neoplasia is synonymous with the term cancer. Malignant neoplasms grow by invasion of surrounding tissues, including blood vessels, lymphatics, and nerves. They metastasize to distant sites by seeding body cavities or by mechanical transportation or via blood vessels and lymphatics. Over time, neoplasms tend to become increasingly autonomous.

1.2. CHANGES TYPIFYING CANCER CELLS

The following characteristics typify cancer cells: uncontrolled and unlimited proliferation at the expense of the host; transmissibility of proliferative abnormality to successive generations (phenotypic change); and, increasing malignancy exhibited by changes in morphology, karyotype, antigen specificity, and metabolism.

1.3. THEORIES OF CARCINOGENESIS

Several theories have been previously postulated to explain carcinogenesis. No single theory can account for the observed characteristics of neoplasia. Through great progress in biological sciences, particularly in molecular biology, all of the previous theories have been replaced with newer concepts and postulates. The previously proposed mechanisms are now only of academic interest.

1.4. MAJOR MECHANISMS FOR CANCER

Currently proposed mechanisms for neoplasia include gene mutation, epigenetic changes, viral transformation, and cell selection; these will be discussed in the following sections.

1.4.1. *Gene Mutation*

Neoplasia might be attributed to abnormalities in one or more of the genes regulating growth and differentiation. The affected individual is therefore more susceptible to cancer by action of carcinogenic agents such as alkylating chemicals or ionizing radiation. The presence of chromosomal abnormalities (H1, H5) in cancer cells supports this mechanism, as in the case of D-deletion syndrome in a high proportion of retinoblastoma; individuals with Down's syndrome (trisomy 21) and Klinefelter's syndrome (XXX) are susceptible to leukemia (K2, K3). Additional supporting evidence includes the presence of the Philadelphia chromosome and the 8;14 chromosomal translocation in chronic myelogenous leukemia (CML) and Burkitt's lymphoma (BL), respectively (B1, Z1).

1.4.2. *Epigenetic Changes*

Neoplasia can arise from a change in the regulation of genes without a change in their structure. Genes responsible for normal differentiation and cell regulation (W1) are intact in some tumor cells, in which they are capable of being expressed if given the appropriate stimulus. The neoplastic phenotype reflects derangement in gene regulation and not a mutation evident as an alteration. Persuasive evidence for this mechanism is the reversibility of cells transformed *in vitro* by cAMP, testosterone, dimethylsulfoxide, and bromodeoxyuridine (F1).

These observations do not suggest that phenotypic reversion is possible in all cancer cells, but it may indicate that many properties of cancer cells are capable of modulation under appropriate conditions. This can be interpreted as indicating that the lesion in cancer cells is at the epigenetic level and not the genetic level, in the form of mutation. The best *in vivo* evidence for cancer regression exists when malignant neuroblastomas evolve into benign ganglioneuromas, presumably through differentiation of neuroblastoma cells into ganglion cells (E1).

1.4.3. *Viral Transformation*

The historical evolution of viral oncology can be divided into four major phases. The first period, from 1910 to 1935, was dominated by studies on avian tumor viruses and efforts to establish etiologic relationship in terms of Koch's postulates. These efforts were often frustrated by expectation now known not to be strictly applicable to oncogenic viruses, namely, that the virus should cause rapid onset of the disease and remain present at the height of the disease. At that time, however, the failure to fulfill Koch's postulates consistently, especially in mammalian tumors, prevented the viral hypothesis from gaining wide credence. The second period spanned the years 1935 to 1945 and saw the discovery of a growing number of mammalian tumor viruses and the important realization that such viruses might not act alone. It was realized that they may in fact require the interaction of specific genetic, physiologic, or environmental cofactors. It was also

recognized during this period that the induced neoplasms might develop after a long latency. During the third era, in the early 1960s, it became apparent that tumorigenesis by DNA viruses was accompanied by integration of viral genes into the genomes of the host cells.

The fourth phase of viral oncology has seen the emergence of the revolutionary concept that RNA tumor viruses (oncornaviruses), like their DNA counterparts, contribute genetic information that becomes part of the genome of the affected host cell. With the latest development in viral oncology, tumor viruses have come to be considered more endogenous than exogenous to the hosts.

With the exception of wart viruses, viral agents have yet to be implicated conclusively in the pathogenesis of neoplastic lesions in humans. Indirect evidence implicating viruses in human neoplasias is mounting, for example, the frequent occurrence of characteristic virus particles in the cells of certain malignancies; the association of group-specific (possibly viral or virus-mediated) antigens with the cells of some neoplasms; the presence of reverse transcriptase of the oncornavirus-type in certain cancer cells; and the presence in certain cancer cell nuclei of DNA base sequences complementary to the base sequences of known or suspected tumor viruses (D2, T2). These and other findings, which are analogous to those associated with virus-induced neoplasms in animals, strongly suggest the involvement of viruses as cofactors in the etiology of certain human cancers (D2).

A virus exerts its oncogenic effect (T3) through integration of genetic information encoded in its nucleic acid into the genome of the infected host cell. In the case of DNA viruses, the integration and subsequent transcription of viral nucleic acid may be analogous to processes that have been best characterized in lysogenic bacteriophages (T5). On the other hand, in the case of RNA viruses, the process of integration is thought to involve a DNA intermediate, synthesized from viral RNA through the action of a virus-specified, RNA-directed DNA polymerase or reverse transcriptase (B1, T2).

1.4.4. *Cell Selection Theory*

In certain situations, stimuli that increase the probability of cancer are thought to do so by favoring proliferation of transformed cells that might not otherwise express their neoplastic proclivities. Although this mechanism is principally invoked to account for the effects of agents that are not carcinogenic by themselves, but which enhance the efficacy of carcinogens, it has been proposed as one of the modes of carcinogenic action (F1, F3). The rationale is partially derived from evidence that under some conditions carcinogenesis can be demonstrated to be a multistage process. Early stages may be reversed or arrested in the absence of further tumor-inciting stimuli. The stimulus required to promote further evolution frequently involves cytotoxic effects that tend to select for cells that have already passed through the initial stages of neoplastic transformation. In this context, the progression of the tumor toward malignancy is viewed as the sequential appear-

ance and selective outgrowth of progressively more autonomous subpopulations of cells evolving through stepwise mutation-like changes and proliferating under the influence of sustained selection pressure (F1, F3).

Another situation favoring selection of dormant tumor cells occurs in immunodeficiency states. Spontaneous or induced immunodeficiency often increases susceptibility to neoplasia (K1, M3). However, the results show more variability than can be explained simply by impairment of immunologic surveillance (M3). In humans the excess is largely due to an increase in the frequency of leukemias and lymphomas, except in those immunodeficiency states associated with chromosomal fragility, such as ataxia telangiectasia (P2, S10).

In an attempt to explain the long latency of neoplasia in terms of stepwise evolution and expression of the neoplastic phenotype, other possible explanations must not be overlooked. One of these is the puzzling phenomenon of tumor cell dormancy. The long interval occasionally intervening between the treatment of a neoplasm and its subsequent recurrence (this may amount to more than a decade) far exceeds the time that would be required merely for the regrowth of the tumor from residual tumor cells. Clearly the factor within the internal environment that accounts for such behavior of tumor cells cannot be adequately characterized at present. The many factors presumably involved include those concerned with the regulation of normal growth and differentiation, such as chalones, morphogenic inducers, and other growth-regulating substances (F2, L5).

1.4.4.1. *Classes of Agents.* The process of carcinogenesis can be divided into at least two steps: initiation and promotion (B6). The changes produced by initiating agents are essentially permanent and irreversible, whereas those produced by promoting agents are transitory and elicit neoplasia only if preceded by appropriate initiating effects (B8). Although the distinction between initiation and promotion is based on the operational differences, many compounds that appear capable of merely initiating carcinogenesis at low doses may behave as complete carcinogens at higher doses or in animals of appropriate susceptibility. Similarly, chemicals that behave as promoting agents under some experimental conditions, failing to cause neoplasia unless preceded by an initiating agent, may under other conditions elicit neoplasia by themselves, possibly because the process of carcinogenesis has been initiated spontaneously.

The nature and mechanisms of the changes responsible for initiation and promotion in carcinogenesis have yet to be established. However, initiation is generally postulated to involve some permanent mutation, such as alteration in DNA, whereas promotion is suspected to involve reversible alterations in epigenetic regulation (B5, V2, W2). This is possibly mediated through the cell membrane. Mutagens as a class tend to behave as initiators whereas hormones and other growth-stimulating factors behave as promoters.

1.4.4.2. *Metabolic Activation and Inactivation.* Most environmental carcinogens are not directly active in their ambient form. They require metabolic activa-

tion in the body in order to become carcinogenic. Derivatives of a parent compound are converted into more active "proximate" carcinogens and finally into the "ultimate" form or forms that react with cellular macromolecular targets to initiate carcinogenesis (M4). Based on present evidence, the ultimate carcinogens as a class are strongly electrophilic (M4, M5). The enzymatic machinery involved in the metabolic activation process resides in multicomponent microsomal mixed-function oxidase systems, notably the cytochrome *P*-450 monooxygenase. Concentrations of carcinogen derivatives formed during metabolism vary markedly under the influence of environmental and genetic factors (A5, K5). This helps to explain age-, species-, and strain-dependent differences in susceptibility to the carcinogenic effects of a given chemical.

2. Retroviruses and Oncogenes

2.1. TUMOR VIRUSES

A virus is little more than a packet of genetic information encased in a protein envelope. The information is encoded by DNA or RNA; in higher organism genetic information is encoded only by DNA. DNA or RNA consists of long strands of four types of nucleotides. The sequence of nucleotides constitutes a coded message comprising the genes. The sequence of nucleotides specifies the order in which amino acids are assembled to form a particular protein. Viruses may have fewer than six genes, whereas the genetic complement (genome) of cells consists of tens of thousands of genes. The reproduction of viruses mimics the process by which cells grow and divide, but the simplicity of viruses makes them much easier to study and understand.

In cells, DNA is transcribed into mRNA, which is translated into proteins. An infecting virus insinuates its genetic information into cellular DNA, so that the cell synthesizes viral proteins as specified by viral genes. The proteins synthesize many copies of the viral genome and execute any other instructions encoded by the viral genes. In some instances the instructions include a command that converts the host cell to a cancerous state.

2.2. TRANSFORMATION

Many tumor viruses have a particularly valuable property. They elicit cancerous changes in cells in an artificial culture medium. This transformation makes it possible to examine thoroughly interactions under controlled conditions, avoiding difficulties associated with experiments on animals, though some viruses are powerful oncogenic agents in animals and do not transform culture cells. The process of transformation includes important morphologic and biochemical

changes. However, the crucial test of malignancy is the ability of cells to grow into tumors *in vivo*. The changes shown by cells that undergo transformation are of two types, alterations of morphology and biochemical changes. Alterations of morphology can result in (1) transformed cells that often have a much more rounded shape than control cells; (2) increased cell density (loss of contact inhibition of growth), i.e., transformed cells often form multilayers, whereas control cells usually form a monolayer; (3) loss of anchorage dependence, i.e., transformed cells can grow without attachment to the surface of the culture dish and will often grow in agar; and (4) loss of contact inhibition of movement, i.e., transformed cells grow over one another, whereas normal cells stop moving when they come into contact with each other.

Biochemical changes can result in (1) an increased rate of glycolysis and alterations of the cell surface (e.g., changes in the composition of glycoproteins or glycosphingolipids and secretion of certain proteases); (2) alterations of cytoskeletal structures such as actin filaments; and (3) diminished requirements for growth factors and often increased secretion of certain growth factors into the surrounding medium.

2.3. RETROVIRUSES

Retroviruses are the only tumor viruses with an RNA genome. Retroviruses derive their name from a feature of their life cycle that makes them unique in biology: their RNA must be transcribed backward into DNA in order to propagate. Retroviruses provide us with the most coherent view of oncogenesis. The following features of retroviruses account for their utility in the study of tumor development:

1. They have been found in a large number of vertebrate species and induce many types of tumors.

2. It is relatively easy to identify and isolate retrovirus oncogenes and their products.

3. Retrovirus oncogenes do not appear to be an endogenous component of the viral genome. They apparently arise from cellular genes (involved in the production of tumors induced by agents other than viruses) of the host in which the virus replicates.

In 1970, the enzyme reverse transcriptase (S17) was discovered in the particles of viruses such as Rous sarcoma virus. During the early hours of infection, the viral RNA genome is transcribed into DNA by reverse transcriptase. The viral DNA is then integrated into the cell's genome.

The retrovirus genome has three regions, *gag*, *pol*, and *env*, that encode proteins, and all of these proteins are found in the virion. The *gag* region encodes a polyprotein that is cleaved to form internal virion structural proteins. The *pol*

region encodes the reverse transcriptase and the *env* region encodes a glycoprotein that covers the virion surface. None of these proteins has a mitogenic effect on cultured fibroblasts.

2.3.1. *Oncogenes of Rous Sarcoma Virus*

The genome of wild-type Rous sarcoma virus (S1) was fragmented by ribonuclease and compared with a deletion mutant of the same virus, to determine which fragment was missing in the latter. It was possible to identify an oncogene, *src*, near one end of the virus genome (Fig. 1). Analysis of the DNA of Rous sarcoma virus has revealed this gene, which is capable of transforming cells. This gene encodes a single protein designated pp 60 v-*src* (L4); the designation signifies that it is a phosphoprotein (pp) with a molecular weight of 60,000; and v-*src* indicates its viral genetic origin.

2.3.2. *Oncogenes of Other Retroviruses*

In addition to the oncogenes of Rous sarcoma virus, dozens of oncogenes of other retroviruses have been recognized. Products of these viral oncogenes are protein kinases, mostly of the tyrosine type. Some viral oncogenes and their products are listed in Table 1.

2.4. THE ORIGINS OF ONCOGENES

It has been suggested that retrovirus oncogenes (S1) are a part of the genetic baggage of all cells, perhaps acquired through viral infection early in evolution. It has been postulated that the oncogenes remain innocuous as long as they remain quiescent. When they are stimulated into activity by a carcinogen, they may convert cells to cancerous growth.

To substantiate this hypothesis a search was conducted to find retroviral oncogenes, such as v-*src*, among the thousands of genes found in the DNA of vertebrates. This was accomplished by a molecular hybridization technique using radioactive DNA copied solely from *src* by reverse transcriptase. DNA related to *src* was found in mammals. On closer inspection the gene discovered in vertebrates proved not to be a retroviral gene at all; it is a cellular gene. The most compelling evidence came from the finding that the protein-encoding information of c-*src* is divided into several separate domains (exons) by intervening regions (introns) and this kind of configuration is typical of vertebrate genes and not of retrovirus genes.

2.5. CELLULAR ONCOGENES

Most of the identified retrovirus oncogenes have close relatives in the normal genomes of vertebrates. Most virologists have accepted the idea that retrovirus

**RETROVIRUS
GENOME
WITHOUT
ONCOGENE**



**GENOME
OF ROUS
SARCOMA**

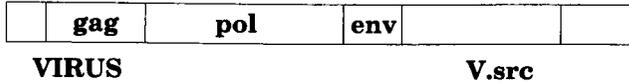


FIG. 1. Rous sarcoma virus deletion mutant genome (top) and wild-type genome (bottom). The *src* oncogene is capable of transforming host cells.

oncogenes are copies of cellular genes. It appears that oncogenes were added to the preexisting retrovirus genome sometime in the not too distant past. How and why this happened is not yet known, but there are reasons to think that copying continues today. The vertebrate genes from which retrovirus oncogenes arose were first called protooncogenes to emphasize their evolutionary significance and to avoid implying that normal cellular genes have oncogenic potential. If viral oncogenes are merely copies, how one can account for their devastating effect on infected cells? The answer to this will be discussed in Section 3.

The study of viruses has brought to light powerful tools for the study of human diseases. Tumor virology has survived its failure to find abundant viral agents of human cancer. The issue now is not whether viruses cause human tumors, as they may, but rather how much can be learned from tumor virology about mechanisms of carcinogenesis.

Cellular oncogenes (V1) can be activated by amplification, by single point mutations, or by transposition. Slowly transforming retroviruses do not contain viral oncogenes but stimulate expression of cellular oncogenes by insertional mutagenicity. Radiation and chemical carcinogens also activate cellular onco-

TABLE 1
SOME ONCOGENES OF RETROVIRUSES

Oncogene	Retrovirus	Product ^a
<i>abl</i>	Abelson murine leukemia virus	Protein tyrosine kinase
<i>erb-B</i>	Avian erythroblastosis virus	Truncated EGF
<i>fes</i>	Feline sarcoma virus	Protein Tyrosine kinase
<i>myc</i>	Myelocytoma virus 29	DNA-binding protein
<i>sis</i>	Simian sarcoma virus	Truncated PDGF (B chain)
<i>src</i>	Rous sarcoma virus	Protein tyrosine kinase
<i>ras</i>	Murine sarcoma virus	Protein with GTPase activity

^aAbbreviations: EGF, epidermal growth factor; PDGE, platelet-derived growth factor.

genes. Expression of multiple oncogenes may be involved. Two groups of oncogenes have been distinguished: one is responsible for establishment (immortalization) and is found in the nucleus; the other is responsible for tumor growth (transformation) and is found in the cell membrane.

3. Oncogene Characterization and Mode of Action

3.1. ONCOGENES

Oncogenes are genes that cause cancer; their evolutionary history implies that abnormal expression of genes of normal cells leads to cancerous growth. The discovery of oncogenes had a major impact on the understanding of fundamental mechanism involved in carcinogenesis. Oncogenes are designated using a three-letter italic abbreviation, e.g., *ras*. The normal cellular gene or protooncogene is given the prefix *c* (*c-src*). Protooncogenes that have been identified so far appear to be genes that are basic to animal life and they have been tightly conserved over eons of evolutionary time. Many are evident in the DNA of arthropods. Protooncogenes have the apparently accidental property of being easily modified into oncogenes. The cellular oncogenes are either identical to normal genes (protooncogenes) or show very small structural differences from their normal counterparts. In the former case, the regulation of their expression may be abnormal in cancer cells.

3.1.1. *Oncogene Proteins*

A number of oncogene products are known: The product of the *erb-B* oncogene is a truncated form of the receptor for epidermal growth factor; the product of the *sis* oncogene is a truncated B chain of platelet-derived growth factor; the product of oncogene *fms* is a macrophage colony-stimulating factor; the product of the *myc* oncogene is a DNA-binding protein that might affect the control of mitosis; the product of the *ras* oncogene has GTPase activity and appears to be related to the proteins regulating the plasma membrane enzyme adenylate cyclase.

Half of the products of oncogenes are protein kinases, mostly of the tyrosine type. This finding was of fundamental importance because it revealed that abnormal phosphorylation of a number of proteins could explain, at least in part, various effects of transformation. The critical cell proteins whose abnormal phosphorylation presumably leads to transformation are still to be defined. However, they include vinculin, which is a protein found in focal adhesion plaques (structures involved in intercellular adhesions). The abnormal phosphorylation of vinculin in focal adhesion plaques could explain the tendency of cells to become round and

their diminished adhesion to substrata and to one another, as has been observed in transformation.

Certain glycolytic enzymes appear to be target proteins for protein-tyrosine kinases. They may be responsible for the increased rate of glycolysis in transformed cells. The protein component of ion pumps may also be involved: activation of Na^+/H^+ antiport systems causing mild alkalization of the cells may play a role in stimulating mitosis.

3.1.2. *Protein Tyrosine Kinase*

A recent finding in a number of laboratories is that phosphotyrosine (S1) is the produce of oncogene protein kinase; this has led to the postulation that phosphorylation of tyrosine might be essential for cell transformation. The amount of phosphotyrosine in most cells is low. It is usually elevated in cells transformed by oncogenes, although the amount is still relatively small, amounting to 1% of the total phosphoamino acids (mainly phosphoserine and phosphothreonine). Certain receptors found in both normal and transformed cells have protein tyrosine kinase activities that are stimulated by interaction with their ligands.

3.2. MECHANISM OF ACTION OF ONCOGENES

There are two working hypotheses to explain how activated cellular oncogenes may be involved in cancer (Fig. 2): (1) the cellular oncogenes may be either amplified and/or overexpressed or expressed at a wrong time and (2) the product of oncogenes may imitate an occupied receptor for a growth factor; (3) the product of oncogenes may act on key intracellular pathways involved in growth control, eliminating the need for an exogenous stimuli.

3.3. MODE OF ACTIVATION OF ONCOGENES

Activation (S8) of oncogenes can result from mutations in the global sense, i.e., point mutations, nucleotide deletions or insertions, and chromosomal translocations. These mutations induce quantitative or qualitative changes in oncogene expression. Several human oncogenes identified in tumors and established cell lines have been cloned and studied in great detail using gene transfer techniques. Evidence has accumulated supporting the view that a single oncogene can be involved at different states or steps in a multistage carcinogenesis process. Moreover, a single properly activated oncogene can trigger the whole process of malignant conversion of a normal cell. thus both the "one gene, one cancer" and "multigene, one cancer" hypotheses may be valid.

Among the modes of *myc* activation are two modes (A3) involving rearrangement close to the gene: retroviral insertion in the murine T and avian B lymphomas

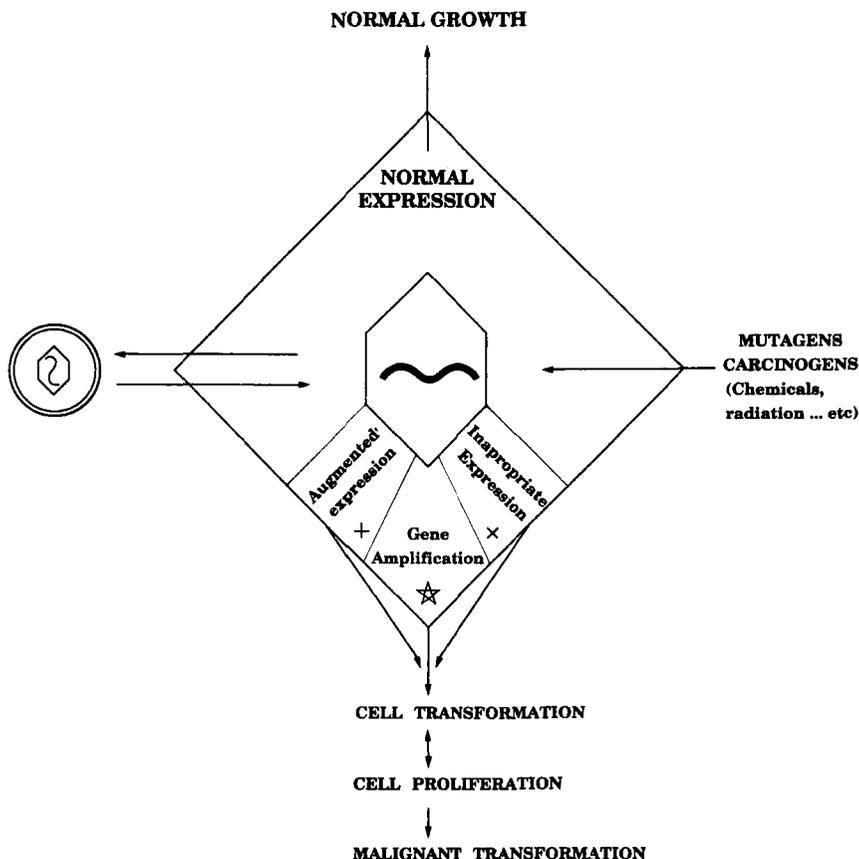


FIG. 2. How cellular oncogenes may be involved in carcinogenesis.

and translocation of nucleotide sequences classes I (within the gene) and II (just 5' to the gene) in the mammalian B lymphoid tumors.

3.4. AMPLIFICATION OF ONCOGENES

The role of amplification (S2) of protooncogenes as a late event during the evolution of cancer cells was studied. Protooncogenes are amplified consistently in animal and human tumors containing double minutes (DMs), c-bandless chromosomes, or homogeneously staining chromosomal regions (HSRs) which are diagnostic of amplified DNA. Amplified protooncogenes identified so far include *c-myc*, *c-abl*, *c-Ha-ras*, *c-Ki-ras*, *N-myc*, *L-myc*, *c-erb-B*, *c-myb*, and *N-ras*. The *myc* genes account for over 90% of the amplification detected so far. Of the *myc*

box genes, the complete structure is known for *N-myc* but not for *L-myc*. Amplification of *N-myc* has been found in neuroblastoma cell lines and in tumors derived directly from patients not subjected to chemotherapy or radiotherapy. DMs and HSRs are the sites of amplified *N-myc*. Amplification of *N-myc* also has been found in cell lines derived from small cell lung cancers, from a few retinoblastoma tumors and cell lines, and from the astrocyte tumor.

3.5. CLASSES OF GENES INVOLVED IN CARCINOGENESIS

The genes involved in carcinogenesis are grouped into various classes: (1) viral genes essential for replication, which also contribute to cell transformation; (2) viral genes superfluous for replication, derived from host cellular genes; (3) cellular genes identified by homology to retrovirus oncogenes; (4) cellular genes at sites of viral integration; (5) cellular genes at sites of chromosome translocation; (6) cellular genes identified by neoplastic transformation and DNA transfer; (7) cellular genes activated or repressed in virus-transformed and other neoplastic cells, and (8) cellular genes whose products interact with those of viral oncogenes.

3.6. SUPPRESSOR GENES

Oncosuppressor genes are a heterogeneous set of genes (A4) that inhibit the cancer-related phenotype of cells. Because they are difficult to identify, only few have been described. Evidence for their existence has mainly been indirect and came from the following types of studies: (1) studies of recessive cancer genes in higher and lower eukaryotes; if both alleles of these genes are deleted or inactivated, cancer develops; (2) studies of cell hybrids have implicated genes that suppress various stages in the malignant conversion of normal cells, e.g., immortalization, morphological conversion, and metastasis; (3) studies of isolation from viral-induced transformation of flat, nontumorigenic revertants in which expression of the transforming genes is not down-regulated, suggesting the presence of genes that suppress the effect of transformation; (4) studies showing that blocks to differentiation can be bypassed by including compounds or differentiation factors; (5) studies of tumor inhibitory factors such as tumor necrosis factor and transforming growth factor- β (TGF- β) show that they have different effects on different types of cells, acting to promote growth in some cases and to inhibit it in others; (6) studies of the discovery of *cis*-acting negative regulatory elements suggest that interaction of such elements with proteins may be important for control of gene expression, particularly of infecting oncogenic viruses; (7) studies of suppression of the transformed phenotype of malignant cells by contact with normal cells that control the growth of neighboring cells; (8) studies of the inhibition of proliferation of transformed cells by transfection with DNA from normal cells may be a useful method for cloning oncosuppressor genes; (9) and

studies of certain preneoplastic clonal cell lines (T1) that are susceptible to transformation by single *ras* oncogenes, whereas others require both *myc* and *ras* oncogenes to induce tumorigenesis. Based on experiments with somatic cell hybrids, the basis of this susceptibility to single oncogene-induced transformation appears to be due to loss of a suppressor gene. Therefore, *myc* must bypass the regulatory effects of this suppressor gene. The mechanism by which cells acquire susceptibility to *ras* is related to responsiveness to epidermal growth factor (EGF) and TGF- α possibly affecting an autocrine mechanism. The suppressor gene may function to regulate signal transduction through EGF receptor.

The types of studies mentioned here represent a new concept for suppressor gene inactivation during a preneoplastic stage progressing toward malignancy.

3.7. GROWTH FACTORS AND ONCOGENES

Normal cells require growth factors (B2) to multiply. One group of growth factors, including platelet-derived growth factor, bombesin, and vasopressin in fibroblasts or antigen in lymphocytes, uses a specific inositol lipid as part of a transduction mechanism for generating intracellular mitogenic signals. These growth factors stimulate the hydrolysis of phosphatidylinositol-4,5-bisphosphate to give diacylglycerol (DG) and inositol-1,4,5-triphosphate (Ins-1,4,5- p^3). The DG remains within the plane of the membrane to activate protein kinase C, one function of which is to increase intracellular pH by switching on a Na^+/H^+ exchange. The other product, Ins-1,4,5- P^3 , functions as a second messenger to mobilize calcium from intracellular stores. These two ionic events, the increase in pH and calcium, contribute to the onset of DNA synthesis. The hydrolysis of an inositol lipid is a key event in this signal pathway, mediating the action of competence factors.

A separate signal pathway, perhaps based on tyrosine phosphorylation, carries out the effects of progression growth factors such as epidermal growth factor and insulin. It is argued that oncogenes may be arranged into groups associated with a specific signal pathway. For example, the *sis* oncogene encodes platelet derived growth factor, which might use the *src* gene product as part of its transduction mechanism to generate the second messenger, DG, Ins-1,4,5- p^3 , and calcium. These last factors then act to stimulate the transcription of *myc* and *fos*. On the other hand, the *erb-B* gene encodes a protein that resembles the receptor for EGF. Recent studies (M1) on p21 (*ras* encoded) proteins involved in signal transduction make comparisons with the G proteins. The G proteins comprise a family of membrane-associated proteins that transduce extracellular signals such as hormones or photons into a diverse array of cellular responses. All these proteins bind and hydrolyze GTP, and GTP in turn regulates their interactions with signal detectors (cell surface receptors) and effectors (membrane-associated enzymes and ion channels). The best characterized members of the G protein family are transducin 1, GS, and Gi. All G proteins are heterotrimers: the α -subunits bind and

hydrolyze GTP and interact with effector elements, whereas the β - and γ -subunits, inseparable in the native state, function biochemically as a β/γ -subunit that anchors the more hydrophilic α -subunits to the plasma membrane. Investigations of light activation of rod cell phosphodiesterase and of hormonal regulation of adenyl cyclase have provided the following general scheme for the function of G proteins in signal transduction: (1) activation of the detector element (by light or hormone) promotes binding of detector to α - and β/γ -subunits and markedly accelerates the rate of exchange of GTP for bound GDP; (2) binding of GTP causes dissociation of α - and β/γ -subunits and detector; (3) free α -GTP then binds to and activates the effector enzyme; (4) effector activation terminates when the α -subunit hydrolyzes the GTP to GDP; and (5) the cycle continues until the activated detector becomes inactivated. With regard to the comparison of p21 *ras*-encoded proteins and the G proteins and the possible roles of p21 proteins in signal transduction, evidence suggests that *ras* oncogene products and their normal counterparts may act as GTP-dependent signal transducers analogous to the G proteins.

3.8. ONCOGENES AND STEROID RECEPTORS

Steroid hormones bind specific cytoplasmic receptor molecules (H3). These complexes are translocated to the nucleus where they bind specific DNA sequences and subsequently induce gene transcription. It has been found that epithelial growth factor, platelet-derived growth factor, and endothelial cell growth supplement are high-affinity and high-capacity receptors of progesterone. Embryonic antigens and carcinoembryonic antigen (CEA) and α -fetoprotein also bind steroid hormones. The mitogens phytohemagglutinin (PHA) and concanavalin A (Con-A) bind progesterone. Significant correlation was observed between nuclear androgen receptor deployment and expression of oncogenes (P4). Expression of *c-fos* was not elevated in prostatic carcinoma in comparison with benign hypertrophic prostate (BHP), whereas expression of *c-myc* was elevated. *H-ras* became increasingly elevated as differentiation was lost.

4. Basic Techniques Used in the Study of Oncogenes

4.1. GENE MANIPULATION

Gene manipulation is defined as the formation of new combinations of inheritable material by the insertion of nucleic acid molecules, produced by whatever means outside the cell, into any virus, bacterial plasmid, or other vector system so as to allow their incorporation into a host organism in which they do not naturally occur, but in which they are capable of continued propagation. By gene manipulation techniques, it is possible to obtain pure DNA fragments in bulk. This opens the door to a range of molecular biological opportunities, including nucleo-

tide sequence determination, site-directed mutagenesis, manipulation of gene sequences, and availability of molecular hybridization probes of absolute sequence purity.

Basic Problems and Solutions

If the exogenous DNA is integrated into the host genome, there is no problem. But if it fails to be integrated, it will probably be lost during subsequent multiplications of the host cells. In order to be replicated, DNA molecules must contain an origin of replication. Bacteria and viruses contain one molecule per genome. Such molecules are termed replicons. Fragments of DNA are not replicons and therefore will be diluted out of the host cells. The obvious solution to this problem is to attach the DNA fragments to suitable replicons, which are known as vectors or cloning vehicles; plasmids and phages are used as vectors.

Composite molecules of foreign DNA that have been inserted into a vector molecule are some times called DNA chimeras. The construction of such artificial recombinant molecules has also been termed genetic engineering, gene manipulation, or molecular cloning. The cloning of foreign or passenger DNA requires the following conditions: (1) the vector DNA must be purified and cut open; (2) the passenger DNA must be inserted into the vector molecule to create an artificial recombinant; (3) the cutting and joining must be monitored by the use of gel electrophoresis or other suitable methods; and (4) the artificial recombinant must be transformed into a host cell such as *Escherichia coli*.

4.2. LABORATORY METHODS

4.2.1. *Gel Electrophoresis*

Progress of the first cutting and joining experiments using DNA molecules was monitored by velocity sedimentation in sucrose gradients. However, this has been entirely superseded by gel electrophoresis. Gel electrophoresis is not only used as an analytical method but it is also used preparatively for the purification of specific DNA fragments. The gel is composed of polyacrylamide or agarose. Agarose is convenient for separating DNA fragments ranging in size from several hundred to about 20,000 base pairs.

Polyacrylamide is preferred for smaller DNA fragments. A gel is a complex network of polymeric molecules. DNA molecules are negatively charged and under an electric field; DNA molecules migrate through the gel at rates dependent upon their sizes; a small DNA molecule can thread its way through the gel easily and hence migrate faster than a larger molecule. Aijj and Borst (A1) showed that the migration rates of DNA molecules were inversely proportional to the logarithms of molecular weights. More recently, Southern (S7) has shown that plotting

fragment length or weight against the reciprocal of mobility give a straight line over a wider range than the semilogarithmic plot. Gel electrophoresis is frequently performed with marker DNA fragments of known size, allowing accurate size determination of unknown DNA by interpolation. DNA bands are stained with ethidium bromide with a sensitivity up to 0.05 μg DNA; the stain appears as a visible fluorescent band under ultraviolet light.

In addition to resolving DNA fragments of different lengths, gel electrophoresis separates the different molecular configurations of DNA—the covalently closed circular, the nicked circular, and the linear forms.

4.2.2. Southern Blotting

Frequently it is necessary to know that sequences in a DNA restriction fragment are transcribed into RNA and to be able to map sequences by hybridization to restriction fragments. The method described here, which is often referred to as Southern blotting, satisfies this purpose—DNA restriction fragments in the gel are denatured by alkali treatment and the gel is then laid on the top of buffer-saturated filter paper. The top surface of the gel is covered with a nitrocellulose filter and overlaid with dry filter paper. Buffer passes through the gel, drawn by the dry filter paper, and carries the single-stranded DNA with it. When the DNA comes into contact with the cellulose nitrate it binds to it strongly. The DNA fragment can be permanently fixed to the cellulose nitrate by backing at 80°C. The filter can then be placed in a solution of radioactive RNA or denatured DNA that is complementary in sequence to the blot-transferred DNA. Conditions are chosen so that the radioactive nucleic acid hybridizes with complementary DNA on the cellulose nitrate. After a washing step the region of hybridization can be detected autoradiographically by placing the cellulose nitrate in contact with photographic film. The method is extremely sensitive and can even be used to map restriction sites around a single-copy gene sequence in a complex genome. Because the radioactive nucleic acid is used here to search out and detect complementary sequences in the presence of a large amount of noncomplementary DNA, it is often referred to as the probe.

4.2.3. Northern Blotting

Southern's technique could not be applied to the blot transfer of RNA separated by gel electrophoresis. Alwine *et al.* (A2) therefore devised a procedure in which RNA bands are blot transferred from the gel onto the chemically reactive paper, where they are bound covalently. The reactive paper is prepared by diazotization of aminobenzyl oxymethyl paper, which can be prepared from Whatman paper by a series of uncomplicated reactions. Once covalently bound, the RNA is available for hybridization with radiolabelled DNA probes. Hybridizing bands are detected autoradiographically. This method is termed Northern blotting.

4.2.4. DNA Sequencing

In the Maxam and Gilbert method (M2) the starting point is a defined DNA restriction fragment. The DNA strand to be sequenced must be radioactively labeled at one end with a ^{32}P -labeled phosphate group. This DNA can be single stranded or of duplex form. The base-specific cleavages depend upon the following points:

1. Chemical reagents that alter one or two bases in DNA have been characterized (Table 2). The reactions are base specific; for example, dimethyl sulfate methylates guanine (at the N7 position).
2. An altered base is then removed from the sugar phosphate backbone of DNA.
3. The strand is cleaved with piperidine at the sugar residue lacking the base. This cleavage is dependent on the previous step.

When each of the base-specific reagents is used in a limited reaction with end-labeled DNA, a nested set of end-labeled fragments of different lengths is generated. It is important to emphasize that the base-specific reactions are deliberately limited to give one or few cleavages per molecule. Sets of fragments are produced by reacting the DNA with each reagent separately. These sequencing reactions are analyzed by running four to five samples side by side on a sequencing gel. The sequence can then be read from a sequencing ladder.

4.2.5. Chemical Synthesis of Genes

The basic method of gene synthesis (C1) is the repetitive formation of an ester linkage between an activated phosphoric acid ester of one nucleotide and the hydroxyl group of another nucleotide, thus forming a characteristic phosphodiester bridge. The major problem is that deoxyribonucleotides are very reactive molecules having a primary and secondary hydroxyl group, a primary amino group, and a phosphate group. Consequently, blocking and unblocking procedures are required.

TABLE 2
REAGENTS FOR MAXAM AND GILBERT DNA SEQUENCING

Base specificity	Base reactions	Altered base removal	Strand cleavage
G	Dimethyl sulfate	Piperidine	Piperidine
G + A	Acid	Acid-catalyzed depurification	Piperidine
T + C	Hydrazine	Piperidine	Piperidine
C	Hydrazine + NaCl	Piperidine	Piperidine
A + C	NaOH	—	Piperidine

The phosphotriester approach solves some of the problems of the phosphodiester method. More recently, the phosphite-triester method has been introduced; this makes use of the extreme reactivity of the phosphite reagent, i.e., the two building blocks are joined in a few minutes compared with the hours required by the phosphodiester approach.

4.3. CUTTING AND JOINING DNA MOLECULES

4.3.1. *Cutting DNA Molecules*

Present DNA technology is totally dependent on our ability to cut DNA molecules at specific sites. When a phage is labeled with ^{32}P , it is apparent that the phage DNA is degraded soon after injection into a bacterial host, for example, *E. coli*, and the endonuclease that is primarily responsible for this degradation is called a restriction endonuclease or restriction enzyme (L2). The host must, of course, protect its own DNA from the potentially lethal effect of the restriction endonuclease, and so its DNA must be appropriately modified. Modification involves methylation, by methylase enzyme, of certain bases at a very limited number of sequences within DNA; these sequences constitute the recognition sequences for the restriction endonucleases. Two types of restriction endonucleases have been identified. Type I endonucleases recognize specific nucleotide sequences. However, they are not particularly useful for gene manipulation because their cleavage sites are nonspecific. The type II enzymes recognize a particular sequence in duplex DNA molecules and break the polynucleotide chains within or near to that sequence, giving rise to discrete DNA fragments of defined length and sequence. Many type II restriction endonucleases have been isolated from a wide variety of bacteria. A large number of enzymes have been characterized and the number continues to grow as more bacterial genera are surveyed for the presence of these enzymes. A system for uniform nomenclature (S6) has been established and is derived as follows. The primary name derives from the first letter of the genus name of the host microorganism and the first two letters of the species name: for example, *Escherichia coli* becomes *Eco*. The host strain designation is added: e.g., *EcoK*. Different restriction sites in the same host strain are indicated by Roman numerals: for example, *Haemophilus influenzae* strain Rd becomes *HindI*, *HindII*, or *HindIII*. Some of the more commonly used restriction endonucleases are listed in Table 3.

4.3.2. *Joining DNA Molecules*

DNA fragments can be joined (G3) to create artificially recombinant molecules. There are currently three methods for joining DNA fragments *in vitro*. The first of these capitalizes on the ability of DNA ligase to join covalently the annealed cohesive ends produced by certain restriction enzymes. The second depends upon the ability of DNA ligase from phage T4-infected *E. coli* to catalyze the formation

TABLE 3
COMMONLY USED RESTRICTION ENDONUCLEASES

Organism	Endonuclease
<i>Anabaena variabilis</i>	AvaI
<i>Bacillus amyloliquifaciens</i> H	BamHI
<i>Escherichia coli</i> RY 13	EcoRI
<i>Escherichia coli</i> R 245	EcoRII
<i>Haemophilus influenza</i> Rd	HindII, HindIII
<i>Haemophilus parainfluenza</i>	HpaI, HpaII
<i>Klebsiella pneumoniae</i>	KpnI

of phosphodiester bonds between blunt-ended fragments. The third utilizes the enzyme terminal deoxynucleotidyl transferase to synthesize homopolymeric 3' single-stranded tails at the ends of fragments.

4.4. PLASMIDS AS CLONING VEHICLES FOR USE IN *E. COLI*

4.4.1. *Properties of Plasmids*

Plasmids are replicons that are stably inherited in the extrachromosomal state. It should be emphasized that extrachromosomal nucleic acid molecules are not necessarily plasmids. The definition given here implies genetic homogeneity, constant monomeric unit size, and the ability to replicate independently of the chromosome. This definition, however, does include the prophages of the temperate phages, e.g., P1, which are maintained in an extrachromosomal state as opposed to those such as λ , which are maintained by integration into the host chromosome. Plasmids are widely distributed throughout the prokaryotes; they vary in size from less than 10^6 Da to greater than 200×10^6 Da and are generally dispensable. Some of the phenotypes that these plasmids confer on their host cells are listed in Table 4.

Plasmids can be categorized into one of two major types, conjugative or non-

TABLE 4
PHENOTYPIC TRAITS EXHIBITED BY PLASMID-CARRIED GENES

Antibiotic resistance	Heavy metal resistance
Antibiotic production	Induction of plant tumors
Degradation of aromatic compounds	Hydrogen sulfide production
Sugar fermentation	Host-controlled restriction and modification
Enterotoxin production	

conjugative, depending on whether or not they carry a set of transfer genes, called *tragenes*, which promote bacterial conjugation. Plasmids can be categorized on the basis of their being maintained as multiple copies per cell (relaxed plasmids) or as a limited number of copies per cell (stringent plasmids). Generally, conjugative plasmids are of relatively high molecular weight and are present as one to three copies per chromosome, whereas nonconjugative plasmids are of low molecular weight and are present as multiple copies per cell. Properties of plasmids of Gram-negative organisms are listed in Table 5.

4.4.2. Purification of Plasmid DNA

An obvious prerequisite for cloning in plasmids is the purification of plasmid DNA. A wide range of plasmid DNAs are now routinely purified. Undoubtedly the trickiest stage of the purification procedure is the lysis of the host cells. Incomplete lysis or total dissolution of the cells results in greatly reduced recoveries of plasmid DNA. The ideal situation occurs when each cell is sufficiently broken to permit the plasmid DNA to escape without too much contaminating chromosomal DNA. Provided the lysis is done gently, most of the chromosomal DNA that is released will be of high molecular weight and can be removed along with cell debris by high-speed centrifugation to yield a cleared lysate. Many methods are available for isolating pure plasmid DNA from cleared lysate; two methods are described here.

4.4.2.1. *Classical Method.* This method (R1) involves isopycnic centrifugation of cleared lysate in a solution of CsCl containing ethidium bromide (EtBr). EtBr binds by intercalating between DNA base pairs, which causes the DNA to unwind. A covalently closed circular (ccc) DNA molecule such as a plasmid has no free ends and can only unwind to a limited extent, thus limiting the amount of bound EtBr. Linear DNA, such as fragmented chromosomal DNA, has no such topological constraints and can therefore bind more of the EtBr molecules. Because the density of the DNA/EtBr complex decreases as more EtBr is bound, and because more EtBr can be bound to a linear molecule than a covalent circle, the

TABLE 5
PROPERTIES OF SOME PLASMIDS OF GRAM-NEGATIVE ORGANISMS

Plasmid	Size (M Da)	Conjugation	No. of copies of chromosome equivalent	Phenotype
Cole1	4.2	No	10-15	Colicin E1 production
RSF1030	5.6	No	20-40	Ampicillin resistance
CloDF13	6	No	10	Cloacin production
R1	62.5	Yes	3-6	Multiple drug resistance
EntP307	65	Yes	1-3	Enterotoxin production

covalent circle has a higher density at a saturating concentration of EtBr. Thus plasmids can be separated from chromosomal DNA.

4.4.2.2. *Birnboim and Doly Method.* Plasmid-containing cells (B7) are treated with lysozyme to weaken the cell walls and then are lysed with sodium hydroxide and sodium dodecyl sulfate (SDS). Chromosomal DNA remains in a high-molecular-weight form, but is denatured. Upon neutralization with acidic sodium acetate, the chromosomal DNA renatures and aggregates to form an insoluble network. The high concentration of sodium acetate causes precipitation of protein-SDS complexes and of high-molecular-weight RNA. Provided the pH of the alkaline denaturation step has been carefully controlled, the covalently closed circular plasmid DNA will remain in a native state and in solution.

4.4.3. *Properties of Plasmid-Cloning Vehicles*

An ideal cloning vehicle would have the following three properties: (1) low molecular weight, (2) ability to confer readily selectable phenotypic traits on host cells, and (3) single sites for a large number of restriction endonucleases. There are several advantages of low molecular weight. First, the plasmid is much easier to handle, i.e., it is more resistant to damage by shearing and is readily isolated from host cells. Second, low-molecular-weight plasmids are usually present as multiple copies and this facilitates their isolation and leads to gene dosage effects for all cloned genes. Finally, with a low molecular weight there is less chance that the vector will have multiple substrate sites for any restriction endonuclease.

4.5. BACTERIOPHAGE AND COSMID VECTORS FOR *E. COLI*

Bacteriophage λ is a genetically complex and extensively studied virus of *E. coli*. Because it has been the object of molecular genetic research, it was investigated and developed as a vector. The DNA of phage λ , in the form in which it is isolated from the phage particle, is a linear duplex molecule of about 45.5 kb pairs. The entire DNA sequence has been determined (S1). At each end are short, single-stranded 5' projections of 12 nucleotides that are complementary in sequence and by which the DNA adopts a circular structure when it is injected into the host cell; i.e., λ DNA naturally has cohesive termini that associate to form the *cos* site.

Functionally related genes of phage λ are clustered together on the map (Fig. 3), except for the two positive regulatory genes, *N* and *Q*. Genes on the left of the conventional linear map code for head and tail proteins of the phage particle. Genes of the central region are concerned with recombination and the process of lysogenization, in which the circularized chromosome is inserted into the host chromosome and stably replicated with it as a prophage. Much of this control region, including these genes, is not essential for phage growth and can be deleted or replaced without seriously impairing the infectious growth cycle. Its dispens-

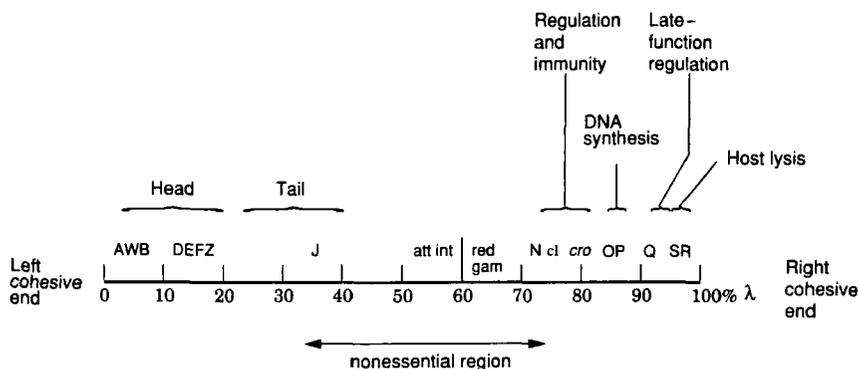


FIG. 3. Gene map of functionally related phage λ genes.

ability is crucially important in the construction of vector derivatives of the phage. To the right of the central region are genes concerned with regulation and pro-phage immunity to superinfection (*N*, *cro*, *cI*), followed by genes for DNA synthesis (*O*, *P*), late-function regulation (*Q*), and host cell lysis (*S*, *R*).

It is possible to insert foreign DNA into the chromosome of phage derivatives and in some cases foreign genes can be expressed efficiently via λ promoters.

4.5.1. Packaging Phage λ DNA *in Vitro*

Manipulated phage DNA can be introduced into competent host bacteria by transfection using freshly prepared DNA that has not been subjected to any gene manipulation procedures: this will result in about 10^5 plaques per microgram of DNA. In a gene manipulation experiment in which the vector DNA is restricted and then ligated with foreign DNA, this figure is reduced to about 10^3 – 10^4 plaques per microgram of vector DNA. In some contexts, 10^6 or more recombinants are required.

The scale of such experiments can be kept within a reasonable limit by packaging the recombinant DNA into mature phage particles *in vitro*. Placing the recombinant DNA in a phage coat allows it to be introduced into the host bacteria by the normal process of phage infection, i.e., phage absorption followed by DNA injection. Depending upon the details of the experimental design, packaging *in vitro* yields about 10^6 plaques per microgram of vector DNA.

4.5.2. Cosmid Vectors

Concatamers of unit length λ DNA molecules can be efficiently packed if the *cos* sites—substrates for the packaging-dependent cleavage—are 37–52 kb apart (75–105% of the size of λ DNA). In fact, only a small region in the proximity of the *cos* site is required for the recognition of the packaging system. Plasmids have

been constructed that contain a fragment of λ DNA including the *cos* site (C3, C4). These plasmids are termed cosmids and can be used as gene-cloning vectors in conjunction with the *in vitro* packaging system.

4.6. DNA TRANSFECTION

The NIH/3T3 cell lines (C5) are commonly used as recipients because of their ability to take up efficiently and integrate exogenous DNA in the form of a calcium phosphate coprecipitate. A gene that induces transformation can then be detected by the appearance of foci of transformed cells in the recipient cell population. These transformed foci can be picked and cultivated for further study. The general procedure for DNA transfection is summarized as follows: Mouse NIH/3T3 cells seeded 24 hours earlier at 1.3×10^6 cells per 10-cm dish were exposed for 18 hours to the DNA-extracted tissues (40 μ g/dish) or cultured cells by the calcium phosphate precipitation technique. Cultures were maintained with twice weekly changes of medium. The transformed foci were counted at 28 days of transfection. Control assays using DNAs from transfectant (positive control) and sonicated human placental DNA (negative control) were included in each assay. Only the foci morphologically different from those in the negative control dishes were counted.

4.7. DETERMINATION OF ONCOGENE PROTEIN PRODUCT

Lysates of frozen tissue containing 900 μ g of protein were immunoprecipitated with specific monoclonal antibody. The resultant immunocomplexes were resolved in 12.5% polyacrylamide slab gels containing SDS; Western blotting was performed. After electrophoretic transfer of protein from the gel onto the nitrocellulose filter, protein was detected by the use of polyclonal antibody and 125 I-labeled protein A. Quantitative differences in the levels of protein are determined by densitometric analysis.

4.8. DETECTION OF POINT MUTATION

One method to detect point mutations involves alterations at specific oncogene codons that lead to the loss of restriction sites upon the action of specific restriction endonucleases; this provides a means for molecular genetic diagnosis of the lesions at these positions. DNAs from tumors under investigation are surveyed for restriction site polymorphisms by digestion with specific restriction endonucleases, using DNA from a tumor with an oncogene of a known point mutation as a control. Fragmented DNAs are separated on 1.4% agarose gel and detected by specific DNA probes.

Another method utilizes altered electrophoretic mobility: faster or slower elec-

trophoretic mobilities of oncogene protein products usually accompany the activating lesion.

5. Oncogenes and Cancer

5.1. ONCOGENES AND MULTISTEP CARCINOGENESIS

It is postulated (S9) that cancer is the result of genetic or epigenetic changes that occur mainly in stem precursor cells of various cell types. Genetic changes, that is, the point mutations, deletions, inversions, amplifications, and chromosome translocations, represent gains or losses in the genes themselves. Epigenetic changes, for example, DNA hypomethylation, represent changes in the gene products (RNA or proteins). Changes in oncogene activity have a genetic or epigenetic origin, or both, and result in quantitative or qualitative differences in the oncogene products. These are involved in changing normal cells into cells with the cancer phenotype (usually a form of dedifferentiated cells) in a multistep process. There are several pathways to cancer and intermediate steps are not necessarily defined in an orderly fashion. Activation of a particular class of oncogenes and inactivation of another class could occur at any step during the development of cancer. Most benign or malignant tumors consist of a heterogeneous mixture of dedifferentiated cells arising from a single cell.

The evolution of a fully malignant tumor is a multistep process resulting from the action of multiple factors (W4), both environmental and endogenous, and involved alterations in the function of multiple cellular genes. Chemical carcinogens that initiate this process appear to do so by damaging cellular DNA. In addition to producing simple point mutations, this damage appears to induce the synthesis of a trans-acting factor that can induce a synchronous DNA replication; a response that may lead to gene amplification and/or gene rearrangement. This phenomenon may also play a role in the synergistic interactions between chemicals and viruses in the development of certain cancers. The primary target for tumor promoters appears to be cell membranes; all of the promoters act at least in part by enhancing the activity of the phospholipid-dependent protein kinase C (PKC). Thus tumor promoters can act synergistically with an activated oncogene to enhance cell transformation.

Among the known etiologies (L1) of human cancers, estimates now indicate that probably the majority of cancers arise by chemical carcinogenesis. Enzymes metabolize unreactive compounds to gene rate active cancer-causing compounds. The result of faulty genetic regulation that appears to cause cancer is the misprogramming of protein synthesis; the polypeptides produced are either atypical of the cell of origin or characteristic of an embryonic or fetal stage of cellular development. The best known example of this phenomenon is the ectopic production of polypeptide hormone by many tumors of nonendocrine origin. Similarly,

a second manifestation of misprogrammed protein synthesis is the disappearance of characteristic cellular antigens and the appearance of tumor-associated antigens, which are often characteristic of fetal rather than adult cells. A third manifestation is an alteration in the isoenzyme composition of the cell.

Part of the metabolic strategy of the cancer cell appears to involve an increase in the pathways directed toward nucleotide synthesis, which is required for rapid cell division.

5.2. ONCOGENES AND CONTROL OF CELL PROLIFERATION

Control of cell proliferation (W3) involves a number of interacting regulatory pathways inside the cell, which can be influenced by a variety of extracellular signals through signal transduction at the cell surface. A structurally diverse set of polypeptide growth factors is involved in the extracellular signaling either alone or through synergistic interactions. These factors can induce DNA synthesis and proliferation of certain target cells. Studies of the structure and function of platelet-derived growth factor (PDGF) and of the receptor for EGF revealed two different but related mechanisms for the supervision of cell proliferation control by oncogenes. The *sis* oncogene encodes a growth factor related to PDGF and thus could stimulate proliferation by an autocrine mechanism, whereas the *erb-B* oncogene encodes a defective EGF receptor that could be delivering a continuous proliferation signal in transformed cells. Other oncogenes may encode proteins that interact at other steps in the growth factor-induced mitogenic cascade.

5.3. ONCOGENES AND METASTATIC PHENOTYPE

The ability of two oncogenes, *src* and *ras* (C2), to convert cells to a metastatic phenotype was studied. It is speculated that a major mechanism by which some oncogenes promote metastatic ability is by subverting a signal transduction process, resulting in activation of a set of genes. Some of these genes appear to promote metastatic ability.

5.4. ONCOGENES IN DIFFERENT CANCERS

5.4.1. Multiple Primary Cancer

Von Hippel–Lindau disease is inherited on an autosomal dominant gene that may show marked expressive variability of cancer phenotype in certain patients and families. A patient with a strongly positive family history of this disease, at age 28, underwent craniotomy with removal of a cystic cerebellar hemangioblastoma. At age 48 he developed syringomyelia of the spinal cord, became quadriplegic, and had a progressive downhill course. At autopsy, hemangioblastomas

of the cerebellum and spinal cord were found as well as a left renal cell carcinoma, an oat cell carcinoma of the lung, a hepatocellular carcinoma, and a typical thyroid adenoma. This tumor spectrum appears to be unique, although chance cannot be excluded. It is possible, however, that these findings might represent an expression of a deleterious genotype that became evident only because of this patient's prolonged survival subsequent to his initial cerebellar hemangioblastoma.

5.4.2. *Bladder Cancer*

The frequency of active *ras* oncogenes in human bladder cancer (F6) associated with schistosomiasis was examined. Of nine squamous cell carcinomas of the bladder, none scored as positive in the regular DNA transfection assay. The restriction fragment polymorphism assay at codon 12 of the H-*ras* gene confirmed the absence of an activating mutation at this site in all samples. Western blotting analyses of the *ras* p21 proteins suggested a point mutation with codon 61 in one sample only. Enhanced expression of the *ras* p21 protein was demonstrated in four samples.

The carcinogenic process involved in endemic bilharzial bladder cancer is thus not associated with detectable point mutations with *ras* genes at a higher frequency than occurs in nonbilharzial bladder cancer.

5.4.3. *Breast Cancer*

Current evidence strongly suggests that the inappropriate expression of genes (P1) normally active in regulating cell proliferation may contribute in a fundamental way to the development of human breast cancer. There are experimental data supporting this hypothesis: (1) the *c-myc* oncogene induces mammary adenocarcinoma in transgenic mice; (2) activation of the c-Ha-*ras* oncogene by a chemical carcinogen induces breast cancer in mice; (3) human breast cancer cell lines have amplified or activated *ras* oncogenes and expression of the mutated *ras* oncogene is sufficient to relieve MCF-7 lines of their dependence on estrogen for tumor formation; and (4) the estrogen receptor is related in structure to the known viral oncogene *erb-A*.

The differential expression (F5) of the *ras* oncogene product p21 in the primary tumor, regional nodes, and distant metastatic sites in the patients with disseminated breast cancer was examined to define the biologic and clinical significance of the *ras* oncogene in the progression of breast cancer.

Expression of *ras* p21 was consistently increased in the invasive component of the primary tumor as compared with the intraductal tumor. In addition, a high level of p21 expression was seen in tumor emboli in lymphatics and blood vessels as compared with the contiguous tumor in parenchymal tissue. It is suggested that markedly enhanced p21 expression is associated with earlier stages (invasion and dissemination) of aggressive breast cancers.

The genomic organization of *c-myc* (B3) was studied in 48 human primary

tumors. Two types of alterations (amplification and rearrangement) were observed in 27 of the tumors studied. The *c-myc* protooncogene appeared to be amplified from 2- to 15-fold in the DNA of 20 tumors. The majority of the tumors analyzed were invasive ductal adenocarcinomas; 58% of these showed *c-myc* locus genetic alterations. The *c-myc* alterations do not appear to correlate with the aggressive behavior of primary breast tumors. They seem to be associated with the development of breast carcinomas.

Regulation of *c-myc* expression (D3) is known to be sensitive to a variety of mitogenic stimuli in various cell types. Because estrogen is a well-documented mitogen of estrogen-responsive human breast cancer (HBC) cells, studies were made of the influence of estradiol and its antagonist, tamoxifen, on the expression of *c-myc* in HBC cell lines. The breast cell lines studied included those that were estrogen responsive, estrogen receptor positive (ER+); estrogen nonresponsive, estrogen receptor negative (ER-); and nontumorous. In ER+ cell lines the addition of estradiol resulted in a noticeable increase in *c-myc* expression after 15 minutes, with a maximal level (greater than 10-fold). In the ER- cell lines the level of *c-myc* mRNA was high and was unaffected by estrogen or tamoxifen. These results suggest that regulation of *c-myc* expression may be an important step in estrogen-induced proliferation of HBC cells.

5.4.4. Skin Cancer

The induction of carcinomas on the skin of experimental animals (Y1) by initiation and promotion protocols involves three operationally distinct stages. Initiation appears to be a genetic change in epidermal basal cells that enables the altered cells to proliferate under conditions in which normal cells are obliged to differentiate. Tumor promotion provokes the clonal expansion of initiated cells through differential growth responses of normal and initiated cells with each promoter exposure. The result of these two stages is the formation of benign tumors—"epidermal papillomas." The third stage, malignant conversion, occurs as a result of further genetic changes in papilloma cells, either spontaneously or at low frequency or by the action of genotoxic carcinogens at a higher frequency. The specific genes involved in any of the stages of skin carcinogenesis have not been elucidated, but activation of a *ras* (Ha) gene may be associated with initiation or malignant conversion.

5.4.5. Colon Cancer

The level of *ras* oncogene protein product p21 (L6) was evaluated in specimens of normal human colonic mucosa, hyperplastic polyps, tubular adenomas, villous adenomas, and the epithelium from a case of ulcerative colitis. Differences in p21 values among all classes of polyps were significant (hyperplastic polyps had values less than tubular adenoma values, which were less than villous adenoma

values). The p21 values for colitis specimens were similar to those obtained from villous adenomas.

It may be concluded that the levels of *ras* oncogene protein product increase with the malignant potential of benign human colonic conditions. These findings suggest that the *ras* oncogene protein product may play an important role in the malignant transformation of benign lesions of the human colon. If these findings are confirmed, as technology progresses to allow molecular probes to measure gene products in biopsy specimens, high-risk patients could be monitored and treated before actual malignant transformation occurs.

5.4.6. *Liver Cancer*

Primary hepatic cancer (PHC) is one of the most prevalent malignancies. Extensive occurrences of PHC (G2) are closely related to hepatitis B virus infection, aflatoxin contamination, and other putative carcinogenic factors in the drinking water. However, direct evidence is still lacking as to which of these factors operate in hepatocarcinogenesis, and how they operate. Therefore, the isolation and identification of oncogenes in PHC will help to elucidate the target genes that carcinogens may attack and the mechanisms of the carcinogens. The *N-ras* genes were overexpressed in six of nine PHC samples. Increased p21 protein synthesis was also noted. The available data strongly imply that *N-ras* is at least one of the transforming genes for PHC.

5.4.7. *Lung Cancer*

Studies on lung cancer suggest that (M6) a number of molecular mechanisms may be important in the pathogenesis of lung cancer, especially small cell lung cancer (SCLC). An inherited predisposition to develop SCLC may correlate with a nonfunctional recessive allele for a gene that maps to chromosome region 3p14–23. Individuals at high risk would be heterozygous for this allele in their germ line, carrying one copy of a normal functional gene and one mutant recessive allele. Exposure to carcinogens, in particular cigarette smoke can produce somatic genetic changes such as chromosomal deletions or gene mutations in the functional allele of this gene, unmasking the nonfunctional allele. Loss of this normal gene may alter the regulation of cell growth, perhaps by allowing deregulated expression of protooncogenes of the *myc* family or autocrine growth factors such as gastrin-releasing peptide (GRP) and/or its receptor. Alternatively, loss of this gene may result in the cell returning to the less differentiated developmental state wherein growth regulation is less stringent. Persons with this mutant gene should be at increased risk to develop SCLC, and further restriction fragment length polymorphism (RFLP) analysis of the 3p region in SCLC may allow identification of specific haplotypes with an increased risk of developing lung cancer. If this notion is correct one might expect to find an increased frequency of second tumors

in lung cancer patients and the presence of similar chromosomal deletions in second tumors arising in SCLC patients. In this regard, cured lung cancer patients, including those with SCLC, have a 10-fold increased risk of developing a second lung cancer. In fact, a chromosome 3p deletion along with other chromosomal abnormalities was identified in acute erythroleukemia cells arising in a long-term survivor of SCLC, implicating this same region in the pathogenesis of both tumors.

Human lung carcinogenesis (H2) is a multistep process involving aberrations in the pathways of growth and differentiation of bronchial epithelial cells. Pathways of growth and differentiation in normal epithelial cells are regulated and balanced. In contrast, carcinoma cells may have a selective clonal expansion advantage by (1) decreased responsiveness to endogenous factors, e.g., epithelial inhibitors (TGF- β) and to inducers of terminal squamous differentiation and/or (2) autocrine production of growth factors, e.g., gastrin-releasing peptide. Three families of protooncogenes, *ras*, *raf*, and *myc*, have so far been associated with human lung cancer.

Human bronchial epithelial cells are being used *in vitro* to investigate the role of specific protooncogenes in carcinogenesis and tumor progression. Using the protoplast fusion method for high-frequency gene transfection, the v-Ha-*ras* oncogenes initiate a cascade of events in normal human bronchial cells, leading to their apparent immortality, aneuploidy, and tumorigenicity with metastasis in athymic nude mice. These and other results indicate that activated protooncogenes can dysregulate pathways of growth and differentiation of human bronchial epithelial cells and play an important role in human lung carcinogenesis.

5.4.8. Cancer of the Testes

Normal testes expressed only small amounts of *myc*-encoded p62 (S4). Seminomas showed increased nuclear and cytoplasmic staining. Undifferentiated teratomas showed little activity, whereas *c-myc*-encoded p62 was abundant in the nuclei of differentiated epithelial structures, yolk sacs, and embryoid bodies.

A modified *ras* gene, either amplified or mutated, or both, is often seen in seminomas. It may be concluded that *ras* gene modification plays a causative role in the development of seminomas. Because the Ki-*ras* gene is located on chromosome 12, amplification of Ki-*ras* may be associated with the specific aberration in chromosome 12 [iso(12p)] that is frequently observed in germ cell tumors.

5.4.9. Neuroblastoma

Embryonic tumors (T4) that arise in the fetus or in young children frequently show a mixed population of differentiated and undifferentiated cells. This evidence suggests that the tumors may result from developmental disturbances during organogenesis that cause arrest of an early stage in the normal differentiation process to adult tissue. Enhanced expression of the N-*myc* oncogene has been

found to be associated with several neuroblastomas and retinoblastomas, and it has been correlated with more advanced stages in these neoplasias.

The analysis of ganglioneuroblastomas and neuroblastomas indicates that the level of *N-myc* expression is highest in the most undifferentiated cells, which show atypical aggressive biological behavior.

5.4.10. *Hematopoietic Malignancy*

Two classes of genes (K3) are apparently critical in the origin of cancers of children. One class, that of oncogenes, acts by the virtue of abnormal or elevated activity. It is operative primarily in leukemias and lymphomas. There is no evidence yet that a genetic predisposition to develop these neoplasms can be imparted by oncogene mutations in the germ line. However, there are conditions that predispose to these tumors, primarily through excessive chromosomal breakage and rearrangement. The other class, that of antioncogenes, is recessive in oncogenesis.

Cancer results when both normal gene copies have been mutated or deleted. Some persons carry one such mutations in the germ line and are highly susceptible to tumor development because only one somatic event is necessary. Some children, even though carrying no such mutation in the germ line, can acquire tumors as a result of two somatic events. Most solid tumors in children seem to involve initiation through the loss of both copies of an antioncogene. Many of the chromosomal aberrations seen in leukemia and lymphoma (R2) involve structural aberrations of chromosomal segments that contain cellular protooncogenes.

In chronic myeloid leukemia, a translocation involving chromosomes 9 and 22 [t(9;22)] is seen in over 90% of the patients who have Philadelphia chromosome. In acute myeloid leukemia, certain structural aberrations are specifically associated with particular morphologic subtypes. Thus in the differentiation of the granulocytic lineage, a t(8;22) is associated, whereas a t(15;17) is seen only in promyelocytic leukemia. Monoblastic leukemia is associated with a translocation or deletion of the long arm of chromosome 12.

The immunoglobulin genes (P3) are clustered in three distinct areas of the genome; the sets of genes encoding the κ light chains, those encoding the λ light chains, and those encoding the H chains are each located on a separate chromosome (2, 22, and 14, respectively). There is a substantial body of evidence indicating that the chromosomal translocations involving the *myc* oncogene and one of the immunoglobulin loci are very common in B lymphoid tumors. In both Burkitt's lymphomas and mouse plasmacytomas, the translocation breakpoints (chromosome 8) and the chromosome bearing the immunoglobulin genes can vary from one tumor to the next. Two different alterations in *myc* expression are likely to occur: (1) an increase in cellular content of *myc* mRNA and (2) qualitative changes in the nature of *myc* transcripts and the types of mRNAs processed from them.

In Burkitt's lymphoma (G1), with the 8;14 chromosomal translocation, the *myc* gene moves into close proximity to the heavy chain locus. In some cases, the oncogene is rearranged and in others it is intact. The structure and function of the *myc* oncogene were examined in a Burkitt's tumor cell line with the usual 8;14 chromosomal translocation and also with the variant 8;22 and 2;8 rearrangements. The translocation of the *myc* gene to the immunoglobulin heavy chain gene cluster or the translocation of either the λ chain locus or the κ chain locus to a region distal (3') to the *myc* oncogene results in transcriptional activation of the *myc* gene involved in the translocation.

Although the *myc* oncogene on the normal chromosome 8 is transcriptionally silent, it appears that the critical association in Burkitt's lymphoma cells is between the oncogene and side of the immunoglobulin constant region genes. Because no differences in the *c-myc* gene product have been detected in Burkitt's lymphoma with or without *c-myc* rearrangements, it appears that the expression of high levels of structurally normal *myc* product is the significant result of translocation.

In view of new knowledge (L3) gained from molecular studies and from studies of acquired immunodeficiency syndrome (AIDS), it has been proposed that the sequence of events leading to the appearance of Burkitt's cells be reconsidered. A three-step model for Burkitt's lymphoma is proposed. The first step is the generation of lymphoid cells that are at high risk for translocations involving the immunoglobulin loci because of the chronic persistent immunological stimulation by malaria or AIDS-associated opportunistic infections that is superimposed on the extremely high rate of physiological turnover of B cells. Step two is the acquisition of the translocation involving *c-myc* and immunoglobulin loci in one or a few B cells. This event, the first genetic event, is not sufficient to transform cells to a neoplastic state, but it renders the lymphoid cells competent and responsive to external growth factors, upon which cell proliferation still depends. The third step is the infection by Epstein-Barr virus (EBV) of a B cell carrying the specific translocation. The acquisition of viral infection renders the cell independent of exogenous growth factors and thus autonomous for its growth. The third step can be achieved by a second cellular event such as activation of another oncogene, and this may be the case in EBV-negative Burkitt's lymphoma.

Despite impressive advances in the clinical management of Hodgkin's disease (S5), little is known about its cellular origin or the mechanisms of its genesis. Recent findings that certain human cellular oncogenes can cause malignant transformation suggest that aberrant activation of these genes may play a role in carcinogenesis. To determine if such genes are operative in Hodgkin's cells, DNA was isolated from splenic nodules of patients with nodular sclerosis Hodgkin's disease. The DNA was tested for its ability to transform mouse NIH/3T3 cells. The genes of these patients had homology with *N-ras* when tested by Southern blot methods (using closed oncogene probes for testing homology). The size of the

restriction fragments was similar to that of transforming genes isolated from patients with acute nonlymphocytic leukemias. The relapse cell population (S3) differs from acute onset clones in patients with acute myelomonocytic leukemia. Follow-up studies tracked a patient with myelomonocytic leukemia exhibiting the *N-ras* mutation (G1n-61/Lys-61); these studies employed the polymerase chain reaction and synthetic oligonucleotide hybridization probes, allowing the detection of as little as 3% of *N-ras*-mutated cells within a population. When the patient went into clinical remission, the mutation became undetectable. When a relapse occurred, the blasts did not carry the *N-ras* mutation. These findings suggest that the relapse cell population and the acute cell population are derived from different clones.

6. Discussion

The burden of cancer falls unequally upon a population of individuals. Some normal persons develop cancer as a result of bad luck; they are victims of background processes such as gene mutations or chromosomal rearrangements. Others are susceptible because they are exposed to environmental agents that increase these background rates.

However, there are individuals who are predisposed to cancer because they carry mutations that encode the pathway to cancer. Two independent lines of work have converged over the past several years. The first focused on the mechanisms by which a variety of animal retroviruses are able to transfer infected cells and induce tumors in their own host species. The other investigated the molecular mechanisms responsible for tumors of nonviral origin, such as those human tumors traceable to chemical causes. It is now realized that common molecular determinants may be responsible for tumors of both classes. These determinants constitute the cellular oncogenes, which are a functionally heterogeneous group of genes. Members of this group, under certain conditions, can achieve the transformation of cells.

The first insight into cellular oncogenes came from the study of a retrovirus, Rous sarcoma virus. This virus has been studied intensively because of its unusual molecular biology involving reverse transcription and the high-efficiency integration of its genome into the cellular chromosome. The genome of Rous sarcoma virus consists of two distinct portions; the first includes the regions *gag*, *pol*, and *env* encoding the glycoprotein of internal virion structure, reverse transcriptase and the glycoprotein covering the virion surface, respectively. The other portion of the Rous sarcoma virus genome contains the *src* gene, which enables the virus to induce sarcomas *in vivo*. The *src* gene is known to encode the tyrosine kinase pp 60. Since the discovery of the first cellular oncogene 16 years ago, several dozen such genes of protooncogenes have been found. They are normally impli-

cated in processes of growth differentiation and/or embryonic development. Because these protooncogenes and the proteins they specify form a structurally and functionally heterogeneous group, it is therefore not surprising that various molecular mechanisms are involved in the activation of these genes, to exhibit their transforming properties.

In fact, five separate mechanisms of protooncogene activation have been found to date. The first mechanism involves overexpression of a protooncogene following acquisition of a novel transcription promoter. The second mechanism involves overexpression due to amplification of the protooncogene or oncogene, which causes corresponding increases in transcript and gene products. The third mechanism depends on the action of enhancer sequences that can increase utilization of the transcriptional promoter to which they become linked. The linked promoter may be as far as several kilobases away and the enhancer may be positioned upstream or downstream of the promoter. The fourth mechanism has demonstrated that the juxtaposition of gene and immunoglobulin domains following chromosomal translocation may result in deregulation of the gene. The gene loses regulatory sequences of its own and acquires normally unlinked sequences involved in immunoglobulin production. The fifth mechanism depends on point mutations in the protooncogenes, which may only serve to inactivate these genes or may affect critical sites converting them into potent oncogenes. An example of this is the point mutation in the *Ha-ras* protooncogene, converting it into a potent oncogene. The mutation involves G to T transfer, causing glycine, normally present in twelfth residue of the encoded p21 protein, to be replaced by valine or aspartate. The study of cellular oncogenes has generated a long list of important agents of cellular transformation. The creation of a tumor cell within a tissue would seem to require far more than the activation of one of these oncogenes within the cell. Spontaneous or chemically induced tumorigenesis is known to be a multistep process, whereas the activation of an oncogene seems to occur as a single, discrete event.

As mentioned earlier, a large number of distinct cellular protooncogenes and associated oncogenes have been discovered. Examples of these genes are *src*, *fes*, *abl*, *fms*, *mos*, *raf*, *ras*, *myc*, *myb*, *fos*, and *sis*. One cannot imagine the existence of a large number of separate physiological functions on comparison of the structures of various genes and their encoded proteins; for the purpose of simplification it may be concluded that structural homology often implies functional analogy. Based on this principle, *src*, *fes*, and *abl* can be grouped together, having a demonstrable tyrosine kinase activity. The large number of oncogenes can be allocated to a smaller number of groups, each group containing the genes functioning on a common regulatory pathway. One aspect of the already established grouping concerns the cellular localization of the encoded gene products. The proteins made by *src*, *abl* and *Ha-ras* are attached to the inner surface of the

plasma membrane, whereas the proteins made by *myc* and *myb* are associated with nuclear structure.

The products of protooncogenes show sequence homology to various components in the mitogenic pathway (growth factors, growth factor receptors, signal-transducing proteins, and nuclear proteins), and it has been postulated that they may cause deregulation of the various components of this pathway. Considering what was mentioned earlier, a single oncogene acting alone has a limited power. The question to be raised, then, concerns how many different oncogene functions must cooperate in order to transform a normal cell or to convert it into a tumor cell, and in the latter state whether additional functions beyond those provided by the oncogene are required. The answer is not simple but it can be deduced from the following considerations:

1. The evolution of a fully malignant tumor is a multistep process resulting from the action of multiple factors, both environmental and endogenous.
2. There are several pathways to cancer, and intermediate steps are not necessarily defined.
3. Synergistic interaction between different factors may occur in the evolution of a fully malignant tumor.
4. Certain preneoplastic clonal cell lines require more than one oncogene for their transformation, whereas others are susceptible to transformation by a single oncogene. The basis for this susceptibility to a single oncogene-induced transformation appears to be due to the loss of a suppressor gene. The necessity for more than one oncogene to induce transformation is to bypass the regulatory effect of the suppressor gene.

7. Prospects

Some peculiarities of the carcinogenic process may be explained in terms of sequential activation of these oncogenes. The progression of tumors from precancerous growths such as papillomas may also have an underlying molecular basis involving oncogenes. However, we cannot say that all aspects of the cancer process will be readily understood in terms of the oncogenes with which we are now familiar. The cancer process underlies complex biological phenomena. Cancer cells can modulate their antigenicity to evade the immune defense. They can also acquire an ability to break off from a primary tumor and seed secondary growths at distant sites. Such cancer phenotypes do not represent initial derangements in growth control but rather secondary adaptations that favor survival and clonal expansion. The presence of the oncogenes leads us to the belief that even these complex biological phenomena will be traced back to an alteration of specific genes.

7.1. DIAGNOSIS AND STAGING OF CANCER

Accurate diagnosis and staging of malignancy commonly depends on a series of diagnostic tests rather than on one individual study. Unfortunately, a single test is not often a precise reflection of the disease state. Whenever a diagnostic examination is performed, the result must be interpreted in the context of how accurately it reflects the presence or the absence of the disease. A large number of biologic markers for cancer have been described. These markers may be classified as tumor-associated oncofetal proteins—carcinoembryonic antigen and α -fetoprotein (AFP)—or ectopic hormones, enzymes, etc. Unfortunately, the specificities of all tumor markers described to date are quite poor; for example, nontumor causes of CEA elevation have been reported, including pancreatitis, recent blood transfusions, ulcerative colitis, and heavy cigarette smoking. Tumor markers have been used as a definitive test through which a clinician can monitor the response of a tumor to therapy, but they are of little or no value in mass screening for cancer. However, they can be of great value for detection of recurrence in a high-risk population, such as postoperative colorectal cancer patients. The prognosis as determined by the principles governing cancer staging, such as the currently employed TNM system, is of paramount importance. The risk-versus-gain judgments often faced in cancer management would be greatly facilitated by accurate staging. The main objective for cancer staging is to assess the eventual risk of metastasis, which, for common adult malignancies, is equivalent to death from the disease. The oncogene products are homologous to growth factors, surface receptors, protein kinases, and cell cycle control proteins. From the available data for DNA sequences, peptides predicted to be exposed within intact molecules can be constructed. These peptides can be used to produce monoclonal antibodies to oncogene products. A battery of antibodies against different oncogene products can be used for early diagnosis of cancer. By this procedure it may be feasible to identify those individuals at highest risk for the development of occupational cancer among cohorts of workers with potential carcinogen exposure. The procedure depends on the application of immunoblotting of urine or serum samples using monoclonal antibodies to various oncogene proteins.

7.2. CANCER TREATMENT

Current data support a clonal origin of most cancers because most tumors arise from a single cell as a result of a spontaneous mutation or after exposure to an oncogenic virus or chemical carcinogen; these tumors then expand to a detectable level while continually shedding viable cells. The ability to cure the cancer then depends on numerous variables, the most important being the presence of viable metastasis. The application of chemotherapy arose out of the appreciation that cancer is not commonly a localized process and is thus not amenable to control

by local means such as surgery or irradiation. The cancer cell presents a variable and moving target to drugs. The interrelationship of anticancer pharmacokinetics and cell kinetics is the mainstay of clinical cancer chemotherapy. Almost all anticancer drugs share two common properties. They work by affecting DNA synthesis and they usually do not kill resting cells unless such cells are destined to divide soon after exposure to the drug. The most important reason for treatment failure is drug resistance. Resistance to drugs occurs in cell populations either because some cells are resistant *de novo* to the agent or agents used or because resistant cell lines develop under the pressure of exposure to drug.

The mechanisms of resistance to anticancer drugs include insufficient drug uptake by the neoplastic cell and increased inactivation of the drug. The lower level of detectability of a mass is when it reaches about 1 cm³ in size; it then weighs about 1 g and contains 10⁹ cancer cells. One cancer cell and its progeny must go through nearly 30 doublings to reach this size; in 10 more doublings the cell number will reach the lethal number, 10¹² cells. Other reasons for drug resistance by cancer cells are decreased requirements for metabolic product, utilization of an alternative biochemical pathway, or rapid cell repair of a drug-induced lesion.

Treatment with a single chemotherapeutic agent is unlikely to produce significant remissions or a cure in patients with cancer. Drug combinations are more effective than single-agent treatments because they provide maximum cell kill within the tolerated range of toxicity, and compensate for acquired or *de novo* drug resistance. One of the most toxic aspects of anticancer drugs is severe marrow hypoplasia, which necessitates prolonged intervals between treatment cycles without considering the risk of tumor regrowth during the increased intervals.

The failure of chemotherapy can be summarized as resistance of tumor cells to therapy and toxic effects on the patient. These problems and others might be solved by lowering the dosage and duration time of chemotherapy through selective use of chemotherapeutic agents. It has been shown (H4) that certain mustards exhibit enhanced reactivities with regions in DNA fragments derived from *H-ras* oncogenes. This finding points to the possibility of the design of alkylating agents to optimize selective reactions with critical DNA regions. An alternative approach presently under investigation has emerged from an understanding of the characteristics of the sequence-specific interactions of the natural oligopeptide antibiotics netropsin and distamycin in the minor groove of DNA. This had led to the synthesis of a novel agent, lexitropsin, in which the binding specificity can be shifted from (AT)_n to (GC)_n in a predictable fashion.

7.3. NEWER METHODS FOR CANCER TREATMENT

Surveillance hypotheses propose that the immune system is capable of controlling malignancy when a small number of malignant cells is present. It can be

stated, however, that there is no conclusive evidence indicating that human tumors, at the time they become clinically apparent, contain surface antigens that can be recognized by the immune system of the tumor-bearing patient. Further, there is no evidence that the immune system develops or can be induced to develop an effective immune response against such tumors. Much of clinical immunotherapy ignores this problem and proceeds on the assumptions that tumor antigens exist and that tumor-bearing patients can make an antitumor immune response. Although neither human tumor antigens nor specific antitumor immune responses have been convincingly demonstrated, there are a number of immune response pathways and effector mechanisms that may be of relevance to immunotherapy. Antibodies can produce antitumor effects by antibody-mediated complement-dependent lysis of malignant cells or antibody-dependent cell-mediated cytotoxicity (ADCC). The effector cells in the case of ADCC are Fc receptor-bearing cells such as macrophages (monocytes) or null lymphocytes (non-T, non-B). Another highly specific immune mechanism for killing tumor cells involves cytotoxic T lymphocytes and a phenomenon termed natural killer (NK) activity. The lineage of NK cells is distinct from that of macrophages and is proposed by some investigators to be of the T cell lineage. The mechanism that permits NK cells and macrophages to discriminate between normal and tumor cells is not known. It has not been established that either of the pathways plays a significant role in the control of malignant cells.

In recent years it has been demonstrated that complex regulatory systems modulate many immune responses; thus even when adequate inducing stimuli are presented, intact effector mechanisms exist. It has been demonstrated that regulatory influences are mainly exerted by suppressor T cells. It has been further demonstrated that concomitant induction of suppressor T cells may be a contributing or permissive factor in tumor induction. Other forms of suppression may be mediated by tumor-specific antibodies either alone or in antigen-antibody complexes. Such antibodies appear to act by interfering with antitumor immune mechanisms having blocking or enhancing effects. Immunotherapy may be designed to activate any one of the effector mechanisms. On one point, all are agreed: if immunotherapy is to succeed, it is essential that the tumor load should first be reduced by surgery, irradiation, or chemotherapy. It is not reasonable to expect the immune system to cope with a large tumor mass; also, the considerable amounts of antigen released by shedding would prevent the generation of a significant response. This leaves the small secondary deposits as the proper target for immunotherapy. The following agents have been tested for immunotherapy:

1. Bacterial preparations, primarily BCG, whether intact, as cell walls, or as extract. These preparations stimulate lymphocytes to release lymphokines, which in turn attract and activate macrophages.
2. Mammalian products, the most important of which is interferon. Interferon

is a 30,000-Da glycoprotein that is secreted by human cells such as lymphocytes and fibroblasts. Interferon augments lymphocyte cytotoxic activity and increases macrophage and NK cell activities. Unfortunately there is no convincing clinical evidence indicating that preparations of interferon are of therapeutic value for any form of human cancer.

3. Synthetic chemicals such as poly(inosinic) poly(cytidilic) acid [poly(IC)] and poly(IC) plus poly(lysine) [poly(ICL)], which are interferon inducers; levamisole also is reported to increase phagocytosis, stimulate lymphoblast transformation, and increase delayed hypersensitivity reactions when their functions are depressed.

Successful immunotherapy depends on reduction of tumor load. It seems logical that this can be more easily accomplished whenever it is feasible to identify precancerous changes such as oncogene activation. Three general methods can be envisioned for blocking oncogene function: (1) the utilization of substances that augment the negative regulatory pathway to antagonize the action of oncogene product; this might induce cell reversion; (2) monoclonal antibodies to growth factors responsible for autocrine stimulation of tumor cells or to transmembrane growth factor receptors; and (3) agents that biochemically inhibit oncogene products such as active-site inhibitors or agents that prevent appropriate localization of the transforming proteins within the cells. A novel approach to the production of antitumor monoclonal antibodies depends on the preparation of monoclonal antibodies to oncogene-related antigens.

Although interferon has not shown significant results in the treatment of cancer, some *in vitro* examples do seem encouraging. Interferon treatment of Rous sarcoma virus-transformed cells (F4) has been reported to result in both reduced synthesis of the *src*-encoded product pp 60 and decreased expression of the transformed phenotype. IFN- α and IFN- β (D1) selectively kill the O6 methyl guanine-deficient human tumor cell line by inactivating the oncogene that allows its repair and growth. It may be concluded that interferon treatment is beneficial in early stages of cell transformation or as a part of a treatment protocol in more advanced cases.

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GASTRIN AND CANCER

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1. Introduction

Gastrin, a well-studied hormone, was first discovered in 1905 as a gastric acid-stimulating factor in extracts of antral mucosa (E1); in 1964 gastrin was purified as a heptadecapeptide (G2, G3). Until the 1970s gastrin was believed to be produced only in antroduodenal G cells (L1), and its main function was assumed to be the regulation of gastric acid secretion. The development of sensitive immunochemical techniques, of efficient methods for peptide purification and sequencing, of recombinant DNA technology, of gastrin-producing cell lines, and of sophisticated physiological models has, however, considerably changed the biological and pathological picture of gastrin during the last decades.

The elaborate biosynthetic pathway of gastrin in its main site of synthesis, the antral G cells, is now well described (B12, H5, J2, S10). The pathway explains the molecular heterogeneity of gastrin in tissue and in circulation (J2, R11, Y1). The structure of the gastrin gene and its widespread but tissue-specific expression and processing in intestinal and other extraantral tissues are now well known (B7, B9, F1, L3, L5, R4, W3, Y4). It has been shown that the most vital function of gastrin

is in mucosal cell growth regulation rather than in gastric acid secretion (J4–J6). Gastrin peptides control the growth of the fundic mucosa and, in addition, significantly influence the growth of colonic mucosa (J4, J6, M3, S4). This trophic effect of gastrin is essential for life, because gastrin maintains the growth of intrinsic factor-producing cells. Humans can live without acid secretion, but not without intrinsic factor and vitamin B₁₂ (J6).

For 20 years the measurement of gastrin has had a small but well-defined place in clinical biochemistry in the diagnosis of pancreatic gastrinomas (J1, T5). The growing recognition of an association between the trophic effect of gastrin and the frequency of carcinomas in the stomach and colon (B11, H10, M3, S4, S5, U1, W1, W2), as well as development of new gastrin assays with improved diagnostic possibilities (B3, H4, S6), suggest that measurement of components of the gastrin system may hold a more central place in cancer diagnosis and therapy. The time is therefore ripe for clinical chemists to consider the gastrin system and its relation to cancer. The present review intends to serve this purpose by an up-to-date description of the biology of the gastrin system and the involvement of gastrin in the development of different forms of cancer. Finally, we will mention new analytical methods for measurement of gastrin in clinical oncology.

2. Definition of the Gastrin System

A prominent feature of bioactive gastrin peptides is the unusually clearly defined active site. All the biological effects of gastrin peptides reside in the common C-terminal tetrapeptide amide (-Trp-Met-Asp-Phe-NH₂). Any modification of this sequence grossly reduces or abolishes binding to its receptors (M4). N-Terminal extensions of the tetrapeptide amide increase the biological activity and the specificity in relation to the homologous cholecystokinin (CCK) peptides. Of particular importance in this respect is the tyrosyl residue in position six as counted from the C-terminal phenylalanyl amide (Fig. 1). Structurally, the gastrins are defined as gastric acid-stimulating peptides having the C-terminal sequence Tyr-X-Trp-Met-Asp-Phe-NH₂, where X in most mammalian species is a glycyl residue.

3. Homology of Human Gastrin with Other Peptides

Most biologically active peptides occur in peptide families whose members display a significant structural homology. The occurrence of peptide families reflects development by gene duplication and subsequent mutations from a single ancestral peptide gene for each family (L2). The homologous structures may cause considerable specificity problems during measurement of the peptides. Moreover,

been elucidated by cDNA deduction of the preprogastrin structure for various mammals (B9, F1, G1, K1, L11, Y4). Comparison of the sequences is quite instructive. It shows how well the active site and sequences around major processing sites are preserved (Fig. 2).

4. Expression and Synthesis of Antral Gastrin

In the normal mammalian organism most gastrin is produced by the antral G cells (L1 and R2). There are also G cells in the proximal duodenum. After antrectomy, duodenal G cells "antralize" and increase their synthesis considerably (B13, N1), but, as mentioned, the normal mammalian organism produces by far most of its gastrin in the antrum. Consequently, gastrin biosynthesis studies have focused on antral tissue (B12, H5, J2, S10). Combination of the results of these studies with general knowledge about peptide hormone synthesis (K3, S2) provides a clear picture of the biosynthetic pathway of antral gastrin (Fig. 3).

After translation of gastrin mRNA in the rough endoplasmatic reticulum (RER) and cotranslational removal of the N-terminal pre- or signal peptide from pre-progastrin, the intact progastrin is transported to the Golgi apparatus. In the trans-Golgi, the first posttranslational modifications occur. These are *O*-sulfation of the tyrosyl residue neighboring the active site, and the first trypsinlike proteolytic cleavages at one monobasic and three dibasic processing sites (Fig. 2). From the trans-Golgi apparatus small vesicles carry the processing intermediates of progastrin toward the basal part of the G cells, where the gastrin peptides are stored in characteristic secretory granules (H1, L3). We assume that the endoproteolytic trypsinlike and exoproteolytic carboxypeptidase E-like processing as well as the subsequent glutamyl cyclization—corresponding to the N-termini of gastrin-34 and gastrin-17 (Figs. 2 and 3)—continue during the transport from the Golgi to the early secretory granules. The last and decisive processing step in the synthesis of gastrins then occurs during storage and maturation in the secretory granules. The membranes of the secretory granules contain the amidation enzyme, peptidyl-glycine α -amidating monooxygenase (PAM) (E2, M6), which removes glyoxylate from the immediate precursors, the glycine-extended gastrins, to complete the synthesis of bioactive α -carboxyamidated peptides (Fig. 3). Amidation of gastrin is the crucial all-or-none activation process, which is carefully controlled (H5, H7, H8, J2). For instance, activation of the amidation enzyme requires copper, oxygen, ascorbic acid, and a pH around 5 (E2, H3).

As a result of the elaborate biosynthetic pathway, the normal antral G cells in humans release a heterogeneous mixture of progastrin products from the mature secretory granules. A small percentage are nonamidated precursors (mainly glycine-extended gastrins), and 98% are α -amidated bioactive gastrins. Of the amidated gastrins, 90% are gastrin-17, 5% are gastrin-34, and the rest is a mixture of

PREPROGASTRIN

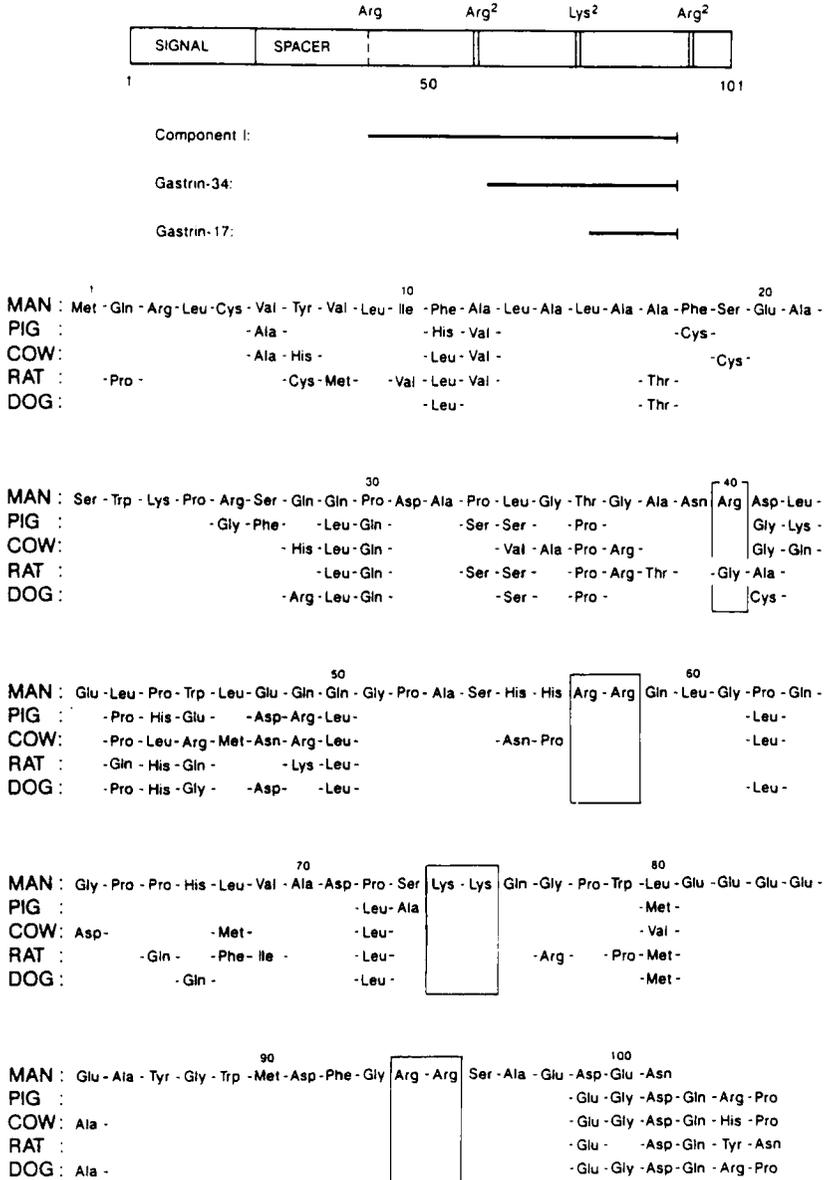


FIG. 2. cDNA-deduced primary structures of mammalian preprogastrins. For porcine, bovine, canine, and rat preprogastrins, only amino acid residues different from those in human preprogastrins are indicated. Presumably, conserved sequences have greater biological significance than do heavily substituted sequences. Note the conservation of the gastrin-17 sequence, and sequences around major processing sites.

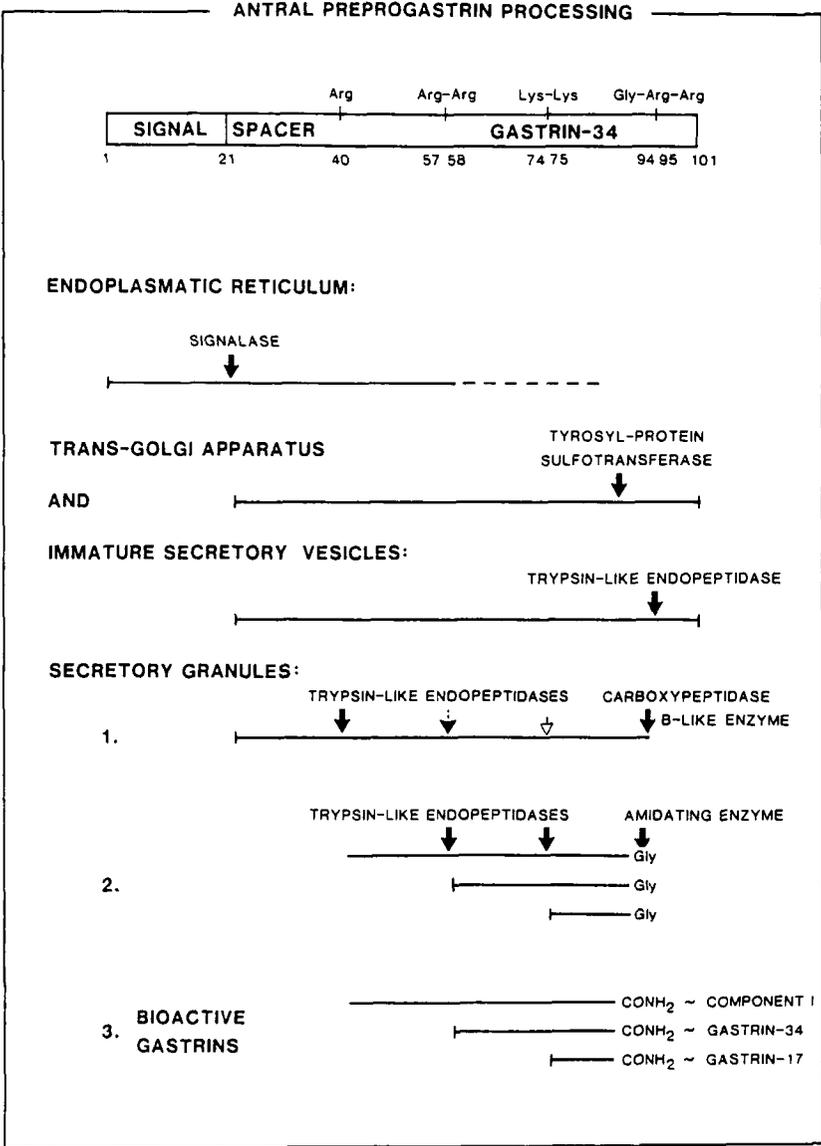


FIG. 3. Scheme of major processing steps for antral preprogastrin to bioactive α -carboxyamidated gastrins. The exact N-terminal cleavage site for gastrin component I is unknown. In addition to component I, gastrin-34, and gastrin-17, the antral G cells also produce small amounts of shorter carboxyamidated peptides such as gastrin-14, -7, -6, and -5.

the long component I of gastrin-14 and of short C-terminal hepta- to tetrapeptide amide fragments (G5, H6, J2, M1, R8, R9). Approximately half of the amidated gastrins are tyrosine sulfated (A1, A2, B12, G3, H5). Due to gross differences of metabolic clearance rates, the distribution of gastrins in peripheral plasma changes so that larger gastrins with long half-lives predominate over gastrin-17 and shorter gastrins (J2).

By increased gastrin synthesis the distribution pattern changes further. Abnormally increased antral synthesis occurs in humans by achlorhydria, as seen, for instance, in pernicious anemia. In antrum-sparing pernicious anemia the translational activity of gastrin mRNA in the G cells seems to be so high that the enzymes responsible for the posttranslational processing of progastrin cannot keep up with the maturation, i.e., the α -carboxyamidation (J2). Consequently, the G cells release more unprocessed and incompletely processed nonamidated progastrin products. Also, the carboxyamidated gastrins are less sulfated (B10) and less cleaved at the N-terminus (J2). Precursors, processing intermediates, and long-chained carboxyamidated gastrins such as component I are cleared from the circulation at a relatively slow rate, and therefore accumulate in plasma, when the synthesis is increased. It follows, therefore, that assays that measure progastrin and its products irrespective of the degree of processing provide a better measure of increased gastrin synthesis than do conventional assays, which recognize only fully processed amidated gastrin (*vide infra*).

5. Expression and Synthesis of Extraantral Gastrin

The gastrin gene is expressed at the peptide level in several other cell types in addition to the antroduodenal G cells. Quantitatively, these other cells contribute very little to the concentration of gastrin in blood. This is partly because the synthesis per cell appears low in comparison with that of the antral G cells, and partly because the secretion seems to serve local purposes rather than a general endocrine purpose by release to the bloodstream. Finally, the biosynthetic processing seems cell specific, i.e., so different from that of the antral G cells that bioactive amidated gastrins may not even be secreted. So far we have encountered expression of progastrin and its products outside antroduodenal mucosa in the TG cell of the small intestine (L3), in endocrine cells in the pancreas (B7, L4), in pituitary corticotrophs and melanotrophs (L4, R1, R4), in oxytocinergic hypothalamo-pituitary neurons (R1, R4, R14), in a few cerebellar neurons (J. F. Rehfeld, unpublished results), in vagal neurons (U2), in the adrenal medulla of some species (J. F. Rehfeld, unpublished results), in the bronchial mucosa (R12), and in spermatogenic cells (S1). As shown in Table 1, the concentrations in the extra-antral tissues are far below that of the antral "main factory."

The function and meaning of gastrin synthesized outside the antroduodenal

TABLE 1
 EXPRESSION OF PROGASTRIN AND ITS PRODUCTS
 IN NORMAL MAMMALIAN TISSUE

Tissue	Total concentration (pmol/g, wet weight)	Progastrin and processing intermediates (% of total)
Antral mucosa	10,000	2
Duodenal mucosa	400	—
Jejunal mucosa	40	—
Ileal mucosa	20	85
Pancreas	2	95
Adenohypophysis	200	98
Neurohypophysis	30	5
Vagal nerve	8	10
Cerebellum	5	20
Adrenal medulla	2	100
Bronchial mucosa	0.3	100
Testicles	6	100
Spermatozoa	2	55

mucosa are largely unknown, but there are several suggestions. First, a most likely explanation includes paracrine or autocrine regulation of growth (Fig. 4). Second, it is possible that the low cellular concentration of peptides is without function in the adult, but reflects a more comprehensive fetal synthesis (B7). Finally, it is also possible that the low cellular concentration is due to constitutive secretion rather than regulated secretion via secretory granules (K3). If so, there is neither storage nor concentration of peptides in the cells in spite of a considerable release of gastrin peptides, per time unit, from the cells.

Although it is possible that the extraantral expression of the gastrin gene is without significant function in the normal adult human organism, recognition of the phenomenon has major clinical relevance. Thus, as described in the following text, tumors originating from tissues and cells that normally express the gastrin gene even at low level may produce gastrin in lethal carcinogenic amounts.

6. Modes of Secretion of Gastrin

In order to understand the normal trophic and possible carcinogenic effects of gastrin, it is necessary to realize that the different types of cells in which the gastrin gene is expressed also secrete gastrin peptides in different ways (Fig. 4). For a long time gastrin was thought to be secreted only as it occurs in the endocrine cells, i.e.,

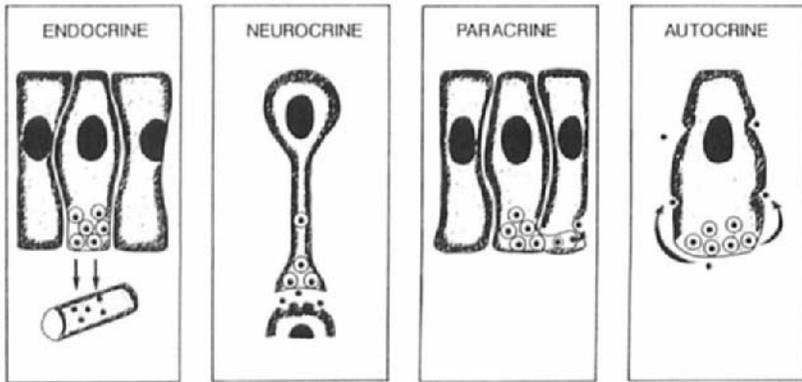


FIG. 4. Four routes of cellular secretion of gastrin. Several other regulatory systems, wherein the same peptide acts as hormone, neurotransmitter, and growth factor, are also released as they occur in these four cell types. Autocrine and paracrine secretion are assumed to play decisive roles for the growth of malignantly transformed cells.

secretory granules from the endocrine G cells in the antroduodenal mucosa empty their peptides into surrounding capillaries following specific stimulations of the G cells.

In contrast to the classical systemic release of gastrin into the circulation from endocrine cells (Fig. 4, first box), three alternative routes of secretion to neighboring cells or to the secretory cell itself have been discovered during the last 15 years. First, the gastrins synthesized in neurons are released from synaptosomal vesicles in the nerve terminal to the receptors of adjacent target cells, i.e., neurotransmitter or neurocrine release (Fig. 4, second box). It is possible that a spillover of neuronally released gastrin may be transported via the bloodstream, in analogy with other neuropeptides. It is also possible that some gastrinergic neurons, for instance the hypothalamo-pituitary neurons, release the peptides directly into blood vessels. Second, in the wake of the discovery of the morphological substrate for paracrine secretion, i.e., short cytoplasmic processes to neighboring cells (L6), it has been shown that there are gastrin-producing paracrine cells in the small intestinal mucosa (L1). These cells carry granules containing gastrin through the cytoplasmic extensions to their specific target cells in the neighboring area. Paracrine cells are hybrids between classical endocrine cells and neurons (Fig. 4, third box). It is consequently possible that a local spillover of gastrin from paracrine cells may also reach the circulation.

The most recently discovered means of secretion is autocrine release (C3, H9, S8), by which cells stimulate their own growth. Trophic peptides bind to specific receptors in the membranes of their own cells (Fig. 4, last box). Autocrine secretion is supposed to play a decisive role in tumor and cancer development. The first

example was small cell bronchogenic cancers, which could be shown to be stimulated by autocrine secretion of gastrin-releasing polypeptide (GRP) (C3, L10). Recent evidence suggests that the growth of cultured colon carcinoma cells (H9, H10) and gastric carcinoma cells (W2) is stimulated by autocrine secretion of gastrin.

7. Expression of Gastrin in Tumors

In 1955 Zollinger and Ellison described a syndrome consisting of severe duodenal ulcer disease, gastric acid hypersecretion, and endocrine tumors in the pancreas (Z1). The syndrome was subsequently shown to be due to hypersecretion of gastrin from pancreatic tumors (G4), which are now called gastrinomas. Although by far most gastrinomas originate in the pancreas, gastrin-producing tumors associated with the Zollinger–Ellison syndrome have also been found in the duodenum and rarely in the antrum and ovaries (H2, L7, P1, T6). Some pancreatic gastrinomas are mixed and also contain, in addition to G cells, cells that produce pancreatic hormones such as insulin, glucagon, pancreatic polypeptide (PP), vasoactive intestinal polypeptide (VIP), and somatostatin (L8). Furthermore, neoplastic G cells may also express peptides encoded by genes for nonpancreatic hormones, especially the proopiomelanocortin (POMC) gene (M2). The Zollinger–Ellison, or gastrinoma, syndrome occurs with a frequency of one new case per million inhabitants of Denmark per year (J1). However, gastrinomas should be suspected among patients with more common severe duodenal ulcer disease and/or diarrhea, thus the requirement for gastrin measurements is significant. In Denmark approximately 400 measurements are required per million inhabitants per year (J1). It is important to diagnose and localize gastrinomas at an early stage, even when the symptoms are relatively mild and transient, because gastrinomas—although often slow growing—are malignant (Z2).

Gastrinomas are the most frequently occurring endocrine tumors in the pancreas (J1). Insulinomas are found slightly less frequently. In contrast, only a few cases of pancreatic glucagonomas, somatostatinomas, VIPomas, and PPomas have been encountered per 5 million among the Danish population during the last decade, in spite of an intensive search. Insulin, glucagon, somatostatin, PP, and VIP are, however, synthesized in the normal human pancreas, where gastrin hitherto could not be detected. This so-called gastrinoma enigma has now been solved by the recent demonstration of low-level expression of progastrin, which is processed only poorly to bioactive gastrin in the adult human pancreas (B7). Consequently, pancreatic gastrinomas cannot be considered ectopic.

Gastrinomas are tumors predominated by G cells and associated with the dramatic symptoms of the Zollinger–Ellison syndrome, which again are caused by

long-lasting and often gross hypergastrinemia. The gastrin gene is, however, also expressed at the peptide level in an array of other tumors and carcinomas, but these carcinomas are without symptoms of the Zollinger–Ellison syndrome and hypergastrinemia. So far we have found expression of gastrin peptides in some brain tumors [acoustic neuromas (R13)], in pituitary adenomas [Nelson and Cushing tumors (B5)], in pheochromocytomas (B6), in bronchogenic carcinomas (R12), and recently also in some colorectal carcinomas (preliminary results). There are several factors that explain the lack of hypergastrinemia and associated symptoms of the Zollinger–Ellison syndrome in all these tumors. First, the level of expression is often so low that gastrins released from the tumors do not contribute significantly to the concentration of gastrin in plasma (Table 2). Second, often only a small fraction of the progastrin is processed to bioactive gastrins. However, as shown in Table 2, the degree of processing varies considerably for each individual tumor. Third, the tumors may synthesize other peptides or substances that interfere with and inhibit gastric acid secretion. However, even though the secretion of gastrin from brain, lung, and colonic tumors is too small to increase the concentration of bioactive, amidated gastrins in plasma significantly, the expression of gastrin may have major carcinogenic significance. Local (autocrine) secretion stimulates growth of tumor cells equipped with receptors for gastrin (B8, H9, H10, I1, S3, W2, Y3). Moreover, it is possible—in some instances even likely—that the release of inactive precursors may contribute to the concentrations of progastrin in plasma. Hence progastrin may serve as a tumor marker (B2, H4, R12, S6) (see Table 3). Progastrin concentrations in plasma even show promise as a prognostic indicator of the degree of malignancy of gastrinomas (B2) (Tables 4 and 5).

TABLE 2
EXPRESSION OF PROGASTRIN AND ITS PRODUCTS IN HUMAN
NEOPLASTIC TISSUE

Tissue	Total concentration range (pmol/g, wet weight)	Progastrin and processing intermediates (% of total)
Pancreatic gastrinomas	400–2,500,000	15–90
Acoustic neuromas	0.2–10	10–100
Pituitary adenomas	0.5–200	20–90
Bronchogenic carcinomas	0.2–20	25–100
Pheochromocytomas	0.2–400	90–100
Colorectal carcinomas	0.2–50	20–100

TABLE 3
CONCENTRATIONS OF α -AMIDATED GASTRINS AND THE TOTAL PROGASTRIN PRODUCTS IN SERUM FROM
NORMAL SUBJECTS, PATIENTS WITH DUODENAL ULCER, AND PATIENTS WITH GASTRINOMA^a

Source	α -Amidated gastrins	Total progastrin products	α -Amidated (% of total)
Normal ($n = 34$)	25 (5-69)	26 (10-92)	87 (27-160)
Duodenal ulcer ($n = 42$)	18 (7-84)	48 (16-106)	39 (15-130)
Gastrinomas ($n = 48$)	390 (24-17,200)	785 (76-84,000)	46 (16-100)

^aValues (medians and ranges) are given in picomoles/liter. The data are from Ref. B2, courtesy of Dr. L. Bardram.

8. Gastrin as Tumor Growth Factor

It is well known that breast cancers can be treated by endocrine manipulation, when receptors for estrogens (which stimulate growth of the normal breast) are present in the tumor. Gastrin has trophic effects on the normal gastric and colorectal mucosae (J4, J6), and several normal gastric and apparently colorectal mucosal cells are equipped with gastrin receptors (B1, S7). By analogy with breast cancers it is consequently possible that the growth of some gastric and colorectal carcinomas is influenced by gastrin. Accordingly, recent studies have indicated that gastrin accelerates the growth of many gastric and colorectal carcinomas (B8, B11, I1, K4, M3, S3-S5, W1).

Different types of studies have been performed to elucidate the effect of gastrin on tumor growth. First, the effect of exogenously administered gastrin has been examined by evaluating the *in vitro* effect either on tumor cell lines, on the growth of tumor cells transplanted into suitable animal models, or on the development of malignant changes induced *in vitro* by carcinogens (B8, I1, K4, M5, S3-S5, S11, T1, T4, W1, W2). Second, some studies have examined the effect of manipulation of endogenous gastrin secretion on the growth of transplanted tumors or on the

TABLE 4
CONCENTRATIONS OF α -AMIDATED GASTRINS IN SERUM AS PERCENTAGES OF TOTAL
PROGASTRIN PRODUCT FROM PATIENTS WITH MALIGNANT AND BENIGN GASTRINOMAS^a

Source	Median (%)	Range (%)
All patients ($n = 48$)	46	16-100
Patients with hepatic metastases ($n = 10$)	23	16-49
Patients with lymph node metastases ($n = 5$)	39	25-66
Patients without metastases ($n = 33$)	54	17-100

^aData from Ref. B2, courtesy of Dr. L. Bardram.

TABLE 5
CONCENTRATIONS OF α -AMIDATED GASTRINS AND THE TOTAL PROGASTRIN PRODUCT IN SERUM
FROM A GASTRINOMA PATIENT AT DIFFERENT CLINICAL STAGES^a

Clinical stage	α -Amidated gastrins	Total progastrin products	α -Amidated (% of total)
Zollinger–Ellison syndrome suspected	27	276	10
3 years later, no tumor found	60	642	9
4 years later, after resection of pancreatic tumor	100	75	13
6 years later, recurrence with hepatic metastases	1500	7200	21

^aData from Ref. B2, courtesy of Dr. L. Bardram.

development of tumoral changes by carcinogens (K2, M3, T2–T4). Changes of endogenous gastrin secretion and plasma concentration have been achieved by feeding of specific nutrients or by surgical interventions on the stomach and/or the small bowel. Third, the effect of growth-inhibiting hormones such as somatostatin and secretin on tumor development in the models mentioned above has also been studied (E3, K4, M5, T1, Y4). Fourth, the effect of specific gastrin receptor antagonists has been examined in the various models (B8, H9, H10, I1). Finally, the occurrence and biochemical nature of gastrin receptors on cancers have been characterized (C2, H10, S3, U1, W2).

Several studies have shown that gastrin stimulates the growth of rat and human gastric cancer cells *in vitro* and promotes the growth of rat and human gastric cancer transplanted into the nude mouse (K4, M5, S11, Y2). Less convincing are the reports on the effect of gastrin on gastric carcinogenesis induced by carcinogens. It has been reported (T1) that administration of gastrin in rats increased the growth of scirrhus gastric cancer produced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). Others, however, were unable to demonstrate such an effect of gastrin in the same model (D1, T3). A later report (T2) emphasized, however, that the timing of gastrin administration is of importance and this may explain earlier discrepancies.

Gastrin seems to promote the *in vitro* growth not only of gastric cancer, but also of some colorectal cancers in man and rat. Furthermore, exogenous gastrin stimulates growth of transplanted mouse and rat colon cancer in mice (B8, M3, S4, W1, W4). The stimulation of colon carcinomas is accompanied by decreased survival due to accelerated tumor growth (W4). Studies on the effect of gastrin on the growth of colon cancer induced by carcinogens are conflicting. One study found increased tumor concentration of DNA, RNA, and protein in gastrin-treated rats after previous tumor induction with methylazoxymethanol (M3), whereas

another group (T4) could not show that gastrin potentiates the growth of colon cancer induced by intrarectal administration of MNNG. Endogenous hypergastrinemia, after antral exclusion or small bowel resection in rats, has been shown to increase DNA synthesis in colon tumors or increase the incidence of tumors in carcinogen-treated rats (E3, K2).

It has recently been shown that long-term hypergastrinemia induces development of carcinoid tumors in the stomach in rats (C1). Because these tumors arise from entero-chromaffin-like (ECL) cells in the gastric body, they are also named ECLomas. The marked hypergastrinemia in these experiments was secondary to achlorhydria induced by long-term administration of high doses of the antisecretory drug, omeprazole. The development of gastric carcinoids was preceded and accompanied by ECL cell hyperplasia. When hypergastrinemia was prevented by antrectomy, omeprazole induced neither ECL cell hyperplasia nor development of carcinoid tumors (S12). In this respect it is interesting to note that the occurrences of ECL cell hyperplasia and gastric carcinoids are extremely rare in normogastrinemic subjects, but occur more regularly in hypergastrinemic patients with pernicious anemia (B11, L9). Moreover, the degree of hypergastrinemia correlates with the degree of ECL cell proliferation (B10).

The overall impression of the many studies reviewed above is that of a likely association between gastrin and carcinomas in the gastric and colorectal mucosae. The association is based on the normal trophic effect of gastrin. However, there has apparently been some confusion regarding the route by which gastrin reaches and stimulates the target cells. Critical evaluation suggests that the gastric carcinomas originate and are stimulated by the circulating gastrins from a hypersecretory antral mucosa, which releases gastrin via the classical endocrine route into the bloodstream. In contrast, the colorectal carcinomas—if influenced by gastrin—are more likely to be influenced by locally released gastrin, i.e., by autocrine and/or paracrine secretion. Understanding the route by which gastrin acts is important both for the conception of the basic carcinogenic mechanisms and for the analytical approach for improving diagnosis and prevention (*vide infra*).

Regarding the cellular molecular mechanisms by which gastrin acts as tumor growth factor, we are still essentially ignorant. Studies of the structure and expression of the gastrin receptor, for instance, and its possible encoding by an oncogene, as well as signal transduction mechanisms in gastrin-sensitive cells, are urgently needed.

9. Requirement for Gastrin Measurements in Clinical Oncology

As already mentioned, measurement of gastrin in plasma has so far had a minor but well-defined place in clinical biochemistry in the diagnosis, localization, and control of the therapy of gastrinomas associated with the Zollinger–Ellison syn-

drome. In order to comply with this purpose, approximately 400 measurements per million people per year have been necessary (J1).

A new indication for gastrin measurements is in the monitoring of treatment of duodenal ulcer patients with omeprazole, as well as with histamine H₂-receptor antagonists (C1, S12). Hypergastrinemia induced by long-term treatment with these drugs—or by nature's own experiment, chronic achlorhydria—involves, as mentioned, a risk of developing gastric malignancies. Until the size of this risk has been evaluated, control of gastrin concentrations in plasma has been advocated. Currently, therefore, there is a substantial demand for gastrin measurements in trials of antiulcer drugs.

In the foreseeable future, however, the requirement for gastrin measurements in clinical oncology may increase even further. As already discussed, gastrin peptides are possible involved in the development and growth of colorectal and other common carcinomas. Colorectal cancers are the most frequently occurring malignant tumors in the western world. A significant percentage seem to have gastrin receptors and to be regulated by autocrine gastrin secretion (H9, H10). Consequently, it is possible that selection of treatment of colorectal carcinomas requires classification with regard to gastrin sensitivity. In other words, we have to decide which tumors synthesize gastrin and/or has gastrin receptors by performing gastrin measurements on extracts of tumor biopsies. It is unlikely that measurement of plasma gastrin concentrations would provide guidance, in spite of the recently described increased plasma gastrin concentration in patients with colorectal cancers (S5). Even if it holds true that plasma gastrin concentrations are increased by carcinomas in the colon, the increase is likely to be due mainly to secondary antroduodenal disturbances. Direct measurements of the concentrations of gastrins, progastrins, or gastrin receptors in extracts of tumor biopsies are probably the most useful parameters.

Although colorectal cancers are immediate targets for application of gastrin or gastrin receptor measurements, there are major cancers in other tissues, such as the stomach, the respiratory tract, and the mammary glands, in which gastrin also may be involved in an autocrine manner (H9, R12). Thus proper oncological diagnosis and treatment may in the near future require that clinical chemistry takes a serious and comprehensive interest in the refined measurement of components of the gastrin system of peptides and receptors.

10. Methods for Measurement of Gastrin

If gastrin measurements in plasma or tissue extracts are to gain wider use as tumor markers, conventional gastrin assays are not ideal. Conventional assays are radioimmunoassays of the principal antral form of gastrin, carboxyamidated gastrin-17. Gastrin-17 has been commercially available for many years. Moreover, it

has long been easy to develop highly sensitive and specific radioimmunoassays for amidated gastrins (R5, R10, S9). The problem is, however, that “sick” gastrin-producing cells process progastrin less well—irrespective of whether they are cancer cells or endocrine G cells in duodenal ulcer patients. In other words, neoplastic gastrin-producing cells release more unprocessed or poorly processed precursor products. These products are not carboxyamidated and consequently are not recognized by the conventional gastrin assays. Therefore, in order to use measurements of the gastrin system as markers of gastrinomas as well as other gastrin-producing carcinomas, it is—as mentioned earlier—necessary to develop assays that measure the entire translation product of gastrin mRNA, independent of the degree of posttranslational processing (B2).

We have developed a library of new assays that recognize glycine-extended processing intermediates (H4), unprocessed precursors in the form of glycine-

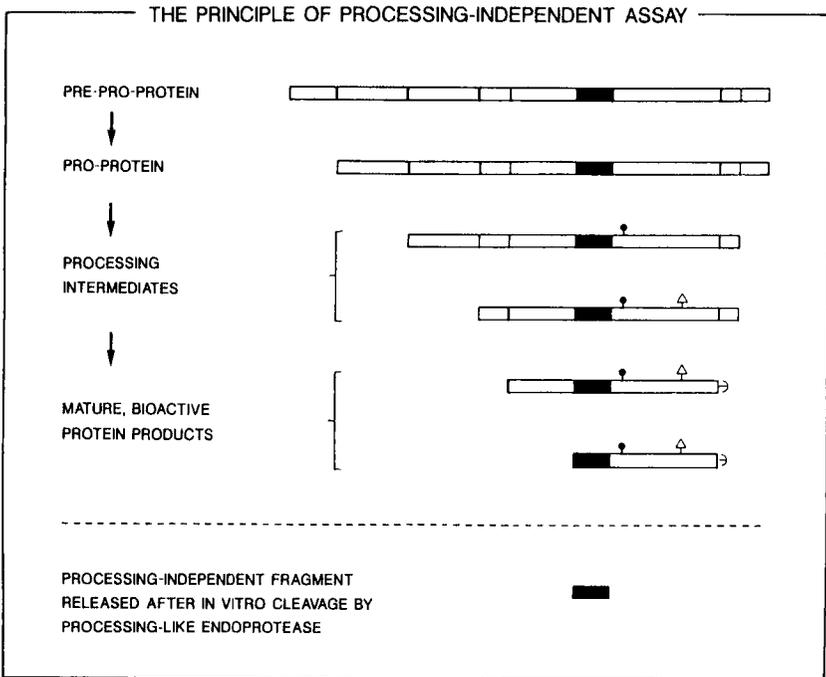


FIG. 5. The principle of processing-independent immunoassays. The scheme illustrates the processing from translation of pre-pro-protein in the endoplasmatic reticulum, via intermediates in the Golgi apparatus and early secretory vesicles, to final maturation (see also Fig. 3). Divisions within the protein bars indicate proteolytic cleavage sites. The protuberances on the tops and ends of the bars illustrate different forms of amino acid derivativization. The filled-in areas of the protein bars are the selected processing-independent sequences, toward which the processing-independent immunoassay is developed.

extended gastrins after trypsin and carboxypeptidase B measurements (H4), or the total translation product, irrespective of the degree of processing (B3, S6). Preliminary results indicate that the latter type of assay can be performed in a simple manner and at the same time considerably improve the diagnostic specificity for tumors (B2, R7, S6). The principle of the assay is illustrated in Fig. 5. The processing-independent assays (PIAs) have been developed by production of

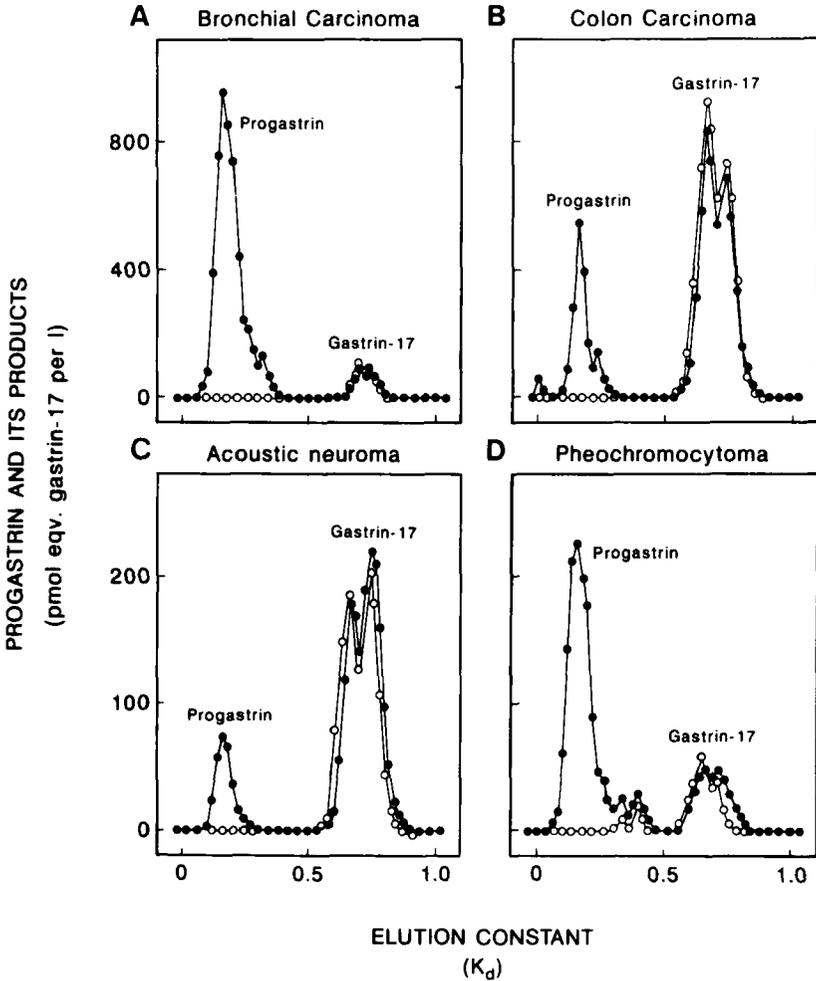


FIG. 6. Gel chromatography (Sephadex G-50 Superfine) of extracts of four different carcinomas: (A) squamous cell bronchogenic carcinoma; (B) colonic adenocarcinoma; (C) acoustic neuroma; (D) pheochromocytoma. The chromatograms show the elution of gastrin peptides as measured using a conventional radioimmunoassay directed against bioactive carboxyamidated gastrins or using a processing-independent radioimmunoassay specific for sequence 55-66 of human progastrin.

high-avidity antibodies, which are monospecific for a suitable progastrin sequence (R7). The sequence has to be located immediately C-terminal to a trypsin-sensitive cleavage site (Figs. 2 and 5). Monospecificity of the antibodies is ensured by immunization with a short synthetic deca- to pentadecapeptide hapten appropriately coupled at its C-terminus to a carrier (B3, B4, S6). A monoiodinated tracer is prepared by labeling a naturally occurring or a synthetically coupled tyrosyl residue in a position C-terminal to the antibody-bound sequence. By incubation of the samples with trypsin prior to measurement, the selected epitope on the precursor is invariably exposed to binding of the antibodies. Moreover, the tryptic cleavage ensures that the peptide fragment to be measured always has the same

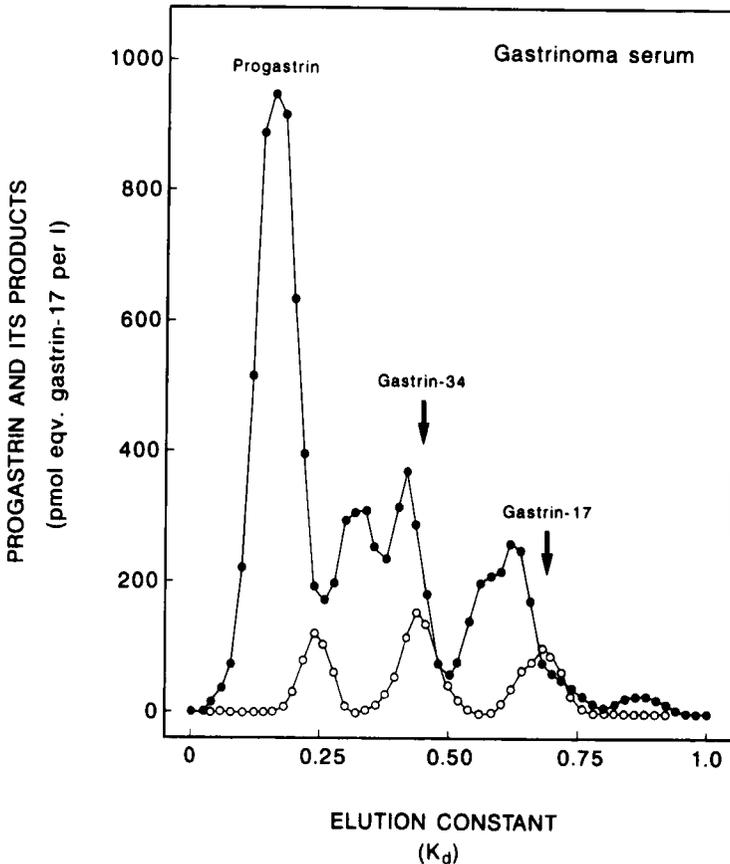


Fig. 7. Gel chromatography (Sephadex G-50 Superfine) of serum from a gastrinoma patient. The chromatogram shows the elution of gastrin peptides as measured using a conventional radioimmunoassay directed against bioactive carboxyamidated gastrins or using a processing-independent radioimmunoassay specific for sequence 55-66 of human progastrin.

size, i.e., the minimal tryptic fragment. Using such a fragment as standard, the trypsin treatment therefore ensures optimal accuracy, because the substance to be measured always corresponds to the standard. As illustrated in Fig. 5, the described approach ensures that the entire translation product is quantitated accurately, irrespective of the degree of processing. In other words, stoichiometrically, one peptide fragment is measured per translated progastrin molecule.

We believe that processing-independent assays may improve early diagnosis of gastrin-producing tumors substantially (R7). For classical pancreatic gastrinomas, evidence of the diagnostic superiority of the PIA is already accumulating (B2). As shown in Tables 2–5 and in Figs. 6 and 7, inclusion of progastrin measurements provides a much better impression of gastrin synthesis in gastrinomas and other and major forms of cancer. Presumably, PIAs may therefore prove helpful in diagnosis and treatment of gastrin-sensitive carcinomas in the stomach, colon, rectum, and respiratory tract.

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A BIOCHEMICAL APPROACH TO RENAL STONE FORMATION

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1. Introduction

The occurrence of renal stone formation might well date back to early man. The oldest urolith ever recovered, from an Egyptian grave, has been estimated as 6000 years old. Although stone formation seems to have been common during medieval times, surgical treatment, performed by the "stone cutters," was difficult if not deadly. Bladder stone formation, as opposed to upper urinary tract stone formation, appears to have been the dominant form of disease during ancient times; the last century has seen a shift in dominance.

Endemic bladder stone disease still occurs in the western population, but a clear relationship exists between a decreasing incidence of bladder stone development and growing prosperity (B1). An increase in the incidence of upper urinary tract stone formation has been seen during recent decades. The diet of an affluent lifestyle induces metabolic changes that increase the risk of calcium-containing stones forming in the urinary tract. Because protein-rich food is more readily available to affluent persons, their urinary excretion of calcium, oxalate, and urate is increased whereas urinary pH and excretion of citrate are reduced (B1). The

typical kidney stone patient would be a middle aged, slightly overweight male who is moderately hypertensive and overstressed in a sedentary occupation (B1). Males have a 4:1 greater risk than females of developing stones. A risk factor model of calcium stone urolithiasis is summarized in Fig. 1.

1.1. DEFINITIONS

Urinary stones are the final product and chief symptom of a many-faceted disease of multifactorial etiology and therefore various definitions of the stones exist. From the mineralogist's viewpoint a stone is merely a solid aggregate of complex composition, precipitated from a supersaturated solution (S4). A more effective definition for urinary calculi is that uroliths are solid structures that arise from disturbances of the physiochemical balance and/or the hydrodynamic system of urine and the urinary tract, from the collecting system down to the urethra. These structures have a minimal size of 1000 μm and consist mainly of crystalline and (to a lesser degree) amorphous organic and/or inorganic components, which may be mixed with a noncrystalline, high-molecular-weight substance (matrix).

The reoccurrence of urolith formation following a primary episode is referred to as recurrent stone disease, irrespective of the stone's composition or localization, or the time interval following the first stone episode. This term does not include continuous crystalluria without clinically manifest concrements (S3).

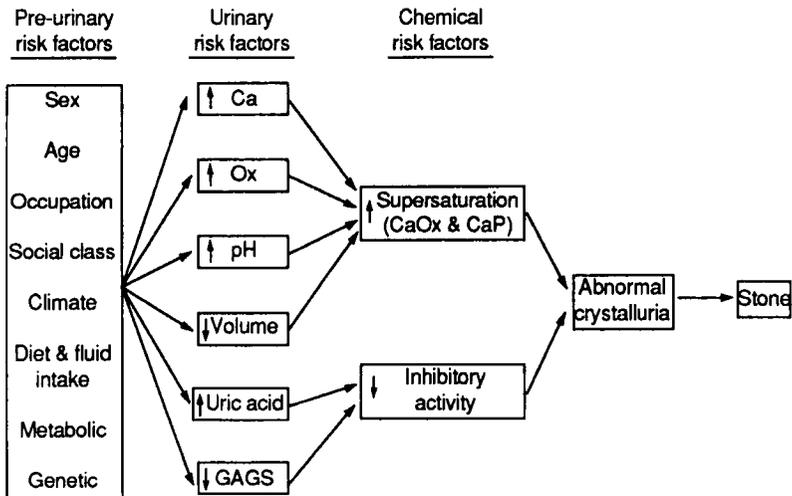


FIG. 1. A risk factor model of calcium stone formation; GAGS, glycosaminoglycans (R2).

1.2. KIDNEY STONE TYPES

More than 30 different types of renal stones have already been identified (S4). The most common types are illustrated in Table 1. Because most renal stones consist of calcium-containing concrements, and especially oxalate (72.4%) (S4), the primary focus of this discussion will be on the formation of calcium oxalate-containing stones.

1.3. THEORIES OF STONE FORMATION

Three main theories exist concerning stone formation in the urinary tract, namely, the precipitation theory, the theory of the lack of inhibitors, and the matrix theory.

According to the precipitation theory, the urine becomes supersaturated with salts, which finally results in the precipitation of crystals that grow and form stones in the urinary tract (Fig. 2). The urinary pH may play an important role in this process. For example, normal urine specimens are supersaturated with uric acid at a pH below 5.5–5.7 (S5). At higher pH values, the solubility increases remarkably. At a pH of approximately 7, the solubility would increase about 10 times. However, a low pH cannot be the sole explanation for uric acid lithiasis, because comparably low urinary pH values are found in healthy subjects (F2). This leads to the second theory on urolithiasis, namely, the theory of the lack of inhibitors.

Inhibitors in the urine prevent the crystallization process—aggregation and growth of crystals (Fig. 2). Two types of inhibitor action mechanisms can be found. Citrate and magnesium, which belong to type one, form soluble complexes with calcium and oxalate, respectively (B1). They then reduce the ionic activity product by complex formation. Compounds such as citrate, pyrophosphate, and glycosaminoglycans belong to the second type of inhibitor. They affect the formation, growth, and aggregation of crystals. However, no significant daily excretion of glycosaminoglycans was initially found among normal subjects and pa-

TABLE 1
THE FREQUENCY OF DIFFERENT TYPES OF KIDNEY STONES^a

Type of stone	Percentage
Calcium oxalate, calcium phosphate, brushite, hydroxyapatite, carbonate, and apatite	80–90
Magnesium and ammonium phosphate	5
Uric acid	3–4
Cystine	0.5–1

^aFrom Backman (B1).

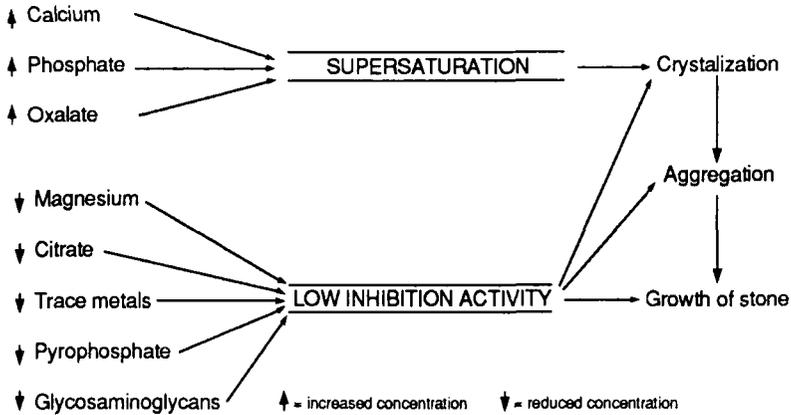


FIG. 2. Schematic model illustrating the possible formation of calcium-containing kidney stones (D1).

tients (S4), but lately a decrease in stone patients has been demonstrated (M3). The relative significance of these inhibitors in crystallization is still not clear.

The last theory on stone formation comprises the matrix theory in which proteins may play an important role in urolithiasis (F1). This theory is based on analyses of many stones, which revealed that the core of these stones contained protein. It had been shown *in vitro* that certain proteins bind calcium and even induce the calcification process (R1). These proteins, also called promoters, were therefore considered to be able to activate the initial crystallization process. However, these results could not be verified (F1). Recently it has been shown that *in vitro* calcium oxalate crystals do contain protein and that the crystallization in urine is not a random event, but rather a selective phenomenon (M4). This supports an earlier statement that stones contain about 1.6% of their weight in nondialyzable extractable protein and that the composition is the same for all stones, regardless of their mineral composition (S7).

The question remains as to which of these three theories is correct. We consider all of them to be applicable, in this multifactorial disease. However, we limit our further discussions to the matrix theory, as it appears to form the basis of urolithiasis.

2. Matrix Theory

2.1. GLYCOPROTEINS AND PROTEOGLYCANS

2.1.1. Composition

Almost all human plasma proteins, except albumin, are glycoproteins (mucoproteins) (M4). Both glycoproteins and proteoglycans are macromolecules con-

sisting of proteins to which oligosaccharide or polysaccharide chains are covalently attached, respectively (M5, S6). A typical glycoprotein contains one or minimal oligosaccharides linked to asparagine side chains of the protein by *N*-glycosidic bonds. Although both glycoproteins and proteoglycans contain *D*-glucosamine or *D*-galactosamine units, uronic acids, such as *L*-glucuronic acid and *L*-iduronic acid, are only present in proteoglycans (M2, M5). In contrast to glycoproteins, proteoglycans contain glycosaminoglycan chains (mucopolysaccharides), to which the core proteins are noncovalently bound at intervals (Fig. 3).

2.1.2. Urinary Glycoproteins

The most well-known urinary glycoprotein is the Tamm–Horsfall mucoprotein (THM). It is argued that the THM and uromucoid are both artifacts from the same native precursor and differ only in their sialic acid content (K1). They are similar immunologically (K1). The molecular mass of THM is 7×10^6 Da (T1), with subunits of about 100,000 Da (F3). THM is synthesized by the cells of the thick ascending limb of Henle in the nephron (L1, R3).

An interesting characteristic of THM is its capacity for gel formation (R3), which agrees with van Hemsbach's view on urolithiasis (V3). This view states that the first step in the pathogenesis of urolithiasis is the precipitation of a mucoid organic matrix followed by mineral deposition. However, much controversy sur-

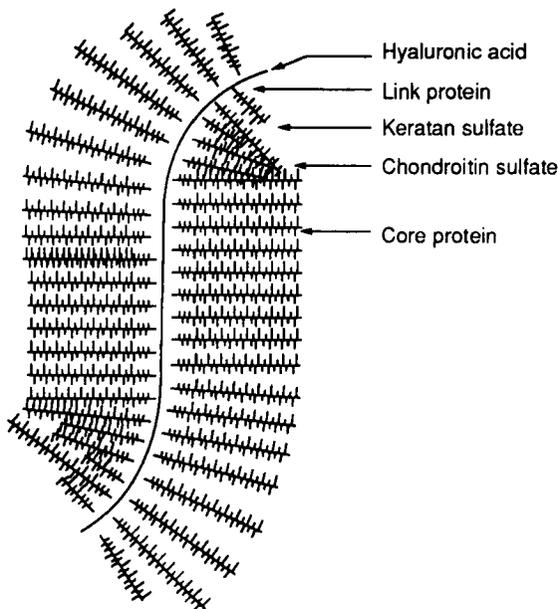


FIG. 3. Schematic diagram of a proteoglycan aggregate (S6).

rounds the possibility that urinary macromolecules consequently promote stone formation (D2, R4, W1).

Another controversy surrounds the amount of glycoprotein (mucoprotein) excreted in the urine of adults with and without stones. Some reports showed that there is no difference in the excretion of uromuoids (B2, S1, S2), whereas others found that the excretion of uromuoid-rich material in calcium stone patients was greater than in their healthy counterparts (B3, K2, M5). This controversy may be attributed to different methods of urine storage, because it has been reported that the physical properties of the mucoproteins are altered by freezing (B3). Nevertheless, urine of black persons, who seldom develop stones, contains no uromuoid (K1, M1).

2.2. URINARY ENZYMES AND UROLITHIASIS

The concentration of urinary glycoproteins may play an important role in stone formation. Therefore, the level and activity of urinary proteases, for example, urokinase and plasmin, might be of great significance. According to this hypothesis, low activities or decreased production of urinary urokinase or plasmin may increase the urinary uromuoid concentrations, as well as stone formation. Increased levels of a urokinase inhibitor, namely urinary trypsin inhibitor (67,000 Da) and decreased urokinase activity in the urine of stone formers are well established (T2). Urinary urokinase activity was therefore studied, as well as the desialylation of urinary glycoproteins. (Fig. 4).

2.2.1. Urokinase

Urokinase is a trypsinlike protease. The inactive single-chain urokinase-type plasminogen activator (high-molecular-weight urokinase, or HMW-UK) has a

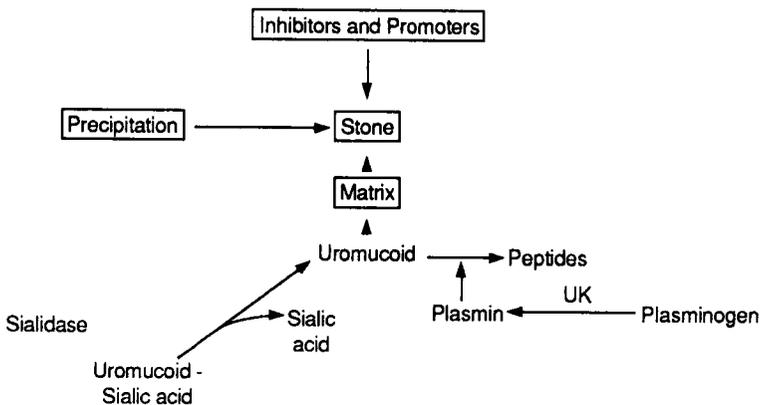


FIG. 4. The possible role of urinary enzymes in urolithiasis; UK, urokinase.

molecular mass of about 54,000 Da (C1, H2) and can be converted by plasmin to an active double-chain urokinase-type plasminogen activator (C1, H2, L2) with the same molecular mass (H2, W2). These two single-polypeptide chains are connected by disulfide bridges (C1, L2). The degradation products of the double-chain type are two low-molecular-weight urokinase (LMW-UK) polypeptide chains of about 34,000 and 20,000 Da (C1). Only the 34,000-Da chain is active (C1).

Spectrophotometric analysis of urinary inhibition on the active forms (HMW-UK and LMW-UK) revealed a significant difference between subjects with and without renal calculi (V1). A positive correlation exists between the percentage of inhibition and the urinary urate concentration. Urate inhibits both the LMW-UK and the HMW-UK, but not plasmin. These results are in perfect agreement with reports that stone patients have higher urinary urate concentrations (C2). These observations may explain why allopurinol is administered to kidney stone patients. Allopurinol causes a decrease in urinary urate excretion by inhibiting the xanthine oxidase, which could cause a higher urinary urokinase activity.

Because age and sex are risk factors of urolithiasis (Fig. 1), radioimmunoassays on males of different ages show that the total urinary testosterone concentrations in stone patients are significantly lower than those in their healthy counterparts (V2). A positive correlation also exists between the total urinary testosterone concentrations and the activity of urokinase (LMW-UK). Steroids such as estradiol, catecholestrogens, and dihydrotestosterone inhibit the urokinase activity *in vitro* at concentrations higher than 10^{-5} M (C. H. van Aswegen and D. J. du Plessis, unpublished data). It therefore seems that the activity of urokinase plays a role in the complex process of stone formation by regulating the concentration of glycoproteins present in urine.

2.2.2. Neuraminidase (Sialidase)

In contrast to urinary uromucoid, which consists of about 9% sialic acid (B3), stone uromucoid contains no sialic acid (K1, M1). This desialylation of mucosubstances to a mineralizable matrix may occur in the urine, because sialidase is one of the renal enzyme systems regularly present in urine (M1). Consequently, significant higher urinary sialidase activities are present in stone patients than are found in healthy males (V4). The age of the patient also correlates with the sialidase activity. The sialidase activity increases with age. Therefore, it is expected that elderly patients may have more desialylated glycoproteins, which increases the risk for stone formation.

It has also been shown that the urinary concentrations of bound and total sialic acid are lower in men with stones than in those without stones. This phenomenon could be attributed to a lower excretion of sialic acid. This decrease in total urinary sialic acid concentration in renal stone patients may cause stone formation. According to the matrix theory it can be explained as follows: Regardless of the fact that renal stone patients excrete more uromucoid-rich material than do their

healthy counterparts (B3,K2,M1), a decrease in the sialidation of these proteins is expected in the presence of less urinary sialic acid. Consequently, this would lead to a possible increase in mineralizable uromucoids, which might result in more urinary stones.

3. Conclusion

The pathogenesis of kidney stones may be attributed to a number of different causes, but the first step in urolithiasis is probably the precipitation of glycoproteins. The cause of this precipitation is still unclear and open to speculation. Nevertheless, whatever the answer to this complex multifactorial disease, the activity and/or concentration of urinary enzymes seem to play an important role and should supplement traditional risk factors and new investigations.

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