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Advances in CLINICAL CHEMISTRY

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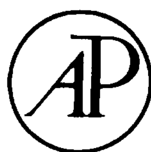

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PREFACE

The scope of clinical chemistry continues to broaden. Accordingly, the Editors have included chapters on cytokines and on human leukocyte antigen and transplantation in this volume. Additionally, chapters on estrogen and progesterone receptors and on glutathione *S*-transferases are included. Chemiluminescence is presented in a chapter covering the theoretical and practical aspects of this field.

Each year we endeavor to expand the intellectual horizons of our discipline by presenting the comments, philosophies, and knowledge of experts in their respective fields. The reception of the readership to this approach has been favorable. We hope to be even more effective in the future. As always, we welcome comments and suggestions for future topics.

The cooperation of a skilled, knowledgeable, and collegial Editorial Board was essential to the production of this volume. Their efforts, along with the competence and willing assistance of the Academic Press staff, made the production of this work the joy that it was. It is my privilege to acknowledge and to work with them. Finally, I thank my wife, Joanne, for her usual good nature and support, and I express my appreciation for the unflagging enthusiasm and generosity of Sister Catherine Sherry.

HERBERT E. SPIEGEL

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THE CYTOKINES: PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL ASPECTS

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1. Introduction

Communication among cells is an essential part of the development, differentiation, and function of all multicellular organisms. Medical science has been greatly influenced by the vast expansion in our knowledge of hormones produced by specialized cells and acting predominantly in an endocrine fashion. However, despite the description by Rich and Lewis in 1928 (R12) of soluble signaling molecules produced by cells of the phagocytic and immune systems, advances in characterizing such molecules have been slow. This has been in large part due to their local production and action and consequently low concentrations in biological fluids. During the last 5 years this field of cytokine biology has seen enormous expansion with the cloning and sequencing of many new molecules. In view of the size and complexity of the topic, we propose to discuss some general principles that underlie cytokines and to review the areas of particular potential importance for clinical chemistry. These are situations in which the laboratory measurement of cytokines may have a role to play in the management of disease, specifically graft rejection, inflammation, and sepsis.

1.1. Nomenclature and Classification

The terminology surrounding the considerable number of peptide mediators of cellular communication is very confused. As with most systems, classification changes as new knowledge is gained but never keeps pace with it.

The first group of mediators recognized to be clearly distinct from the peptide hormones were the lymphokines. These were followed by the interferons, substances that inhibit viral replication in cells and also generally inhibit cellular replication. Other mediators were soon described: monokines produced by cells of the monocyte macrophage lineage, colony stimulating factors promoting growth of hemopoietic cells in culture, and several growth factors produced by and acting on a range of cell types.

The late 1970s and 1980s saw the cloning of the genes for many of these factors and with this came the realization that single peptides may have several biological activities. In 1972 the term interleukin was invented and applied to two lymphokines, lymphocyte activating factor—interleukin-1 (IL-1)—and T-cell growth factor—interleukin-2 (IL-2) (A1). The term cytokine was first used in 1974 to describe mediators produced by nonlymphoid cells but soon was used to describe all factors driving the differentiation of cells involved in the immune system. Thus the interleukins and the interferons were included in the cytokines. More recently, it has become clear that the colony stimulating factors and growth factors share some functions and interact with the cytokines. This gave rise to the more general term polypeptide regulatory factor, describing molecules of low molecular weight (less than 80 kDa) with a short or intermediate range of action acting on specific

receptors to control differentiation or proliferation of cells. On the face of it the "classical" polypeptide hormones differ in being produced by cells localized in an anatomical "gland" and acting at distant sites. On reflection, however, it is clear that many of the hormones are produced by other cells and act locally, for example, in the brain or gut. Conversely, some of the cytokines, described on the basis of local actions, are now known to act on distant sites such as the brain, liver, and adrenal. Many of the molecules considered to be cytokines are listed in Table 1 and properties of known cytokine receptors are summarized in Table 2.

1.2. General Characteristics

Despite the fact that many of the molecules were described on the basis of a single biological function, several subsequently turned out to be pleiotropic molecules with a wide range of actions and target cells. All the cytokines act locally, modulating the behavior of adjacent cells in a manner described as paracrine. Many act on cells that secrete them (autocrine) or cells at distant sites (endocrine). An important action of some cytokines is modulation of the function of other cytokines as well as the classical neurological and endocrine systems. The actions of an individual cytokine on a particular cell type may critically depend on the extracellular milieu of other mediators and on the modulation of intracellular signaling pathways. All of these factors give rise to the concept of a complex network of interactions, which means that the study of the effects of single cytokines on cellular function may be misleading.

Another interesting aspect of many cytokines is the occurrence of redundancy, which is more than one cytokine exhibiting the same biological activity. One extreme example of cytokine redundancy is tumor necrosis factor (TNF). Two forms of TNF, TNFα and TNFβ, have been described. TNFα is a monocyte product synthesized with a conventional secretory protein signal sequence, while TNFβ is a T-lymphocyte product and is synthesized without a signal sequence. At the amino acid level TNFα and TNFβ exhibit only 30% homology, although the genomic organization of these molecules suggests that they are derived from a common ancestral gene. Both TNF molecules bind to the same cellular receptors and biologically, at least *in vitro,* these two cytokines are indistinguishable (A7).

In a fashion similar to other polypeptide regulatory factors, cytokines act on cells via specific receptors. Cytokine receptors are membrane proteins, many of which appear, at least at the DNA level, to be related (B17, D11, L24). Of the hemopoietic family the receptor for IL-2 (IL-2) has been most extensively investigated, two molecules, p55 (also called the low-affinity receptor or TAC) and p75, compose the IL-2 binding site. Both molecules are required to generate a high-affinity receptor and for IL-2 to deliver a signal to the cell (L16, R16, S44, W8). A soluble form of the TAC molecule is released by activated T lymphocytes, and serum levels of this molecule have been reported to reflect the extent of T-cell

TABLE 1

CHARACTERISTICS OF THE CYTOKINES

Cytokine	Propeptide	Mature peptide	Chromosome location	Some major cellular sources	Some major biological activities
IL-1α	271[a]	159 (17.5)[b]	2q13	Monocytes	T-cell activation
				Lymphocytes	B-cell immunoglobulin synthesis
IL-1β	269	153 (17.3)	2q13–21	Endothelial cells	Monocyte activation
				Smooth muscle cells	PMN adhesion to endothelial cells
				Keratinocytes	PMN release from bone marrow
				Astrocytes	Acute phase protein synthesis
				Microglia	Osteoclast activation
					Decreased proteoglycan synthesis
					Fibroblast proliferation
					Muscle proteolysis
					Endothelial cell activation
IL-1ra		(17.5–22)		Monocytes	IL-1 antagonist (inhibits bioligical activities associated with IL-1α and -β
IL-2	153	133 (17.5)	4q26–27	T lymphocytes	T-lymphocyte growth and differentiation
					B-lymphocyte growth and differentiation
IL-3	152	134/140 (28)	5q23–31	T lymphocytes	Hemopoietic cell differentiation
					Mature basophils
IL-4	153	129 (16–20)	5q23–32	T lymphocytes	T-lymphocyte growth and differentiation
					B-lymphocyte growth and differentiation
IL-5	134	123 (20)	5q23.3–32	T lymphocytes	Eosinophil differentiation
					B-cell growth and differentiation
IL-6	212	184 (21–26)	7p15–21	Monocytes	Hepatocyte stimulation
				Endothelial cells	B-cell differentiation
				Keratinocytes	T-cell differentiation
IL-7	177	152 (25)		Bone marrow stromal cells	Immature B and T lymphocytes
IL-8α	99	79 } (10)	4q21	Monocytes	Neutrophil chemoattractant
IL-8β		77 } (10)		Epithelial cells	Neutrophil activation

IL-9	202	179 (58)	22q12	T lymphocytes Fibroblasts Hepatocytes	Hepatocyte stimulation Myeloid cell differentiation Mast cell growth factor Inhibits adipocyte lipoprotein lipase activity
IL-10				T lymphocytes	T-lymphocyte growth and differentiation factor Inhibitor of cytokine synthesis
TNFα	233	157 (17)	6p21.3	Monocytes T lymphocytes	Interferon enhanced cytotoxicity Enhanced class II antigen expression Endothelial cell activation
TNFβ	205	171 (20–25)	6p21.3		Monocyte activation Fibroblast proliferation
GM-CSF	152	127 (35)	5q33.1	Monocytes Lymphocytes Fibroblasts Endothelial cells	Differentiation of early progenitor cell toward granulocytes and macrophages
G-CSF	204	174 (20)	17q11.2–21	Monocytes Endothelial cells	Differentiation of committed stem cell toward granulocytes
M-CSF	190–221	158–189 (35–45)	5q23–33	Monocytes Fibroblasts Endothelial cells	Differentiation of committed stem cell toward monocytes
TGFβ1	391	112	19q13.1–13.2		Hepatocyte stimulation Plasma cell IgA synthesis
TGFβ2	414	112	1q41		Inhibits T lymphocytes
TGFβ3	410	112	14q24		
IFNα	189	166	9p22	Leukocytes	Antiviral activities on nucleated cells
IFNα_2	188	165			
IFNβ	187	166	9p22	Fibroblasts	Antiviral activities on nucleated cells
IFNγ	166	143 (40)	12q24.1	T lymphocytes	MHC Class II antigen expression to enhance antigen presentation, synergistic action with cytostatic and cytolytic activities of TNF

[a]Number of amino acids.
[b]MW in kDa.

TABLE 2
CHARACTERISTICS OF CYTOKINE RECEPTORS

Cytokine	Receptor type	Subunit	Molecular weight (kDa)	Chromosome location	Affinities (*M*)
IL-1	I		80		} 2×10^{-7}–1×10^{-9}
	II		68		
IL-2		α	55	10p15	} 1×10^{-8}–1×10^{-11}
		β	75	22	
IL-3			140		1×10^{-8}–1×10^{-10}
IL-4			80		} 1×10^{-10}
			140		
IL-6		IL-6R	80		} 1×10^{-9}–1×10^{-11}
		gp130	130		
TNF	I		80		7×10^{-12}
TNF	II		60		3×10^{-10}
GM-CSF			84		} 1×10^{-8}–5×10^{-10}
			120		
G-CSF					5×10^{-10}
M-CSF		*c-fms*	165	5q33–34	1×10^{-11}
TGFβ	I		53		
TGFβ	II		70–100		5×10^{-8}–5×10^{-9}
IFNα/β			130	21q21	1×10^{-9}–1×10^{-11}
IFNγ			105–130	6q15–21	1×10^{-9}–1×10^{-11}

activation (R30). Relatively little is known of the signal transduction systems associated with cytokine receptors, although there is growing evidence that protein kinase systems are important. In particular, protein kinase C has been associated with IL-1, TNF, and IL-2 signal transduction (F9, G27, M36, Z10). However, some cytokine receptors, such as the macrophage-colony stimulating factor (M-CSF) and epidermal growth factor (EGF) receptors, have an intracellular tyrosine kinase domain, and the hemopoietic family of receptors, which includes IL-2, appears to indirectly activate tyrosine kinases (D11, F11, T4).

It is possible to demonstrate specific effects of individual cytokines on cells *in vitro.* However, it is becoming increasingly clear that cells are affected by many different molecules and that the effect a cytokine has on a cell will depend to a large extent on which other signals have been delivered to the cell. Interaction of the immune, neurological, and endocrine systems has been demonstrated at several levels (B35, C4, R22, S45, S47, W5). In particular the expression of functional receptors at the cell surface is regulated by a variety of cytokine and noncytokine mediators. It is also recognized that programmed events such as stages in the cell cycle and differentiation state determine the level of receptor expression and cytokine responsiveness of a cell (C8, K27, P4). Cytokine signals are integrated

at the signal transduction stage with signals from other systems so that the effect on a cell reflects the sum of all signals acting on the cell.

2. Biochemistry

2.1. Interleukin-1

2.1.1. *Structure Aspects of Interleukin-1*

Interleukin-1 (IL-1) was the first leucocyte-derived cytokine to be extensively studied (D12, D19). It was recognized at an early stage that at least two distinct forms of IL-1, IL-1α and IL-1β, existed. Biologically these two cytokines are closely related; however, a number of differences in both *in vivo* and *in vitro* assays have been described (B42, B53, D12, D23, F15, G13, G18). More recently a third IL-1-like molecule has been described, although so far this molecule appears to act only as an antagonist of IL-1α and IL-1β and is called the IL-1-receptor antagonist (IL-1ra) (C14, L4, M17, W22). The mature 17-kDa form of each protein is produced by an unusual proteolytic cleavage of the respective 31-kDa precursor molecules (pro-IL-1α and pro-IL-1β). None of the members of this family of molecules possess a secretory signal peptide and the mechanisms of release are not understood. Indeed there is some evidence that IL-1 may function as a cell-associated molecule (M12). The genes coding for IL-1α and IL-1β in humans are located on chromosome 2 and have a similar molecular organization (7 exons) (Fig. 1) (A17, C26, F32). Analysis of the IL-1 genomic sequences reveals many potential regulatory regions. Interestingly IL-1β but not IL-1α contains TATA and CAAT boxes immediately 5′ to the transcription initiation site (Fig. 2) (C26, F32). The IL-1β gene also contains regions that might function as glucocorticoid, SP1, and viral enhancer binding sites (C26). There is some evidence of polymorphism in the IL-1β gene (D15), and abnormal gene regulation has been associated with gene rearrangement (S35). An SP1 binding region has also been identified in the IL-1α gene and reported to be polymorphic (H8). The three-dimensional structure of IL-1α and IL-1β have also been determined by X-ray crystallography (F18, G21, H6). The suggested structure of both molecules is a globular protein composed of six strands arranged as antiparallel pleated β sheets with three-fold symmetry and capped at one end with six additional β strands. The N-terminal strand appears to fold in close association with one strand of the antiparallel β sheet. The observations that only the mature form of IL-1β is active, that antibodies against N-terminal peptides inhibit biological activity, and that a peptide from the N-terminal region of mature IL-1β competitively inhibits IL-1 receptor binding suggest that it is the N-terminal region of the molecule that participates in receptor interaction (B52, H12, T3, Z9). It has also been recently reported that a single point mutation close to the N terminus uncouples IL-1β–receptor interaction (G8).

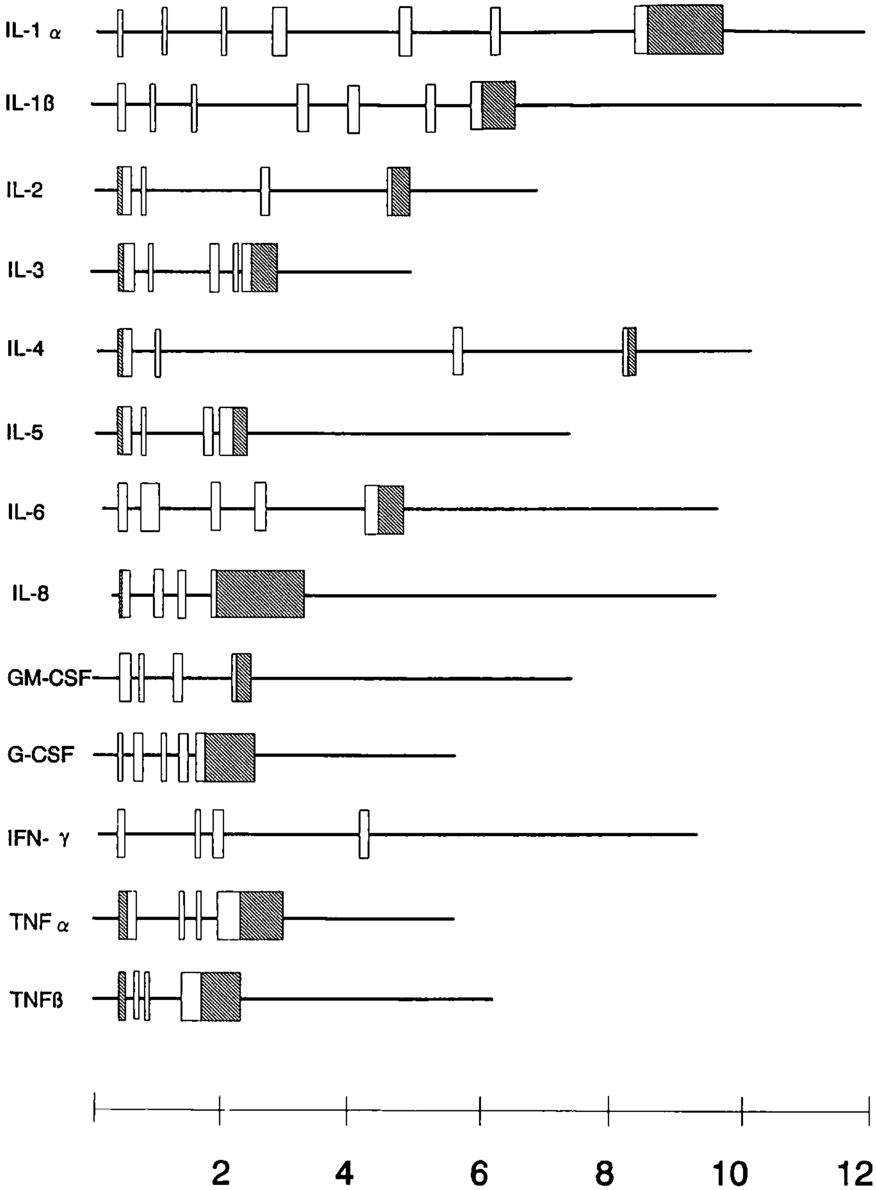

FIG. 1. Genomic organization of members of the cytokine family. Boxes represent exons. The cytokines can be grouped according to their genomic organization. IL-1α and IL-1β are composed of seven exons and six introns. IL-2, IL-4, IL-5, IL-8, GM-CSF, IFNγ, TNFα, and TNFβ genes are composed of four exons and three introns. IL-3, IL-6, and G-CSF are composed of five exons and four introns.

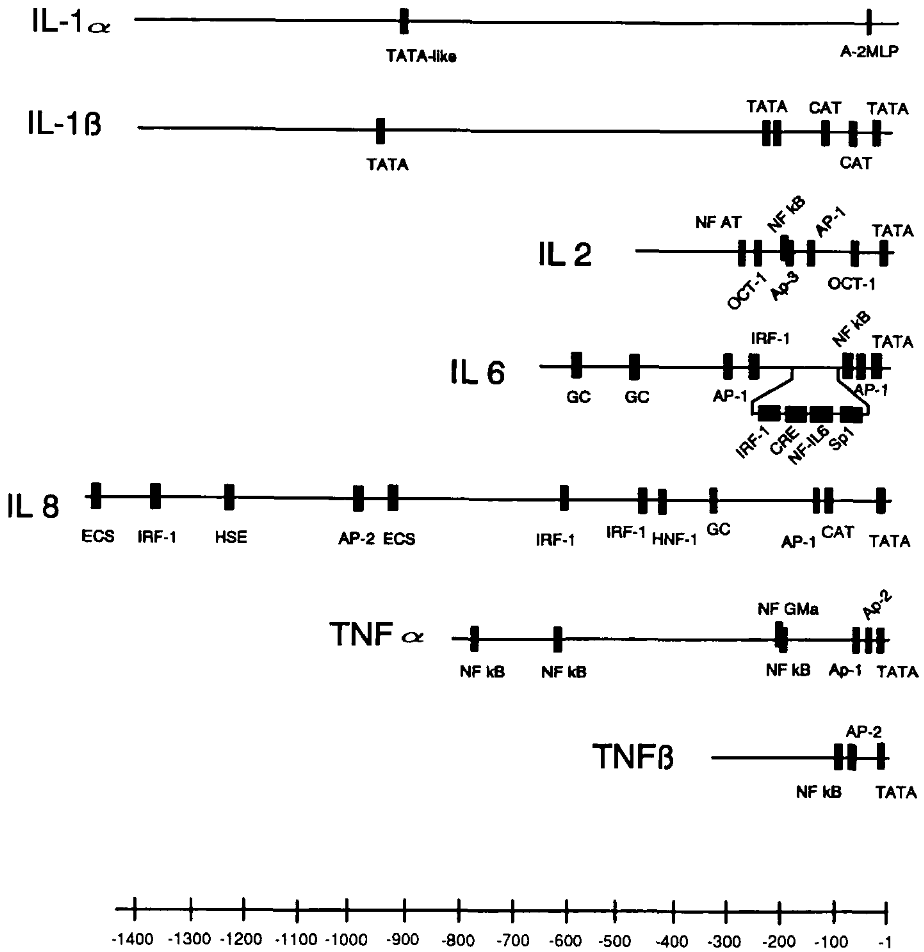

FIG. 2. Organization of the upstream (5′) regulatory region of some cytokine genes considered to play an important role in the pathophysiology of inflammation and the immune response.

IL-1α and IL-1β are produced by several nucleated cell types, including blood monocytes, tissue macrophages, polymorphonuclear leukocytes (PMN), endothelial cells, and fibroblasts. Different cells will produce IL-1α and IL-1β in different ratios; for instance, in peripheral blood mononuclear cells IL-1β is preferentially released, whereas in endothelial cells and fibroblasts IL-1α and IL-1β are produced in a more equal ratio. This suggests that these related cytokines are controlled by different mechanisms (L26, T20). Production of IL-1 is regulated at both transcriptional and translational levels. Peripheral blood mononuclear cell

adhesion or exposure to either C5a or β-glucan polymers provides sufficient stimulus to induce IL-1β gene expression but not translation (S14, S15). Endotoxin and a variety of other bacterial products rapidly stimulate additional transcription as well as translation of IL-1β mRNA in primed cells (S14). IL-1 has also been demonstrated to stimulate its own gene expression, although this is a relatively slow and apparently distinct event (S16).

2.1.2. *Receptor and Signal Transduction*

Two distinct membrane-associated receptors for IL-1 have been described (B50, C23). An 80-kDa receptor (IL-1RI) is expressed on fibroblasts, keratinocytes, endothelial cells, and hepatocytes, whereas a 68-kDa receptor (IL-1RII) is expressed on B-cell lines, PMN, and bone marrow cells (D25, S39). Both types of IL-1 (α and β) will interact functionally with either receptor and the IL-1ra can inhibit the activity of IL-1 on either receptor. However, whereas both forms of IL-1 (α and β) bind to each receptor, there is evidence that IL-1α and IL-1β have different biological activities, suggesting different binding properties for cytokine–receptor combinations or different receptor accessory components involved in mediating signal transduction (B45, G13). A number of different signal transduction pathways have been associated with IL-1–receptor interaction. Several groups have reported that IL-1 stimulates G-protein activity, including GTP binding and hydrolysis (O5, S3). In some studies IL-1 has been suggested to activate protein kinase A via G-protein activation of adenylate cyclase (C19, M39, Z2). Other studies have suggested that novel protein kinases and possibly protein kinase C are activated by IL-1 perhaps via modulation of phospholipase C to produce diacylglycerol or release glycosyl–phosphatidylinositol-anchored membrane proteins (B46, O5, R25).

2.1.3. *Biological Activities of Interleukin-1*

IL-1 plays an important role in the activation of T lymphocytes (B5, R9, W15). It stimulates the production of IL-2 and expression of the IL-2 receptor in antigen-activated cells (E8, F5, V9). IL-1 also has important activities on B cells, co-operating with other cytokines in the stimulation of immunoglobulin synthesis (F6, H26, K32). IL-1 may also play a role in maintaining B tolerance (D27).

A major role for IL-1 is as a mediator of inflammation. The activities of IL-1 as a proinflammatory cytokine are discussed in detail elsewhere, however; these include induction of eicosanoids, proteinases, and a variety of enzyme activities involved in generating inflammatory mediators (e.g., phospholipase-A2) (B63, D9). IL-1 is also recognized as a hepatocyte stimulating factor and, although it is not as potent as IL-6 in this regard, it does appear to have a role in inducing synthesis of some acute phase proteins (B15, G5, K23). Fibroblast proliferation is stimulated by IL-1, although this is antagonized by IL-6 (K22). IL-1 also induces hypoglycemia perhaps in part due to its ability to stimulate increased glucose transport (B44).

On its own IL-1 does not appear to stimulate hemopoietic colony growth. However, IL-1 stimulates release of cytokines that have hemopoietic activities (B2, B3, B24, N3) and also appears to synergize with other cytokines in mediating development of hemopoietic colonies (K15, K31, M46, S50).

2.2. Interleukin-2

2.2.1. *Structural Aspects of Interleukin-2*

The first T-cell growth factor [later called IL-2 (A1)] was originally described as a factor produced by phytohemagglutinin-stimulated normal human lymphocytes that allowed the long-term growth of normal T cells *in vitro* (M47). A single gene, located on chromosome 4 in humans, codes for a 133-amino-acid mature IL-2 protein. The structure of IL-2 has been predicted from X-ray crystallography data and appears to resemble that of growth hormone. Although the natural IL-2 molecule can be O-glycosylated (C31), carbohydrate groups do not appear to contribute to the biological activity of this cytokine. IL-2 is produced by antigen- or mitogen-activated T lymphocytes and hence acts as an autocrine or paracrine growth factor. Multiple regulatory sequences 5′ to the IL-2 gene have been identified, including glucocorticoid core binding sequences, an NFkB-like binding sequence, and at least two nuclear factor binding sequences (K1, S32, V1). At least two distinct events controlling IL-2 synthesis have been distinguished (J6). These act via modulating the binding of nuclear factors (NFIL-2E and NFIL-2A) to three distinct regulatory regions (A, D, and E) 5′ to the transcription initiation site. NFIL-2E (or NFIIa) binds site A and E (D34), and NFIL-2A (NFIIIe) binds site A and site D (S31). An NFkB-like protein binds to a region 5′ to the gene (S32).

2.2.2. *Interleukin-2 Receptor and Signal Transduction*

The IL-2 receptor consists of two chains, α (also called CD25 and TAC) and β, located on chromosome 10 and 22, respectively (G16, L16, L17). The relatively abundant α chain has an apparent molecular weight of 55 kDa and binds IL-2 with low affinity (10^{-8} *M*). Preliminary X-ray crystallography data have been reported for the α chain (L1). The β chain has an apparent molecular weight of 75 kDa and, in association with the α chain, constitutes a single high-affinity binding site for IL-2 (10^{-11} *M*) (W8). Both α and β chains are required to generate a functional IL-2 receptor (S44). A restricted region of the IL-2 receptor β chain has been demonstrated to be essential for growth signal transduction (H7). Expression of the α chain appears complex but is at least partly under the control of an NFkB-like sequence (L30, P12, T11, W8). Much less is known about control of expression of the IL-2 β chain (W8). Activated T cells release a soluble form of the IL-2 receptor α chain (C25). The mechanism by which this extracellular domain of the α chain is released and the biological relevance of this event are not understood.

Signal transduction events associated with IL-2 receptor interaction have been extensively studied. Surprisingly, since the IL-2 receptor does not contain a tyrosine kinase domain, one of the earliest events observed is tyrosine phosphorylation of the IL-2 receptor β chain (F11). Later events include the protein kinase C-dependent phosphorylation of a 68-kDa protein (E11) with apparent identity to the cytoskeletal protein *l*-plastin (Z6) and a signal transduction event shared by several hematopoietic growth factor systems (E13). Phosphorylation of the ribosomal S6 protein and increased protein synthesis also follow IL-2 receptor interaction (E12). Negative signals appear to involve cAMP-mediated events (F9, F10).

2.2.3. *Biological Activities of Interleukin-2*

IL-2 was originally identified as a T-cell growth factor (M47) and this remains the principal biological function of this cytokine. However, it has subsequently been demonstrated that IL-2 also activates macrophages, stimulates proliferation of B lymphocytes, and augments the cytolytic activity of NK cells and cytotoxic T lymphocytes (B21, G14, H16, M43, R3).

2.3. Interleukin-6

2.3.1. *Structural Aspects of Interleukin-6*

Interleukin-6 (IL-6) is a pleiotropic glycoprotein with an apparent molecular weight of between 21 and 26 kDa as determined by SDS–PAGE (H22, W29). The molecule consists of 212 amino acids, of which 28 constitute a hydrophobic secretory signal sequence (H24). Two potential N-linked and several potential O-linked glycosylation sites have been suggested together with phosphorylation to account for the observed heterogeneity of natural IL-6 (G24, M16). In humans the gene for IL-6 is found on chromosome 7 and occupies approximately 5 kb (B59). The gene consists of five exons and four introns, closely resembling those of growth hormone, prolactin, erythropoietin, and G-CSF (B18, B19). There are at least three polymorphic forms of IL-6, identified by *Msp*I, *Bgl*I, and *Bst*NI restriction sites (three, two, and four allele polymorphism, respectively) (B48, B60, B61). The *Msp*I and *Bgl*I polymorphisms are probably due to base pair substitutions, *Msp*I in the vicinity of the fifth exon and *Bgl*I in the 5′ flanking region, while the *Bst*NI polymorphism appears to be due to an insertion/deletion event 3′ of the fifth exon.

Sequence analysis of the 5′ flanking region of the IL-6 gene suggests that several different signal transduction pathways, including both cAMP-dependent and protein kinase C-activating systems, could participate in the regulation of IL-6 gene expression (H13, H22, S25). Indeed numerous stimuli have been shown to induce IL-6 synthesis. The most important regulatory region appears to be a c-*fos*-like serum-responsive element (SRE) containing two important cis-acting regulatory elements (CRE) controlling IL-6 gene transcription NFIL-6, and the

multiple response element (MRE) (A10, H22, I5, P17, R5). The c-*fos* SRE-like sequence has been located 5′ (-173 to -145) to the IL-6 gene. Other regulatory sequences are located on either side of the SRE region, 5′—two glucocorticoid response elements [interesting because it has been shown that dexamethasone inhibits IL-6 production (Z1)] and an AP-1 binding sequence—and 3′—one NFkB binding site and one TATA box (H22, R4, R5, Z2).

2.3.2. *Receptor and Signal Transduction*

Specific receptors for IL-6 have been demonstrated on a variety of normal and transformed cell types (T2). Initially a receptor with a single binding affinity of between 1.3 and 6.4×10^{-10} *M* was described (T2). Some subsequent reports have suggested both high (1×10^{-11} *M*)- and low (1×10^{-9} *M*)-affinity forms of the IL-6 receptor on a human myeloma cell line (H24). A membrane-associated receptor for IL-6 has been identified and the gene encoding this protein cloned (H24). A single polypeptide chain constitutes the ligand binding site and the transmembrane region of the receptor. This 468-amino-acid protein, which has an apparent molecular weight of 80 kDa and six potential N-linked glycosylation sites, confers both high- and low-affinity binding to sites when it is transfected into cells (H24). In contrast to IL-6, IL-6 receptor expression is increased by glucocorticoids (S45). At the molecular level the IL-6 receptor resembles the receptors for growth hormone, prolactin, erythropoietin, and the p75 chain of the IL-2 receptor (B17). Sequence homology with immunoglobulin light chain, CD4, poly-Ig receptor, and α_1B-glycoprotein suggests that this molecule might also be classified as belonging to the immunoglobulin supergene family. Binding of IL-6 to its receptor is thought to involve association of the receptor with an accessory protein, gp130 (T1). It is possible that gp130 plays a role in signal transduction, although the nature of that role is not known. It has been reported that extracellular calcium is required for IL-6-stimulated growth of plasmacytoma cells (N10). An important role for calcium is also suggested by the ability of lead and cadmium, competitive inhibitors of calcium-dependent processes, to inhibit IL-6-stimulated proliferation of a hybridoma cell line and IL-6-stimulated synthesis of α_1-antichymotrypsin (S. W. Evans, unpublished observation). Phobol esters stimulate the accumulation of acute phase protein mRNA in hepatoma cells, and protein kinase C "burnout" experiments ablate IL-6-stimulated accumulation of acute phase protein mRNA, suggesting but not establishing a role for the calcium-dependent protein kinase C in IL-6 signal transduction (B14, E10).

2.3.3. *Biological Activities of Interleukin-6*

IL-6 has a wide variety of different biological activities *in vitro.* At least two distinct types of properties can be discerned, differentiation and proliferation. The wide variety of cells that can be activated or that differentiate in response to IL-6 suggests an important *in vitro* regulatory role for this cytokine. Using a variety of

different cells, including primary hepatocytes and hepatoma cell lines, recombinant IL-6, and antibodies specific for IL-6, it has now been clearly established that this cytokine is one of the predominant mediators controlling acute phase protein production (H11).

A number of cytokines have been implicated as endogenous pyrogens, including IL-1, TNFα, interferon-γ, IFN-γ, IFNα, IL-8, and IL-6, with IL-1 and IL-6 being the most potent (B68, R27). Since IL-1β will induce IL-6 production and circulating levels of IL-1β appear not to be sufficient to account for fever, it is possible that IL-6 alone or stimulated by low concentrations of IL-1β is the endogenous pyrogen (L14).

It has been shown that several monocyte-derived cytokines can act on the hypothalamic–pituitary–adrenal axis, stimulating adrenal cells to release corticosteroid hormones (B31, N2). Injection of IL-6 into rats results in an increase in concentrations of ACTH in the plasma (N2). IL-6 can also act directly on adrenal cells to induce corticosteroid release (S4, S5). IL-6 interacts with a number of cytokines to enhance the growth and differentiation of multipotent progenitor, erythroid, myeloid, and megakaryocytic cells (R10, R11, S60).

IL-6 has been identified as an essential and late acting factor in mitogen-activated immunoglobulin production, stimulating the synthesis of IgM, IgG, and IgA and inhibiting B-cell proliferation (B34, V5). IL-6 has also been shown to act as a growth factor for plasmacytoma cells, myeloma cells, EBV-transformed cells, and B-cell hybridoma (K7, R15, V5). A number of reports suggest that IL-6 also plays a role in the generation and activation of cytotoxic T lymphocytes, LAK cells, NK cells, and macrophages (G1, L31, M37, S55). IL-6 has also been shown to have an antitumor activity when it is injected into mice (M54).

2.4. Tumor Necrosis Factor

2.4.1. *Structural Aspects of Tumor Necrosis Factor*

Tumor necrosis factor (TNF) was originally described as a leucocyte-derived endotoxin-induced factor responsible for *in vivo* and *in vitro* tumor necrosis (C13) and as cachectin, a molecule that caused the wasting associated with chronic infection (R28). Amino acid sequencing and cDNA cloning for the genes of these two activities revealed their identity, encoding a protein of 17 kDa. More recently it was found that this molecule shares 30% homology, some biological activities, and common receptors with a slightly larger molecule, lymphotoxin (G19). Cachectin/TNF is predominantly macrophage derived and is termed TNFα; lymphotoxin is T cell derived and is known as TNFβ. The TNFα molecule is secreted as a nonglycosylated molecule, whereas the TNFβ molecule is glycosylated. The genomic sequences coding for TNFα and TNFβ are located within the major histocompatibility locus on chromosome 6. There is some evidence of polymorphism in the TNFα gene (F31). The three-dimensional struc-

ture of TNF has been determined and surprisingly resembles a viral coat protein (J4). TNF readily associates as a trimeric molecule, in which form it appears most biologically active.

2.4.2. *Receptor and Signal Transduction*

All nucleated cells express TNF receptors and at least two different types exist (B64). The 80-kDa (TNF-RI) and 60-kDa (TNF-RII) receptors have been cloned from myeloid and epithelial cells, respectively (L24, S13, S41). The two receptors have different affinities for TNF, 7×10^{-12} *M* for the 80-kDa receptor and 3×10^{-10} *M* for the 60-kDa receptor (B64). The extracellular domain of the TNF receptors shows homology with the NGF receptor (J3) and the B-cell surface antigen CD40 (S41).

Signal transduction mediated by TNF is at least as confusing as that mediated by IL-1. GTP binding and hydrolysis have been reported to occur following TNF receptor interaction (I2). Specific phosphorylation events have also been described and attributed in part to the activation of protein kinase C (M27, S20, S21). Later events include induction of transcriptional activators c-*fos* and c-*myc* and transcriptional factors that bind to the NFkB site (D31, L22).

2.4.3. *Biological Activities of Tumor Necrosis Factor*

The biological activity most frequently attributed to TNF is IFNγ-enhanced tumor cell cytotoxicity (O4, S6, S57). In the presence of IFNγ normal cells such as epithelial and endothelial cells may also be susceptible to the cytotoxic and cytostatic action of TNF (S9, T5). In the absence of IFNγ, however, TNF appears to stimulate proliferation of several cell types, including monocyte/macrophages and fibroblasts. The major biological activity of this cytokine, however, appears to be as a proinflammatory molecule stimulating the release of a variety of locally acting mediators of inflammation (R24). TNF stimulates the release of IL-1, colony stimulating factors, and leucocyte chemotactic factors from endothelial and monocytic cells (D20, E4, L5, S34); the release of prostaglandins from macrophages fibroblast, chondrocytes, and mesangial and synovial cells (B1, C6, D5, P9); and enhanced expression of MHC class II antigens on many different cell types (P8). Fibroblasts also synthesize proteinases and proteinase inhibitors in response to TNF (I6). TNF also inhibits adipocyte lipoprotein lipase activity (F30) and stimulates release of lipoprotein lipase from endothelial cells (S12).

2.5. Interferon-γ

2.5.1. *Structural Aspects*

IFNγ, originally described as immune interferon, is synthesized and proteolytically processed in activated proliferating T lymphocytes (T19). T lymphocytes were stimulated by a variety of different agents, including IL-2 and virus-

synthesized IFNγ. The mature form of IFNγ is a 143-amino-acid polypeptide with two potential N-glycosylation sites and is encoded by a gene located on chromosome 12. The three-dimensional structure of IFNγ has been predicted to resemble that of IL-2 (J2), a member of the hematopoietic growth factor family. The predicted structure of IFNγ is of six α helixes (A–F) connected by five turns. Four of the helixes (B, C, E, and F) resemble the core of the IL-6 molecule. One helix (A) is found at the N terminus and a small helix (D) links helixes C and E. Antibodies raised against N- or C-terminal peptides are inhibitory, and the N-terminal 39 amino acids compete with IFNγ for receptor binding (L27). However, more recently it has been demonstrated using mutant molecules that the N-terminal but not the C-terminal sequences are essential for biological activity (H27, L32).

2.5.2. *Receptor and Signal Transduction*

A specific membrane receptor for IFNγ has been identified and cloned (A8). IFNγ–receptor interaction is associated with phosphatidyl-serine metabolism (Y7) and rapid protein phosphorylation (H21). Induction of MHC class II antigens by IFNγ is associated with modulation of cis-acting molecules (F29).

2.5.3. *Biological Activities of Interferon-γ*

The major biological activity attributed to IFNγ is enhanced antigen presentation. This activity is effected by the induction of MHC class II antigens on a variety of cells, including epithelial, endothelial, and connective tissue cells, and monocytes (B32, P16, S51, V7). IFNγ also acts as a macrophage activating factor (A6, Y10) and synergizes with TNF to effect tumor cell lysis and inhibit cell proliferation (S6).

2.6. Other Cytokines

2.6.1. *Colony Stimulating Factors*

Multicolony stimulating factor [interleukin-3 (IL-3)] appears to have the widest range of targets, stimulating proliferation of macrophages, PMN, eosinophils, megakaryocytes, early erythroid cells, and mast cells (M53, Y1). The major cellular source of IL-3 appears to be the T lymphocyte. Encoded by a single gene on chromosome 5 (5q23–31) and transcribed as a single 0.95-kb mRNA species, this 140-amino-acid molecule is produced as a glycoprotein with an apparent molecular weight of 28 kDa (L9, Y1, Y2). In addition to its role as a differentiation factor for early stem cells, IL-3 activates mature basophils (V2).

GM-CSF acts on virtually the same set of cells as IL-3, but is unable to stimulate mast cell proliferation. In addition to T cells, endothelial cells and fibroblasts are also important sources of GM-CSF (G23). GM-CSF is a 127-amino-acid molecule

encoded as a single gene closely linked to the IL-3 gene on chromosome 5 (5q23–33) (Y3). It is translated from a 0.9-kb mRNA molecule and is glycosylated, having an apparent molecular weight of 35 kDa. Multiple mechanisms control the expression of GM-CSF and this is reflected by the many different mediators that can induce its synthesis (N7). As well as acting as a differentiation factor on early stem cell development, GM-CSF enhances adhesion of mature monocytes to endothelial cells (G3) and activates neutrophils (C29, C33).

The gene for human G-CSF, located on chromosome 17 (17q11.2–21), encodes a relatively hydrophobic protein that is O-glycosylated and has an apparent molecular weight of 20 kDa (S36, W18). G-CSF is produced by stromal cells, macrophages, PMN, and endothelial cells (H19, K6, Z5). In macrophages G-CSF mRNA levels are regulated primarily at a post-translational rather than a transcriptional level (E7). G-CSF acts on determined stem cells, driving them toward a granulocyte differentiation path, and also on mature granulocytes, inducing enhanced antibody-directed cell cytotoxicity and expression of receptors for chemotactic factors (P14, P15).

Unlike the other CSFs that are produced as single-chain polypeptides, M-CSF is produced as a dimer of two identical subunits. Alternative RNA splicing sites and N-glycosylation events give rise to a heterogeneous population of molecules, with the apparent molecular weight of each subunit being between 35 and 45 kDa (A14). The gene encoding M-CSF has been cloned (K8) and is located on chromosome 5 (5q33.1) in humans, close to those of IL-3 and GM-CSF. In addition to those cells producing G-CSF, fibroblasts are an important source of M-CSF. As for G-CSF, M-CSF mRNA levels are regulated primarily at a post-translational level (E7). The main activities of this cytokine appear to be induction of determined stem cells toward the macrophage linage and activation of mature macrophages (H38, L20).

2.6.2. *Interleukin-4*

Interleukin 4 (IL-4) was originally described as a costimulator, with anti-immunoglobulin antibodies, of B-cell proliferation (H35). The gene for IL-4 is located on chromosome 5 and only one active gene has been identified (C15). The mature gene product is a glycoprotein with an apparent molecular weight of 16–20 kDa. A high-affinity receptor for IL-4 has been described ($1–2 \times 10^{-10}$ *M*). [Cross-linking and immunoprecipitation studies suggest that the IL-4 receptor is a 140-kDa protein and is associated with membrane-bound accessory proteins (F25, M50, P22, Z7, Z8).] Originally identified as a B-cell growth factor (H35) it has become apparent that this cytokine exerts other effects on B cells. IL-4 induces MHC class II antigen expression and enhanced IgG1 immunoglobulin synthesis in mice (A16, F16, G12) and enhanced IgA synthesis by human B cells (F20). Another important property of IL-4 is its T-cell growth-factor-like activity (M33, V3).

2.6.3. *Interleukin-5*

Interleukin 5 (IL-5) was originally described as a B-cell differentiation antigen called T-cell replacing factor. IL-5 is a 20-kDa glycoprotein, the mature protein comprising 123 amino acids (Y4). It has been suggested that the carbohydrate component of IL-5 is important in its biological activity (T12). A single IL-5 gene has been described and gives rise to a 0.9-kb mRNA transcript (L11). Specific high-affinity receptors for IL-5 have been identified on IL-5-dependent cell lines and on human eosinophils (C22, M38, P13). IL-5 has two major activities in the mouse, B-lymphocyte stimulation (R2, R19, S8, S18, S27) and eosinophil stimulation (C28, E5, S23, W9). In humans B-cell stimulatory activity of IL-5 is controversial.

2.6.4. *Interleukin-7*

Interleukin-7 (IL-7) plays an important role in stimulating the growth and differentiation of pre-B cells in the bone marrow (H15, S56). The major source of IL-7 in the bone marrow appears to be stromal cells (H10, K14), upon which B-cell development in the bone marrow appears to be dependent (S56). IL-7 may also play a role in stimulating development of thymocytes (E14, O3, S2) and also appears to stimulate proliferation and differentiation of mature T lymphocytes (B33, C18). Receptors for human IL-7 have been identified, cloned, and assigned to the hemopoietic growth factor receptor family (G17).

2.6.5. *Interleukin-8 and Related Cytokines*

Early studies suggested that IL-1 and TNF were chemotactic for PMN. However, it has become apparent that this activity resides in a family of molecules of 10 kDa, called neutrophil activating peptides (NAP) or IL-8 (L15). IL-1 and TNF induce the release of these molecules from mononuclear cells, fibroblasts, endothelial cells, and keratinocytes and they act on a specific receptor expressed by PMN. The main activity ascribed to these molecules is PMN chemotaxis, but the molecules may cause oxygen radical and enzyme release by PMN and mobilize PMN into the circulation. The common feature that identifies members of this family of peptides is alignment of four cysteine residues. IL-8 is the most potent chemotactic member of this family. Synthesized as a 99-amino-acid precursor, IL-8 is released as a 79-amino-acid molecule, IL-8α, which itself is further cleaved to 77- and 72-amino-acid forms, IL-8β and IL-8γ. IL-8γ is predominantly found to be associated with macrophages and may reflect the proteolytic enzymes released by macrophages. Receptors for IL-8 have been identified on PMN and approximately 20% of lymphocytes. The three-dimensional structure of IL-8 (NAP-1) has been reported (C27).

2.6.6. *Interleukin-9*

Interleukin-9 (IL-9) is a glycoprotein with an apparent molecular weight of 58 kDa and is encoded by a gene on chromosome 22 in humans. IL-9 was originally identified and characterized as a macrophage differentiation factor that inhibited proliferation of leukemic progenitor cells and was called leukemia inhibitory factor (LIF) (M29). It has since been demonstrated that this cytokine has a number of other activities. IL-9 inhibits differentiation of embryonic stem cells, allowing the long-term maintenance of these cells in culture (P5). IL-9 was demonstrated to be identical to the third hepatocyte stimulating factor (HSF III), immunochemically distinct from IL-6 and IL-1 but biologically indistinguishable from IL-6 (B16). Because of this reported biological identity with IL-6, the expression of IL-9 receptors on osteoblasts (A13), and the ability of this cytokine to induce calcium release from bone (R8), as well as its action as a lipoprotein lipase inhibitor (M48), IL-9 has been suggested to have an important role in the regulation of acute inflammation.

2.6.7. *Interleukin-10*

Interleukin-10 (IL-10)—originally called cytokine synthesis inhibitory factor—appears to act chiefly as an inhibitor of cytokine synthesis (M45). However, it has also been reported that IL-10 is a growth factor for immature and mature T cells (M2) and stimulates cytotoxic T-cell differentiation (C21).

2.6.8. *Transforming Growth Factor-β*

Transforming growth factor-β (TGFβ) inhibits the proliferation of T lymphocytes, B lymphocytes, NK cells, and hemopoietic cells (K9, K10, L28, R23). TGFβ is a potent inhibitor of IL-1 activity and it has been suggested that this effect is mediated by inhibition of IL-1 receptor expression (D28).

2.7. Cytokine Inhibitors

Cytokine inhibitors can be loosely divided into three groups: cytokines themselves, cytokine receptor antagonists, and cytokine binding molecules. Many cytokines can inhibit or antagonize the activity of other cytokines. Two important examples are TGFβ and IL-10, described above. The only authentic cytokine receptor antagonist so far described binds the IL-1 receptor (see Section 2.7.1.). Many molecules exhibit cytokine binding activity, including soluble receptor proteins as well as plasma proteins (see Section 2.7.2.).

2.7.1. *Interleukin-1 Receptor Antagonist*

An IL-1 receptor antagonist (IL-1ra) has been purified from human monocyte-conditioned medium (22 kDa) (E3, H4) and the human U937 monocyte cell line

(25 kDa). The latter antagonist has been found to bind selectively to the high-affinity IL-1 receptor on T lymphocytes and fibroblasts but not to the receptor, of slightly lower affinity, on bone marrow granulocytes, pre-B cells, and macrophages (C14). *In vitro,* the receptor antagonist has been shown to inhibit IL-1-induced adhesion of neutrophils and eosinophils to endothelial cells in a concentration-dependent fashion and to inhibit both IL-1α and -β activities in the LAF assay. *In vivo,* the receptor antagonist has been found to suppress corticosterone release when it is injected subcutaneously into the mouse hind paw together with IL-1α and -β. In contrast an 18- to 25-kDa inhibitor purified from the urine of patients with monocytic leukemia (B6) was able to block fibroblast proliferation, was effective against both IL-1α and -β, and appeared to act at the cell surface or at the receptor.

2.7.2. *Soluble Cytokine Binding Proteins*

Two types of soluble binding protein have been described, nonspecific serum proteins and cytokine-specific soluble receptor proteins. The major nonspecific serum protein capable of binding cytokines appears to be α_2-macroglobulin (B57, B58, M11). A number of soluble cytokine inhibitors related to the relevant receptor have been described. The soluble form of the IL-2R α chain has been found in the serum of apparently normal individuals and is increased in the serum of individuals with inflammatory diseases such as rheumatoid arthritis (W31). Similar molecules have been described for IL-1, IL-6, IFNγ, and IL-7 (F7, N14, S62). The mechanism of release has not been properly established but appears to require proteolytic cleavage of the membrane-bound receptor. Soluble inhibitors for two other cytokines, IL-4 and TNF, appear to be derived by alternative RNA splicing sites that give rise to receptors lacking a transmembrane sequence and that are secreted (M50, S22).

3. Pathophysiology

3.1. Inflammation and the Acute Phase Response

Inflammation involves a series of cellular changes that facilitate phagocytosis and the killing of microorganisms or the digestion of cell debris. This is followed by proliferation of connective tissue cells and repair. Both of these events require the complex control of various cell types at a local level. There is increasing evidence that, while many different molecules mediate effects such as vascular permeability and chemotaxis, cytokines control many of the cellular processes of inflammation.

The local processes of inflammation are facilitated by systemic metabolic changes that mobilize energy as glucose, fatty acids, and amino acids. Fever potentiates several enzymatic reactions in inflammatory and tissue cells that,

perhaps because they may be damaging to host tissues, operate at a suboptimal level at normal body temperature. Leukocytosis provides an increased supply of phagocytic cells. Suppressive effects such as the release of cortisol may curtail the potentially destructive effects of inflammation. Production of acute phase proteins by the liver provides an increased tissue supply of certain inflammatory mediators and inhibitors. All these events are controlled by the same multifunctional cytokines, which exert key local controls.

The systemic responses that accompany trauma and inflammation are known as the acute phase response (D16). The present evidence supports the idea that this is an adaptive response conditioning the "milieu interieur" so that inflammation and healing may progress optimally. A complex array of hormones and cytokines induces and controls the components of this response and it is rapidly becoming clear that these and the nervous system are closely integrated.

3.1.1. *Acute Phase Proteins*

Acute phase proteins are a family of approximately 30 plasma proteins produced in increased amounts by the liver in inflammation. They may all have roles to play in inflammation or the healing process that follows (Table 3). The rate of increase in their plasma concentration and the incremental changes that occur following inflammation vary considerably among them and reflect their induction by different cytokines and their molecular size, volume of distribution, and rate of catabolism both in the circulation and at the site of inflammation. Inflammation complicated by other processes such as intravascular coagulation may thus induce a different pattern of acute phase protein elevation, although in simple acute inflammation it is very stereotyped for a particular species of animal.

Since the liver was discovered to be the major site of acute phase protein synthesis, a vast amount of experimental work has been directed toward elucidation of the chemical messengers involved in synthesis and regulation. Although several cytokines have been implicated, the complexity of the acute phase response is only just becoming apparent and it is likely that synergistic interaction takes place not only between cytokines, but also between cytokines, hormones, and other molecules.

In the 1980s IL-1 was generally believed to be the major inducer of acute phase protein production. However, it is now apparent that IL-1 can induce only a limited number of acute phase protein genes and it has also been shown to act in a negative manner by suppressing the induction of a number of acute phase proteins (including fibrinogen in rat and humans as well as α_2-macroglobulin and cysteine proteinase inhibitor in the rat) caused by other mediators such as IL-6 (A15, D2, K23). Apart from suggestions of differential production of α_1-acid glycoprotein (α_1-AGP) (G9), both the α and the β forms of IL-1, encoded by separate genes, are equipotent in their actions on hepatocytes, and their receptors on human and murine lymphocytes have been shown to be identical (D24).

TABLE 3
ACUTE PHASE PROTEINS

Protein	Result
Inflammatory mediators	
Complement components	Opsonization, chemotaxis, and mast cell degranulation
C-reactive protein	Phosphorylcholine binding, complement activation, and oponization
Factor VIII (with fibrinogen and prothrombin)	Fibrin matrix formation (clotting)
Kallikrein (kininogenase)	Vascular permeability and dilatation
Plasminogen	Activation of complement, clotting, and fibrinolysis
Proteinase inhibitors	
Antithrombin III, C1-1NH, factor 1, and factor H	Control of mediator pathways
α1-Antitrypsin	Elastase and collagenase
α1-Antichymotrypsin	Cathepsin G
Haptoglobin	Cathepsins B, H, and L?
Thiol proteinase inhibitors	Cysteine proteinases
Scavengers	
Ceruloplasmin	$[O_2^-]$?
C-reactive protein	DNA?
Haptoglobin	Hemoglobin
Serum amyloid A	Cholesterol
Immune regulation	
α1-Acid Glycoprotein	Monocyte production of the IL-1ra
C-reactive protein	T- and B-cell interaction
Repair and resolution	
α1-Acid glycoprotein	Promotes fibroblast growth
α1-Antitrypsin	Binds to elastic fibers
α1-Antichymotrypsin	Inhibits remodeling by leukocyte proteinases?

In some circumstances, TNFα and IL-1β, molecules with the same spectrum of activities at other cellular target sites, have similar acute phase protein inducing capabilities. In various rat hepatoma cell lines both molecules have been shown to increase synthesis of α_1-AGP, haptoglobin, and C3 (B15), and to inhibit production of fibrinogen, thiostatin, and α_2-macroglobulin (W26). In some cell systems and *in vivo,* the effect of recombinant TNF (rTNF) is not as potent as that of recombinant IL-1 (rIL-1) on the induction of acute phase proteins (D2, K23, S40), although it is generally thought that the same genes are affected.

Gauldie and others (G4) have suggested that IL-6, in combination with IL-1β and glucocorticoid, accounts for all the hepatic-specific stimulation by monocytes, and Baumann and co-workers have described an additive effect of IL-6, IL-1, and glucocorticoids on α_1-AGP and haptoglobin synthesis in rat hepatoma H-35 cells. In contrast, IL-1 was found to have a negative additive effect on IL-6 induction of fibrinogen in rat and human cells and on thiostatin and α_2-macroglobulin in the rat (G4). These effects—the collated results using several cell lines—are summarized in Table 4, which shows the acute phase proteins as distinct subsets based on differential induction by combinations of cytokines. More recently it has become clear that IL-9 is also an important acute phase protein, inducing cytokine (see earlier), and it would not be surprising if additional hepatocyte regulating factors were rapidly identified.

IL-6 is known to be produced by a wide variety of cells, including fibroblasts, synovial cells, endothelial cells, and hepatocytes themselves (L29) and reports of significant expression of IL-6 in normal human hepatocytes (T14) and IL-1-stimulated hepatoma cells (N6) suggest that an autocrine mechanism may be involved in liver stimulation. However, IL-6 mRNA expression was not detected in the liver of normal rats or those undergoing an acute phase response (B13) and suggested caution when interpreting data using isolated hepatocytes cultured under nonphysiologic conditions such as in the absence of corticosteroids.

Conflicting evidence exists regarding the effect of IFNγ on the acute phase response, and lack of effect (G4) and inhibitory activity for some proteins and enhancement of others (M4) have been reported in the HepG2 cell line. Although the genes of complement components appear to be sensitive to IFNγ when they are transfected into fibroblasts, the greatest contribution of IFNγ to the acute phase

TABLE 4
CYTOKINE INDUCTION OF ACUTE PHASE PROTEINS

Group	Protein
	I
Induced by IL-1, TNF, or IL-6	α1-Acid glycoprotein, complement C3, complement factor B, haptoglobin, serum amyloid
	II
Induced by IL-6 only	α1-Antitrypsin, α1-antichymotrypsin, ceruloplasmin, C-reactive protein, cysteine proteinase inhibitor, fibrinogen
	III
Coopertion between IL-1 and IL-6	Synergize: α1-acid glycoprotein, complement component C3, haptoglobin Antagonize: fibrinogen, cysteine protease inhibitor

response is likely to be via modulation of macrophage production of IL-1 and TNFα (D26).

Although the direct effects of IL-1, TNFα, and IFNγ on the liver appear to be rather limited, these cytokines affect other cells involved in the acute phase response, most notably endothelial cells and fibroblasts. IL-1 and TNFα have been shown to induce the production of IL-6 in fibroblasts (K22), each is involved in the production of the other (B1, D20), and IL-1 can also induce its own gene expression (W11). In addition IL-1, acting directly on the pituitary to release adrenocorticotrophic hormone (ACTH) or indirectly through the hypothalamus to induce corticotrophin releasing hormones, can cause the increase in glucocorticoid necessary for the hepatic responses (B29, B35, L33).

The network of cytokines may well be viewed as an amplification system that controls acute phase protein production. Although the pattern of proteins synthesized is genetically programmed, it may be "finely tuned" by the cytokines, IL-6 being the main signal, with other cytokines and hormones acting as accessory modulators (Fig. 3). Proteins synthesized would therefore be determined by the sequence of exposure of the hepatocyte to the various regulating molecules. In this way the most effective homeostatic response to infection, inflammation, or tissue damage may be achieved.

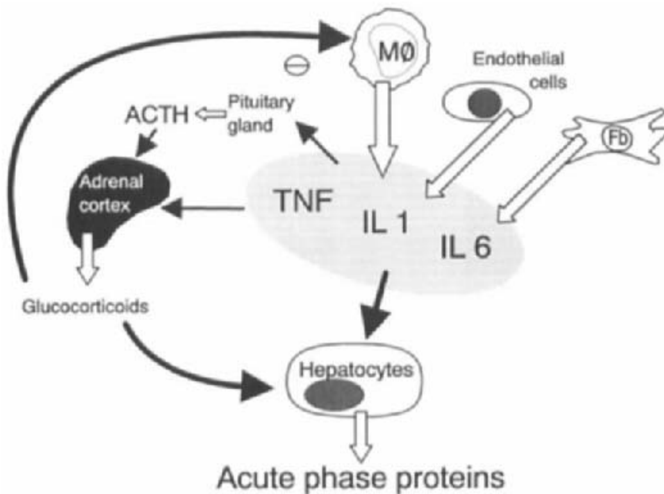

FIG. 3. Cytokine regulation of acute phase protein synthesis. IL-1, TNF, and IL-6 produced by macrophages, fibroblasts, and endothelial cells at the site of inflammation stimulate hepatocytes to synthesize acute phase proteins. These cytokines also potentiate the release of glucocorticoids by adrenal cells. Glucocorticoids synergize with cytokines to stimulate acute phase protein synthesis but inhibit macrophage synthesis of cytokines.

3.1.2. *Metals*

Inflammation is often associated with decreased serum levels of iron and zinc and increased levels of copper. The mechanism of these effects are entirely different.

Plasma iron levels fall within the first hour following operative trauma, paralleling transferrin, to which all iron in plasma is bound. This is probably due to alterations in vascular permeability mediated by a multiplicity of factors (M58). Iron levels, however, continue to fall and transferrin saturation decreases, reaching about 20% of initial value 12 hr following the onset of trauma. Iron is rapidly sequestered in damaged tissue, probably as a result of its complexing to lactoferrin, an iron binding protein released from polymorphonuclear leukocytes (B67). The iron lactoferrin complex is thus sequestered in macrophages as hemosiderin, a complex with ferritin. This iron is in a dynamic state and will subsequently reenter the transferrin-bound pool for participation in hemopoiesis.

Degranulation of PMN, releasing lactoferrin, can be induced by purified preparations of endogenous pyrogen and the activity may well reside within IL-1 (K18). Iron is essential for bacterial growth and the rapid sequestration of iron at inflammatory sites may serve as an antimicrobial mechanism and even the low-plasma iron may confer a protective effect (B67).

Following acute trauma in humans plasma zinc levels fall by about 40% during the first 8 hr. The initial fall parallels that of albumin (the major zinc binding protein of plasma), which stops at about 2 to 4 hr, whereas zinc continues to fall, reducing the amount carried on the protein. As with iron, the first phase is due to loss of albumin from the vascular to the extravascular compartment.

The second phase of zinc reduction, which persists for the duration of inflammation, can be induced *in vivo* by endogenous pyrogen administration (K2). *In vitro* rIL-6 but not rIL-1 increases hepatocyte levels of metallothionine (S19), which, under normal conditions, is transcriptionally regulated by dietary zinc. Induction of metallothionine synthesis by glucocorticoids, catecholamines, glucagon, and IL-6 results in rapid transfer of zinc from plasma to the hepatocyte. The role of the response is unclear, although zinc is necessary for microbial growth. The increase in copper as seen in inflammation is a consequence of cytokine-induced secretion from the liver into the plasma of the copper containing protein ceruloplasmin (see later).

3.1.3. *Leucocytosis*

There is a well-known association between increased numbers of immature peripheral blood PMN and the acute response to infection, trauma, and inflammation. PMN enter the circulation from the marginal pool in response to many signals such as complement factors, catecholamines, prostaglandins, and cytokines, whereas release from the bone marrow of immature cells and accelerated turnover

are mediated by cytokines. IL-1 induces PMN released from the marrow (U3) and, along with IL-6 and IL-3, production of specific granulocyte and macrophage replication factors (colony stimulating factors from various cell types) (S28).

3.1.4. *The Pituitary–Adrenal Axis and Cortisol Release*

Exposure to bacterial toxins has been long known to stimulate release of ACTH from the anterior pituitary. Indeed the administration of endotoxin was used as a clinical test of anterior pituitary function. Experiments with hypophysectomized animals show that most corticosterone release caused by endotoxin is mediated by ACTH but that a significant extrapituitary mechanism also exists.

Besedovsky first showed in 1986 (B35) that rIL-1β increased ACTH and cortisol levels in mice. Corticotrophic cells from pituitaries incubated with IL-1 confirmed that it is a potent secretagogue for ACTH. *In vivo* increases in ACTH and corticosterone were blocked by antibodies to CRF, implying a hypothalamic site of action. IL-1 is also able to induce corticosteroine release directly from adrenal gland cells (S4, S5) but rIL-6 is even more potent in this respect and it synergizes with low concentrations of ACTH (S5).

Glucocorticoids have strong "antishock" and "antiendotoxin" effects and critically ill patients who fail to manifest an appropriate response have a poor prognosis (F17). The immediate cortisol increase of trauma is undoubtedly mediated by neural pathways; however, IL-1 and IL-6 may play an important role in sepsis. There is poor correlation between levels of ACTH and continuing high levels of corticosteroid several days after the onset of trauma and, since serum levels of IL-1 and IL-6 have been shown to rise and remain elevated for several days following severe trauma, these cytokines are strong candidates as factors controlling the long-term adrenal response.

3.1.5. *Fever*

It has been shown experimentally that increased temperature, within the physiological range of fever, greatly enhances a number of cellular functions integral to inflammation and host survival. It has been suggested that some aspects of the immune response and inflammation do not function optimally at normal body temperature and, by optimizing these responses at temperatures reached only "in disease," these powerful and potentially damaging systems can be brought into play when their protective effect is required. *In vitro* effects enhanced by temperatures at or above 40°C include enhanced bactericidal activity of human PMN against certain gram-negative bacteria and the antiviral effects of IFNγ (H18); enhanced proliferative responses of T cells to mitogen, IL-1, and IL-2 (B56, D30); and increased B-cell antibody production in response to T-cell cytokines (H5). Thus phagocytosis and bacterial and viral killing are potentiated. In addition, metabolic changes in other cells, such as prostaglandin production, muscle proteolysis, and oxygen radical production, are increased.

In vivo many experiments have shown the beneficial effect of increased body temperature against viral and bacterial infection. Increased survival from bacterial infection (K20) in lizards was shown to be due to more rapid recruitment of leukocytes to the site of infection and containment of spread (B30) at higher induced temperature. In infected fish, fever was associated with significantly enhanced survival (C36). Enhanced survival from infection at increased body temperature has been observed in mice infected with herpes simplex and other viruses (S17), puppies with herpes virus (C12), piglets with gastroenteritis virus (F33), rabbits with pneumococcal pneumonia (R13), and humans with bacterial peritonitis (W17). Experimental work over many years has shown that fever is mediated by peptides released from inflammatory cells, collectively known as endogenous pyrogen. The main pyrogenic cytokines appear to be IL-1, TNF, and IL-6. Dinarello (D18), in attempting to identify the pyrogen in leukocyte supernatants, has concluded that activity is due to a mixture of all of them. A 1°C fever in rabbits is induced by about 20 ng/kg of IL-1, 200 ng/kg of TNF, and 1 μg/kg of IL-6. Because all the molecules have a similar molecular weight, IL-1 is more potent on a molar basis, and in fever IL-1 rises to 1 ng/ml, TNF to 3 ng/ml, and IL-6 to 300 pg/ml in plasma.

Injection of endogenous pyrogen into the brain suggests that the sole site of action is the anterior hypothalamus. Certain prostaglandins, notably PGE_1, induce an immediate fever when they are injected into the same areas but are not blocked by prostaglandin synthetase inhibitors, such as aspirin, whereas endogenous pyrogen is. This has led to the conclusion that PGE_1 is the final common pathway in pyrogenesis. Several monoamines, including catecholamines and serotonin, may also be pyrogenic.

3.1.6. *Sleep and Analgesia*

IL-1 acts on the brain to induce slow-wave sleep (and analgesia), which is not blocked by salicylates that prevent the concomitant fever (K30, T10). In humans plasma IL-1 levels are higher during slow-wave sleep than in waking (M42). IL-1 is secreted by astrocytes in response to bacterial products, probably explaining the sleep-inducing effects of endotoxin and muramyl dipeptide (F23).

Purified human IFNγ causes naloxone-reversible analgesia in mice (B47) and rats (N4), suggesting opiate agonist effects. rIL-1α and -β have analgesic actions that are not naloxone reversible but are partly prevented by intravenous injection of antibody against corticotrophin releasing factor, again suggesting that opioid peptides may be induced.

3.1.7. *Energy Metabolism*

There is evidence for cytokine-mediated effects on the hormones of intermediary metabolism in the acute phase response but it is conflicting. The early work showed that leukocyte supernatants caused insulin and glucagon release in

the pancreas (G11), although more recently IL-1 appeared to be cytotoxic for pancreatic islet cells (D17). Other work suggests that IL-1 may increase production of both insulin and glucagon (B27). Studies of the hyperlipidemia accompanying parasitic infection have led to the observation that TNF, and to a lesser extent IL-1, inhibits lipoprotein lipase and enzymes responsible for the conversion of glucose to fatty acid (B37, B38). Decreased glucose utilization for fat synthesis results in greater availability of glucose for metabolism by peripheral tissues, and the unopposed action of fat cell lipase releases fatty acids from fat stores for similar use. The resulting weight loss and cachexia give the name cachectin to TNFα.

3.1.8. *Pathological Aspects*

A number of cytokines have been implicated in the pathology of inflammatory disease. In particular the role of IL-1 has been extensively investigated. Intraarticular administration of IL-1 induces inflammatory changes identical to those seen in some animal models of chronic arthritis (P10). IL-1 has been shown to mediate cartilage matrix degradation, inhibit synthesis of proteoglycans, induce the breakdown of bone matrix, and stimulate PGE_2 synthesis (D5, D12, M40). More recently IL-6 and TNF have been shown to have many of the properties attributed to IL-1 and it remains to be established which cytokines exert an effect *in vivo.* Increased levels of all three of these cytokines, IL-1, TNF, and IL-6, have also been reported in the synovial fluid from arthritic joints (N12).

A number of reports have suggested that a peripheral IL-2 T-cell defect exists in patients with rheumatoid arthritis, SLE, and other inflammatory disease (I3, K17). On the other hand, sera from individuals with RA or SLE have elevated levels of soluble IL-2 receptor, which has been postulated to act as a carrier protein for IL-2 (R31). Other investigators have shown that, if rested prior to *in vitro* stimulation, T cells from RA and SLE patients do not exhibit the IL-2 defect (H37) and that cells removed before disease manifestation actually hypersecrete IL-2 (W20). More recently it has been suggested that rather than an IL-2 defect, many inflammatory (and autoimmune) diseases might be IL-2 driven (K29).

The role of three cytokines, IL-1β, and TNFα, and IL-6, in mediating the acute phase response has been described above. Levels of circulating IL-1β, TNFα, and IL-6 have been measured in individuals suffering severe trauma. In burn patients, levels of IL-6 are raised significantly above those of controls (N6) and similar increases have also been reported in a more controlled study of patients undergoing elective surgery (C38). Another consequence of severe trauma is immunosuppression (F1). The mechanism of this immunosuppression is not clear, although it has been suggested that increased serum levels of the soluble IL-2 receptor (TAC) and production of TGFβ by monocytes from burn patients may be responsible (T8, W6, W7). Another possibility is that IL-6 induces the release of corticosteroids from the adrenal gland (S4, S5) and that this suppresses the im-

mune response by inhibiting the synthesis of cytokines such as IL-1 and IL-6 (B35, W5, Z1).

Systemic sclerosis (SS) is one connective tissue disease in which the role of cytokines has been investigated. IL-1 has been shown to both stimulate and suppress fibroblast proliferation (K24, P20). It has been reported that spontaneous IL-1 production by monocytes from SS patients is higher than that in normal cells (A11). It has also been reported that leukocytes from SS patients produce abnormal amounts of the cytokines that stimulate fibroblasts but normal amounts of INFγ and INFα (i.e., cytokines that can inhibit collagenase synthesis). This suggests that the defect in SS might be the overstimulation of collagenase synthesis by fibroblasts and not loss of a negative signal (D33). On the other hand it has also been reported that monocytes from SS patients produce reduced levels of IL-1β (B28), as well as an IL-1 inhibitor (S7) upon stimulation.

Anomalies in the acute phase protein response have been reported in various diseases. Studies in systemic lupus erythematosus, dermatomyositis, systemic sclerosis, mixed connective tissue disease, ulcerative colitis, primary biliary cirrhosis, and chronic active hepatitis have shown low acute phase protein responses for the amount of inflammatory activity present (P6). This may be due either to disease-related unresponsiveness or to "down-regulation" of the acute phase response in chronic inflammation (M1, W16). Patients with systemic sclerosis do not mount an appreciable acute phase response to therapeutic infusion of PGE, compared with patients with atherosclerosis (W24), although they do to infections (C20).

Unresponsiveness in scleroderma was later shown to be associated with disease duration (S42). Increased spontaneous IL-1 activity and production of an IL-1 inhibitor have been shown in patients with this disease (see earlier), although decreased IL-1 monocyte production has been observed in a number of chronic diseases such as cancer, leprosy, and SLE (W27). Patients with osteoarthritis, SLE, and scleroderma were, however, found to respond to urate crystal injection in a manner comparable to that in controls (H40).

Decreased monocyte production of TNF also has been reported in SLE (Y9), as has decreased IL-6 production in patients with chronic lymphocytic leukemia, whereas Yorston *et al.* (Y5) showed that patients with recurrent iritis are progressively less likely to show acute phase responses with increasing numbers of previous attacks. In some conditions the acute phase response mechanisms may become refractory to certain stimuli or it may be that a greater degree of stimulation is required in chronic disease. Defective host response to tissue damage or injury could therefore enhance or perpetuate inflammation.

3.2. Cytokines and Leukocyte Biology

In addition to the macrophage and macrophage-derived cytokines that play a central role in controlling inflammation, the antigen-specific immune response

utilizes T and B lymphocytes (Fig. 4). The role of cytokines in controlling the proliferation, differentiation, and interaction of these cells is described below.

3.2.1. *T-Cell Physiology*

A number of cytokines are involved in the development, clonal expansion, and activation of T lymphocytes. Many cytokines including IL-1, IL-2, IL-4, and IL-6 have a potential role in controlling early T-cell development (B9, E14, G2, K13, K25, O3, R26, W14, W32, Z4). IL-1 and IL-6 appear to play distinct but related roles as accessory factors in antigen-mediated T-cell activation (A2, C7, F5, H34, K4, V6, W15). The main growth factors stimulating T-cell proliferation or clonal expansion are IL-2 and IL-4. Both IL-2 and IL-4 are produced by activated T lymphocytes. In the mouse distinct populations of helper T cells produce IL-2 or IL-4, T_H1, and T_H2 (B10, M51). There is growing evidence that a similar distinction occurs in humans. Receptors for IL-2 and IL-4 are expressed on a variety of

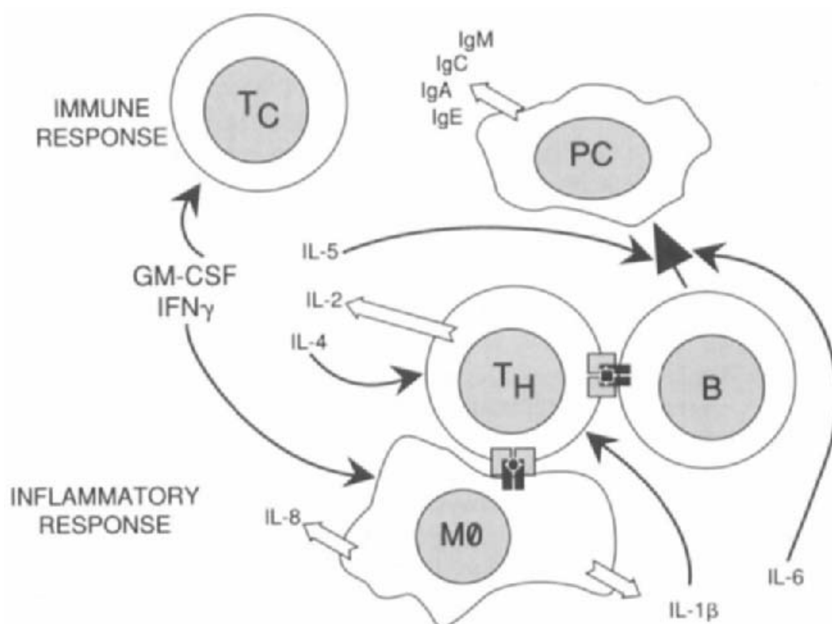

FIG. 4. The role of cytokines in controlling cellular interactions involved in the immune and inflammatory responses. In an immune response, cells present antigens and release IL-1 to activate appropriate T-helper cells and IL-6, which acts as a B-cell differentiation factor. Activated T-helper cells produce a variety of cytokines, including lymphocyte growth (IL-2 and IL-4) and differentiation (IFNγ and IL-10) factors and hemopoietic growth factors (IL-3 and GM-CSF). Macrophages can also be activated by antigen nonspecific mitogens such as LPS. Cytokines released include those with proinflammatory activities such as IL-1β, TNFα, and IL-8, as well as the anti-inflammatory IL-6.

different cells, including activated T cells, resting and activated B cells, macrophages, and mast cells (B20, B41, C2, E9, L17, R16, R17, W8, Z7). Interestingly, IL-2 and IL-4 act as autocrine growth factors, in that T cells secreting IL-2 express IL-2 receptors and proliferate in response to IL-2 but not to IL-4. The situation is reversed for IL-4. Indeed, IL-4 will inhibit the response of T cells to IL-2 (M9). Both IL-2 and IL-4 also stimulate the proliferation of T_C cells although apparently by distinct mechanisms. It would seem then that these two cytokines have complementary but subtly different roles, activating and stimulating proliferation in distinct populations of T cells, as a result of which the nature of an immune response to a particular antigen is subtly adjusted (L21, M52). There is evidence that preferential stimulation of particular T-cell subsets can contribute to the pathology of a disease. Different mouse strains infected with *Leishmania* exhibit different forms of disease. One strain with high IL-4 and IgE but low IFNγ shows poor delayed-type hypersensitivity responses, suggesting overactive T_H2 cells, and develops a severe generalized form of disease. Another strain with low IL-4 and IgE has a milder localized infection. Injection of antigen-specific T_H1 clones into infected mice clears infection, whereas injection of T_H2 cells exacerbates infection (L21, M52).

3.2.2. *B-Cell Physiology*

Cytokines from both types of activated T cells, as well as those from antigen presenting cells, act in concert to regulate B-lymphocyte proliferation and differentiation (C3, O2). As described earlier T_H2 cells produce IL-4 and IL-5, which together influence the switch toward IgE and IgA (R20, Y4). On the other hand the T_H1 cytokines IFNγ and IL-2 also influence immunoglobulin production. IFNγ antagonizes the action of IL-4, so that IL-2 and IFNγ acting together can induce production of IgG (K16). The macrophage-derived cytokine IL-6 also acts on activated B cells, stimulating the general synthesis of immunoglobulin (R6). It therefore seems likely that the nature of an antibody response is determined by the cytokine profile associated with a particular immune response; T_H1 cells will tend to stimulate IgM and IgG antibodies, whereas T_H2 cells will stimulate IgA and IgE antibody synthesis.

3.2.3. *Myelopoiesis*

Many cytokines play a role in myelopoiesis (Fig. 5). An important group includes the CSFs that stimulate proliferation of developing macrophage and granulocyte populations (M23). In addition to their proliferative role, these cytokines may be involved in triggering differentiation, maintaining cell viability, and functional stimulation of mature macrophage and granulocytic cells. The action of these cytokines has been interpreted in terms of the differentiation stage of the responsive cells, so that pluripotent stem cells respond to IL-3, giving rise to a wide range of cell types, whereas M-CSF and G-CSF act on relatively late-stage

cells, which have only the potential to develop into a macrophage or a granulocyte (M30, S50). While it is clear that each CSF can act on its own specific receptor (M56, P3), there are reports describing the cross-reactivity of colony stimulating factors on each other's receptors (P2). It is also clear that different CSF receptors can be expressed at the same time on the same cell (C8, P4) and that they regulate expression of each other's receptors (K27, P3). Evidence suggesting receptor heterogeneity for some CSF receptors is also beginning to accumulate (C8, P2). It is thus likely that different CSFs act in a complex but coordinated fashion to control differentiation and proliferation of myeloid cells.

Several cytokines other than the CSFs also play a role in myelopoiesis; for instance, IL-6 has been shown to hasten the emergence of blast cell colony forming cells in the presence of IL-3 (L8). It has also been demonstrated that all of the CSFs can induce production of IL-6 in normal myeloid cells and that IL-6 can induce production of GM-CSF and expression of receptors for GM-CSF, IL-3, and G-CSF in leukemic cells (S1). Cytokines that do not have a direct effect on myeloid cells can still effect myelopoiesis indirectly by inducing synthesis of other cytokines. IL-1 has been shown to induce production of IL-6 and CSFs from a variety of cell types, including macrophages, lymphocytes, stromal cells, and endothelial cells present in the bone marrow (S28). A stromal cell factor that supports the long-term culture of B cells has been characterized and called IL-7 (H39). This cytokine plays an important role in maintaining a population of pre-B lymphocytes, although it does not appear to stimulate differentiation of pre-B cells to B cells. In summary, combinations of cytokines acting in both an autocrine and a paracrine fashion may be required for properly regulated proliferation and differentiation of myelopoietic cells (D10, K14, P18).

3.2.4. *Pathological Aspects*

In malignant disease, the major interest lies in the suggestion that IL-6 may be a growth factor for plasma cells in myeloma. At the moment the evidence is interesting, but the conclusions are controversial.

In 1972 Namba and Hanaoka (N5) showed that pristane-induced plasmacytomas in BALB/C mice depended for their *in vitro* growth on a monocyte-derived factor, at that time unidentified, but subsequently shown to be IL-6. A murine plasmacytoma cell line with a gene insertion upstream of the IL-6 gene was found to constitutively produce IL-6. Further studies showed that in culture mouse plasmacytomas could be rendered dependent on IL-6 for growth, which could be blocked by antibodies to IL-6 or its receptor. If such cells were transfected with IL-6 cDNA, they showed autonomous growth and tumorigenicity when they were transplanted in mice. Growth of subsequent tumors *in vivo* could be inhibited and even reversed by monoclonal antibodies capable of blocking the binding of IL-6 to its receptor. Further evidence was provided by the finding that massive lethal plasmacytomas occurred spontaneously in transgenic mice bearing a human IL-6

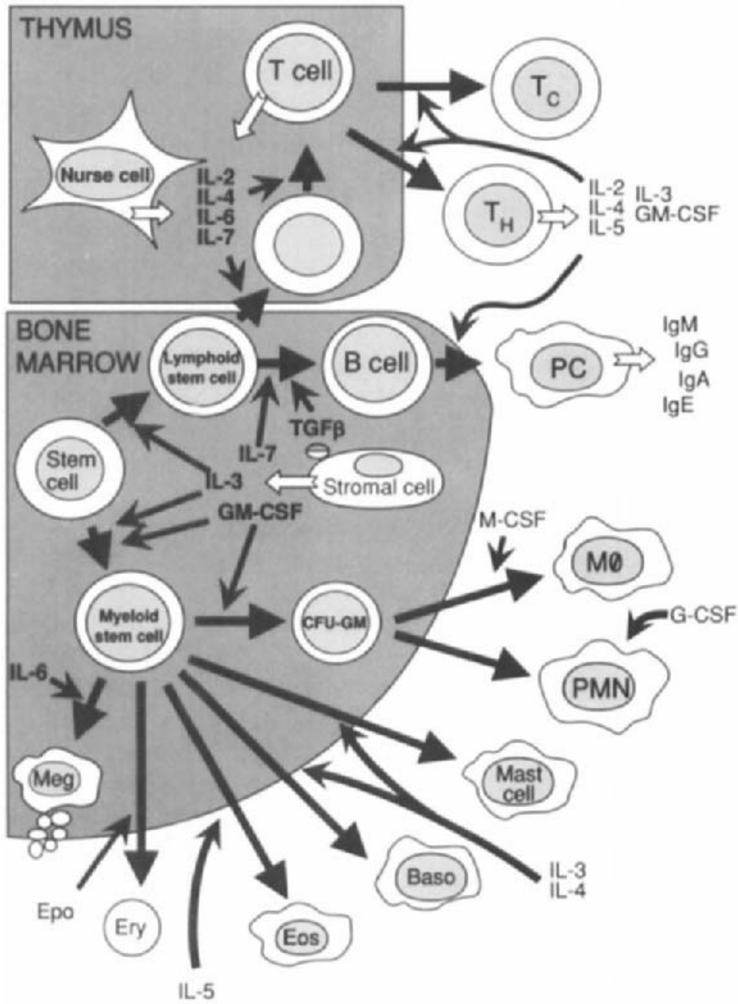

FIG. 5. The role of cytokines in controlling hemopoiesis. Many cytokines play a role in hemopoiesis by stimulating the proliferation and differentiation of cells derived from a pluripotent stem cell population. Early differentiation events take place in the bone marrow, where IL-3, GM-CSF, and IL-7 appear to have important stimulatory roles. TGFβ is a negative regulator of early cell differentiation. B-lymphocyte and myeloid cell development continues in the bone marrow while T-lymphocyte development is completed in the thymus. A large number of cytokines have been suggested to act on immature cells to encourage linage specific development.

gene fused with the immunoglobulin heavy-chain enhancer, giving rise to high levels of constitutive IL-6 production. However, such plasma cells did not produce transplantable tumors, nor did they contain apparent c-*myc* gene rearrangements, which are observed in almost all pristane-induced plasmacytoma cells that form transplantable tumors. Such evidence strongly suggested a possible role for IL-6 in plasmacytomas, although clearly other factors or events were required for full tumorigenesis.

Freeman *et al.* (F26) demonstrated that in four cases of myeloma that they studied, IL-6 mRNA was expressed in the myeloma cells. In addition cytoplasmic IL-6 was detectable. These findings have been difficult to repeat and other workers have suggested that bone marrow-adherent cells (monocytes) rather than plasma cells are the source of IL-6 in the bone marrow of myeloma patients. However, further studies have shown that the plasma cells from about 50% of myeloma patients are responsive to IL-6 in short-term culture and that antibodies specific for IL-6 inhibit spontaneous uptake of tritiated thymidine, thus suggesting that IL-6 may be acting as an autocrine growth factor. Furthermore, the growth inducing activity of IL-1 and TNF on freshly isolated myeloma cells is blocked by antibodies to IL-6. Cytokines other than IL-6 may also play a role in myeloma. IL-3 appears to act synergistically with IL-6 (B26), whereas IL-4 acts to inhibit the growth of freshly isolated myeloma cells (T6). Klein (B11) from Montpelier was able to show that the *in vitro* IL-6 response of myeloma cells obtained from patients with multiple myeloma was directly correlated with the *in vivo* labeling index of these tumors and that serum IL-6 levels, which were frequently raised in these patients, correlated well with disease severity.

The experimental analogue of the transgenic mouse in humans is cardiac myxoma. Patients with this condition frequently show evidence of autoimmune disease, polyclonal hypergammaglobulinemia, fever, and increases in acute phase proteins. These changes are associated with very high serum levels of IL-6, which has been shown to be expressed by the myxoma cells themselves. Extensive marrow plasmacytosis occurs in this condition, which is reversed upon removal of the tumor. However, malignant transformation and myelomatosis have not been described (J5). A comparable but more complex condition is Castleman's disease, in which activated B cells in the germinal centers of hyperplastic lymph nodes produced IL-6. Symptoms similar to those of cardiac myxoma were seen and, in one case, malignant myeloma developed. The nature of Castleman's disease is, however, unclear, and viruses or other factors activating the lymph node B cells are likely.

There is thus tantalizing evidence to suggest that deregulation of gene expression for IL-6 could be instrumental in the polyclonal plasmacytoma process and the generation of blood cell neoplasias. In many malignant lymphomas, as with pristane-induced plasmacytomas in mice, there is translocation of c-*myc* to the chromosomal location of the immunoglobulin genes, which are normally highly

transcribed, and the c-*myc* protein is thus constitutively produced at high levels. In normal B lymphocytes c-*myc* is only transiently expressed early in the cell cycle and in response to growth promoting cytokines. The c-*myc* gene product is a DNA binding nuclear transcription factor. In developing follicular B cells IL-6 appears to induce expression of the *Bcl*II oncogene product that is associated with the mitochondria. The questions that arise are thus of great interest. Is this mutation necessary for IL-6 response? As with many tumors, is the progress from plasmacytoma to malignant transformation a multistep process? Why are the data from different groups apparently in contradiction? Does the newly described IL-6 inhibitor have a role to play? It is produced along with IL-6 by granuloma-derived macrophages. Could it be that, under some circumstances of culture, it is produced by myeloma cells to inhibit responsiveness to exogenous IL-6 and could it play a role in therapy?

In man a surprising number of myelopoietic growth factors have been mapped to a region on the long arm of chromosome 5, frequently associated with translocation events or deletions seen in leukemia (R29). Genes coding for GM-CSF, M-CSF, IL-3, IL-4, IL-5, and the cellular receptor for M-CSF have been located in the 5p23–35 region. Evidence from both *in vitro* and *in vivo* systems suggests that these cytokines, which play a central role in the up-regulation of myelopoiesis and in increasing the rate of release of blood cells into the circulation, may contribute to leukemia if they are inappropriately expressed as a consequence of gene translocation or deletion (R29).

3.3. Cytokines and Sepsis

Cytokines are peptide molecules that are produced by or act on cells of the immune and inflammatory systems. These molecules regulate the local and systemic events of the immune response, inflammation, hematopoiesis, and wound healing (B22, B65, H17, L10, W25). It is not surprising then to find cytokines playing an important role in those processes, following trauma, that lead to multiple organ failure (MOF) (Fig. 6). At the simplest level there are two major factors that contribute independently to MOF, shock, and septicemia. The role of cytokines in septicemia has been extensively examined and is considered later. The role of cytokines in shock is less well understood and is not considered any further here, except to note a recent report suggesting the presence of high levels of TNF when hemorrhage accompanies trauma but not following trauma alone (A18, A19).

3.3.1. *Trauma-Induced Immune Suppression*

In spite of aggressive antibiotic therapy and the practice of good hygiene, systemic bacterial infection is frequently a serious complication following trauma and one that can result in MOF (F1). Perhaps the most important factor contribut-

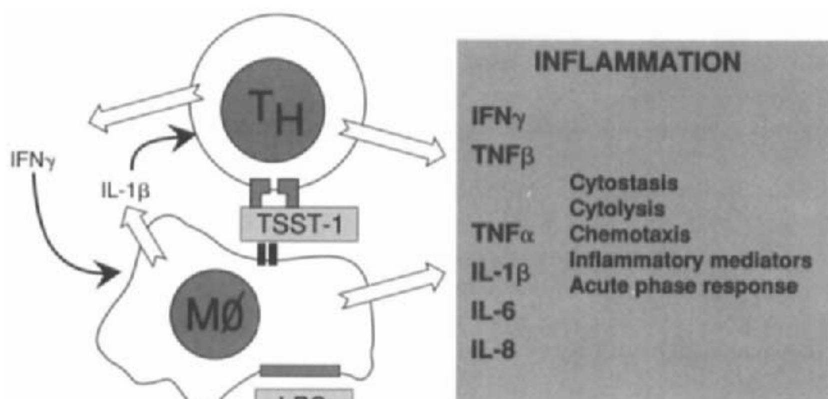

FIG. 6. The role of cytokines in the antigen-independent activation of monocytes and T lymphocytes. *Gram-negative endotoxins.* Cells of the monocyte/macrophage linage can be activated by bacterial endotoxins such as LPS to produce proinflammatory cytokines, especially IL-1β, TNFα, and IL-8. IL-1β produced by activated macrophages will stimulate T-helper cells to produce IFNγ and TNFβ. *Gram-positive enterotoxins.* Many enterotoxins such as toxic shock syndrome toxin-1 can cross-link the T-cell receptor and macrophage MHC class II antigens activating both cells to produce the same spectrum of cytokines as endotoxin activated cells. The proinflammatory cytokines IL-1 and TNF stimulate production of inflammatory mediators such as reactive oxygen species, platelet activating factor, eicosanoids, and proteolytic enzymes. IL-8 is chemotactic for PMN. In concert with IFNγ, TNF is cytostatic and cytotoxic for cells. IL-6 acts as an anti-inflammatory molecule stimulating the production of glucocorticoids and acute phase proteins.

ing to serious infection in traumatized individuals is the immunosuppression that occurs as a result of trauma (A3, F1, H28). Defects in T-cell-mediated immunity are readily demonstrated in trauma victims and there is also some evidence that PMN and macrophage functions are also suppressed (F2, M24, M35, R18, S46). The effect of such events is to weaken defenses against invading microorganisms. In particular the loss of PMN and macrophage function will predispose an individual to bacterial infection, whereas the loss of appropriate T-cell function will lead to a general inability to respond to the majority of invading microorganisms.

The mechanism by which immune suppression is brought about is not well understood, although it has been suggested that important mediators of T-cell suppression include the steroid hormones such as cortisol, soluble IL-2 receptor, trauma-derived peptides, and acute phase proteins (C35, E1, H28, O6, R18, S30, T7, T13). It is also possible that the impaired PMN and macrophage functions observed in trauma patients are not entirely the result of suppression but may be the result of excessive activation and depletion of functional cells from the circulation.

IL-6 has been suggested to play a major role in down-regulating inflammation, one consequence of which is immunosuppression. Important immunosuppressive actions of IL-6 are the induction of corticosteroids and acute phase protein synthesis. Corticosteroids are synthesized by the adrenal cortex largely under the control of pituitary ACTH. However, there is growing evidence that cytokines such as IL-6 can also regulate cortisol production and that T cells make an ACTH-like molecule. IL-6 is able to act both at the hypothalamic level to induce corticotrophin releasing factor (CRF) and directly on the adrenal to induce cortisol synthesis, and it has been suggested that IL-6 is particularly important in maintaining the long-term adrenal response to trauma (S5). Levels of IL-6 following trauma appear to correlate at least as well with cortisol concentrations, which remain elevated after ACTH levels return to normal, as with CRP levels (P1). Corticosteroids such as cortisol can potentially inhibit many aspects of the immune response; frequently these involve inhibition of cytokine synthesis (F13).

IL-6 appears to be the major mediator responsible for the control of acute phase protein synthesis (H11, L7). Acute phase proteins contribute in many ways to the down-regulation of inflammation, inhibition of proteinases, mopping up of reactive molecules, and immunoregulation. One protein in particular has been suggested to be immunosuppressive, AGP (B25, C35). A possible mechanism by which AGP can mediate immunosuppression is by stimulating synthesis by macrophages of an inhibitor of IL-1ra (see Section 2.7.1.) (B54).

A number of cytokines also play important roles in regulating other processes that might contribute to immunosuppression. SIL-2R is released by T lymphocytes stimulated to proliferate by IL-2. IL-1 and TNF induce the synthesis and release of proteinases that might contribute to the production of trauma-derived peptides. The effects of sIL-2R and trauma-derived peptides are probably particularly important at or close to the site of production; that is they are acting in a paracrine fashion.

3.3.2. *Gram-Negative Septicemia*

Gram-negative bacteria produce a cell wall component lipopolysaccharide (LPS) that, when purified and administered to animals, induces an acute phase response. If administered by an appropriate route and in sufficient quantity LPS can cause MOF (B4, N9) (Fig. 6). Cells of the macrophage/monocyte linage have been identified as important cellular targets for LPS (A6, B7, L18, M26, M49). Isolated macrophages stimulated with LPS produce a variety of cytokines including IL-6, IL-1β, and TNFα. Two of these cytokines, IL-1 and TNF, are mediators of the local and systemic effects occurring during infection (B36, B39, D12, D21, K33, W12). The role of IL-6 was discussed earlier (see Section 3.3.1.). Administration of either IL-1 or TNF to animals has been shown to induce many of the effects observed in bacterial sepsis (T15). Elevated TNF levels have been reported in acute lethal sepsis. These observations have led to the suggestion that TNF may mediate MOF

(H2, I4, K28, L25, M49). However, there is growing evidence that suggests that the role of TNF in MOF is not straightforward. In particular the reported levels of TNF and efficacy of antibodies against TNF are different in different models of lethal sepsis (B4). Systemic *Escherichia coli* infection induces high levels of circulating TNF, and antibodies to TNF inhibit the lethal consequences of infection. On the other hand, in compartmentalized infection, rat cecal ligation-induced peritonitis, no circulating or peritoneal TNF was detected and antibodies to TNF failed to inhibit lethal outcome. In another model, which used fecal material to infect the peritoneal cavity, circulating TNF levels were elevated but again antibodies failed to protect against lethal outcome (B4). Circulating TNF and IL-1 have been reliably detected only in the serum or plasma of individuals with severe acute sepsis. This may, however, be due to the presence of serum binding proteins masking lower levels of this cytokine (E6, G22, L3). Chronic administration of low doses of TNF does not mediate the effects of sepsis and when carefully investigated in animal models the effects of LPS and TNF are sometimes found to be different (K28, M55, R7). A more cautious view would be that TNF plays a major role only in some forms of sepsis and MOF. Interestingly it has been reported that the metabolic effects of TNF are different, depending on the site of production (T18). Another example of MOF resembling compartmentalized sepsis but in which bacterial infection is not apparent is severe acute pancreatitis. Recent investigations suggest that excessive PMN activation is a characteristic of this disease but that circulating levels of TNFα are not significantly different from those of TNFα with mild disease (B8). The implication being either than macrophages are not activated to produce TNFα or that any TNF produced is utilized locally.

3.3.3. *Gram-Positive Septicemia*

Gram-positive bacteria can also give rise to septicemia and multiple organ failure. Whereas this path to MOF may be less dramatic and more easily controlled, the cellular basis of gram-positive septicemia provides an interesting adjunct to that of gram-negative sepsis. Gram-positive bacteria produce toxins, so-called "super antigens," which act by cross-linking MHC antigens on macrophages with the T-cell antigen receptor (L2, L12, R14, U2) (Fig. 6). This cross-linking stimulates the T cells to produce mediators such as IL-1, TNF, and IFNγ (F19, M32, U1) that participate directly in mediating an inflammatory response and stimulate macrophages to produce IL-1β and TNFα as well as other mediators that amplify the inflammatory stimulus (D6, G25). Subsequent events probably resemble those of gram-negative-induced MOF.

3.3.4. *Cytokines in the Activation of Polymorphonuclear Leukocytes and Macrophages*

Whereas the evidence that TNF acts as a systemic mediator of MOF is controversial, the role of this cytokine along with IL-1 in mediating local tissue

damage is now attracting the attention of investigators. A number of different cytokines are potentially involved in the recruitment and activation of PMN and macrophages, including the proinflammatory cytokines, IL-1, TNF, and IL-8. Of particular interest are IL-1 and TNF, which can be produced by macrophages and other cells in, and close to, traumatized tissues or by LPS-stimulated macrophages at distant sites. These cytokines have multiple roles in cell activation. They induce expression of adhesion molecules on endothelial cells, facilitating recruitment of leukocytes from the circulation (C30, L5, L34, Z3). Many of the products produced by cells or the reticuloendothelial system in response to TNF can contribute to MOF: reactive oxygen species (D32, K3, W10), proteolytic enzymes, complement components (D4, H36, K5), eicosanoids (K29, L19, M44, M57), and platelet activating factor (M28, R1, S54). TNF and IL-1 induce the synthesis of themselves and each other by a variety of different cells, effectively amplifying the inflammatory response (A9, C32, H25, L23, S53, W21).

There is considerable evidence that TNF can effect many leukocyte activities and might be regarded as a systemic effector molecule for MOF. However, as mentioned earlier, the correlation of circulating TNF levels and MOF in some animal models with severe acute pancreatitis is not convincing. It remains possible that another mediator produced by LPS-stimulated macrophages is responsible for the events leading to MOF (M25, T9). Whatever the role of TNF as a systemic mediator, TNF may act together with other cytokines such as IL-1 and IL-8 in closed compartments to regulate many of the tissue damaging effects contributing to MOF.

4. Cytokine Measurements in Disease

4.1. Inflammation

The key role of the macrophage-derived cytokines in inflammation and the acute phase response has already been discussed. On a theoretical basis it may be expected that their measurement in sera or other biological fluids would be a more sensitive or rapid indication of inflammatory activity than the secondary effects such as acute phase protein production.

4.1.1. *Interleukin-1*

Elevated plasma levels of IL-1 have been detected in a wide range of different conditions that are characterized by inflammation such as rheumatoid arthritis (RA), acute arthritides (D13, E2, M5), Crohns disease (S10), periodontitis (C16), sunburn (G20), burns (K34), endometriosis (F3), psoriasis (C5), gram-positive meningitis (S11), and extended exercise in healthy volunteers (C9). In RA there is some correlation with disease activity (E2). In synovial fluid, biologically active

and immunoreactive IL-1 are detectable in rheumatoid arthritis, osteoarthritis, and other arthritides, and inhibitory activity in biological assays is present in many cases (F22, H30, N13, S43, W30). In a study of synovial fluid from various arthritides, 49% of 111 fluids contained immunoreactive IL-1; of these 74% were from RA and 15% from seronegative arthritides, whereas 11% were from patients with noninflammatory arthritis (W19). Syntheses of IL-1β mRNA (B66) and the IL-1β molecule have been demonstrated in synovia (D29). Raised ventricular fluid IL-1 levels have been found in head injury (M18). IL-1 has been detected in the CSF of patients with multiple sclerosis (H9). TNF was also found to be present in some cases, but IL-6 was found very rarely. It is interesting that no correlation between plasma IL-1 levels and acute phase proteins has been found and there is, as yet, little evidence to suggest that IL-1 measurements will find a place in clinical practice.

4.1.2. *Tumor Necrosis Factor*

Plasma measurement of TNF has been overshadowed by the finding of massively high levels of this cytokine in both patients and animals with bacteremia and endotoxemia (see later). In experimental animals sterile inflammation induces increases in IL-1 but not in TNF (M41). Surgery causes an increase in IL-6, with TNF rising only after significant hemorrhage in mice (A19). In humans, similar studies suggest that trauma in the absence of bacterial infection is not a significant stimulus to TNF production (P21). Inflammation does, however, appear to be a stimulus. Maury (M13) showed raised plasma levels in RA, myocardial infarction, and HIV infection. The highest levels were seen in myocardial infarction, but in most cases the correlation with C-reactive protein was poor. Significant elevations of TNFα and IL-1α but not of IL-6 have been reported in alcoholic cirrhosis and acute alcoholic hepatitis (B43, F12).

In synovial fluid Hopkins and Meager (H31) found that TNFα was consistently elevated in patients with seropositive RA as opposed to other arthritides. TNFβ was not present. Westacott *et al.* (W19) found that synovial fluid levels of TNFα were similar in seropositive RA and seronegative spondarthritides but higher in patients with osteoarthritis. Interestingly, there was a correlation between levels of IL-2, a stimulus to TNF production, and TNF itself. Further studies in osteoarthritis have shown that TNF levels are related to disease duration (D14).

4.1.3. *Interleukin-6*

Elevated levels of IL-6 have been found in cardiac myxoma (J5) and Castleman's disease (Y8). In both cases IL-6 is elaborated by tissues affected by the disease and gives rise to fever, acute phase protein production, and hypergammaglobulinemia, features typical of the acute phase response elicited by this cytokine. Raised serum levels of IL-6 have been detected in patients with burns (N6, P19) and following surgical procedures (C38, N8, P21). Nijsten (N6) showed

that IL-6 detected in plasma within hours after burn injuries was 2 to 100 times the normal level whereas increases in CRP and AAT followed more gradually. In postoperative patients IL-6 was detectable in serum within 3 hr, reached a maximum at 24 hr, and leveled off by 48 hr, whereas CRP could not be detected during the first 6 hr but gradually increased over the next 48 hr. Other acute phase proteins, haptoglobin, AAT, and AGP, increased gradually over a longer time course.

Not surprisingly RA, a disease strongly associated with an acute phase response, was found to have a raised level of plasma IL-6 in many cases (B40, G28, H23). Levels were also raised in polymalgia rheumatica and giant cell arthritis and remained elevated for some time in response to steroids despite a prompt fall in ESR (D3). IL-6 has also been reported in the serum and synovial fluid of patients with various rheumatic diseases (S61). Synovial fluid levels of IL-6 were found to be 1000-fold higher and to correlate positively with those in serum, suggesting localized production in the joint. Synovial fluid levels of IL-6 could also be correlated with articular index, a clinical measure of local inflammation, whereas serum IL-6 levels were positively correlated with CRP and negatively correlated with albumin in RA. Raised levels have been observed in the plasma of patients with cirrhosis (K12), psoriasis (G26), and mesangial proliferative glomerulonephritis (H32).

Many tumors contain IL-6 detected by immunohistochemical techniques and elevated serum levels have been reported in various solid tumors (S24). In mice the growth of several transplantable tumors is associated with increased plasma levels of IL-6, which is correlated with cachexia, one of the effects of this cytokine (see earlier) (G10, M19).

IL-6 is a ubiquitous cytokine whose message can be elaborated by most tissues. It is perhaps not surprising that IL-6 may appear in the plasma of many inflammatory conditions especially if B-cell stimulation is a feature.

4.2. Infection, Sepsis, Endotoxemia, and Bacterial Shock

4.2.1. *Tumor Necrosis Factor*

A considerable body of evidence implicates TNF as a major mediator of mischief in bacterial sepsis and endotoxic shock. It is produced in large quantities after the administration of endotoxins and enterotoxins such as TSST-1 (D6, F14, F19). After binding to high-affinity receptors expressed on all nucleated cells, TNF induces physiological responses such as capillary permeability and endothelial coagulability. These give rise to the hypotension, pulmonary edema, and disseminated hemorrhagic necrosis typical of septic shock.

High levels of TNF are demonstrable in the plasma of rabbits and baboons administered lethal doses of endotoxin within 45–100 min (H20, M10). The

administration of human recombinant TNF to rodents produces circulatory collapse with clinical and postmortem findings indistinguishable from those for septic shock (T14). Monoclonal antibodies [F(ab')2 fragments] directed against TNF, which neutralize the bioactivity in tissue culture, protect against shock, organ injury, and death following lethal doses of endotoxin (T17). Mice of the C3H/HeJ strain, long known to be resistant to the effects of endotoxin, have recently been demonstrated to possess a genetic defect in TNF synthesis (B37).

In humans TNF can be demonstrated in the plasma following the administration of minute doses of endotoxin within 2 hr, and symptoms of fever, headache, malaise, and nausea are correlated with its appearance (H20, M31). Phase 1 trials using recombinant TNF as an anticancer agent have graphically demonstrated that its toxic effects are very similar to those of septic shock (W13).

In very severe gram-negative sepsis TNF concentrations are increased in most patients, sometimes to a very high level, whereas IL-1 and IFNγ are increased in fewer than 20% of cases (D1, G15, W3, W4). Systemic meningococcal disease in children seems to be one of the strongest stimuli to increased plasma TNF levels (B62, G15, W4). Such a situation mimics much of the experimental work that has been undertaken in animal models. In many clinical situations, however, the rapid use of antibiotics makes the clinical course of septicemia a less dramatic and sometimes more protracted one. It is thus clear from a number of studies that findings are quite variable, owing to the difficulty of defining septic shock. At the time of diagnosis of sepsis, Debets (D7) found that only 25% of cases had raised TNF levels but that they had a mortality twice that of patients with normal TNF levels. De Groote *et al.* (D8) found raised levels in only 16% of patients with gram-negative sepsis. Offner *et al.* (O1), on the other hand, found that TNF levels at the onset of septic shock were raised to some degree in all patients but increased thereafter and in some cases remained moderately elevated for a prolonged period. Extremely high levels were found in patients who died within 24 hr. In a study of burn victims who had repeated episodes of bacteremia, TNF was seen transiently and repeatedly in the circulation. The frequency of TNF peaks correlated with both infection and mortality rate (M7).

The evidence incriminating TNF in the pathogenesis of septic shock is now considerable. However, other cytokines may be involved, perhaps in amplifying TNF production or responses. TNF induces IL-1 synthesis in macrophages and endothelial cells and IL-1 administration enhances the lethal effects of recombinant TNF (W2). More recently it has been shown that the administration of recombinant human IL-1 receptor antagonist IL-1ra improves survival in lethal endotoxemia in mice (A12). Indeed the administration of endotoxin alone induces inhibitors of both TNF and IL-1 in human plasma (S48). These findings yet again underline the complexity of the network of cytokines and their inhibition.

Therapeutic strategies based on inhibiting TNF have not yet emerged in clinical practice, although it is interesting to note that glucocorticoids are potent inhibitors

of TNF synthesis and release (H3) and that in humans they are effective very early on in the clinical course (B51, S33). Cycloxygenase inhibitors are currently of interest in this context (S49).

Many studies have now measured TNF in plasma with a view to its use as a marker of the process of septic shock or its severity. Most studies agree that there is a relationship between the level or duration of elevated TNF values and the mortality in septic shock. However, it is difficult to define a value above which prognosis declines markedly, particularly because the concentrations seen in most patients are lower than those in human volunteers after injection of endotoxin without harmful consequences. It is clear from experimental studies (see earlier) that TNF is not the only determinant of outcome. Although peak TNF levels after endotoxin infusion are 15 times higher than peak IL-1β levels and are obtained more rapidly (C10), the presence of raised IL-1 is an independent variable in predicting outcome (D7). As is the case with graft rejection, too much weight must not be placed on cytokine measurements when simple clinical measurements may have more to offer. Calandra *et al.* (C1) in a recent study showed that the clinical severity, patient's age, culture of bacteria from the blood, urine output, and arterial pH contributed more significantly to prediction of patient outcome than serum levels of TNF. They did, however, find that after 10 days TNF was undetectable in survivors but raised (305 pg/ml) in nonsurvivors. It is also clear that gram-positive sepsis may give rise to levels of TNF similar to those in gram-negative infections (M8).

Apart from gram-positive and gram-negative septicemia, raised TNF levels have been found in a number of other infectious conditions, including several parasitic diseases (T16), malaria (K11), leprosy (P11), and AIDS (K20). In malaria TNF levels are elevated in severe malaria, correlating with disease severity and parasite density (S29, S52).

Measurement of TNF in cerebrospinal fluid is of some interest because in both mice and humans considerable levels of TNF are present in the early stages of bacterial meningitis but are not found in viral infection (L13). Unfortunately unmeasurable levels of TNF may be found in bacterial meningitis. However, despite this, discrimination among the conditions is good (N1).

4.2.2. *Interleukin-6*

Gram-positive and gram-negative bacteremia results in elevated plasma levels of biologically active and immunoreactive IL-6. Plasma levels range from 5 to 100 ng/ml (F21, H1, H14, W1). Comparable levels can be induced by administration of LPS or TNF to human volunteers, in which measurable increases take around 30 min, peaking at 2 hr (J1). Unlike TNF, IL-6 appears to be more consistently raised in septicemia. Hack *et al.* (H1) found that levels were increased in 32 of 37 patients with positive blood cultures. Levels were similar in gram-positive and gram-negative sepsis and the degree of elevation was highly correlated with

mortality. Eighty-nine percent of patients with levels above 7.5 mg/dl died, whereas all of those with levels of less than 40 pg/ml survived. In a controlled group of subjects no IL-6 was detectable. In contrast, Calandra *et al.* (C1) found that levels of TNF and IL-1 but not IL-6, on admission, were associated with patient outcome for gram-negative septic shock. In neonates and children Sullivan *et al.* (S58, S59) showed that IL-6 is raised in septicemia and that the levels correlate with mortality. They were unable to detect the cytokine in normal cord blood.

In patients with meningitis cerebrospinal fluid levels of IL-6 may be very high (greater than 500 ng/ml) and appear to be raised in bacterial and viral meningitis and encephalitis (F28, H33). Waage *et al.* (W1) found that IL-6 was detectable in 98% of patients with meningococcal meningitis. In patients with both meningitis and bacteremia, concentrations can be 10- to 100-fold higher in cerebrospinal fluid than those in serum. Raised levels of IL-6 have been detected in the cerebrospinal fluid of patients with multiple sclerosis (L6) but the findings are contradicted by Hauser *et al.* (H9), who found IL-1 and TNF but not IL-6. Another study (F27) reported raised cerebrospinal fluid levels of IL-6 in multiple sclerosis together with levels in plasma that were 17 times higher than those in cerebrospinal fluid. There was no correlation with disease activity or duration and these strange findings were suggested to reflect systemic B-cell responses in multiple sclerosis. IL-6 levels are elevated in amniotic fluid and fetal plasma in neonatal and intraamniotic infection. Second and third trimester amniotic fluid contains low but detectable levels of IL-6 in the 50- to 1500-pg/ml range but no IL-1. Normal parturition results in a modest increase. Romero *et al.* (R21) showed that 10 out of 56 consecutive patients with markedly elevated IL-6 levels delivered preterm and 4 of these had microbiological evidence of infection. In another study, IL-6 levels in infants with isolated perinatal infections were found to be significantly elevated, whereas TNF levels were not (M34).

Whether IL-6 or TNF is the most useful cytokine to detect local and systemic sepsis is yet to be established. The marked elevation of IL-6 in many conditions characterized by macrophage and B-cell activity and its systemic hormone-like role suggest that it may be a hormone of infection, useful as a measurable marker in plasma.

4.3. Allograft Rejection

The process of rejecting a grafted organ represents a massive stimulation of the immune system, involving T cells, B cells, and macrophages. Not surprisingly, therefore, many workers have examined the possibility that measuring cytokines will provide an earlier prediction of impending rejection than current measurements.

Detecting graft rejection has depended largely upon measurements of graft function, although some indices of the immune activation that precedes graft

rejection have been investigated in the past, e.g., β2-microglobulin shed from activated lymphocytes and C-reactive protein produced from the liver as a result of cytokine release. Both showed changes in graft rejection, although neither was found to have a place in its management. It is important to be able to distinguish concomitant infection and cyclosporin toxicity from rejection itself. Unfortunately the immune activation of viral infection is not dissimilar to that of rejection and has proved a major limitation for cytokine measurements in predicting rejection episodes.

4.3.1. *Interleukin-2 and the Soluble Interleukin-2 Receptor (sIL-2R)*

As described, IL-2 binds rapidly to its cellular receptor, which may then be shed from the cell surface into the tissue fluid and from there to the peripheral blood. IL-2 itself has a much shorter half-life than that of the shed receptor. The presence of the soluble receptor is thus a better measure of IL-2 activity than levels of the cytokine itself (R30). IL-2 activity has been a prime target for research into better markers of rejection as it is a key component in T-cell priming for allograft rejection and could be expected to change at a very early stage.

Cytokines have been studied most in renal transplantation and there is a considerable body of literature for both IL-2 and sIL-2R. IL-2 is normally detectable in the serum. It is, however, not detectable by current assays in normal urine (S37) and its presence has been taken to suggest immune attack on the graft. It may, however, imply very mild rejection not requiring specific therapy and should be used in combination with indices of graft function (M3). Raised serum levels of IL-2 do, however, accompany a proportion of rejection episodes (M21). More interestingly, several authors have found that raised levels of IL-2, prior to transplantation, predict a high rate of rejection (C34, S38). This is an interesting observation and suggests that grafting a patient where the immune system is activated enhances recognition and rejection of the graft. The evidence is sufficiently strong to lead these authors to suggest that preoperative IL-2 levels should be measured and transplantation delayed if high levels are found. The findings of all these workers suggest that acute viral infection is extremely difficult to distinguish from rejection but that cyclosporin toxicity does not provide a significant IL-2 response.

Measurement of sIL-2R may be a more sensitive indicator of impending rejection than IL-2, although recent publications suggest that the predictive value of isolated results does not exceed that of creatinine. Levels are increased in chronic renal failure, bacterial and viral infection, and treatment with ATT or OKT3 cells (Y11). Malcus *et al.* (M6) found that serum creatinine and sIL-2R rose over the course of 5 days prior to a rejection episode. Increased sIL-2R in plasma had a sensitivity of 73% and a specificity of 87% for acute rejection, whereas creatinine showed a sensitivity of 70% and a specificity of 84%. A number of authors (D22, N11, S52) have found that it is possible to distinguish viral infection and cyclo-

sporin toxicity from acute rejection on the basis of the degree of elevation of sIL-2R. For example, Forsythe (F24) found that levels of sIL-2R greater than a thousand units/ml or a rise greater than 400 units/ml was invariably associated with rejection. As with many biochemical markers aimed at detecting specific pathological processes, sequential values are more useful than single measurements. A reasonable conclusion to draw from the existing publications is that sIL-2R may have a place in detecting unsuspected acute rejection during the first 1–2 weeks after transplantation.

In liver allograft rejection sIL-2R rises on average 7 days prior to other evidence of rejection or organ dysfunction (P7) and rises higher than sIL-2R in renal allograft rejection (K26). Serum sIL-2R rises to levels comparable to those in rejection by cytomegalovirus infection (P7). Biliary sIL-2R may have some promise as a more sensitive and specific measure. Adams *et al.* (A5) found that levels above 65 units/ml had a sensitivity of 94% and a specificity of 84% for acute rejection.

In heart transplantation Jutte (J7) found raised levels of sIL-2R in 2 of 33 cases during acute rejection and similar levels were also found in 25 out of 172 patients in the absence of signs of rejection. These authors reasonably concluded that such measurements have no part to play in cardiac transplantation.

4.3.2. *Macrophage-Derived Cytokines*

Mononuclear phagocytes in the tissues of rejecting grafts are evident early and inevitably publications have appeared on the measurement of cytokines derived from them. Renal allograft rejection is associated with marked elevations of serum TNF to levels somewhat lower than those seen in severe sepsis (M14). McLaughlin *et al.* (M22) found that increased levels were seen in 24 of 37 cases of acute renal allograft rejection. In contrast sIL-2R levels were not increased in viral infection. Noronha (N11) found increased levels in rejection and viral infection but not in cyclosporin toxicity. In a recent study White and Obers (W28) found that only in patients immunosuppressed with cyclosporin did TNF show consistent changes related to rejection. In patients immunosuppressed with azothiaprine and steroids, levels varied widely from day to day despite lack of any documented rejection. They concluded that IL-1 levels had no value, whereas TNF levels are indicative of immune activation in cyclosporin-suppressed transplant patients. TNF increase is detectable 1–2 days prior to rejection in liver graft recipients. As would be expected, levels are also increased in hepatic artery thrombosis, liver infarction, and endotoxic shock. Raised TNF shows a sensitivity of 67% and a specificity of 95% for acute rejection. If, however, a higher level of 408 ng/ml is taken, the sensitivity is 89% and the specificity 88% (I1). In cardiac transplantation raised levels are associated with rejection, rising several days before other evidence. Cyclosporin and glucocorticoids have no effect (C24).

IL-6 is detectable in the plasma of normal individuals by bioassay or immunoassay, although it is not detectable in normal urine. In uncomplicated renal transplantation, peak levels occur 2–3 days after surgery, falling to normal within 3 weeks. In rejection both serum and urine levels start to increase 2–3 days before clinical evidence and rejection, urinary changes being greater (V4). As with other cytokines sequential measurements provide more useful information (Y6). The systemic reaction of fever, nausea, and lung edema associated with use of murine anti-CD3 monoclonal antibodies (OKT3) as an immunosuppressant is accompanied by a marked increase in serum IL-6 (B49), TNF, IL-2, and IFNγ (A4, C17). Maury and Teppo (M15) found that IL-1, measured by immunoassay, showed a 10- to 20-fold elevation in the majority of graft rejection episodes, predicting clinical rejection by about 1 day. In contrast White and Obers (W28) found that IL-1 failed to give any indication of changes in immune status. Variations in assay characteristics may account for this.

The place of cytokine measurement in monitoring graft rejection is far from clear. At best such measurement may provide an additional early indication of impending rejection. At worst it may add nothing. Further detailed studies are required to address this issue.

5. Cytokine Assays in Body Fluids

Almost without exception, cytokines have been discovered and characterized on the basis of their biological actions. Bioassays have thus preceded the development of immunoassays and in many cases both have been designed for use with tissue culture supernatants. Developing such assays for the measurement of cytokines in biological fluids is not straightforward. Interference from other cytokines or inhibitors and nonspecific interference from binding proteins, interfering antibodies, or matrix effects is a common problem. Unfortunately assays have not always been fully validated for use with biological fluids before being made commercially available or being used to acquire data for publication. Some characteristics of cytokine assays are shown in Table 5. Table 5 shows the factors to be considered in developing assays for use with biological fluids.

5.1. Specificity

The multiple actions of individual cytokines mean that cell lines rarely respond only to one cytokine. This is well illustrated by the efforts to develop specific bioassays for IL-1. Although the original mouse thymocyte proliferation assay was thought to be IL-1 specific, it is now clear that many cytokines influence the assay. IL-6 and TNF can replace IL-1 (G7, U4), and IL-2 and IL-4 can sygerize with

TABLE 5
CHARACTERISTICS OF CYTOKINE ASSAYS

	Bioassay	Immunoassay
Measurement	Biologically active molecules	Numbers of available epitopes (protein or peptide)
Specificity	May be high using specific antibodies	High but may be inappropriate
Interference	Native inhibitors, anti-cytokine antibodies	Serum proteins, anti-cytokine and heterophile antibodies, rheumatoid factors
Sensitivity	High	Reasonably high
Precision	Poor (10–100% coefficient of variation)	Should be good (5–10% coefficient of variation)
Performance	Difficult (1–4 days)	Easy (4–24 hr)

Source. Reproduced with permission from "Cytokines and Inflammation," J. Bienvenu and D. Fradelezi (Eds.), John Libbey, Eurotext, Paris.

TABLE 6
CRITERIA TO BE CONSIDERED IN CYTOKINE ASSAYS

	Bioassay	Immunoassay
Specificity	Confirm with neutralizing antibodies	Dependent on antibody
Accuracy	Specific and nonspecific inhibitors, cytokine binding proteins, antibodies	Protein matrix effect, complement, cytokine binding proteins, rheumatoid factors, heterophile antibodies
	Examine for nonparallel dilution curves of test and standard	
	Assess blood collection procedure (cytokine release by leukocytes and cytokine stability in biological fluids)	
	Determine recovery from whole blood and other body fluids at multiple dilutions	
Sensitivity	Effects of body fluid on cell growth	High background due to matrix
Precision	Between and within assay variation	
Standardization	Refer to international standards	

IL-1 (F4). Many modifications have been developed in an attempt to avoid these problems. Neutralizing antibodies have been used to show that the activity resides in IL-1 or IL-6 (K21). Unfortunately these may contain contaminating cytokines (G6). The assay may be saturated with IL-2 or IL-4 to remove the influence of these cytokines in the test sample (H29). Appropriate serum dilutions may allow IL-1 to be measured, even though at a lower dilution TNFγ would interfere (G7) (Fig. 7). Unfortunately, these strategies do not allow for cytokines that have not yet been described and assays have rarely been tested for the influence of other biologically active molecules such as steroids. Assays do have the advantage that the epitope reactivity can be characterized to some extent if monoclonal antibodies are used. The antibodies can be unequivocally demonstrated to react only with a certain cytokine. Unfortunately monoclonal antibodies raised to recombinant (frequently nonglycosylated) antigens may fail to recognize polymorphic forms of the cytokine and reactivity with precursor molecules or fragments is unpredictable. In biological fluids it may be important to measure molecular and antigenic variants of cytokines, which are different from those of the original cell-derived product.

5.2. Accuracy

Specific and nonspecific inhibitors have now been described for many cytokines. Specific inhibitors such as the IL-1ra (H4) or soluble receptor components shed from cells may be inhibitory in bioassays (F7). IL-1 and IL-6 in plasma may be bound to carrier proteins such as α_2-macroglobulin (B57, M11). In addition circulating autoantibodies to cytokines have been found in normal individuals,

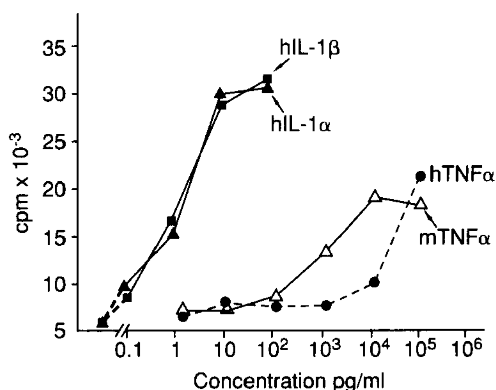

Fig. 7. Dilution curves of human (h) IL-1α, IL-1β, and TNFα and mouse (m) TNFα in an EL46.1 assay for IL-1, reproduced with permission from A. J. F. Gearing and R. Thorpe, Assay of interleukin-1, *in* "Interleukin 1: Inflammation and Disease" (R. Bomford and B. Henderson, Eds.), pp. 79–91, Elsevier, Amsterdam, 1989.

although the autoantibodies' implications for assays are unknown (B23). Such cytokine-specific effects may alter biological activity and possibly immunological reactivity if the epitopes are masked. Although it is obvious, it is important to emphasize that bioassays measure activity and immunoassays measure epitopes on proteins. Both types of information are essential for understanding the role of cytokines in disease, as the amount of biologically active cytokines and the total amount of cytokines in biological fluids may differ considerably. In most of the studies that have been published immunoassays recognize more cytokines than bioassays for biological fluids, and recombinant cytokines added to samples are frequently only partly recovered by bioassays. Figure 8 shows the effect of sample dilution on the inhibitory activity of synovial fluid to which IL-1 has been added (H30). In many bioassays it is necessary to measure a range of dilutions when a bell-shaped curve is seen. Various strategies such as chloroform extraction (C11) and silica adsorption (S26) have been used to remove inhibitory activity from bioassays for IL-1.

Immunoassays established to measure the very low (ng/ml) concentrations of cytokines in body fluids are uniquely susceptible to nonspecific interference from plasma or serum and from interfering antibodies or proteins that may cross-react with those used in the assay. Rheumatoid factors are well known to cause interference in solid phase assays such as ELISA (B8). Heterophile antibodies that react with animal immunoglobulins present a problem in assays in which serum or plasma is used at low dilution (V8) (Table 7). Serum contains a complement, which may interfere with solid phase immunoassays unless inactivation has taken place (B55). In designing assays controls must be employed to test for these effects

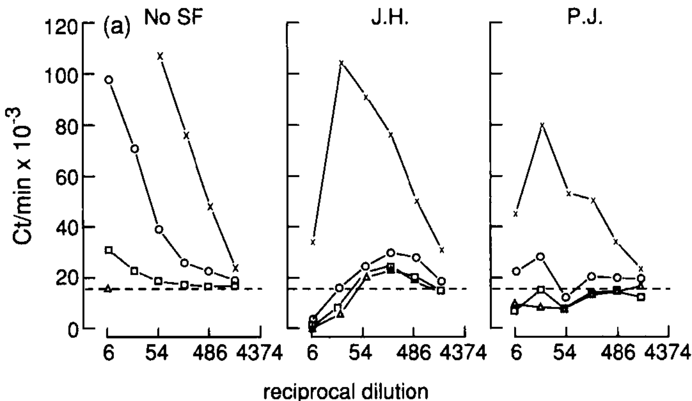

FIG. 8. Purified IL-1β added to synovial fluid (SF) dilutions (J.H. and P.J.) or a medium (No SF) to give an equivalent of 1233 pg/ml (×), 123 pg/ml (○), 12 pg/ml (□), or no (Δ) exogenous IL-1β in the undiluted SF. Reproduced with permission from *Hopkins Clin. Exp. Immunol.* **72,** 422–427.

in samples from both normal subjects and patients, and appropriate strategies such as adsorption of serum samples with animal immunoglobulins must be employed if necessary.

5.3. Sensitivity

Bioassays are generally more sensitive than immunoassays for the measurement of cytokines in cell culture supernatants. The characteristics of calibration curves vary among different assays (Fig. 9) and limits of sensitivity must be set to use only the part of the curve that provides adequate precision.

Biological fluids, plasma in particular, may be inhibitory or cytotoxic to cell lines used in bioassays. In many cases this may be obviated by sample dilution but at the cost of loss of sensitivity (B11). In immunoassays, the protein matrix in plasma and synovial fluid may give a high background signal due to nonspecific binding.

5.4. Precision

Precision in bioassays is almost invariably poor. Considerable variation in the shape of the calibration curve occurs among assays. Immunoassays have better reproducibility if signal levels are sufficiently high above background. High-affinity antisera improve the signal-to-noise ratio and it is worth expending con-

TABLE 7
The Effect of a Second Antibody from an IL-6 ELISA on Eight Human Sera

Patients	Absorbance	Absorbance (second antibody omitted)
1	0.068	0.098
2	0.080	0.077
3	0.789	0.773
4	1.244	1.33
5	0.064	0.070
6	0.222	0.114
7	0.544	0.56

Source. Reproduced with permission from J. T. Whicher and E. Inghram, Cytokine measurements in body fluids, *in* "Cytokines and Inflammation" (J. Bienvenu and D. Fradelezi, Eds.), John Libbey, Eurotext, Paris, 1991.

Note. Samples 3, 4, and 7 contain heterophile antibodies that bound the first and third antibodies. Sample 7 contained, in addition, measurable IL-6 activity. The IL-6 ELISA employs a mouse monoclonal capture antibody, aBSF2-166 (a generous gift from Professor T. Hirano, Osaka University, Japan), coated to a microtiter plate, and a goat polyclonal anti-IL-6 second antibody (a generous gift from Dr. S. Poole, NIBSC), followed by a biotin-labeled mouse monoclonal anti-goat IgG (Sigma) and avidin–horse radish peroxidase (Dako).

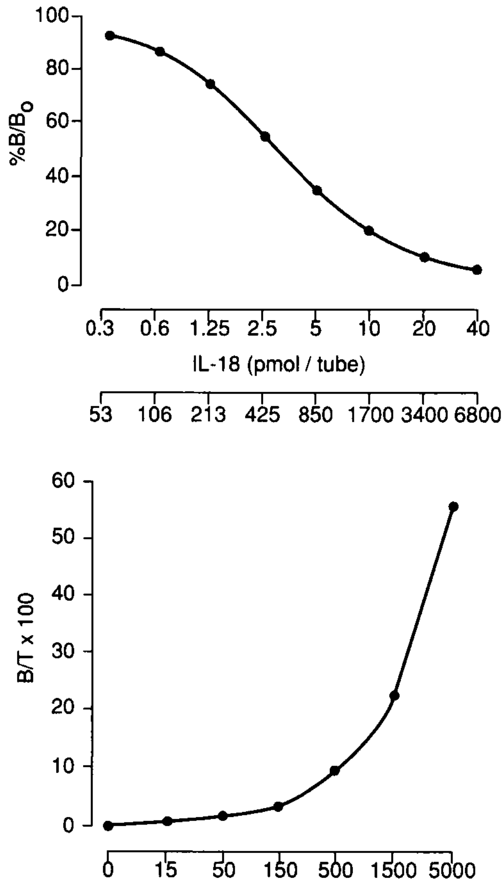

FIG. 9. The calibration curves of two commercial IL-1β immunoassays. Bottom axes represent IL-1β in pg/ml. Reproduced with permission from J. T. Whicher and E. Ingham, Cytokine measurements in body fluids, *Eur. Cytokine Net.* **1**, 239–244.

siderable effort in acquiring polyclonal and monoclonal antibodies of good affinity. Many antisera that provide adequate assays for use with the relatively high levels of cytokines in cell culture supernatants are inadequate for use in body fluids.

5.5. STANDARDIZATION

Standardization of cytokine assays presents the same problems as that of assays for all proteins in biological fluids (W23). Many different biological activities and

TABLE 8
AVAILABLE STANDARD CYTOKINE PREPARATIONS

Preparation	Status	Code	Units/ampule
IL-1α	Interim standard	86/632	117,000
IL-1β	Interim standard	86/680	100,000
IL-2	Interim standard	86/504	100
IL-2 rDNA	NIBSC reference	86/564	202
IL-3	Interim standard	88/780	1,000
IL-4	Interim standard	88/656	1,000
IL-6	Interim standard	8/514	6,250
M-CSF	Interim standard	89/512	60,000
G-CSF	Interim standard	88/502	10,000
GM-CSF	Interim standard	88/646	10,000
TGFβ1 rDNA	Interim standard	89/514	3,000
TGFβ1 (bovine)	None	89/516	
TGFβ2	None	89/518	
TNFα	Interim standard	87/650	40,000
TNFβ	None	87/640	
TNFα (murine)	None	88/532	
GM-CSF (murine)	None	89/612	

Source. Compiled by Dr. R. Thorpe, Division of Immunology, National Institute of Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3 QC, United Kingdom.

numerous preparations of the cytokine may be available: natural and recombinant proteins made in bacteria, yeasts, and mammalian cells. Such materials may vary in potency in different bio- or immunoassays. Comparing data from different laboratories is thus impossible unless proper cytokine standards have been used and their behavior in relation to natural cytokines has been established in a given assay. If standards and samples do not show parallel dilution curves, accuracy will vary according to concentration. Nonparallelism commonly renders an assay inherently invalid due to plasma protein matrix effects. International standards are available for several cytokines (Table 8).

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ANALYTICAL APPLICATIONS OF CHEMILUMINESCENCE

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1. Introduction and Scope of the Article

Of the optical techniques that have become part of the analyst's armory, chemiluminescence is, perhaps, the latest to have come of age. Historically, absorbance spectrophotometry with chromogenic labels has offered the clinical analyst convenience and universality, since UV/visible spectrophotometers are both ubiquitous and amenable to automation. However, direct detection of chromophores without enzyme amplification is limited in sensitivity to 0.1–1.0 μM, since few molar extinction coefficients are greater than 10^5. Radiolabel techniques do offer far superior sensitivity, but as the demands for highly sensitive analyses become more voluminous and more widespread in terms of their setting (i.e., both health-

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related and industrial environments), there will be an ever-increasing drive to adopt alternative nonisotopic analytical methods.

This article is designed to provide readers who are unfamiliar with chemiluminescence with a sufficient theoretical basis to understand the phenomenon and to extend that basis with a survey of its evolution in terms of chemistry, analytical applications, and recent innovations both in high sensitivity clinical assays and in nucleic acid detection methods.

Many recent applications of chemiluminescence enjoy the protection of patents and some of these applications have been commercialized, e.g., novel chemiluminescent compounds, detectors, and complete assay systems. It is not, however, the purpose of this article to focus on such commercialization and, in keeping with this precept, no patent citations will appear in the reference list. Because of space limitations, certain topics such as bioluminescence, electrochemiluminescence [recently reviewed by Greenway (G12)], and cellular chemiluminescence [see Stanley and Kricka (S41) for an update] will not be covered in this article. Even with the above omissions, it is difficult to provide a comprehensive review of all aspects of chemiluminescence, and one has to exercise a degree of selectivity. The focus will be on what is important in the field of clinical analysis, and an attempt will be made to cover the representative literature.

1.1. Why Chemiluminescence?

Among nonisotopic techniques, fluorescence (both intrinsic and extrinsic) offers a convenient mode of detection, and the sensitivity of some fluorescent labels is comparable to that of radiolabeled iodine. Recent innovations include the use of polarized light for excitation, such that the degree of polarization of the emission as well as its intensity can provide information about the concentration and size-related behavior (e.g., rotational diffusion) of the fluorescent-labeled molecule. One disadvantage of steady-state fluorescence techniques is that many analytical samples either autofluoresce or quench the fluorescence of the substance of interest. A recent development that circumvents this problem utilizes long-lived fluorophores such as the lanthanide metal ions as labels. Detection is time resolved and data are collected *after* the decay of spurious or otherwise unwanted fluorescence, i.e., after 100–200 μsec.

Although it is not the purpose of this article to champion one technology over another, it is fair to say that chemiluminescence offers the sensitivity of fluorescence detection without some of the attendant problems of luminescent emission from analytical samples. Thus, chemiluminescence detection can be comparable to and, with the aid of enzyme amplification, even superior to that of ^{125}I. This is not to say that there are no problems associated with chemiluminescence-based analytical techniques. As we shall see later (Section 2.5), the actual signal from chemiluminescent molecules is, in most cases, a transient flash, lasting

for a second to a few minutes, depending on the nature of the chemiluminescent substance. Specialized detection equipment (c.f., scintillation counters) is, therefore, required (see Section 2.6.).

The above caveats notwithstanding, since the first reports of analytical applications of chemiluminescence in the late 1970s (S18, S20, S21, S25, S42), such applications have increased rapidly in number and scope (e.g., flow-through reactors, immunoassays, gene-probe assays, DNA sequencing, Southern blotting, and HPLC), such that chemiluminescence will very likely become the dominant detection technology for solution-phase analytes in the 1990s.

1.2. The Chemiluminescence Literature

Since their inception in 1978, the biennial International Symposia on Bioluminescence and Chemiluminescence have provided an excellent forum for presenting novel data and applications in the chemiluminescence field. The published proceedings from these meetings provide a comprehensive survey of the state of the art, together with relevant literature citations (D8, P5, S17, S41). The trend in the symposia has changed from the early meetings, when there was little emphasis on analysis, to a preponderance of immunoassay and gene probe applications, as well as innovations in detection technologies, such as charge-coupled imaging devices.

Notable books, belonging to series that have focused on chemiluminescence, include the "Critical Reviews," edited by Knox Van Dyke, on instrumentation and applications (V2), cellular chemiluminescence (V4), and immunoassays and molecular applications (V6). The series "Clinical and Biochemical Analysis" has one volume devoted entirely to chemi- and bioluminescence (B34), and a subsequent volume on ligand-binder assays has one chapter focused specifically on chemiluminescent immunoassays (K25). Two volumes of the "Methods in Enzymology" series are devoted to chemiluminescence and bioluminescence (D7, D9). A recent volume in the series "Practical Spectroscopy" provides an excellent survey of analytical techniques involving fluorescence and chemiluminescence (B4). Probably the most readable and all-encompassing book to have been written in recent years is Campbell's "Chemiluminescence: Principles and Applications in Biology and Medicine" (C3), and it is to be highly recommended as a basic text. Whereas chemiluminescence articles are dispersed among dozens of scientific journals, one of these (*The Journal of Bioluminescence and Chemiluminescence*) is devoted entirely to the subject. One measure of the degree of interest in chemiluminescence can be seen in Fig. 1, which shows the growth of published articles in the 1980s, from 50 in 1980 to ca. 450 in 1991. The data were compiled from a computer search of titles or key descriptors containing words associated either with chemiluminescence or with specific chemiluminescent compounds.

In the last few years, a number of useful reviews on chemiluminescence have

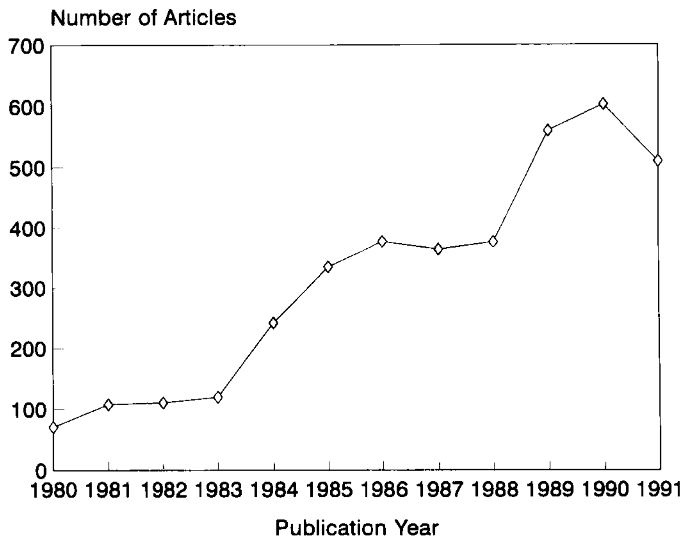


FIG. 1. Growth in chemiluminescence publications since 1980. These data were obtained by a computer search for titles and key descriptors containing the word chemiluminescence or the name of any specific chemiluminescent compound.

appeared. These have described basic theory (M27), immunoassays (D16, H21, M26, M27, S48, W23), enzyme assays (B19, K28), general clinical and analytical applications (B5, G6, S39, W25), chromatography (B4, Y1), gene probes (B7, N5), and industrial flow systems (I1, I2). Stanley has described the setting up of a data bank on chemi- and bioluminescence (S40), and both he and Kricka are responsible for publishing an extremely valuable annual bibliography; e.g., for 1990 see Kricka and Stanley (K27, K28) and for part of 1991 see Kricka and Stanley (K29).

1.3. CHEMILUMINESCENCE AS AN ANALYTICAL TOOL

The analytical applications of chemiluminescence fall into three broad categories. First, there are some chemiluminescent reactions that are catalyzed by specific compounds and that can, therefore, be diagnostic for the presence or quantitation of those compounds. For example, the hydrogen peroxide oxidation of the cyclic luminescent hydrazide luminol is catalyzed by transition metal ions such as Co(II), which can thereby be detected at a 10 p*M* concentration in a flow injection system (B33) and even down to 1 p*M* when the chemiluminescence is induced ultrasonically (K19). Other transition metal ions that have been similarly detected (in the 1–10 nM range) are Cr(III) (C13), Cu(II), and Ni(II) (S26). Other

examples of direct detection using luminol chemiluminescence are described in Section 3.1.5.

1.3.1. *Chemiluminescent Labels*

By far the most popular current method of utilizing chemiluminescence as an analytical tool is to employ chemiluminescent compounds as labels in so-called ligand binder assays such as immunoassays. Such assays were originally designed to detect substances in biological fluids such as blood, serum, urine, or saliva, but are increasingly being adapted to environmental media [such as drinking water or seawater (C19) and flow-through bioreactors (N9)]. Labels are prepared by covalently attaching chemiluminescent compounds either to the hapten (or antigen)

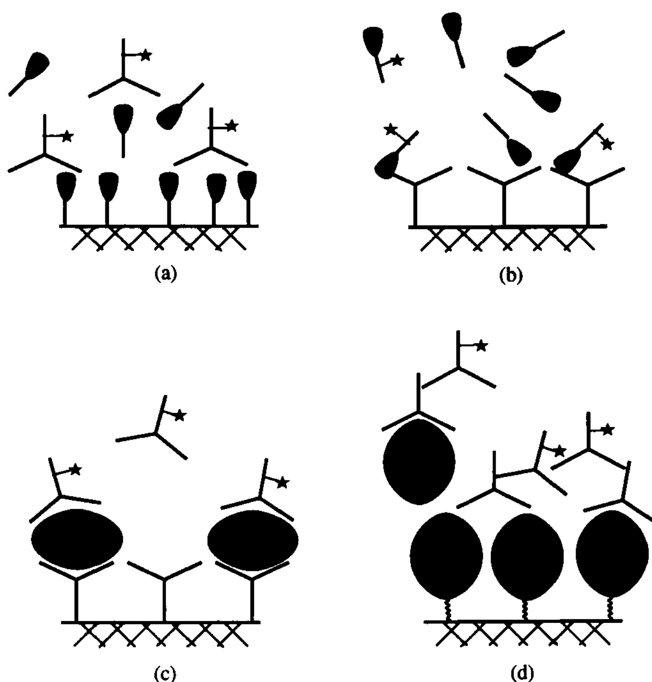


FIG. 2. Solid phase immunoassay configurations using chemiluminescent labels. (a) Small molecular weight analyte (hapten) is immobilized onto a solid phase; labeled antibody in solution partitions between immobilized and sample analyte. (b) Hapten-specific antibody is immobilized onto a solid phase, and sample hapten competes for antibody binding sites with labeled hapten. (c) Protein antigen-specific antibody is immobilized onto a solid phase; antigen and labeled antibody form sandwich complexes on the solid phase. (d) Protein antigen is immobilized onto the solid phase; sandwich complexes are formed between solid phase, antigen-specific monoclonal antibodies, and species-specific (e.g., goat anti-mouse) labeled antibodies; solution phase antigen competes with solid phase antigen for sandwich complexes.

or to the hapten-specific antibody. Some possible configurations of heterogeneous or separation-based assays are illustrated in Fig. 2, which is by no means comprehensive. The general principal of all these so-called solid-phase (separation-based) immunoassays is that a suitable surface (test tube, ELISA well, latex bead, glass bead, or magnetic microsphere) is chemically coupled either to the substance to be detected (hapten or antigen) or to antibodies specific for that substance. In the latter case, for example, as a result of immunoconjugation to form a so-called sandwich complex, the chemiluminescent-labeled moiety will be captured by the solid phase in an amount determined by the concentration of analyte in the sample. After removing unbound material by mechanical separation (particle-based assays) or decantation (coated tubes or wells), the solid-phase surface is treated with the reagents that initiate chemiluminescence, and the resulting signal is measured in a suitable detecting device. In some cases, it is possible to utilize chemiluminescent labels in homogenous, or non-separation-based, ligand binder assays. For example, if the chemiluminescent properties of a labeled analyte (hapten or antigen) are directly modified by interaction with a binder (antibody), then the degree of modification will be determined by the amount of competing, unlabeled analyte that is present in the biological sample. Specific examples of homogenous chemiluminescent assays are described in Section 3.1.4.1.

1.3.2. *Enzyme Labels*

A more recent development in chemiluminescence, as applied to diagnostic assays, is to utilize an enzyme as a label. In this case, it is the enzyme that is captured by an immunoconjugation event at a solid-phase surface and that is subsequently detected by chemiluminescence. This can be achieved by using suitable enzyme/substrate pairs that generate hydrogen peroxide, which, in turn, can be detected by luminol or isoluminol chemiluminescence (see Section 3.1). Alternatively, the enzyme may convert a nonchemiluminescent substrate into a product that spontaneously emits light. This latter method, using, for example, adamantyl dioxetane luminescence, is finding increasing application in both immunoassays and DNA-probe assays (see Sections 3.3.4–3.3.6).

1.4. Types of Chemiluminescent Compounds

The literature on organic chemistry abounds with examples of molecules that, in appropriate conditions, will exhibit chemiluminescence (A1, M17, M22). Nevertheless, the development of chemiluminescence as a diagnostic tool in immunology, clinical chemistry, and molecular biology has resulted almost entirely from the use of three or four compounds and their respective derivatives. There are several reasons for this dichotomy. First, many chemiluminescent compounds are inherently unstable and difficult to control. Conversely, the light-producing reactions of extremely stable compounds (such as some of the dioxetanes) may be so

slow (months to years) that exploitation of such compounds is completely impractical. The third, and perhaps most serious, drawback of the majority of chemiluminescent reactions is that they are inefficient and give rise to very weak or ultraweak chemiluminescence. The significance of this will become apparent in Section 2, in which the theory behind chemiluminescent processes is described.

1.4.1. *Specific Examples*

For the purposes of this section, a brief, and by no means exhaustive, survey of some chemiluminescent reactions is presented. Thus, the thermal decomposition of cyclic peroxides has frequently been accompanied by light generation (K22, M20, W20) according to the general scheme in Fig. 3. Peroxides may also be formed by autooxidation of hydrocarbons, and the transient peroxy-intermediate may disproportionate with concomittant light emission as in Fig. 4 (K4). A postulated precursor of thyroxine has similarly been shown to emit weak light during oxidation at high pH to form a peroxy-intermediate that disproportionates to the corresponding aldehyde plus oxalic acid as in Fig. 5 (C16). The base-catalyzed oxidation of the cyanomethyl indole in Fig. 6a has been shown to be weakly chemiluminescent, and the light-emitting reaction possibly proceeds via the intermediate shown in Fig. 6b (D18, D19).

Unsaturated compounds are also susceptible to chemiluminescent reactions. The liquid olefin shown in Fig. 7 undergoes spontaneous aerobic oxidation, presumably via the cyclic peroxy-intermediate also shown in the figure. This reaction is accompanied by the emission of green light.

In many cases of chemiluminescence involving autooxidation, the intermediate dioxetans cannot be formed. If, for example, tetralin hydroperoxide is thermally converted to tetralone in the presence of the metalloporphyrin zinc tetraphenylporphyrin (Fig. 8), this reaction is accompanied by light emission (L12). Peroxy esters of the form $R \cdot CO \cdot OOR$, either straight chain (D12) or cyclic (A5), have been shown to decompose with light emission, and even Grignard reagents such as *p*-Cl–Ph–MgBr emit light when treated with oxygenated solvent (F2).

While they are all undoubtedly chemiluminescent, the compounds and reactions illustrated above, with few exceptions, generate extremely weak light emission. Even when the emission is amplified by the presence of fluorescent molecules, as in reactions involving chemically initiated electron-exchange luminescence

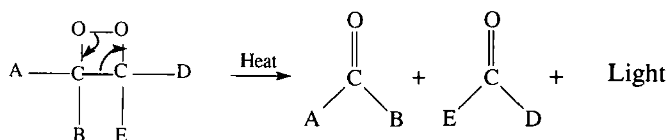


FIG. 3. Generation of chemiluminescence by thermal decomposition of substituted dioxetanes.

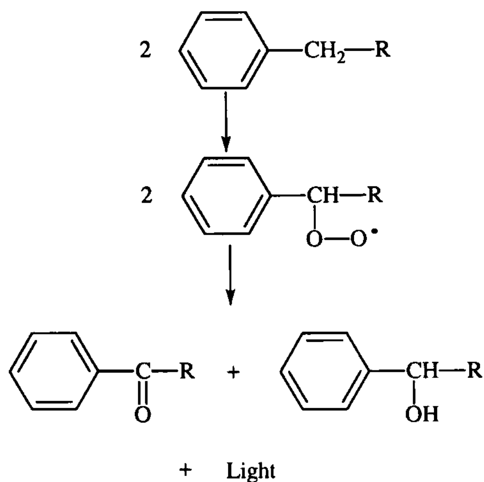


FIG. 4. Autooxidation of hydrocarbons and chemiluminescent decomposition of intermediate peroxide.

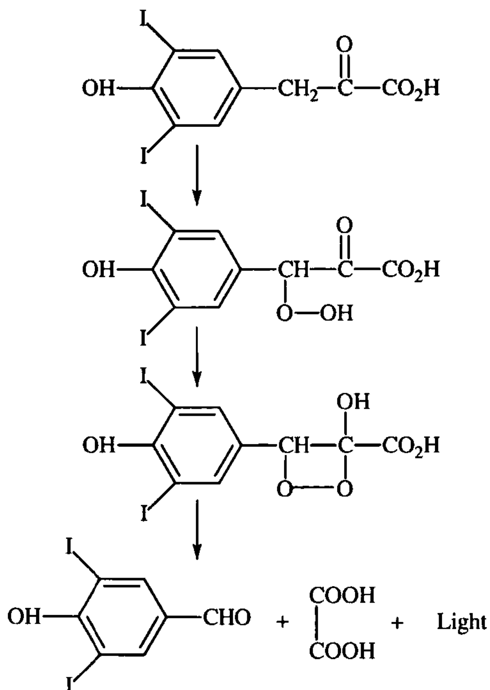


FIG. 5. Formation and subsequent chemiluminescent decomposition of a peroxy intermediate derived from a precursor of thyroxine.

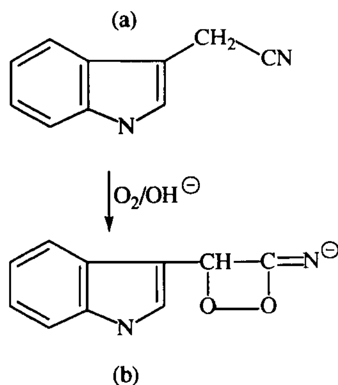


FIG. 6. Base-catalyzed formation of a peroxy intermediate from cyanomethyl indole.

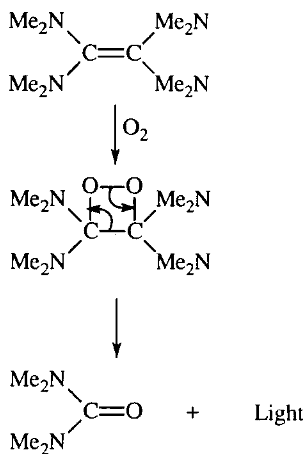


FIG. 7. Chemiluminescent oxidation of *tetrakis* (dimethylamino) ethylene.

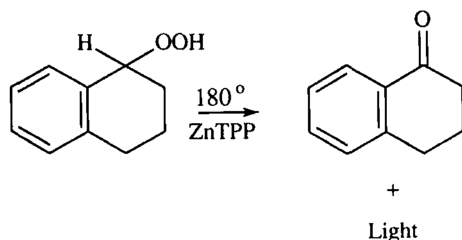


FIG. 8. Zinc tetraphenylporphyrin-catalyzed conversion of tetralin hydroperoxide to tetralone with accompanying luminescence.

(CIEEL) (A10, S14, S23), the light output is still too weak for any practical applications. It is, however, possible that those reactions currently deemed impractical may, by means of innovative chemistry, see the "light of day" in some future analytical application.

1.4.2. *Analytically Important Chemiluminescent Compounds*

To date, the vast majority of analytical and diagnostic applications of chemiluminescence are derived from four types of organic molecule, whose importance stems from a fortuitous combination of properties, viz., high light-emitting efficiency, chemical stability, and amenity to chemical modification in the form of covalent coupling to analytes of interest or analyte-specific antibodies. The general structures of these four classes of chemiluminescent molecule are shown in Fig. 9, and it is the main purpose of this article to focus on each of them in terms of their chemistry, mechanism of chemiluminescence, and contributions to clinical chemistry, immunodiagnostics, and nucleic acid detection. Where data are available, the merits and limitations of each type of compound will be discussed, and some prognosis for future developments will be presented.

Ironically, the synthesis and chemiluminescent properties of some of these important compounds were first reported in the 1920s; i.e., acridine-9-carboxylic acid, a precursor of acridinium esters, was synthesized in 1928 (L7), which was the same year that Albrecht described the chemiluminescence of luminol (A8). The emission from lucigenin, a *bis*-acridinium analog, was reported 7 years later by Gleu and Petsch (G7).

2. Theoretical Background

The following description provides a theoretical framework for understanding the excitation process whereby molecules can be induced to emit light. The

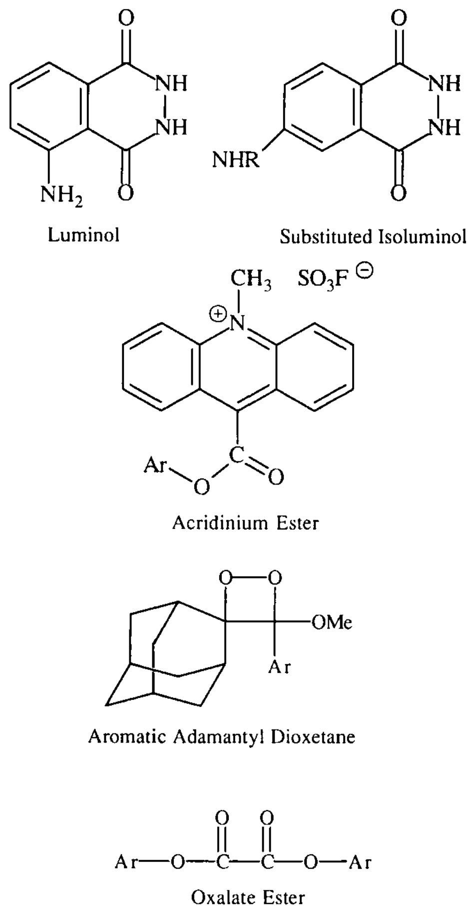


FIG. 9. Chemical structures of chemiluminescent molecules used in diagnostic assays.

efficiency of the process will be discussed as well as ways in which the light can be quantitated. The section will conclude with a brief survey of different types of light-measuring devices.

2.1. The Energetics of Chemiluminescence

Chemiluminescence, as its name suggests, refers to the emission of light by a substance as a result of a chemical reaction. As such, it belongs to a much larger class of light-emitting phenomena in which the distinguishing features of each are primarily (although not entirely) the means by which the molecules of the emitting

substance are supplied with energy. In this case, then, the energy is in the form of the thermodynamic enthalpy change (ΔH) during the chemical conversion of a reactant, A, to a product, B, via a light-emitting intermediate species, B^*, i.e.,

$$A \xrightarrow{k_c} B^* \xrightarrow{k_e} B, \tag{1}$$

where k_c and k_e are the rate constants for conversion of A to B^* and the decay of the light emitting species B^* to the product B, respectively. The species B^* is in an electronically excited state relative to that of the product B, which is referred to as being in its ground state. It may of course be possible to convert ground state A to ground state B without formation of a metastable excited state, i.e., with no light emission. One can easily visualize these processes on a potential energy

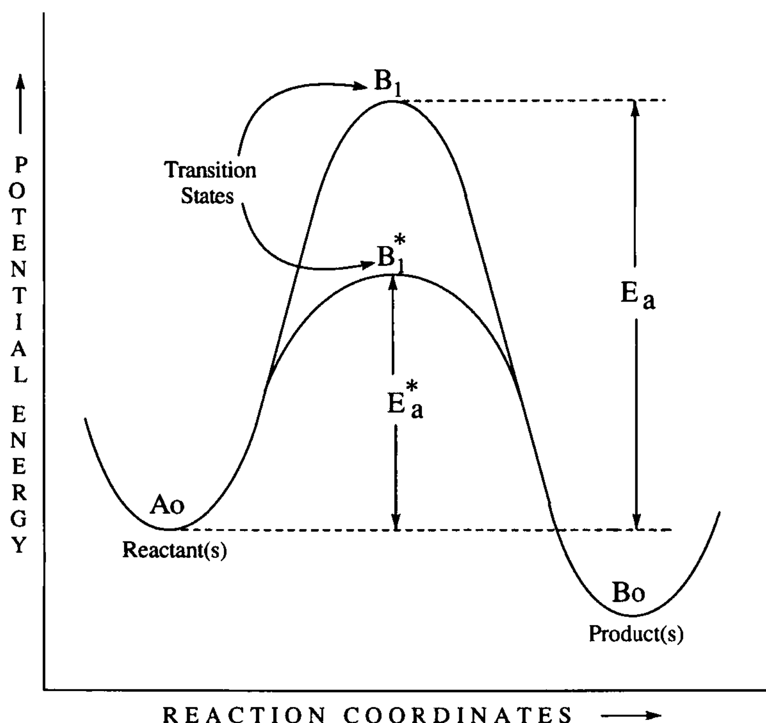


FIG. 10. Arrhenius energy profile showing the energy barrier between reactant(s), A_0, and product(s), B_0. Activation energies E_a and E_a^* are required to elevate reactant(s) to transition state, B_1, or electronically excited transition state, B_1^*. Only molecules that can pass through electronically excited transition state B_1^* will exhibit chemiluminescence.

diagram (Fig. 10) in which, according to the Årrhenius formalism, activation energies E_a and E_a* produce the necessary transition states B_1 and B_1* required for the formation of product B. Since only B_1* is a light-emitting species, it follows that, for efficient chemiluminescence, E_a* must be less than E_a (as in Fig. 10) rather than the converse. Figure 10 is, however, an oversimplification, since it gives no indication of the nature of the energy levels involved. The equilibrium state for any molecule is its ground electronic state. Within that state, vibrational and rotational energy levels exist such that the wave function for any ground state molecule (χ) can be represented as the product of electronic, vibrational, and rotational wave functions, ϕ_e, ϕ_v, and ϕ_r, respectively; viz.,

$$\chi^0 = \phi_e^0 \cdot \phi_v^0 \cdot \phi_r^0, \tag{2}$$

Since electrons in filled orbitals are normally paired (i.e., with opposite spins), the ground electronic state is also referred to as a singlet state. The absorption of chemical energy can produce electronically excited singlet states whose wave function (χ^1) can then be represented as

$$\chi^1 = \phi_e^1 \cdot \phi_v^1 \cdot \phi_r^1 \tag{3}$$

In the case of fluorescence, the transition between states χ^0 and χ^1 is produced by light itself, i.e., by electromagnetic radiation, and the energy difference ΔE between ground and excited singlet states is given by

$$\Delta E = h \cdot \nu, \tag{4}$$

where ν is the frequency of the radiation and h is Planck's constant (6.63×10^{-27} ergs/sec). A clearer picture of the various processes involved in chemiluminescence, from the initial energy produced by a chemical reaction to the final emission of visible photons, is provided by the well-known Jablonski diagram, shown in Fig. 11. Molecules in the ground electronic state, S_0, absorb enthalpic energy which elevates them to higher electronic singlet states, S_1 or S_2. Dissipation of this excess energy can occur in a variety of ways. Internal conversion from S_2 to S_1 can occur, as can deactivation from higher to lower vibrational levels within each singlet state. Electron spins can become unpaired (i.e., parallel), in which case the excited molecules cross over to the triplet state in a process known as intersystem crossing. After losing sufficient energy to occupy the lowest vibrational level of the first excited singlet state S_1 (or corresponding triplet state T_1), molecules may still return to the ground state without any light emission, in which case the decay is radiationless and takes the form of heat exchange to surrounding molecules or solvent molecules. On the other hand, the excess energy may be dissipated in the form of light emission. Decay from singlet states occurs in the nsec time scale and is referred to as fluorescence or chemiluminescence, depending on the original source of excitation energy. Decay from triplet states is a much slower process and occurs in the µsec–msec time scale. This long-lived emission

is referred to as phosphorescence, and it has found applications both in clinical diagnostics [time-resolved fluorescence of lanthanides (D10, S34)] and as a method to study the slow rotational diffusion of proteins in cell membranes, whereby one measures the decay of phosphorescence anisotropy from a polarized excitation source.

If light emission does occur, the energy difference between the lowest vibrational level of the first excited singlet or triplet state and the ground state S_0 determines the frequency (ν) of that emission, by Eq. (4). Further, since

$$\nu = c/\lambda, \tag{5}$$

where c is the velocity of the light *in vacuo* (3×10^{10} cm/sec) and λ is its wavelength, one can relate the energy difference ΔE to the wavelength of the emission by combining Eqs. (4) and (5) into the so-called Planck–Einstein relationship.

$$\Delta E = h \cdot \nu = h \cdot c/\lambda. \tag{6}$$

To appreciate the magnitude of the energy involved in luminescence emission,

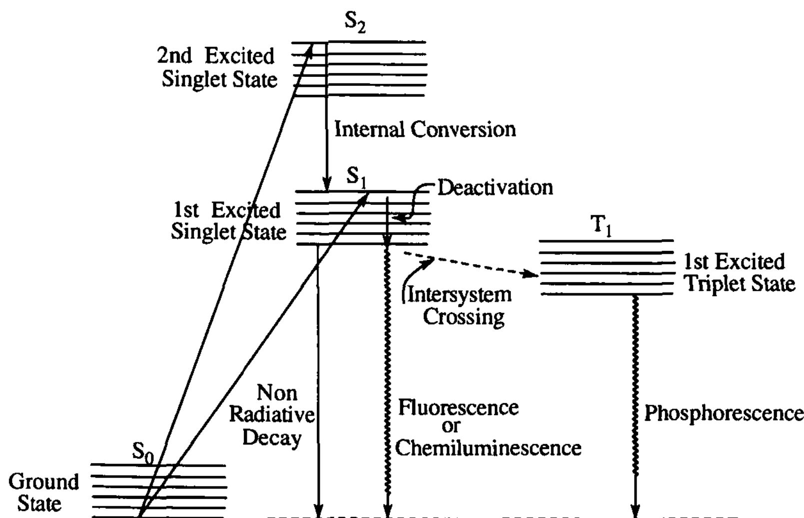


FIG. 11. Jablonski diagram showing how the absorption of electromagnetic or chemical energy by a molecule can lead to electronically excited states and showing that the dissipation of such energy can result in luminescence emission. Maximal fluorescence/chemiluminescence emission will occur from the lowest vibrational level of the first excited singlet state, S_1, to the same vibrational level in the ground state, S_0. Intersystem crossing to the triplet state, T_1, can give rise to long-lived emission or phosphorescence.

we can calculate the range of energies required to produce emission in the visible spectrum, i.e., from 400 to 750 nm. For photons of violet light (λ_1 = 400 nm), we have from Eq. (6),

$$\Delta E_1 = \frac{h \cdot c}{\lambda_1} = \frac{6.63 \times 10^{-27} \text{ erg/sec} \times 3 \times 10^8 \text{m/sec}}{400 \times 10^{-9} \text{ m}}$$

$$= 4.97 \times 10^{-12} \text{ ergs}$$

and since 1 erg = 2.39×10^{-11} kcal,

$$\Delta E_1 = 11.88 \times 10^{-23} \text{ kcal/photon.}$$

Further, since 1 "mole" of photons (defined by modern convention as 1 Einstein) contains N photons (where N is Avogadro's number, 6×10^{23}), then

$$\Delta E_1 = 71.27 \text{ kcal/mole.}$$

In a similar fashion, it can be shown that photons in the far red (λ_2 = 750 nm) would require an energy excess (ΔE_2), where

$$\Delta E_2 = 38.01 \text{ kcal/mole.}$$

Thus, the energy spread for visible emission is from 38 to 71 kcal/mole. An important point to make at this juncture is that the energy of the emission (ΔE) has *nothing* to do with the intensity of that emission. It merely defines its frequency and, therefore, its wavelength. The fact that blue photons are more *energetic* than red ones (as the example above showed) has implications for the way in which photons are detected; i.e., there is usually a degree of chromatic dispersion in detector response (see Section 2.6.).

If the energy is supplied by a chemical reaction, then, for a given wavelength (λ), the combined enthalpy and activation energy for the formation of the electronically excited transition state (Fig. 10) will be given by

$$-\Delta H + \Delta E^* \geq h \cdot c/\lambda. \tag{7}$$

A consideration of Figs. 10 and 11, and Eq. (7) can provide some information on the molecular structure of potentially chemiluminescent organic molecules and may provide a framework for devising novel ones. Nonproductive energy losses will be minimized in molecules with few vibrational states in any given electronic state. This would dictate that small, rigid, planar structures with restricted rotational mobility between bonded atoms, i.e., condensed aromatic or heteroaromatic molecules, may be potential chemiluminescent compounds. The actual chemical reaction itself should be exothermic (large negative ΔH) and have a small Årrhenius activation energy (ΔE^*) for producing the transition state of the electronically excited species (B_1* in Fig. 10). The latter implies rapid molecular rearrangements such as fragmentation and electron transfer. Indeed, the fragmen-

tation of peroxides (either as starting materials or as metastable reaction intermediates) accounts for a high proportion of known chemiluminescent processes. Chemiluminescence from electron transfer processes normally requires electrogeneration of the excited species and the resulting emission is known as electrochemiluminescence (ECL). ECL applied to clinical diagnostics is relatively new, but may well achieve prominance during the next decade as biosensor technology matures.

A low activation energy, ΔE^* (or E_a^* in Fig. 10), also dictates that the geometry of the transition state should closely resemble that of the excited singlet state. For further consideration of the relationship between the thermodynamics of chemiluminescence and molecular structure, the reader is referred to McCapra (M17) as well as the review article by McCapra and Perring (M22).

2.2 Chemiluminescence Efficiency

In the worked examples above, there was a tacit assumption that 1 Einstein (1 mole of photons) is produced by 1 mole of chemiluminescent reactant. However, a reference to Fig. 11 shows that this cannot be the case. Only a fraction of the energy supplied to ground-state reactant molecules will finally be converted into photon emission. A parameter of major importance, then, in the field of chemiluminescence is the quantum yield of the process. Stated simply, the quantum yield, Q relates the photon output to some reference input such as the number of ground-state molecules in the chemiluminescent reaction; i.e., its absolute value will be in Einsteins per mole of reactant. Clearly, Q must reflect the variety of means whereby molecules are able to dissipate the excess energy absorbed in the chemical reaction (Fig. 11). It is generally accepted that Q is the product of three separate components, viz.,

$$Q = q_r \cdot q_{ex} \cdot q_{em}, \tag{8}$$

where q_r is the fraction of molecules undergoing a potentially light-producing reaction (i.e., leading to B_1^* not B_1 in Fig. 10), q_{ex} is the fraction of those molecules that are elevated to the excited singlet state, and q_{em} is that fraction of molecules in excited states that actually emits light. In this context, the role played by the solvent may be crucial in determining the magnitude of q_{em}, since the nonradiative decay of excited molecules to the ground state is often influenced by the nature of the surrounding solvent molecules. In general, quantum yields are higher in organic solvents than in water, and, in such solvents, Q is often inversely related to the solvent polarity or dielectric constant. The explanation for this presumably reflects the ease with which excess energy from electronically excited states can be transferred to polar molecules (e.g., water) rather than apolar ones (e.g., benzene).

2.3. Units of Light Measurement

The number of photons produced radially per unit time from a point-source emitter is denoted as the flux. The intensity of the emission, on the other hand, is the flux per unit solid angle (or steradian). However, the term intensity is frequently used without any reference to which portion of space relative to the point source is being considered. In fact, from the point of view of detecting the emission, the important quantity is the rate of photon emission per unit area of detector face located some distance away from the source. This quantity, too, is loosely referred to as intensity. I shall consider the characteristics of a detector and some recent innovations in chemiluminescence detection in Section 2.6. With reference to units, though, one can describe the rate of light generation in terms of the number of photons (or Einsteins) per second or in terms of their corresponding rates of energy production [Eq. (2)], i.e., in ergs per second. Since the rate of change of energy is in fact the power sustained by the source and since 10^7 erg/sec is equal to 1 W, we can interconvert photon flux with the power, in watts, sensed by a detector. For example, if a solution of acridinium ester (see Section 3.2) is chemically excited to produce 10^6 photons/sec of blue light at 430 nm, then by Eq. (6),

$$E = \frac{h \cdot c \times 10^6}{430 \text{ nm}} \text{ erg/sec} = \frac{h \cdot c \times 0.1}{430 \text{ nm}} \text{ W}$$

$$= \frac{6.63 \times 10^{-27} \times 3 \times 10^8 \times 0.1}{430 \times 10^{-9}} \text{ W}$$

$$= 0.46 \times 10^{-12} \text{ W}.$$

2.4. Label Detectability

The driving force for the development of chemiluminescence-based assays (as well as any other optical or electrical detection methodology) is the replacement of radiolabels both for safety reasons and because of their intrinsic instability. Because the earliest high sensitivity immunoassays utilized antibodies with covalently attached ^{125}I as the label, this has served as a yardstick against which all subsequent assay technologies are measured. For this reason, it is important to understand the detection limits for ^{125}I. Radioactive iodine is a γ-emitter that eventually decays to a stable isotope of lead. The decay process exhibits first-order kinetics so that we can write

$$-\frac{dI}{dt} = k \cdot I_0 \quad \text{and} \quad I = I_0 \cdot e^{-kt}, \tag{9}$$

where I is the amount of label at time t (sec), I_0 is the initial amount, and k is the

first-order rate constant. When the radiolabel has decayed to half the original amount, t is equal to $t_{1/2}$, the decay half-life, i.e., from Eq. (9),

$$I_0/2 = I_0 \cdot e^{-kt_{1/2}} \quad \text{or} \quad \ln 2 = kt_{1/2}.$$

Therefore,

$$k = \ln 2/t_{1/2}. \tag{10}$$

Equation (9) states that the rate of decay of ^{125}I is the product of the initial amount of radiolabel and the rate constant for decay. The minimum amount of ^{125}I that can be detected corresponds to the lowest detectable decay rate. If we assume this to be 1 dps (ca. 50 cpm), then

$$1 = k \cdot I_{\min} \quad \text{or} \quad I_{\min} = 1/k \tag{11}$$

and, from Eq. (10),

$$I_{\min} = t_{1/2}/\ln 2 \text{ molecules.} \tag{12}$$

Therefore,

$$\begin{aligned} I_{\min} &= 60 \times 24 \times 3600/\ln 2 \text{ molecules} \\ &= 7.48 \times 10^6 \text{ molecules} \\ &= \frac{7.48 \times 10^6}{6 \times 10^{23}} \text{ moles} \\ &= 1.25 \times 10^{-17} \text{ moles.} \end{aligned}$$

If the assay volume is 1 ml, then

$$I_{\min} = 1.25 \times 10^{-14} \text{ moles/liter.}$$

This, then, represents the minimum *label* detectability, which means that the minimum *analyte* detectability cannot be lower than this and will almost certainly be higher.

2.5. Kinetics of Light Emission

Before considering detector characteristics and some recent developments in chemiluminescence detection, it should be noted that analytical applications of chemiluminescence involve two types of chemiluminescent response. In the first type, the chemiluminescent molecule is used as a detection label and is, therefore, present in limiting concentration relative to the reagents used to initiate the chemiluminescent reaction. The chemical reaction will therefore be "pseudo" first order. The slowest process in the sequence of events leading to light emission is the reaction itself, e.g., hydrolysis, bond-breaking, and rearrangements. From Eq. (1), one can therefore write that the intensity I_{cl} will be given by

$$I_{cl} = Q \cdot dB/dt; \tag{13}$$

i.e., it is a product of the rate of formation of product, B, and the quantum yield of the process. The kinetic profile of the light output will have the form shown in Fig. 12a, i.e., an initial lag phase followed by a rapid increase in the rate of product formation up to a certain maximum, after which there is no more reactant to replace the excited-state product, and the intensity will decay monoexponentially, provided a single substrate is being consumed. The emission will, therefore, be in the form of a flash lasting from 1 sec to tens of seconds.

In the second application of chemiluminescence, an enzyme is used as a label, i.e., in limiting amount, and the enzyme is detected by a large excess of substrate that becomes chemiluminescent upon reaction with the enzyme. Again, light intensity is proportional to the reaction rate, which in this case is the substrate turnover rate. The kinetic profile of light intensity will therefore reflect the enzyme kinetics and will take the form shown in Fig. 12b. Since the emission of light cannot take place before the formation of the excited state, there will again be a lag phase. However, when the enzyme is turning over substrate at a constant rate, i.e., in the steady state, the light output will plateau and also remain constant, for

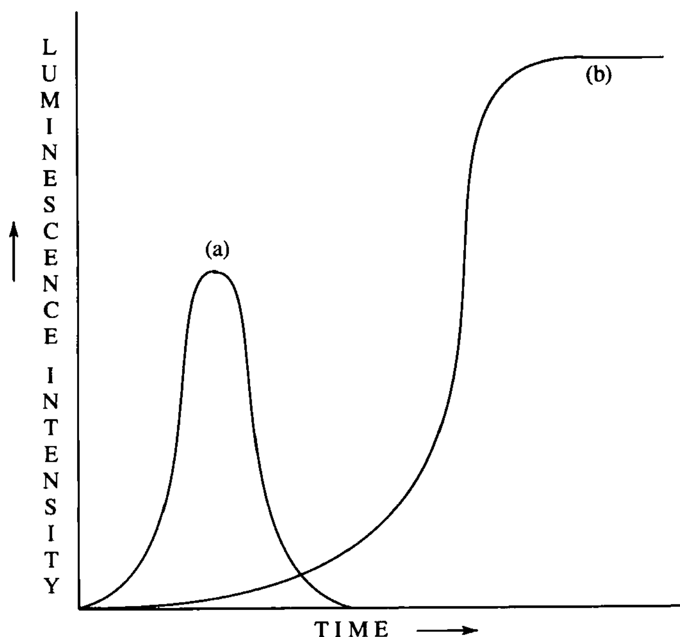


FIG. 12. Kinetic profile of chemiluminescence emission. (a) Flash-type kinetics from chemiluminescent labels; (b) glow-type kinetics from enzyme labels with chemiluminescent substrates.

tens of minutes or even hours. Such an emission is referred to as a glow, to distinguish it from the transient output produced by chemiluminescent labels.

2.6. Chemiluminescence Instrumentation

The light output from a chemiluminescent reaction is measured in a luminometer. These range from simple manual instruments capable of taking a single reaction tube, through semiautomated instruments with capacities of up to 250 tubes, to high-throughput automated systems that conduct complete assays, including reagent pipetting, separations, and final readout.

Three major innovations in instrumentation have occurred within the last decade. First, photomultipliers have been improved with respect to sensitivity, noise, and uniformity of spectral response. Second, there has been a parallel development of, on the one hand, large, fully automated, complete analytical systems and, on the other hand, small, battery-powered instruments employing silicon photodiode detectors. Third, there has been a revolution in various imaging devices, motivated, in large measure, by the analytical demands of recombinant DNA technology. Thus, camera luminometers and charge-coupled imaging devices are finding increasing application in semiquantitative analyses of microtiter plates, sequencing gels, and both Western and Southern blotting techniques. It is outside the scope of this chapter to discuss detection instrumentation in any detail. However, the interested reader can find a wealth of theoretical and practical information in some older reviews (A11, S36, S37, W1) as well as some more recent discussions (B17, C3, S38, T22).

There are several important differences between solid-state detectors, such as photodiodes, and vacuum devices, such as photomultipliers. These are highlighted in Table 1, from which it can be concluded that the choice between the two really depends on the type of analysis one wishes to carry out. Large, high-sensitivity instruments designed for the clinical laboratory invariably employ photomultipliers for detection. Recent innovations in photomultiplier design include the use of

TABLE 1
The Main Differences Between Solid-State and Vacuum Tube Detectors

Properties	Photomultiplier	Photodiode
Quantum efficiency	Low	High
Physical characteristics	Fragile	Rugged
Spectral response	Low in red	Low in blue
Relative sensitivity	1000	1
Power source	High voltage	Battery
Cost	Expensive	Cheap

bi-, tri-, or multialkali photocathodes, which partially alleviate the fall-off in response in the red region of the spectrum (>600 nm). Cooling of the tube is being increasingly adopted to reduce noise from thermally produced electrons.

However, if size, portability, and ease of use are important criteria for a specific application, then the new generation of solid-state photodiode detectors may be ideal. One such instrument has recently been described for measuring ATP down to 0.5 pmole, using the power from four AA alkaline batteries (M3). Detection on instant film in photocassettes (B30, M33), or in so-called camera luminometers (B32, K30), is rapidly expanding the scope of luminescence measurement and can be exemplified by an assay for hepatitis B viral DNA (see Section 3.3.6.1) (B26).

There are two ways to increase the amount of detectable signal from a chemiluminescent label. First, one can improve the quantum efficiency of the light-emitting reaction by chemically modifying the label itself, or its microenvironment. Second, one can increase the sensitivity of the detecting instrument such that low, hitherto undetectable, light sources are now measurable. This latter approach has engendered much effort in low-level imaging technology from early work with TV vidicons (R4) to the use of charge-coupled devices, or CCDs (H1, H14, L3, W17). These are solid-state detectors containing a matrix (e.g., 256 × 256) of photosensitive pixels. Incident photons from a chemiluminescent sample liberate charge within the pixels and this charge can be accumulated before it is discharged (by charge coupling) to give a readout proportional to the incident photon flux. Multisample analyses are possible, e.g., simultaneous quantitation of samples in microtiter plates or petri dishes (H14, M1). By the same token, multiband gels or blots (protein and nucleic acid) can also be visualized by CCDs (B28, H8, H9, P12, T18).

An overview of the various types of instrument currently used for chemiluminescent assays has been given by Van Dyke (V3) and more recently by Bronstein and Kricka (B20). Table 2 is a compilation of several commercial instruments with a brief description of the key features of each. An automated flow-through system for clinical chemistry assays utilizing luminol chemiluminescence (see Section 3.1) has been described by Tabata *et al.* (T4). Semiautomated (B14) and fully automated (K5,K34) immunoassay instruments using acridinium esters (see Section 3.2) have been developed and evaluated (C6,Z4).

Before we examine chemiluminescent compounds in detail, mention should be made of biosensor devices that utilize chemiluminescent detection. As early as 1978, Freeman and Seitz (F3) described a fiber-optic system using luminol to detect hydrogen peroxide at micromolar levels. More recently, the technology has been vigorously pursued by Coulet and co-workers in France [see Coulet and Blum (C21) and Gautier *et al.* (G3) for an update on the state of the art]. Of particular interest in this context (although it is outside the scope of this article) is the development of a "reagentless" fiber-optic bioluminescent sensor for detecting ATP and NADH at picomole levels (G1, G2).

TABLE 2
A SELECTION OF COMMERCIAL LUMINOMETERS[a]

Company	Country	Model	Sample Type/Detector	Applications
Amersham	United Kingdom	RPN 5104 (5106 in United States)	Microtiter plate/PMT	Immunoassay; DNA/protein blotting
Analytical Luminescence Laboratory	United States	Camlight 500	Multiwell/film	ATP/immunoassay
		Camlight 501	Single well (gels)/film	ATP/immunoassay
		Moonlight 2010	Single tube/PMT	ATP/immunoassay
Astromed	United Kingdom	CCD3200	Charge-coupled device	Low-level imaging
Berthold Analytical Instruments	Germany	Clini-Lumat	Single tube/PMT	Immunoassay
		LB950	Multi-/PMT	Immunoassay
		LB952T	Multi-/PMT	Immunoassay
		LB9500	Single/PMT	Immunoassay
		LB9501	Single/PMT	Immunoassay
		LB9505	Six-tube/PMT	Immunoassay
BioChemMack	Russia	EMILITE	Single tube/photodiode	Immunoassay
Bio-orbit	Finland	1251	Multitube/PMT	Immunoassay
C-Gem Biomedical	United States	Optocamp I	Single tube/PMT	Immunoassay
		Optocamp II	Multitube/PMT	Immunoassay
Ciba–Corning Diagnostics	United States	MLA I	Single tube/PMT	Immunoassay
		MLA II	Multitube/PMT	Immunoassay
		ACS 180	Automated assay system	Immunoassay
Dynatech	United States	CL 1050	Microtiter plate/film	Immunoassay
		ML 1000	Microtiter plate/PMT	Immunoassay
		TL 1	Single tube/photodiode	Immunoassay
Hamilton	United States	Lumicon	Six-tube	Immunoassay
High-Tech Scientific	United Kingdom	RML-10	Cuvette/PMT	Rapid mixing kinetics
Hoeffer Scientific Instruments	United States	Photocassette	Microtiter plate/film	Immunoassay
LKB Wallac	Finland	1251	Multitube/PMT	Immunoassay
London Diagnostics	United States	Luma-Tag Analyzer	Single tube/PMT	Immunoassay
		Luma-Tag Auto/Analyzer	Multitube/PMT	Immunoassay
Los Alamos Diagnostics	United States	633	Multitube/PMT	Immunoassay
		6500	Multitube/PMT	Immunoassay
Shodex	Japan	CL-2	Flow-through/PMT	HPLC detection
Soma	Japan	S-3400	Flow-through/PMT	HPLC detection

(continued)

TABLE 2 *(Continued)*

Company	Country	Model	Sample Type/Detector	Applications
Tropix	United States	ICL 901 ICL 902 ILA 911 ILA 912	Microtiter plate/film Membrane blot/film Single tube/PMT Multitube/PMT	Immunoassay DNA/protein blotting Immunoassay Immunoassay
Turner Design	United States	20e	Cuvette; scintillation vials/PMT	Research; immunoassay

[a]Adapted from the data of Bronstein and Kricka (B20), together with more recent material.

3. Applications of Specific Chemiluminescent Compounds

The following account presents a more detailed look at those chemiluminescent compounds and their derivatives that are currently regarded as useful in clinical assays. An attempt will be made to evaluate the literature and to highlight the strengths and limitations of each class of compound.

3.1. Luminol and Isoluminol

The oxidations of the aminophthallic hydrazides luminol and isoluminol (Fig. 13) are among the earliest chemiluminescent reactions to have been studied. Albrecht (A8) reported in 1928 that luminol, when oxidized with hydrogen peroxide or hypochlorite ions, emitted a blue light. Drew *et al.* (D13–D15) showed that derivatizing the heterocyclic ring abolished the luminescence of luminol and that any substitution of the 5-amino group lowered the quantum yield of the light

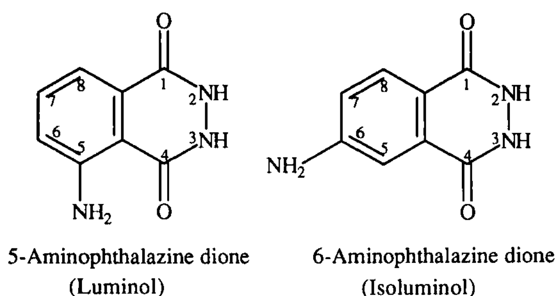


FIG. 13. Chemical structures of the chemiluminescent cyclic hydrazides luminol and isoluminol.

emission to a degree determined by the electron-withdrawing properties of the substituent. Thus, a 5-NO_2 group instead of a 5-NH_2 group lowered the light emission by a factor of 10^4 (W11). In aqueous systems, the quantum yield of luminol is reportedly 25 times (W11) or 10 times (B31) that of isoluminol (1.5 and 0.15%, respectively). However, unlike luminol, derivatization of the amino group in isoluminol by alkylation is beneficial, provided that steric hindrance of the alkylamino group does not distort electron delocalization from the nitrogen lone pair to the aromatic ring. Thus the quantum yield of *N, N*-diethyl isoluminol was shown in the mid-1960s to be similar to that of luminol (G17, G19). More recently, derivatives containing terminal amino groups for conjugating to haptens or antibodies have been prepared, and one of the most popular, aminobutylethyl isoluminol, or ABEI (S20, S21), is shown in Fig. 14.

3.1.1. *Mechanism of Chemiluminescence*

In aqueous solution, the light-emitting reaction of luminol has several stringent requirements, viz., a high pH, a strong oxidant, and a catalyst or initiator. Typical oxidizing catalysts are hypochlorite (S26), transition metal complex ions such as $Fe(CN)_6^{3-}$, and a variety of iron-containing compounds such as porphyrins, hemoglobin, peroxidases, and catalase (G10, S20). Oxygen-containing species include molecular oxygen, singlet oxygen, ozone, and hydrogen peroxide (A15, B16).

Thus a typical chemiluminescent reaction of luminol can be represented by Fig. 15. In this mechanism, which is not yet completely established (M21), the ferricyanide is acting as a one-electron oxidant. The final step in the reaction is the formation of an excited-state aminophthalate dianion, which decays to the ground state, emitting blue light at 425 nm. In DMSO, the emission is yellow–green at 500 nm, and, in aqueous DMSO, both emissions occur in a ratio that is dependent upon solvent composition (W12, W15). Although both emissions proceed via an excited-state aminophthalate dianion, it is thought that a rapid tautomerization and proton exchange to an excited-state quinoid form occurs in the aprotic polar

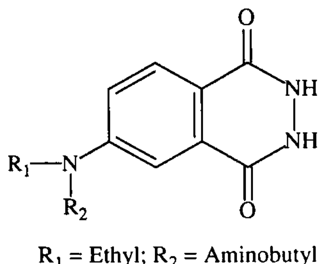


FIG. 14. Chemical structure of the popular isoluminol label ABEI (*N*-aminobutyl-*N*-ethylisoluminol), synthesized by Schroeder and Yeager (S20).

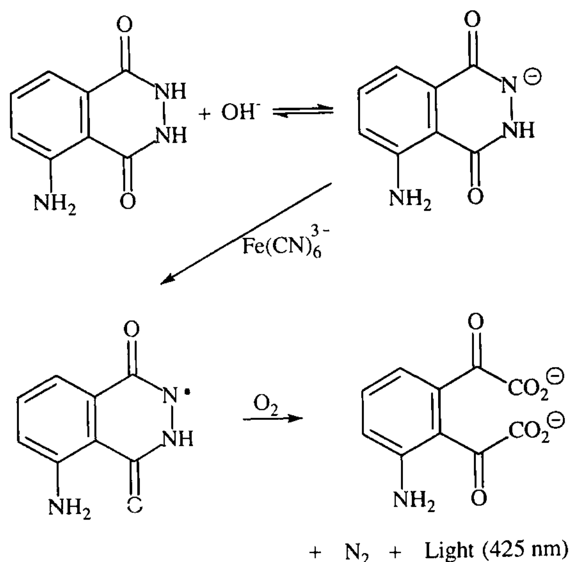


FIG. 15. Postulated mechanism for the chemiluminescence of luminol, taken from McCapra and Beheshti (M21). In this sequence, ferricyanide acts as a one-electron oxidant. The products are nitrogen and an excited-state aminophthalate dianion that decays to the ground state with concomittant light emission.

solvent (W13, W15). This could not occur with isoluminol (Fig. 13) and, experimentally, isoluminol emission is not seen to be redshifted in DMSO. Some recent work of Metelitza *et al.* (M30) has shown that it is possible to observe luminol/peroxide chemiluminescence at moderate pH (8.5–9.5) in the absence of any conventional catalyst or enzyme. This alternative involves carrying out the reactions in an organic solvent (octane/chloroform, 1:1) in reverse micelles of a long-chain quaternary ammonium surfactant (CTAB). The catalytic effect of reverse micelles had also been shown earlier in chloroform/cyclohexane (H17).

3.1.2. *Use of Luminol for Calibration*

In Section 2.6, there was a brief discussion of the issues involved in efficient detection of chemiluminescence. Because it has been so thoroughly studied, the luminol/peroxide system has been employed as a chemiluminescent standard (with accurately determined quantum yield), either for instruments (L4) or for other chemiluminescent reactions (H7, N1). A recent determination of its quantum yield by Lind and Merenyi (L11) gives a value of 1.2%, which is identical to a much earlier measurement (L4). Interestingly, the quantum yield (although not the emission wavelength) was found to be the same in aqueous media and in DMSO when a photodiode detector was employed (close source) (M10), but required a

correction factor when a photomultiplier was used (remote point source) (L5). A detailed description of how to use luminol as a calibrating tool has been recently described (O2).

3.1.3. *Cyclic Hydrazides as Chemiluminescent Labels*

As mentioned in Section 3.1, the derivatization of luminol results in a much reduced quantum yield. On the other hand, isoluminol compounds (derivatized at the 6-amino position) exhibit higher quantum yields than the parent compound. This fact dictates the way in which each compound is utilized as an analytical agent. Compounds such as ABEI (Fig. 14) can be covalently conjugated to a variety of substances, ranging in size and complexity from steroid hormones to immunoglobulins. These chemiluminescent labels can be subsequently utilized in immunoassays according to the general scheme outlined in Section 1.3.1.

As mentioned previously, luminol and isoluminol in aqueous media require a base, an oxidant such as H_2O_2, and a catalytic initiator such as ferricyanide or hemin in order to exhibit chemiluminescence. This is not the case in aprotic media such as DMSO, DMF, or hexamethylphosphoric acid triamide, where a strong base and solvated air are the only requirements (W13). However, since most clinical analyses are carried out in aqueous media and since luminol cannot be derivatized without a serious reduction in quantum yield, an alternative strategy has been devised to utilize the chemiluminescent potential of luminol in clinical assays. This strategy utilizes the enzyme horseradish peroxidase as the label and luminol/H_2O_2 as the substrate. The peroxidase is a 40-kDa glycoprotein containing the Fe^{3+}-coordinated heme protoporphyrin IX as a prosthetic group. At high pH, the enzyme can function simply as a heme catalyst for the chemiluminescence of either luminol itself or isoluminol derivatives. Highly basic solutions will render the enzyme inactive, but dissociate the catalytic heme group (E5). Under these conditions, the light emission from luminol or substituted isoluminol derivatives is high and is relatively short-lived (flash-type kinetics). However, when used in conjunction with biological fluids, endogenous heme in hemoglobin, or other transition metal catalysts, can interfere with the emission intensity (O3). At a more moderate pH, i.e., 8–9, the enzyme is active and catalyzes the oxidation of luminol to an excited-state aminophthalate dianion, which subsequently reverts to the ground state with the emission of a photon. The mechanism, although not clearly understood, is thought to involve a two-electron oxidation of the iron in the peroxidase to Fe^{IV} (Compound I), which subsequently undergoes two one-electron reductions, to Compound II, and finally to the ground-state enzyme (D3, D17, P9). A plausible scheme has been described by Thorpe and Kricka (T7), based on earlier studies of Misra and Squatrito (M34), whereby Compound I (formed by the peroxide oxidation of peroxidase) produces a luminol radical and is reduced to Compound II. Compound II also produces a luminol radical and is reduced back to peroxidase. The reactive luminol radicals combine with oxygen in a two-step

reaction to form luminol endoperoxide, which finally decomposes to give nitrogen plus the excited-state 3-aminophthalate dianion (T7). This peroxidase-mediated emission of light from luminol, at a slightly alkaline pH, is not subject to the same degree of interference from substances present in analytical samples, as is seen at very high pH. The fact that luminol derivatives are nonchemiluminescent has been exploited in a recent study in which luminol was converted into a substrate for *N*-acetyl β-D-glucosaminidase (S4). As expected, the substrate was nonchemiluminescent but could be enzymatically triggered to produce luminol emission. The technique has not been exploited in an immunoassay, but the potential is there for using antibody-labeled *N*-acetyl-β-D-glucosaminidase in the same way that horseradish peroxidase or alkaline phosphatase labels are used.

3.1.4. *Analytical Applications of Isoluminol*

The first assay using isoluminol was described in 1976 by Schroeder *et al.*, who used an isoluminol label to monitor serum biotin in a direct, competitive assay (S22). A number of aminoalkyl derivatives of isoluminol (such as ABEI in Fig. 14) have been subsequently synthesized (S20) and covalently attached to steroid hormones such as cortisol and progesterone. Substitution of both amino hydrogen atoms in isoluminol affords greater detection sensitivity; i.e., *N*-cortisol-aminobutylisoluminol is detected down to 1 fmole (10^{-15} mole), whereas *N*-cortisol-aminobutyl-*N*-ethylisoluminol (the cortisol conjugate of ABEI) can be detected down to 200 amole, i.e., 200×10^{-18} mole (P7). The length of the spacer between the steroid and the isoluminol is also a factor in the latter's luminescence properties, but the relationship is not simple; i.e., it depends on the nature of the steroid. Thus, in Fig. 16, where the steroid is progesterone, the optimal light intensity occurs when $n = 6$ (K15). However, when the steroid is estrone, the detection limit for $n = 4$ is ca. 250 amole and for $n = 2$ it is ca. 150 amole (W10). A summary of label detectibilities as a function of spacer length and steroid structure is presented in Table 3, where the labels are derived from the generalized structure in Fig. 16.

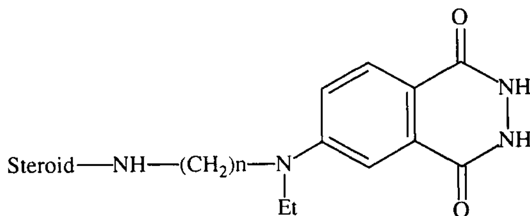


FIG. 16. Steroid conjugates of *N*-ethylisoluminol, with variable length spacers.

TABLE 3
DETECTABILITY OF ISOLUMINOL DERIVATIVES COVALENTLY COUPLED TO STEROID HORMONES[a]

Linker length (n)	Steroid	Label detectability	Reference
2	None	100 amole	K17
2	None	100 amole	S28
2	Estrone	250 amole	W10
3	Cortisol	5 fmole	P7
4	None	50 amole	P4
4	Progesterone	150 amole	P6
4	Estrone	100 amole	W10
6	None	500 amole	S28
6	Progesterone	140 fmole	K17

[a]Linker lengths are taken from Fig. 16. The data are adapted from Kricka (K25).

Most of the assays now carried out with isoluminol derivatives are solid-phase competitive assays (Fig. 2), in which the analyte to be determined competes with the labeled analyte for the available binding sites on antibodies that are immobilized on the solid phase. Thus, plastic microspheres have been used to immobilize antibodies for estriol (K12) or thyroxine (W9). Estradiol antibodies have been immobilized onto plastic tubes (K7) or beads (K16). In all of the above cases, the amount of immunoconjugated hormone label is inversely proportional to the amount of free hormone in the analytical sample. Unconjugated labels are removed by decantation or aspiration from the solid phase, and the specific, immunoconjugated labels are measured in a luminometer. This approach has been successfully applied to progesterone analyses in either serum (D6) or saliva (D5). A review of several separation-based assays with isoluminol analogs was presented a few years ago by Kohen *et al.* (K18).

More recent work with isoluminol has extended the scope of clinical assays to include dual epitope protein antigens using the "sandwich" approach, i.e., employing an immobilized antibody, the antigen to be assayed, and a second isoluminol-labeled antibody (see Fig. 2), which is captured on the solid phase only at sites occupied by antigen bound to the primary antibody (apart from nonspecifically bound label). A recent report describes the above protocol for a prolactin assay in the range 3–500 ng/ml (M29). The same report describes a variant of the sandwich assay for prolactin, in which the second antibody is biotinylated and the label proper consists of ABEI-conjugated streptavidin. However, from the available data, this latter assay appeared to be less sensitive than the former by a factor of 2 (M29). Preliminary data have been presented to show that isoluminol solid phase assays might be applicable to the measurement of microbial food toxins (K6).

Homogeneous Isoluminol Assays The very first isoluminol assay (for biotin) was homogenous (S22), and, although intrinsically less sensitive than their heterogeneous counterparts, such assays continue to attract attention because of their simplicity and ease of use. Homogenous assays are applicable in those cases where the conjugation between labeled analyte (isoluminol derivative) and analyte antibodies directly modulates the label chemiluminescence. For example, it was found that the chemiluminescence of an ABEI–progesterone label was enhanced upon binding to progesterone antibodies and that this enhancement could be used to generate a homogeneous, nonseparation assay for progesterone (K17). Although the assay undoubtedly worked, it was about 10-fold less sensitive than the corresponding solid phase assay, i.e., 25 pg per tube compared with 2–5 pg per tube (D6). Moreover, the progesterone had to be extracted from the serum before the assay (K17), since serum components can interfere with isoluminol chemiluminescence. The homogeneous approach to isoluminol-based assays may, therefore, be appropriate for nonserum samples where sensitivity is not of primary concern, e.g., urinary estrogens (C2, M28). A useful review of the current state of isoluminol-based assays for steroid hormones in serum, urine, and saliva has been presented by De Boever *et al.* (D4). As a semantic digression, the word "direct" is frequently applied to isoluminol-based steroid assays and can be misleading. Direct assays are interchangeably defined as homogeneous, nonseparation assays *or* assays that do not require prior extraction of the steroid from serum samples. In practice, because serum can induce quenching of chemiluminescence, the latter assays are seldom homogeneous, or direct, in the sense of not requiring a separation between free and bound label.

A novel homogeneous assay for urinary estrone-3-glucuronide has been reported by Kim *et al.* (K8) using an *N*-aminoethyl-*N*-ethyl isoluminol derivative (see Table 3, $n = 2$). In contrast to antibody enhancement, this assay exploits the fact that 2*N* NaOH enhances free label chemiluminescence, and this is *reduced* in the presence of specific antibodies. Unlabeled estrone-3-glucuronide reverses the inhibition in a concentration-dependent manner and 8 pg per tube can be minimally detected (K8).

The assays described above are possible only because of a fortuitous effect of the reagents on free and bound labels. Two further homogeneous methods involve a more sophisticated approach. The first employs chemiluminescence energy transfer from a chemiluminescent donor to a fluorescent acceptor. For readers unfamiliar with the phenomenon of energy transfer, an excellent review by Stryer is recommended (S49). Briefly, in the conventional case of *fluorescence* energy transfer, when the emission wavelength of one fluorophore (the donor) is in the same spectral region as the excitation wavelength of a second fluorophore (the acceptor) and the two molecules are no more than ca. 100 Å apart, then it is possible to observe emission from the acceptor and a corresponding quenching from the donor molecule. If donor and acceptor are attached to an antigen and its

antibody, respectively, then the immunoconjugation event can be observed by virtue of the energy transferred from donor to acceptor. Such a scheme is the basis for a homogeneous fluorescence assay requiring no separation of free from bound label, since only donor/acceptor immunoconjugates will exhibit energy transfer.

In the first reported example of *chemiluminescence* energy transfer, the isoluminol derivative ABEI (Fig. 14) was covalently attached via its isothiocyanate to either rabbit immunoglobulin or cyclic AMP. Antibodies to these molecules were labeled with fluorescein. Homogeneous competitive immunoassays were conducted for both analytes and, in order to maximize the combined effects of fluorescein emission and isoluminol quenching, the parameter measured, as a function of analyte concentration, was the intensity ratio I_{460}/I_{525}, at the wavelengths corresponding to isoluminol and fluorescein emission, respectively (C4, C5, P3). Sensitivities for the analytes were in the sub nanomolar range; i.e., they were as good if not better than those observed for other homogeneous immunoassays.

The second of these assays was a liposome-based assay for digoxin (N2). Liposomes were filled with glucose oxidase and they could be lysed with a conjugate of mellitin (a lytic component of bee venom) and ouabain (a digoxin analog). The released enzyme produced hydrogen peroxide, which was detected by isoluminol. Antibodies to digoxin bound the mellitin–ouabain conjugate and inhibited its lytic effect. Free digoxin in the assay medium competed with the liposomes for antibody binding and reversed the inhibition of lysis in a dose-dependent manner (N2).

Future Outlook Mechanistic studies continue to attract attention, and the effect of a reverse micellar environment (quaternary alkylammonium) on luminol and an isoluminol–cortisol conjugate in nonaqueous media is being examined in ongoing work by Metelitza *et al.* (M30). It should be evident from the foregoing that isoluminol derivatives have provided many useful chemiluminescence assays for steroid hormones. In addition, they are now being increasingly examined as tools for protein assays. Much of the innovation derives from designing the appropriate isoluminol conjugates, and this, in turn, stems from some pioneering work in the synthesis and characterization of tracer molecules (P8, S20, S21). With the advent of HPLC methods for purification and quality control of isoluminol labels (J4), there is no doubt that isoluminol chemiluminescence will continue to play an important role in the development of novel clinical assays. As a note of caution, though, when labeling macromolecular analytes (irrespective of the label), better detectability through multiple labels may be accompanied by impaired antigenic reactivity (C1, M29).

3.1.5. *Analytical Applications of Luminol*

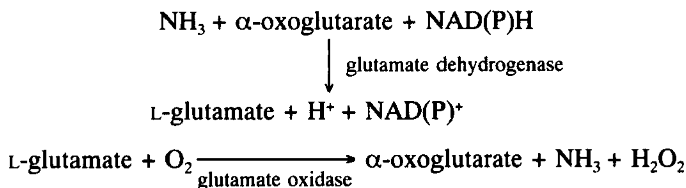
In Section 3.1.3, reference was made to the fact that luminol/peroxide chemiluminescence can be initiated by horseradish peroxidase at high pH, at which the

enzyme is inactive and the catalytic heme moiety is dissociated from it. Alternatively, at pH 8.5 the enzyme acts catalytically to produce luminol radicals and luminol endoperoxides as possible intermediates (M34, T7). In this manner, the enzyme itself can be used as a "label," i.e., in limiting concentrations, and luminol and peroxide can be employed as substrates. Background emission is much lower than that at high pH, but specific emission catalyzed by the peroxididase label is also very low.

Despite these qualifications, horseradish peroxidase labels have been used with H_2O_2/luminol at pH 7.0 for cortisol (A12, T21), dehydroepiandrosterone (A13), and estriol (R9) assays. At a slightly higher pH (9.0), a protein antigen from *Candida albicans* was detected (S43), and at pH 10.5 both albumin and IgG were detected in the nanogram range (P14, P15). Although it does not really belong in this section, one might mention that peroxidase-labeled antibodies have also been used in conjunction with the chemiluminescent luciferin from *Pholas dactylus* and molecular oxygen (instead of luminol and peroxide). Label detectability is reportedly 100 amoles and a sensitive sandwich assay for human chorionogonadotrophin (HCG) has been described (L14).

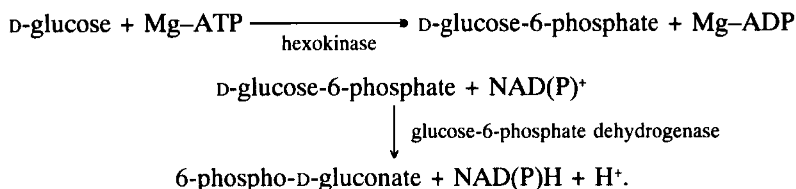
As a hybrid between the *isoluminol-based* assays described in Section 3.1.4 and the horseradish peroxidase-catalyzed, *luminol-based* assays described here, Gundermann *et al.* have described a high quantum-yield naphthalene cyclic hydrazide (7-dimethylaminonaphthalene-1,2-dicarboxylic acid hydrazide) and its use in assays catalyzed by horseradish peroxidase (G20).

In Section 1.3, reference was made to flow injection techniques for detecting transition metal ions as catalysts in the peroxide oxidation of luminol. Other flow injection techniques are being developed in which analytical quantities of hydrogen peroxide are determined in an excess of luminol plus catalyst (ferricyanide or peroxidase). Thus, Tabata's group has pioneered flow injection technology with in-line immobilized enzymes for chemiluminescent clinical chemistry assays. For example, luminol and ferricyanide, in conjunction with immobilized glucose oxidase or uricase, can be used to generate chemiluminescence from the H_2O_2 produced from glucose or uric acid, so that the latter compounds can be quantitatively determined (T2, W26). More recent efforts have resulted in methods for determining ammonia, NAD(P)H, and Mg, using glutamate dehydrogenase, glutamate oxidase, and hexokinase, respectively, immobilized on glass beads (M36, T1, T3, T4). The metabolic reactions for these schemes are summarized below, viz.,



$$NH_3 + \alpha\text{-oxoglutarate} + NAD(P)H \xrightarrow{\text{glutamate dehydrogenase}} \text{L-glutamate} + H^+ + NAD(P)^+$$

$$\text{L-glutamate} + O_2 \xrightarrow[\text{glutamate oxidase}]{} \alpha\text{-oxoglutarate} + NH_3 + H_2O_2$$



A similar scheme on a dedicated instrument allows one to carry out four assays from a 10-μl serum sample, i.e., glucose, lactate, uric acid, and BUN (O1, T4). Free fatty acids from isolated fat cells have also been determined by chemiluminescence assays based on enzymatic peroxide generation (N3, N4).

3.1.6. *Enhanced Chemiluminescence of Luminol*

As we have seen, a peroxidase label with excess luminol/peroxide offers the possibility of chemiluminescence at moderate pH (8–9), with reduced background and with an emission intensity that allows one to measure a number of analytes in assays that are not too demanding in terms of sensitivity. The breakthrough for this chemiluminescent system came with the discovery that firefly luciferin (a 6-hydroxybenzothiazole derivative) increased the light emission from a horseradish peroxidase-catalyzed luminol reaction by two to three orders of magnitude *and* reduced the background signal to ca. 1% (W18). This observation was followed by an intense screening of a large number of compounds, several of which proved to be effective enhancers (T7, T11, T12). Figure 17 shows the structures of some of the compounds that have been found to be most potent as enhancing agents.

The enhancers fall into three groups, viz., benzothiazole derivatives, substituted phenols, and substituted naphthols. Unfortunately, none of the comparative studies cited above have used consistent protocols for assessing the efficacy of putative enhancers. Thus, it is difficult to compare, say, a specific phenol with a specific benzothiazole. However, within each structural group, ranking orders have been established, as shown in Table 4 for the benzothiazoles. Even for a homologous group of compounds, the published data (e.g., columns 1 and 2 in Table 4) are somewhat misleading in that 6-hydroxybenzothiazole would seem to be the most efficient enhancer. However, if one normalizes the data in terms of molecular weights (Table 4, column 3), then dehydroluciferin is clearly the best enhancer, and luciferin itself is about 60% as efficient as 6-hydroxybenzothiazole. From these and other data, it has been established that the 6-hydroxy group is essential for enhancement (cf. 6-ethoxybenzothiazole and benzothiazole in Table 4).

In the substituted phenol/napthol group, *p*-phenylphenol is an extremely potent enhancer, with a reported amplification factor of 530 under experimental conditions in which 6-hydroxybenzothiazole (at double the phenol concentration) had an amplification factor of only 27 (T7) *Para*-substituted halogenophenols are also

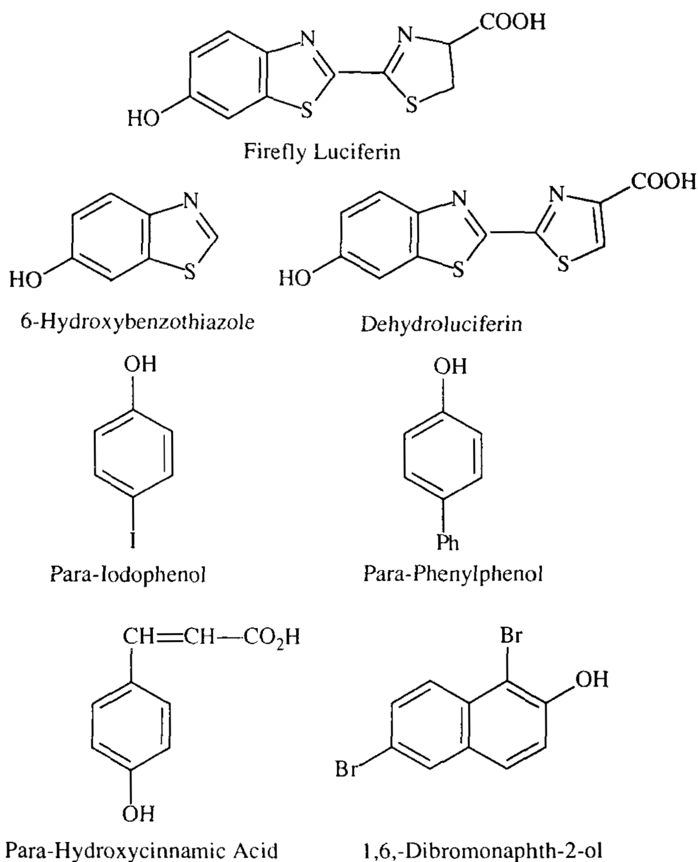


FIG. 17. Representative compounds that have been shown to enhance peroxidase-catalyzed luminescence from a mixture of luminol and peroxide.

efficient enhancers, with potencies related to the size of the halogen atom, i.e., iodo > bromo > chloro. Both *p*-hydroxycinnamic acid and 1,6-dibromonaphth-2-ol would seem to have potencies intermediate between those of *p*-phenylphenol and *p*-iodophenol. Although these data have been variously reported (K31, K32, K33, T7, T8, T11, T12, T13), there has been no attempt to standardize the experimental protocols in terms of reagent concentrations and molar concentrations of enhancers. Even the optimal concentration of enhancer is dependent upon the other reagent concentrations. Figure 18 illustrates the effect of 6-hydroxybenzothiazole, under conditions that are described in the figure legend.

Given the above caveats, the most potent enhancer so far tested is *p*-phenyl-

TABLE 4
ENHANCEMENT OF PEROXIDASE-CATALYZED LUMINOL CHEMILUMINESCENCE EFFECTED BY BENZOTHIAZOLE DERIVATIVES[a]

	Amplification factor	
Benzothiazole derivative	Measured	Normalized[b]
Firefly luciferin	224	415
6-Ethoxy luciferin	0.4	0.7
Dehydroluciferin	635	1170
Benzothiazole	0.7	0.6
6-Hydroxybenzothiazole	667	667

Note. A peroxidase–antibody conjugate (10^5-fold dilution from stock) was treated with enhancer (10 μl of 500 μg/ml in DMSO) plus luminol (56 μm) and hydrogen peroxide (1.87 m*M*) in 1 ml Tris buffer at pH 8.0. Luminescence intensity was integrated over 60 sec and expressed relative to a DMSO control.

[a]See Fig. 17. Adapted from Thorpe *et al.* (T11) with permission.

[b]A molar amplification factor obtained by normalizing the data in column 2 (T11) to the molecular weight of 6-hydroxybenzothiazole.

phenol. As far as one can determine from the available literature, the next group of enhancers, in order of decreasing efficacy, is as follows: 1,6-dibromonaphth-2-ol, *p*-hydroxycinnamic acid, *p*-iodophenol, dehydroluciferin, 6-hydroxybenzothiazole, and, lastly, firefly luciferin, which was the compound first shown to exhibit the enhancing effect (W18).

3.1.7. *Mechanism of Enhanced Chemiluminescence*

Since the emission spectra of enhanced and unenhanced luminol oxidations are similar, it seems clear that emission is from the excited-state aminophthalate derived from luminol itself and not from the enhancer. The enhancement is also specific for peroxidase; i.e., it does not occur under conditions in which the heme would dissociate from the enzyme. Furthermore, luminol chemiluminescence triggered by heme-containing compounds, such as hemoglobin or cytochrome c, is actually reduced by enhancers such as *p*-iodophenol (T7). This latter phenomenon explains the potential usefulness of enhanced chemiluminescence, i.e., an amplified signal combined with a reduced background and reduced interference from the reagents as well as from endogenous heme compounds.

Although the precise mechanism for the enhancement remains unknown, Thorpe and Kricka (T8) have proposed that enhancers render the sequence of events for unenhanced luminol oxidation (see Section 3.1.3) more efficient. This would be consistent with kinetic studies on the reactivity of phenol enhancers with the horseradish peroxidase intermediates Compounds I and II (H11, V10). Specifically, the hypothesis is that (a) horseradish peroxidase Compounds I and II

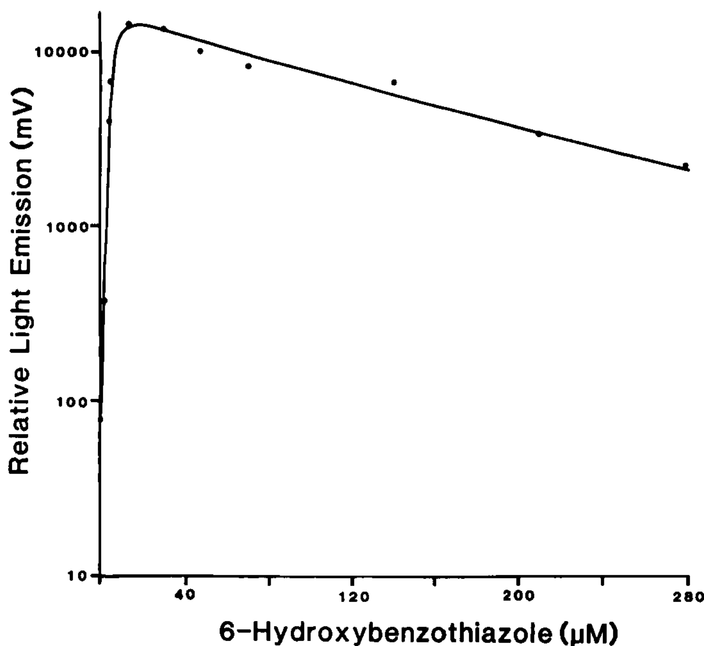
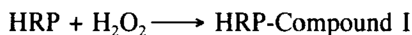
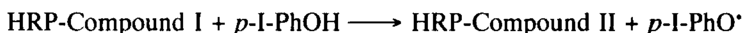


FIG. 18. Influence of 6-hydroxybenzothiazole on light emission from the peroxidase conjugate-catalyzed oxidation of luminol. Anti-AFP–HRP conjugate (10 μl) and the enhancer (20 μl of a DMSO solution) were placed in separate corners of a cuvette. Luminescence was triggered by adding 1 ml of luminol (60 μM) and hydrogen peroxide (2 m*M*) in Tris buffer at pH 8.5. Data points represent peak light emissions from intensity–time curves. Taken from Thorpe *et al.* (T11) with permission.

react faster with enhancers than with luminol and (b) the enhancer radicals, so formed, react very rapidly with luminol to produce luminol radicals as essential intermediates in the formation of excited-state aminophthalate dianions. Enhancers such as *p*-iodophenol are therefore acting as electron transfer mediators in a scheme outlined by Candy and Jones (C7), viz.,

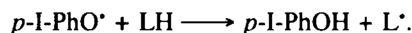


$$\text{HRP} + H_2O_2 \longrightarrow \text{HRP-Compound I}$$



$$\text{HRP-Compound I} + p\text{-I-PhOH} \longrightarrow \text{HRP-Compound II} + p\text{-I-PhO}^{\bullet}$$

$$\text{HRP-Compound II} + p\text{-I-PhOH} \longrightarrow \text{HRP} + p\text{-I-PhO}^{\bullet}$$



$$p\text{-I-PhO}^{\bullet} + \text{LH} \longrightarrow p\text{-I-PhOH} + \text{L}^{\bullet}.$$

One implication of this scheme is that nonenhancer phenols would be unable to produce luminol radicals even though they could still form phenoxy radicals. Although the reasons for this inability are unclear, one might envisage that in the presence of a nonenhancer, the effect of an enhancer would be attenuated; i.e., they

would both be competing for horseradish peroxidase Compounds I and II. This is indeed the case and, for example, *p*-cresol significantly reduces the enhancing effect of *p*-iodophenol on peroxidase-catalyzed luminol oxidation (C7).

The effect of nonenhancers has been exploited in an assay for tyrosine in which the attenuation of luminol oxidation enhanced by *p*-iodophenol was proportional to the concentration of added tyrosine (C8). As a validation of the method, the number of tyrosines in lysozyme was found to be 3, which is in accord with its known amino acid sequence.

3.1.8. *Analytical Applications of Enhanced Luminol Chemiluminescence*

Peroxidase-catalyzed luminol oxidation, enhanced by phenols, naphthols, or luciferin analogs, has some attractive features for clinical assays. The emission is prolonged ("glow" type) and constant, allowing for ease of measurement; i.e., reactions can be carried out in batches away from the luminometer and read later as end-point determinations when the emission intensity is constant. The emission is also intense, so that detection is facilitated and can even be accomplished by silicon diode detectors or photographic film.

Many solid-phase assays have been described using the so-called enhanced technology (T15). Solid phases have ranged from 6-mm-diameter beads (M32, T10, T11, W18), coated tubes (T10–T12), microtiter plates (O6, S3, T14, T16, W2), magnetizable latex particles (H12), micropins (T8, T15), and nitrocellulose discs (H16).

Since the reports on enhanced chemiluminescence in the mid-1980s (e.g., T10, T12–T14, T16, T17), numerous assays have been described in the literature. Table 5 represents a summary of the types of analyte that have more recently proved amenable to detection by enhanced luminol oxidation via horseradish peroxidase labels. This table is meant to be illustrative rather than comprehensive.

Other applications of this type of detection include Western blotting procedures for the identification and visualization of protein antigens (L1, L8, L9). One such recent assay for herpes simplex virus Type 2 showed that enhanced chemiluminescence is 500 times more sensitive than a colorimetric method (D1). Antibodies to several of the HIV viral proteins have also been detected in chemiluminescent Western blots (S16).

Areas in which technological innovations have been accomplished include detection instruments [ranging from small portable luminometers (M3) to camera-type detectors (B32, K30) and charge-coupled devices (L3)], data analysis (E3, S12), and assay formatting, e.g., immunofiltration (H16). The so-called camera luminometers (B32) offer the joint advantages of ease of use, portability, and detectability without an electrical power supply. With fast film such as Polaroid 612 (20,000 ASA), exposure times can be as short as 5 sec. Examples of assays using enhanced chemiluminescence and Polaroid 612 film include α-fetoprotein (L9), albumin (L8), immunoglobulins and transferrin (J3), and ferritin (O7).

TABLE 5
A SELECTION OF IMMUNOASSAYS REPORTED SINCE 1986 IN WHICH ENHANCED LUMINOL CHEMILUMINESCENCE WAS EMPLOYED[a]

Analyte	Reference
Steroid hormones	
Estradiol	M32,S3,S47
Cortisol	M5
Progesterone	M32
Aldosterone	H18
Thyroid hormones	
Total T4	G5
Free T4	A14,B11,B12,M7
T3 uptake	G5
Free T3	C23
Protein hormones	
Follicle stimulating hormone	M9
Luteinizing hormone	B15
Human chorionic gonadotropin	B12,M6
Thyroid stimulating hormone	D11,J5,P2,S13
Proteins	
Albumin	K23
CA-15-3	M31
Carcinoembryonic antigen	C20,H2,H10
Ferritin	A14
α-Fetoprotein	T19
β_2-Microglobulin	K26
Sex hormone binding globulin	K24
Creatine kinase MB	B10
Thyroid binding globulin	W22
Antibodies	
Total IgG	B15,H16
Total IgE	S44
Hepatitis B surface antigen	I3
HIV	S16
Rubella	H12,W16
Malaria	H3
Respiratory syncytial virus	H15
Therapeutic drugs	
Digoxin	C22
Isometamidium	S33
Miscellaneous	
Cyclic AMP	O6
Triazine pesticides	C12,H5
Environmental monitoring	A7

[a]The data represent a compilation adapted from a survey by Kricka (K26), plus material from more recent sources.

3.1.9. *Enhanced Chemiluminescence and Nucleic Acid Detection*

Nucleic acid blotting techniques are analogous to Western blotting procedures with proteins. If no DNA separation is involved and one simply wishes to identify a target sequence, then a labeled probe is allowed to hybridize to the target, which is itself immobilized on a nylon or nitrocellulose membrane support, i.e., the so-called dot–blot assays (K1). If one wishes to probe for specific sequences in a mixture of DNA fragments, then the capture of label (for signal generation) is carried out after an electrophoretic separation followed by electroelution of the bands onto a membrane support, where the whole procedure is known as Southern blotting (S35).

DNA Southern blots using enhanced chemiluminescence date from 1985 (M11). In that first report, a plasmid target (pBR322) was biotinylated by nick translation (R7), hybridized to a nitrocellulose membrane, incubated with a streptavidin–horseradish peroxidase conjugate, and detected by enhanced luminol chemiluminescence. Hybridization probes can also be directly conjugated to the peroxidase enzyme, although the probe synthesis and purification are more complex. After hybridization to the target nucleic acid, detection is again accomplished by enhanced chemiluminescence (U2, U3). The light output can be recorded on blue-sensitive X-ray film or fast Polaroid film (P11).

3.2. Acridinium Esters

While luminol and isoluminol require an oxidant plus a catalyst for initiation of the chemiluminescent reaction, esters derived from *N*-methyl acridinium carboxylic acid require only alkaline hydrogen peroxide (W4). Acridinium esters were first introduced by McCapra's group (M23, M25, S32), based on earlier work on the bioluminescence of the lucigenin/luciferase system (G18), and reviewed in McCapra and Beheshti (M21). From Fig. 19, one can see the structural similarity between lucigenin and a typical acridinium ester.

The main starting material for this group of chemiluminescent molecules is acridine-9-carboxylic acid, which can be prepared by the original method of Stollé (S46). The overall synthetic scheme is shown in Fig. 20, taken from Zomer *et al.* (Z6), which also provides comprehensive experimental details. An alternative route, starting from commercially available acridine, is described by Weeks *et al.* (W6) and outlined in Fig. 21. From the acridine carboxylic acid, acridinium esters are subsequently formed by esterifying the carboxyl group via the acid chloride and quaternizing the tertiary amino group with methyl iodide or methyl fluorsulfonic acid as in Fig. 22.

3.2.1. *Mechanisms of Chemiluminescence*

We have already seen in Section 1.4.1 that cyclic peroxides (dioxetans) can be thermally decomposed to give excited state ketones that revert to the ground state

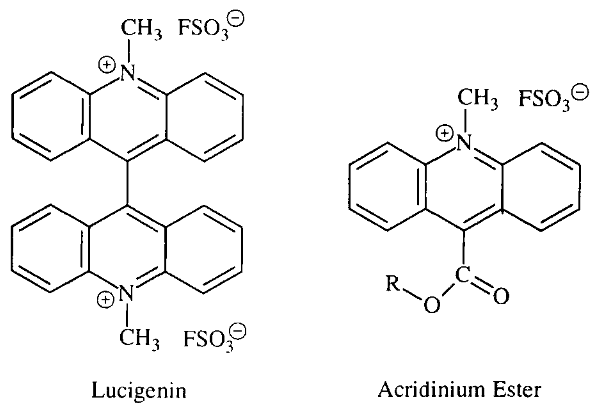


FIG. 19. Structural similarity between lucigenin and an acridinium ester.

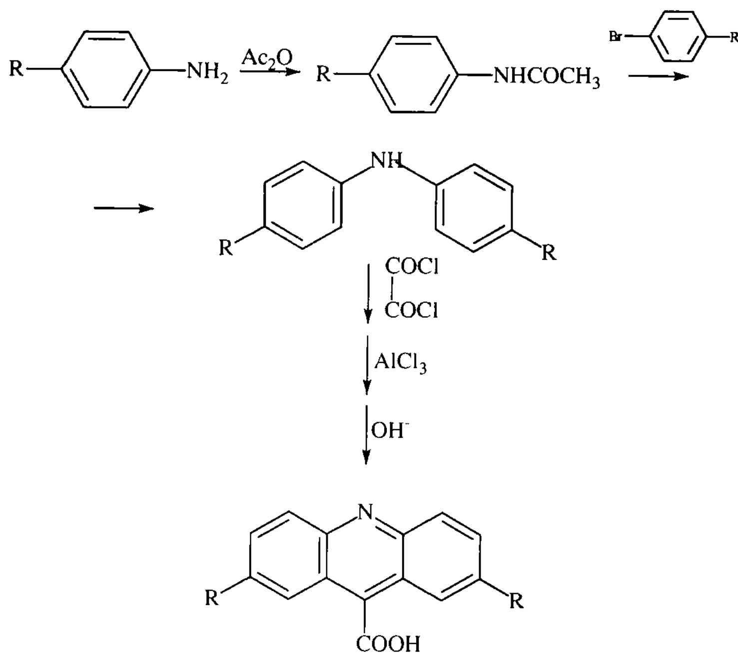


FIG. 20. Synthesis of substituted acridine-9-carboxylic acids according to the method of Stollé (S46), where *R* = H or MeO [adapted from Zomer *et al.* (Z6)].

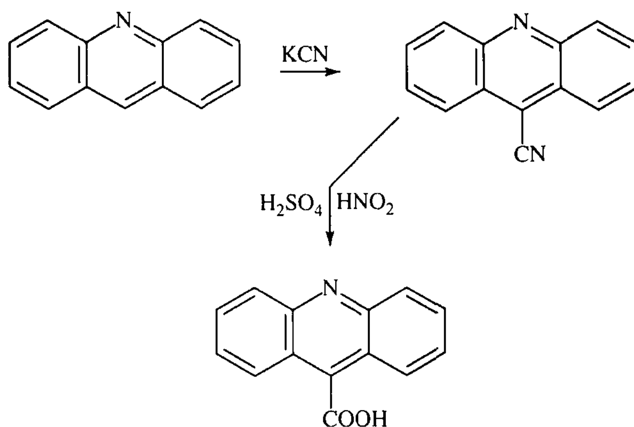


FIG. 21. Synthesis of acridine-9-carboxylic acid in a two-stage reaction starting with acridine. This method was adopted by Weeks *et al.* (W6), based on the original scheme by Lehmstedt and Wirth (L7).

with light emission (Fig. 3). Acridinium ester chemiluminescence is initiated by alkaline hydrogen peroxide, and McCapra and Beheshti (M21) have suggested that a metastable dioxetaneone is formed by attack of the peroxide anion HOO^- at the ester-containing carbon atom, where the driving force is the electron-deficient quaternary nitrogen atom and the possibility of charge delocalization through the conjugated π system. The scheme is outlined in Fig. 23, which shows that the initial peroxy-intermediate undergoes an internal cyclization to the postulated dioxetaneone, which spontaneously decarboxylates to give an excited-state N-substituted acridone. Fluorescence emission spectra of isolated acridones provide good evidence that they are indeed the light-emitting species in the chemiluminescence of acridinium esters (M16, M17, M22). An alternative scheme has the initial peroxide attack at the ester carbonyl (W14, Z6), although there is no

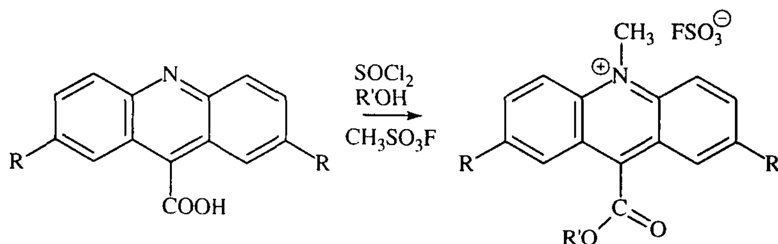


FIG. 22. Conversion via the acid chloride of a substituted acridine-9-carboxylic acid into an ester of 10-methylacridinium fluorosulfonate.

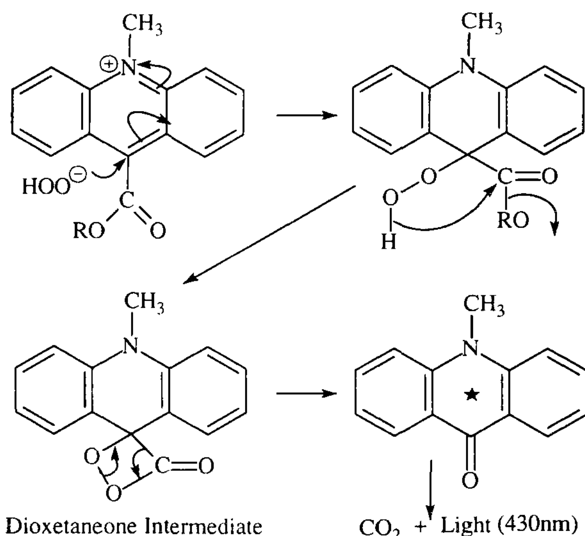


FIG. 23. Mechanism for the peroxide-induced chemiluminescence of acridinium esters. The dioxetaneone is a postulated metastable intermediate.

compelling evidence for such a mechanism. As previously mentioned, the conditions for initiating chemiluminescence from acridinium esters are a high pH (e.g., 200 m*M* NaOH) and hydrogen peroxide (ca. 0.5%). However, a complicating factor is that at pH>7.0, the acridinium ester exists as a carbinol or "pseudobase" (Fig. 24). Whereas, the final product of hydrogen peroxide and the carbinol (in alkaline solution) is still an *N*-methyl acridone, there is no possibility of forming the dioxetaneone intermediate and the subsequent electronically excited state. Such a reaction does not afford chemiluminescence, i.e.; it is a "dark" reaction (Z1). Thus, for efficient chemiluminescence, it is important that the

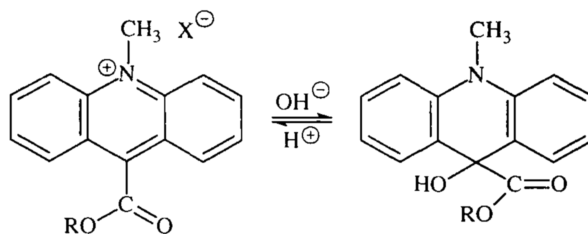


FIG. 24. Acid–base equilibrium of an acridinium ester, showing formation of inactive "pseudobase" (carbinol) at pH > 7.0.

acridinium ester is initially at an acidic pH, i.e., to push the equilibrium away from the pseudo-base formation.

The kinetics of light emission are those of a transient flash (see Section 2.5), where the time course is a function of the reagents and the molecular structure of the ester. The reagent composition needs to be optimized for each individual acridinium ester, and, in a recent study (Z5) on a series of novel acridinium esters, optimal reagent concentrations were found to be 100 m*M* NaOH and 0.015% hydrogen peroxide (Z6).

Both quantum yield and emission kinetics are influenced by the nature of the leaving group, –OR, in Fig. 19. A strong correlation between the pK_a of the conjugate acid of the leaving group and quantum yield has been observed (M18, M26, S45). Thus, phenyl acridinium esters (with relatively strong conjugate acids, e.g., ϕ-OH) are superior to methyl acridinium esters, presumable because of electron delocalization through the aromatic π system in the phenoxy anion. By the same token, compound (a) in Fig. 25 reaches maximum emission in 600 msec, at which point it decays with a $t_{½}$ of 300 msec, whereas compound (b) reaches its maximum chemiluminescence intensity at 1.0 sec and decays with a $t_{½}$ of 2.0 sec (K10, Z5). Although no supporting data were presented, it seems reasonable to propose that the *N*-tosyl-*N*-phenoxy conjugate acid from hydrolysis of (a) has a lower pK_a than the corresponding cyclic sulfonamide formed from (b). The more efficient of the acridinium esters are reported to have quantum yields in the range of 0.01–0.10 (1–10%) (C3, M22). Apart from the structure of the acridinium ester itself, the emission is also influenced by the microenvironment (cf. luminol,

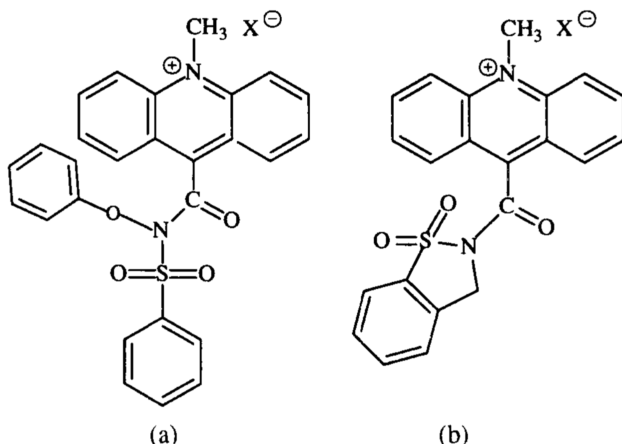


FIG. 25. Two sulfonyl derivatives of an acridinium amide, which illustrate the importance of leaving group on chemiluminescent properties (K10,Z5).

Section 3.1.4) (H18, P1). In a recent study of surfactants (B1), hexadecyl trimethyl ammonium chloride (CTAC) produced a significant increase in the emission intensity of acridinium ester-labeled albumin, but Triton X-100 exerted a larger enhancing effect than CTAC on a similarly labeled antibody.

3.2.2. *Acridinium Esters as Labels*

In order to utilize an acridinium ester as a label, it must be covalently coupled to the antigen or hapten that is being analyzed, or to an antibody of that analyte, in such a way that the electron-withdrawing nature of the ester leaving group is unaffected. The first report of such a label was from Simpson *et al.* (S31, S32), who coupled a carboxyphenyl acridinium ester to antibody lysine groups using mixed anhydride or carbodiimide chemistry. The labeling efficiency was, however, rather poor, and it was not until the advent of the *N*-hydroxysuccinimidyl ester in Fig. 26 (W4) that reproducible labeling of antibodies (via their lysine ε-amino groups) was feasible (W5). Similar labels, with methyl groups flanking the ester function on the aromatic ring, were found to offer superior stability against hydrolysis (Fig. 27). Thus, an immunoglobulin conjugate of Fig. 27a showed no loss of chemiluminescence activity after a week at 37°C, whereas the ester in Fig. 26 lost 90% of its activity over the same time period (L2).

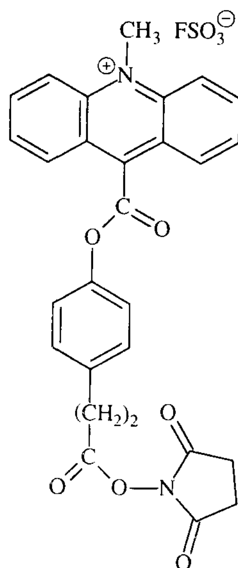


FIG. 26. An activated acridinium ester for covalent labeling, viz., 4-(2-succinimidyloxycarbonylmethyl)phenyl-10-methylacridinium-9-carboxylate fluorosulfonate. After Weeks *et al.* (W4,W5).

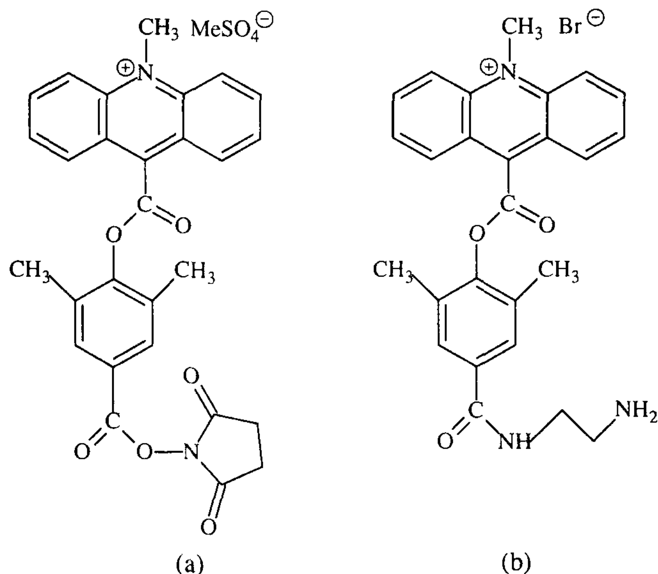


FIG. 27. Two acridinium esters with increased stability for covalent labeling of (a) lysine groups, viz., 2′,6′-dimethyl-4′-(N-succinimidyloxycarbonyl)phenyl-10-methylacridinium-9-carboxylate methosulfate; (b) carboxyl groups, viz., 2′,6′-dimethyl-4′-[*N*-(2-aminoethyl)carbamoyl]phenyl-10-methylacridinium-9-carboxylate bromide. Taken from Law *et al.* (L2).

Other workers have also tried to modify the stability and flash kinetics of the basic acridinium ester. Since the conjugate base of the leaving group should have lower basicity than HOO^-, Kinkel *et al.* considered thiophenols and sulfonamides (pK_a 6–8 and 5.5–10.5, respectively; cf. H_2O_2, pK_a 12) and synthesized the labels shown in Fig. 28 (K10). Both of the above compounds were reported to give fivefold higher chemiluminescent quantum yields than the ester devised by Weeks *et al.* (W4). In terms of kinetics, the emission from antibodies labeled with the sulfonamide derivative (Figure 28b) was much larger than that of the thiocarboxylic acid derivative shown in Fig. 28a (K10). Consistent with the emission kinetics, solutions of the sulfonamide-labeled antibody showed much greater longer-term stability toward hydrolysis than the thiocarboxylate.

3.2.3. *Clinical Applications*

The first immunoassay utilizing acridinium esters was described in 1981 (S31, S32). The *p*-carboxyphenyl ester of 9-carboxy-*N*-methyl-acridinium bromide (Fig. 24; R = HOOC–ϕ\X = Br) was coupled by carbodiimide chemistry to the lysine residues of sheep α-fetoprotein (AFP) antibody. Unlabeled antibodies were immobilized onto plastic tubes, and the AFP antigen was assayed in a direct two-site

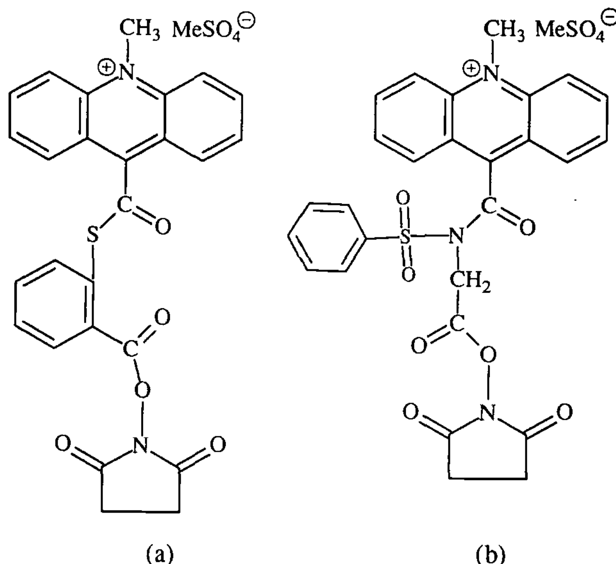


FIG. 28. Novel thiocarboxylate (a) and sulfonamide (b) acridinium esters synthesized by Kinkel *et al.* (K10).

sandwich assay, whereby the labeled antibody was captured by antigen that had been bound by immunoconjugation to the immobilized antibody. Chemiluminescence was initiated by the addition of alkaline peroxide, and the signal was proportional to the amount of antigen present in the sample (S32). Poor coupling efficiency of this carboxy derivative led to the development of the *N*-hydroxysuccinimidyl derivative in Fig. 26 as the first acridinium ester derivative designed for protein coupling. This compound reacted more reproducibly with antibodies, and successful two-site direct assays were carried out for AFP and TSH, using cellulose as the solid phase to which the primary antibody was covalently attached (W3). Separation of free label from immunoconjugated label was achieved by centrifugation. The label detectibility was reported to be 1 amole (10^{-18} mole) of AE-labeled immunoglobulin. In the AFP assay, the antigen was simultaneously incubated for 1 hr with the solid-phase antibody and the labeled, secondary, antibody. In the case of TSH, the antigen was incubated for 2 hr with the labeled antibody and the complex was further incubated for 1 hr with the immobilized antibody (W3, W7). Such an assay protocol is sometimes referred to as a "reverse sandwich." The minimal detectable dose in the above assay was 4 μIU/liter which is equivalent to a molar concentration of 1.6×10^{-14} *M*. In a more recent formatting of these assays, magnetic particles were employed as the solid phase and a commercial magnetic separator was used for separating free from

immunoconjugated label (W6). Labeling stoichiometry was reported to be three AEs per antibody.

The utility of acridinium esters as labels for antibodies is dependent upon the ease with which they can be covalently attached and the degree of labeling that is achievable, i.e., the number of labels per macromolecule. Some attempt to control and to optimize the labeling procedure has been made using an activated *N*-hydroxysuccinimidyl derivative (Z7). However, there are really no hard and fast rules, and the problem remains largely an empirical one. Overlabeling can lead to antibody precipitation, depending on the hydrophobic nature of the label used. The desired goal is to achieve maximal labeling (specific activity) without protein precipitation *and* without the resulting conjugate having increased nonspecific binding to the solid phase. The optimal label stoichiometry, then, will vary from one chemiluminescent molecule to another and will result from a process of trial and error.

Hart and Taaffe produced a "universal" label by attaching acridinium ester molecules to streptavidin, with a labeling efficiency of up to eight labels per protein (H6). A sandwich assay for mouse IgG was described, in which the second antibody was a biotinylated goat anti-mouse IgG, and this, in turn, captured the acridinium ester conjugate by means of streptavidin–biotin coupling. The thermal stability of the label was, however, rather poor (H6).

Further improvements in label stability (resistance to hydrolysis) were made by Law *et al.* (L2), who introduced the dimethyl phenyl ester derivative already referred to in Figs. 27a and 27b. Magnetic separation, as described above, was employed with these labels, and assays for free thyroxine (free T4) and TSH, were described (B13, L2). Chemiluminescence was initiated by two reagents, viz., nitric acid/peroxide, followed by excess sodium hydroxide. The nitric acid played a dual role of displacing the equilibrium away from the inactive carbinol form (Fig. 24) and of stabilizing the hydrogen peroxide. In the free T4 assay, a competitive format was adopted (Fig. 2) in which a carrier protein covalently labeled with T4 *and* acridinium ester competed with T4 in a serum sample for binding sites on T4 antibodies immobilized on magnetic particles (L2). The use of acridinium esters for determining human parathyrin and its N-terminal fragments has recently been described (K11), as have assays for tumor necrosis factor (N7) and hepatitis B surface antigen (W8).

In an interesting variation of the technology, Law *et al.*, (L2) described a liposome-based TSH assay in which the liposomal membrane surface contained covalently attached antibodies, and a modified membrane-impermeable acridinium ester derivative was encapsulated inside the vesicles. A normal sandwich assay was conducted using magnetic particles as the solid phase and antibody-sensitized liposomes as the label. The total assay time was 5 min, compared with the normal 2.5 hr, and showed superior sensitivity.

A completely new way of triggering acridinium ester chemiluminescence has

been devised by Littig and Nieman (L13). The authors were motivated by a desire to minimize the number of reagents and to utilize the label in a flow-through cell. The hydrogen peroxide required for the chemiluminescent reaction was generated electrochemically by voltammetric reduction of oxygen. In this manner, a lysine–AE conjugate was detected at 10^{-14} mole (L13).

3.2.4. *Novel Acridinium Esters*

The factors that affect the usefulness of acridinium esters as analytical labels for diagnostic assays include solubility, emission wavelength, quantum yield, and the rate of emission. Reference has already been made to a membrane-impermeant, disulfonate, acridinium ester (L2) for liposome-based applications. Other workers have focused on the quantum yield and flash kinetics. In an attempt to increase the former and to reduce the latter, the conventional methyl group on the quaternary nitrogen atom of the acridinium ring system was replaced by other alkyl or substituted alkyl groups (Fig. 29a) (B2). Unfortunately, none of these derivatives showed any enhancement in quantum yield or faster emission kinetics than the parent *N*-methyl analogue. In a potentially more significant study, the acridine nitrogen atom was quaternized in such a way that it, rather than the ester function, could serve as the point of attachment for immobilization or protein labeling (Z5, Z6). This was achieved by synthesizing the *N*-carboxymethyl derivative shown in Fig. 29b. As the authors point out, the significance of attaching the acridinium ester to a solid phase or protein via its quaternary nitrogen atom is that the molecule will remain covalently attached during light emission (refer to Section 3.2.1 for the mechanism) and may, therefore, be utilized in a chemiluminescence energy transfer assay (C4). Such a possibility has, however, not yet been realized.

Whereas a water-soluble acridinium ester was described by Law *et al.* (L2), the

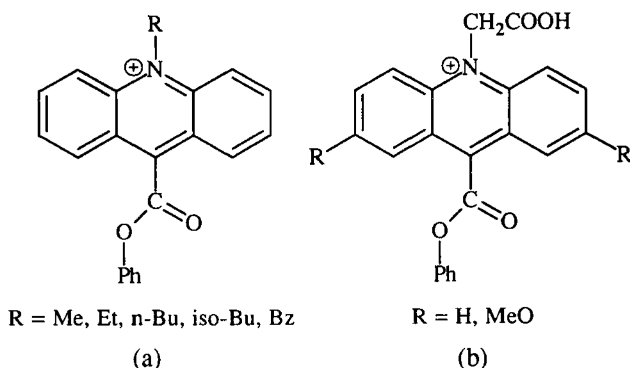


FIG. 29. Novel *N*-alkyl derivatives of phenyl acridinium esters. (a) Taken from Batmangelich *et al.* (B2); (b) taken from Zomer *et al.* (Z6).

compound had no functional group for hapten or antibody labeling. In an attempt to overcome this problem, Mayer *et al.* (M14) extended previous work on *N*-hydroxysuccinimide sulfonamide derivatives (K10) and prepared a morpholinium analog, a hapten conjugate of which, the authors claim, has improved solubility and immunoreactivity (M14). The article fails to provide any luminescence data for this novel derivative, and its utility must, therefore, remain questionable. In a comparative study of novel halogenophenyl acridinium esters (extending some early work by McCapra (M18) and a new sulfonamide derivative, the latter was reported to have the highest integrated emission intensity (G15). However, since luminometer integration times are normally longer than the flash duration, this result may merely reflect the slower (as reported) emission decay kinetics of the sulfonamide.

3.2.5. *Instrumentation Developments for Acridinium Esters*

Two automated complete assay systems, utilizing acridinium esters and particle solid phases, have been recently described. A high-throughput bench-top system is able to process 180 samples per hour and the time to the first assay result is 15 min. Actual assay times are 7 min per sample (K34). The dimethyl acridinium ester label in this system has already been described (L2). The second system employs an acridinium sulfonamide (M12) and coated ¼-in. polystyrene beads or latex microparticles as solid phases. Assays for β-HCG, CEA, and TSH have been reported (K5).

The application of acridinium esters to microtiter plate assays has been recently developed, and assays for TSH and AFP were presented (W24). Since the emission from these labels is transient, i.e., completed in about 1 sec, this represents a novel development in microtiter plate detection. In a comparison between luminol and acridinium ester, a 96-well plate was processed in 15 min for the former label and in 4 min for the latter (V1).

3.2.6. *Nucleic Acid Detection*

The ability to synthesize short oligonucleotide DNA probes with an acridinium ester attached at any defined position within the sequence (A16, N5) has allowed for the use of such probes in hybridization-based assays for RNA and DNA target sequences. Two techniques have recently emerged, together with a combination of the two (for enhanced sensitivity).

The first, and simplest of these, exploits the fact that cationically charged microspheres (which can be magnetic particles) selectively bind *hybridized* AE probes. Thus, after a solution-phase hybridization between a DNA AE probe and a target ribosomal RNA, containing a sequence complementary to the probe, the hybridized probe can be removed in a 10-min separation step and quantitated in a luminometer (G11, T6). Assays have been described for a number of bacterial

infections, including *Campylobacter jejuni*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* (N5).

The second technique uses a homogeneous assay format with no physical separation between hybridized and nonhybridized probes. The method is the result of a discovery that for a suitably positioned acridinium ester, within an oligonucleotide sequence, hybridization prevents hydrolytic attack on the ester by hydroxide ions. Such hydrolysis would lead to the inactivation of the chemiluminescent label (see Section 3.2.1). This differential susceptibility to hydrolysis forms the basis for a so-called hybridization-protection assay (HPA), which is schematically illustrated in Fig. 30.

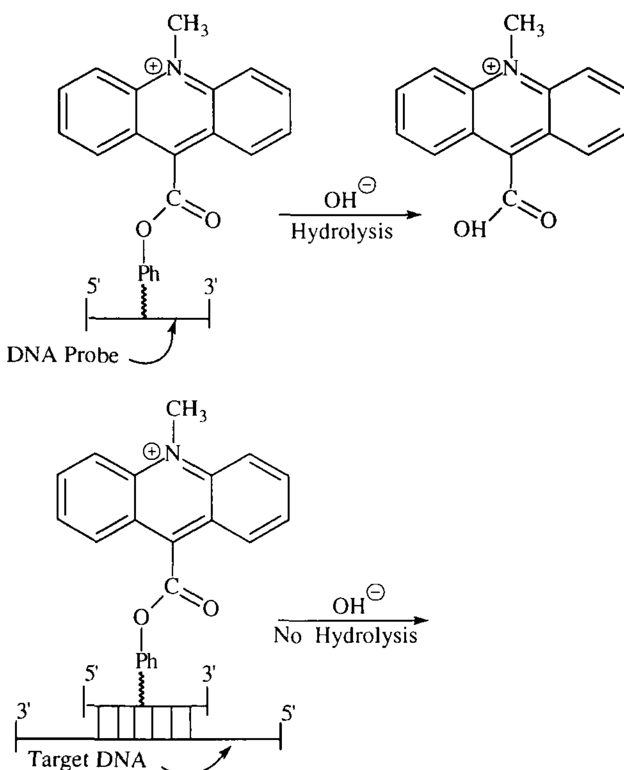


FIG. 30. Schematic illustration of the hybridization protection assay (HPA). The acridinium ester is covalently attached to a DNA probe. Upon hydrolysis of the phenyl ester group, there is no possibility of forming a dioxetaneone intermediate and hence the reaction is "dark"; i.e., there is no light emission. By contrast, chemiluminescence of the hybridized probe (shown in the lower part of the figure) is minimally affected. Adapted from Nelson *et al.* (N6), with permission.

The mechanism for the protection remains unknown, although there is some speculation that the acridinium ester intercalates between the strands of the hybridized duplex, within which it remains impervious to hydrolysis (A16). The assay protocol involves a hybridization step, followed by hydrolysis between pH 7.5 and 8.5 and, finally, the initiation of chemiluminescence. The greater the signal, the more hybridized AE probe there is and, hence, the higher the concentration of target RNA. Ribosomal RNA from *Chlamydia* has been detected down to 50 μg (equivalent to 1000 bacterial organisms), and a single-base mismatch between *Neisseria gonnorhoeae* and *Neisseria meningitidis* was correctly distinguished (A16, N6). An assay for mycobacteria in culture showed similar sensitivity to radiolabel methods (G9). By combining the HPA assay with a separation step using cationic microspheres, the background signal could be reduced to the extent that 6 amoles (10^{-18}) of target ribosomal RNA could be detected (A16, N5, N6).

In an alternative method for labeling oligonucleotides, Septak (S27) employed a modified nucleotide base (analog-C). He was able to show that dual labeling of an oligonucleotide with acridinium esters did not lead to self-quenching of the chemiluminescence provided that there were at least seven bases between the two labels (S27).

In assays involving the detection of double-stranded cDNA, probe hybridization can occur only after melting of the target duplexes. Unfortunately, the conditions for achieving this (elevated temperature and pH) are also those that favor hydrolytic inactivation of the AE probe. A way of minimizing this loss is to form a stable adduct of the acridinium ester. Bisulfite, for example, affords total protection from hydrolysis, but its reversal (by dilution) is relatively slow, which means that the assay becomes accordingly longer (H4). The article cited also contains a discussion of other adduct-forming compounds for acridiniums.

3.3. Dioxetanes

In Section 1.4.1, we saw that many of the compounds that exhibit chemiluminescence undergo reactions in which a cyclic peroxy intermediate, or dioxetane (strictly speaking, dioxetan), is formed, and this subsequently breaks down to give an excited-state product. The existence of such 1,2-dioxetanes as metastable intermediates in chemiluminescent reactions was first proposed in 1968 by McCapra (M15). In the following year, Kopecky and Mumford (K22) were able to synthesize and isolate a stable dioxetane, 3,3,4-trimethyl-1,2-dioxetane (Fig. 31), which decomposed upon heating at 50°C to give acetone, acetaldehyde, and luminescence. From this work, together with that of other laboratories, it has been established that the substituents attached to the peroxide ring may include alkyl, aryl, spiroalkyl, alkoxy, or aryloxy groups. In this section, we shall examine both the chemistry of dioxetanes and the way in which the structures influence their

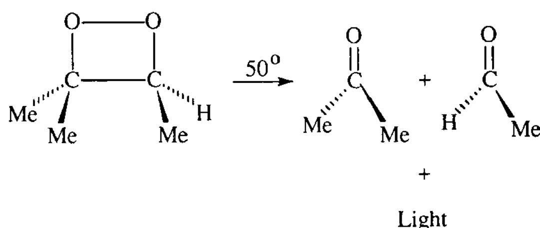


FIG. 31. Thermal decomposition of the cyclic peroxide, 3,3,4-trimethyl-1,2-dioxetane, to give chemiluminescence.

luminescent properties. As analytical tools, dioxetanes can be employed either as labels in ligand binder assays or as high-sensitivity substrates in assays that utilize enzymes as labels. In the former case, luminescence is triggered chemically or thermally, in which case it is referred to as thermochemiluminescence. In the latter case, chemiluminescence is produced by reactions with a stable nonluminescent substrate that is enzymatically destabilized.

3.3.1. *Mechanisms of Thermochemiluminescence*

Figure 32 shows two postulated pathways for the thermal decomposition of uncharged dioxetanes. The concerted mechanism in Fig. 32a was first proposed by McCapra (M15, M16) and elaborated upon by others (K2, K3, T23, T25). Which of the two carbonyl products is in the ground state and which is in the excited singlet or triplet states depends upon the nature of the substituents R1–R4. Emission from the triplet state (T_1) is unlikely because of collisional quenching, unless energy transfer to a fluorescent acceptor such as 9,10-dibromoanthracene is possible. The emission wavelength is determined by the fluorescence emission of the carbonyl excited singlet state (S_1) and is generally in the region of 425 nm. This concerted mechanism would seem to apply to simple aklyl-substituted dioxetanes, including alicyclic-substituted ones.

Figure 32b shows an alternative decomposition pathway, which is probably the one adopted by neutral aromatic substituted dioxetanes (O4, R5, R6, S15). In this scheme, the peroxy bond is symmetrically stretched until it forms a biradical, which can be in either the singlet state (antiparallel electron spins) or the triplet state (parallel electron spins). A subsequent fission of the C–C bond leads to excited-singlet (S_1)-state or excited-triplet (T_1)-state carbonyl products, together with the ground-state carbonyls from the other halves of the biradicals. Emission is from the excited states and, as before, triplet-state emission is unlikely.

Simple alkyl dioxetanes have an activation energy for decomposition in the range 20–26 kcal/mole, which means that they rapidly decompose at, or even below, room temperature (L6, T24). Clearly, such compounds are of little value

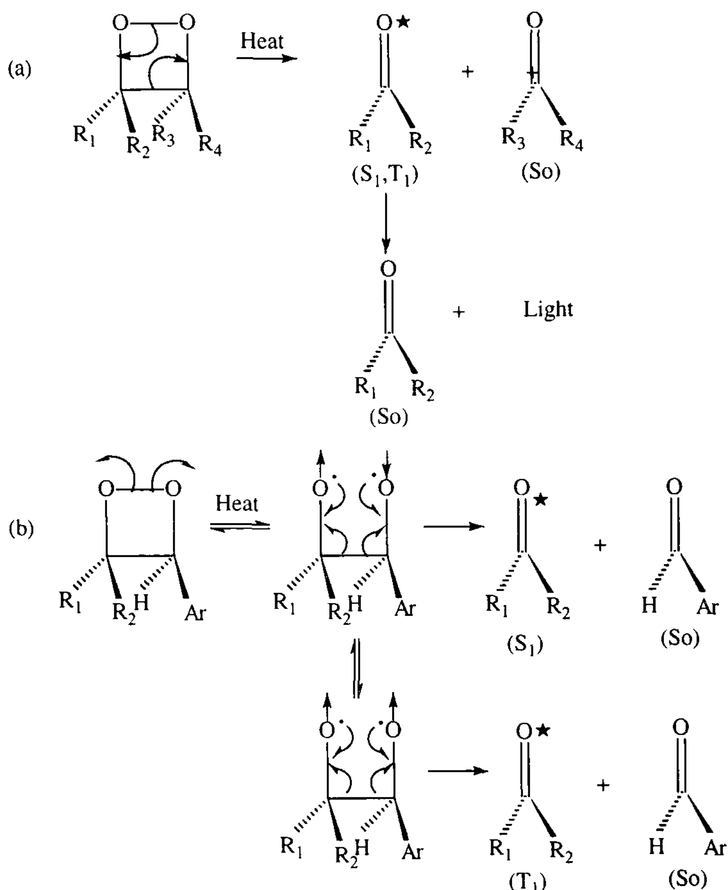


FIG. 32. Possible mechanisms for the thermal decomposition of dioxetanes [adapted from Hummelen *et al.* (H21), with permission]. In (a), a concerted bond cleavage leads directly to two carbonyl products, one of which is in the excited state and emits light (M15, M16). The substituents R_1–R_4 can be simple alkyl or alicyclic groups. In (b), homolytic bond cleavage leads to a biradical that exists as an equilibrium mixture of singlet-state (↑↓) and triplet-state (↑↑) forms. As before, chemiluminescence emission probably occurs via the excited singlet-state carbonyl product arising from the homolytic bond cleavage of the intermediate biradical (R5, R6). The substituents *R* and Ar can include uncharged alkyl, alicyclic, and aromatic groups.

as analytical tools because of their intrinsic instability. However, by varying the nature of the substituents attached to the dioxetane ring, one can modulate the thermal stability. The discovery in 1972 by Wieringa *et al.* (W19) of a highly stable dioxetane, namely *bis*-adamantyl 1,2-dioxetane, laid the foundation for a

new class of stable dioxetanes that could be triggered by heat to produce chemiluminescence. This fact, together with the development of a variety of synthetic methods, has enabled hapten derivatives to be prepared and utilized in sensitive immunoassays.

The thermal decomposition of dioxetanes is a first-order process that can be written as

$$A = A_0 \cdot e^{-kt}, \tag{14}$$

where A is the concentration of dioxetane at times t, A_0 is the initial concentration, and k is the first-order rate constant. We can combine this with the Arrhenius equation for the temperature dependance of the rate, viz.,

$$k = A_0 \cdot e^{-E_a/RT}, \tag{15}$$

where E_a is the activation energy in cal/mole, R is the molar gas constant, i.e., 1.98 cal/mole, and T is the temperature in degrees Kelvin.

For a first-order process as shown in Eq. (14), the half-time Γ is related to the rate constant k, i.e.,

$$k = \ln 2/\Gamma. \tag{16}$$

By combining Eqs. (15) and (16), we have

$$\ln 2/\Gamma = A_0 \cdot e^{-E_a/RT} \tag{17}$$

or

$$2.3 \log(\ln 2/\Gamma) = 2.3 \log A_0 - E_a/1.98 \cdot T. \tag{18}$$

Using the data of Wieringa *et al.* (W19) for the compound shown in Fig. 35, the activation energy is 35.2 kcal/mole and $\log A_0$ is equal to 14.2. Therefore at room temperature, i.e., 25°C or 298 K, we have

$$2.3 \log (0.69/\Gamma) = 2.3 \times 14.2 - \frac{35.2 \times 10^3}{1.98 \times 298}.$$

Therefore,

$$2.3 \log (0.693/\Gamma) = -27$$

$$\log (0.693/\Gamma) = -11.7$$

$$0.693/\Gamma = 10^{-11.7} = 2 \times 10^{-12}$$

$$\Gamma = 0.693/2 \times 10^{-12} = 3.47 \times 10^{11} \text{ sec}$$

$$\Gamma = 1.1 \times 10^4 \text{ years.}$$

The interested reader can verify by a similar calculation that for a temperature of 250°C (523 K), the corresponding half-life for decomposition is reduced to 50 sec!

3.3.2. *Analytical Applications of Thermochemiluminescence*

This remarkable thermoselectivity affords compounds such as *bis*-adamantyl dioxetane the potential for being used as labels in thermochemiluminescent immunoassays. Such assays have been pioneered by Hummelen and co-workers at Groningen University (H20, H21, L16). However, before such potential could be realized, two other features of thermostable dioxetanes needed to be addressed. First, synthetic methods had to be developed whereby dioxetanes could be covalently attached to clinically relevant molecules. A convenient way of achieving this is to first functionalize a dioxetane precursor, then convert it into a dioxetane, and finally attach a hapten or protein (antibody) by covalent coupling via the functional group. This sequence is illustrated in Fig. 33, which is taken from work published by Hummelen's group (H19). Various derivatives of the simple alkyl dioxetanes have now been described (A2–A4, A6). Despite this variety, to date those compounds that have been used as labels in immunoassays are all derivatives of *bis*-adamantyl dioxetane, or, more correctly, adamantylidene adamantane 1,2-dioxetane (H20). Figure 34 shows four functionalized derivatives of this compound and these labels can all be covalently attached to free amino or thiol groups in any desired macromolecule (protein carrier or antibody) or hapten. By attaching such labels to antibodies (with a labeling stoichiometry of 5–15), it has been found possible to conduct sandwich immunoassays of the type described in Section 1.4.1 (L15).

Second, the chemiluminescence quantum yields of dioxetanes that decompose by the mechanisms shown in Figure 32 are very low. This is because (a) the excited singlet yield is very low (Fig. 35) and (b) the excited-state carbonyl compounds produced by the thermal cleavage have relatively poor fluorescence quantum yields; e.g., ϕ_F for adamantanone is only 0.0052. With regard to the quantum efficiency of dioxetane thermochemiluminescence, it was shown that the poor emission from excited-singlet-state carbonyl compounds could be amplified by an energy transfer process (B9). In this context, 9,10-diphenylanthracene (DPA) has been shown to be a good fluorescent acceptor for 1,2-dioxetanes (W21) with a fluorescence quantum yield of 0.8–1.0 (E4) and a Förster donor–acceptor distance, R_0, of 15.3 Å (H20). The spectral characteristics of DPA are such that the emission is at a wavelength that is only slightly redshifted with respect to the dioxetane donor emission. This is due to the fact that DPA has absorbance maxima at 375 and 400 nm, both of which are encompassed by the wide spectral range of *bis*-adamantyl dioxetane emission. Furthermore, the Stokes shift for DPA emission is only about 30 nm. By attaching both *bis*-adamantyl dioxetane *and* DPA to antibodies, one has a labeled antibody with an isochromic internal signal amplification feature (L16, H20, H21). This is illustrated in Fig. 36, which shows

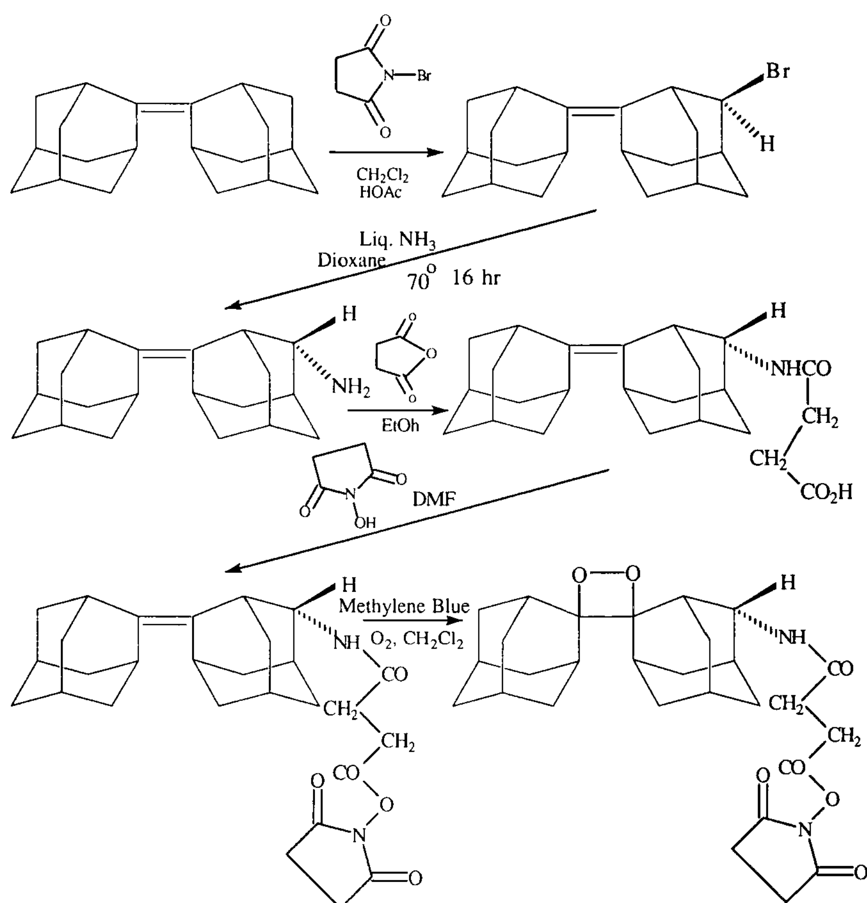


FIG. 33. Synthesis of an *N*-hydroxysuccinimidyl derivative of *bis*-adamantyl dioxetane, suitable for covalently labeling the amino groups of hapten derivatives or protein lysine residues. Adapted from Hummelen *et al.* (H19), with permission.

how *N*-hydroxysuccinimidyl (NHS) derivatives of DPA and dioxetanes can be coupled in various stoichiometries to an antibody, which can then be employed as the labeled second antibody in a sandwich-type immunoassay.

In a model system, it was shown that BSA labeled with 17 molecules of dioxetane had its quantum yield increased by a factor of 40 when DPA was attached to the protein at a 30:1 stoichiometry (H20). Coupling of the labeled BSA to monoclonal antibodies enabled Hummelen and co-workers to use the resulting conjugates in sandwich immunoassays, which were referred to initially by the

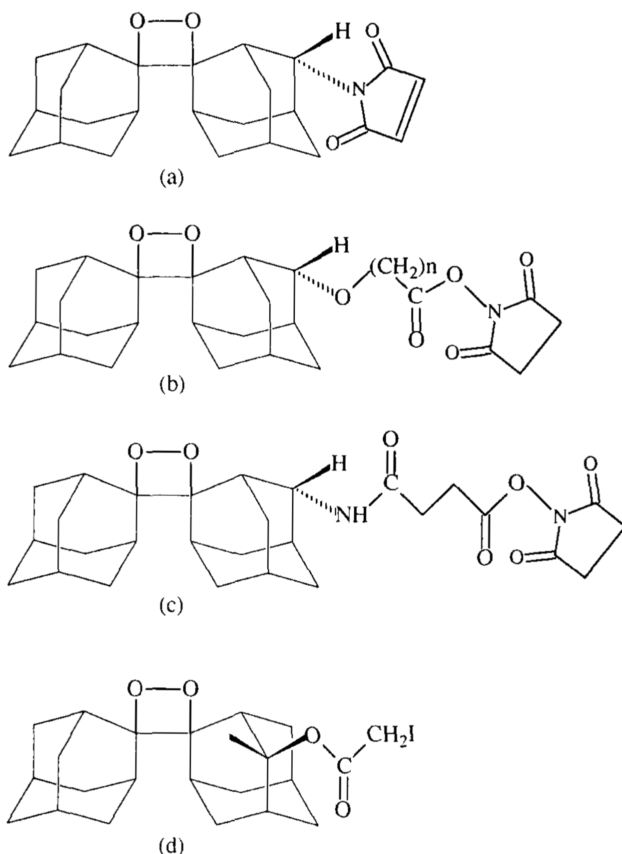


FIG. 34. A selection of functionalized *bis*-adamantyl dioxetanes, useful as covalent labels [adapted from Hummelen *et al.* (H19), with permission]. Compound (a) is designed for labeling thiol groups; compounds (b) and (c) are suitable for amino groups; and compound (d) can react with either thiol or amino groups, depending on the experimental conditions.

lengthy acronym FATIMA (fluorescence-amplified thermochemiluminescent immunoassays), e.g., for carcinoembryonic antigen and human IgG (H20). A subsequent report on applying this technique to an assay for HCG referred to it simply as FTIA (fluorescent thermochemiluminescent immunoassay), and the detection limit for the hormone was found to be ca. 5 mIU/ml (L16). The aforementioned article focuses on the merits of fluorescent-labeled versus "conventional" thermochemiluminescent assays and also on multiple labeling strategies. However, contrary to the authors' claims, it is not obvious from the data presented that

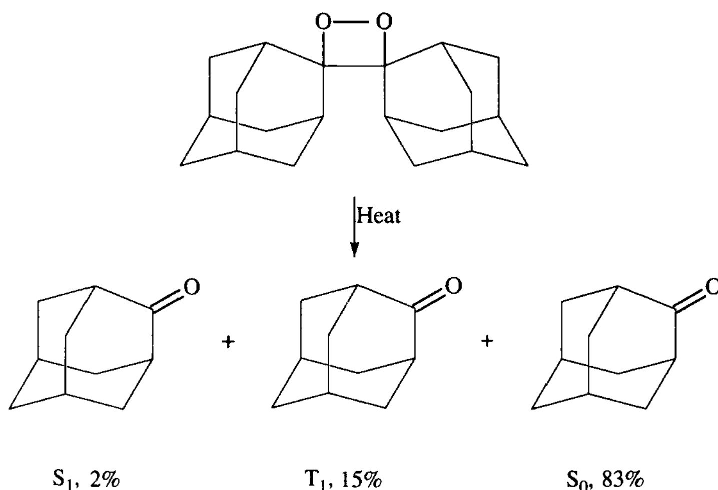


FIG. 35. Distribution of singlet- and triplet-state carbonyl products arising from the thermal decomposition of *bis*-adamantyl 1,2-dioxetane. Taken from Hummelen *et al.* (H20), with permission.

fluorescence offers any additional advantage in terms of reducing the minimal detectable analyte concentration.

One further aspect of thermochemiluminescence needs to be addressed in order that assays can be carried out at the temperatures required to trigger the emission of luminescence. The solid phase carrier has to be stable over the temperature range used to trigger chemiluminescence, i.e., 200–250°C. Two solid phase templates that meet these requirements are Kapten 500H and Teflon.

As already mentioned, the chemiluminescence emission in the above assays is essentially isochromic with the dioxetane itself. However, it is possible to observe more conventional redshifted emission by energy transfer from 1,2-dioxetanes to acceptors such as anthracene (in contrast to diphenyl anthracene), fluorescein, and rhodamine B. It is not obvious, though, how one might exploit such a phenomenon in an analytical assay, given the requirement for a 250°C heating step and the energy transfer requirement that donor and acceptor be no more than ca. 100 Å apart. Indeed, the high temperature required for triggering chemiluminescence, together with the rather specialized equipment needed, has precluded a more widespread adoption of thermochemiluminescence as a routine technique for clinical analysis.

3.3.3. *Nonthermal Triggering of Dioxetane Emission*

The real breakthrough in exploiting dioxetanes as analytical tools stems from an initial idea by McCapra (M17, M19, M24) to explain firefly bioluminescence.

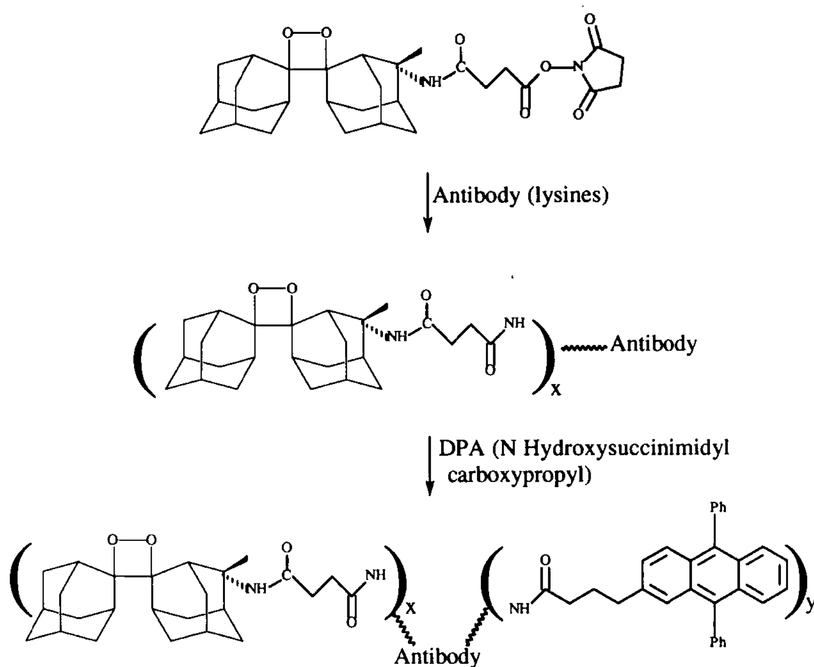


FIG. 36. Covalent attachment of *bis*-adamantyl dioxetane and the fluorophore, 2-(*N*-succinimidyloxycarbonylpropyl)-9,10-diphenylanthracene, to an antibody. Chemiluminescence efficiency is enhanced by intermolecular energy transfer.

This was further elaborated upon by Schuster and co-workers (K21, S23, S24) and by Schaap's group at Wayne State University (S6, S8, S10, S11, Z2, Z3). Thus, the observation that some hydroxy-substituted aromatic dioxetanes show high chemiluminescent efficiencies at alkaline pH (phenolic anionic form) led to the formulation of a third mechanism for chemiluminescent decomposition of dioxetanes. This mechanism, known initially as intramolecular electron transfer (M19, Z2) and subsequently as chemically initiated electron exchange luminescence, or CIEEL (F1, K20), can be best illustrated by reference to the dioxetane shown in Fig. 37, where the chemiluminescence is triggered by the addition of fluoride ions.

The mechanism is thought to involve the intramolecular donation of electronic charge from the phenolic oxy-anion to the peroxide σ^* orbital (S6, S8). The aromatic systems that have been examined to date are either phenyl or naphthyl, and it has been found that the position of the substituent phenol group is critical in determining both the chemiluminescent efficiency (E1, E2, S8, S9, S11) and the wavelength of the emission (E1, E2).

An example that illustrates both of the above parameters is shown in Fig. 38. Not only is the emission from the 2,7-disubstituted naphthalene derivative

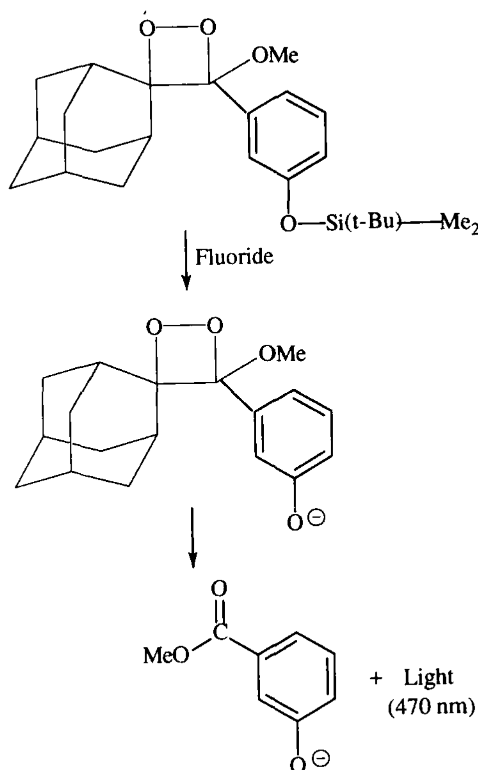


FIG. 37. Scheme for the fluoride-induced decomposition of 4-(3-*tert*-butyldimethylsilyloxy-phenyl)-4-methoxyspiro[1,2-dioxetane-3,2'-adamantane] with subsequent light emission. Taken from Schaap *et al.* (S8), with permission.

(Fig. 38a) 150-fold larger than that from the 2,6-disubstituted analogue (Fig. 38b), but also the wavelength is almost 100 nm redshifted from the normal dioxetane emission maximum. Computer-assisted molecular orbital calculations of the AM1-type have been used to rationalize the above data (B25, E1), whereas similar theoretical treatments have been applied to other dioxetanes (R3). However, based on a suggestion by Hummelen and co-workers (H21) that an efficient CIEEL process requires close proximity of the phenolic electronic charge to the peroxy σ^* orbital, one can intuitively rationalize all the published data on phenyl and naphthyl-substituted adamantyl dioxetanes from both Schaap's group and Bronstein's group (B25, E1, E2, S9–S11). A schematic explanation is illustrated in Fig. 39, which shows how the phenoxy electron can be delocalized to give either an α or a β aromatic carbanion, and that only in the latter case does rotation of the

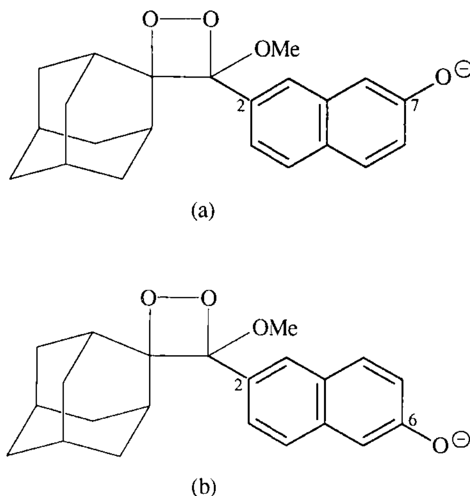


FIG. 38. Chemiluminescence properties of the phenolic anions of two substituted adamantyl naphthyl dioxetanes. The emission wavelength and half-life of compound (a) are 550 nm and 23 min, respectively, while the corresponding values for compound (b) are 459 nm and 9 sec. These data are taken from Edwards *et al.* (E1).

aromatic ring(s) afford efficient charge transfer to the peroxy bond. Thus, the 2,7-disubstituted derivative in Fig. 38a is much more efficient as a luminescent compound than the 2,6-analogue shown in Fig. 38b. A similar consideration might also apply to the emission wavelengths of such compounds. Again, referring to Fig. 39, the 2,7-derivative affords an excited-state carbonyl compound that is highly conjugated, hence the long emission wavelength of 550 nm. The 2,6-derivative, on the other hand, yields an unconjugated excited-state carbonyl compound that emits in the normal dioxetane emission range of 460–470 nm.

3.3.4. *Dioxetanes as Enzyme Substrates*

Dioxetanes are beginning to have a tremendous impact on chemiluminescent methodologies for clinically relevant analytes. This impact is due to the development of dioxetanes as enzyme substrates in enzyme-amplified assays similar to the type described in Section 1.3.2. The evolution of the current generation of highly efficient substrates merits some comment. Schaap and co-workers (S8) showed that the naphthalene silyl ether shown in Fig. 40a could be triggered by fluoride ions to give chemiluminescence with a quantum yield of 0.01. On the other hand, the *meta*-disubstituted phenyl derivative in Fig. 40b is extremely efficient in a CIEEL chemiluminescence process (for reasons outlined in the previous section) with a quantum efficiency of 0.45. Subsequent studies showed that the naphthalene derivative in Fig. 40a with an *O*-acetyl group instead of the silyl ether, could

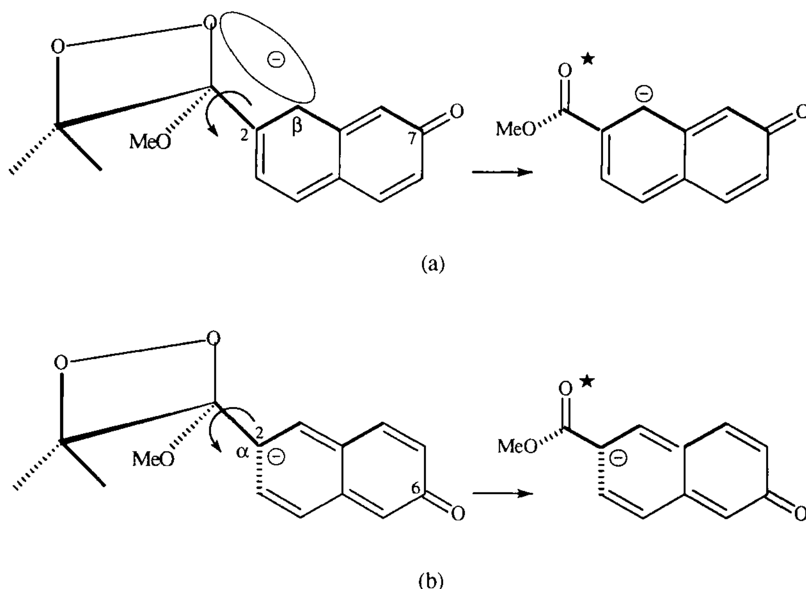


FIG. 39. Illustration of how the delocalization of electronic charge in disubstituted naphthylate adamantyl dioxetanes can give rise to β-carbanions [2,7-derivative shown in (a)] or α-carbanions [2,6-derivative shown in (b)]. Only in the former case is there an efficient interaction between the electron cloud and the peroxy group.

be enzymatically cleaved by an acyl esterase to produce the light-emitting phenoxy derivative (S10). In the third of three key papers, Schaap acknowledged the utility of alkaline phosphatase for enzyme-amplified immunoassays and described the synthesis of a phosphate-substituted adamantyl xanthene dioxetane (S11). In the presence of alkaline phosphatase at pH 10.3, the compound produced the phenoxy analogue, which subsequently decomposed to produce light with a chemiluminescent quantum yield of 10^{-6}. This represented the first, albeit inefficient, chemiluminescent substrate for alkaline phosphatase. The coup de grace came when the phosphate analogue of the highly efficient *meta*-substituted phenyl dioxetane (S8) shown in Fig. 40b was synthesized as an alkaline phosphatase substrate (B21, B28, S5, S7, V11). This compound, as previously mentioned, has a quantum efficiency of 0.45, irrespective of whether it is derived from a phosphate ester, an acetate, or a silyl ether (S8). The enzyme-catalyzed reaction for this dioxetane is shown schematically in Fig. 41. Since the substrate is present in excess and the enzyme is present in limiting amounts, the kinetics of light emission are those of the glow-type described in Section 2.4 (Fig. 12b), where the light intensity reaches a plateau after 10–20 min and remains stable for an hour or so (provided enough enzyme substrate is present). The detection limit for alkaline

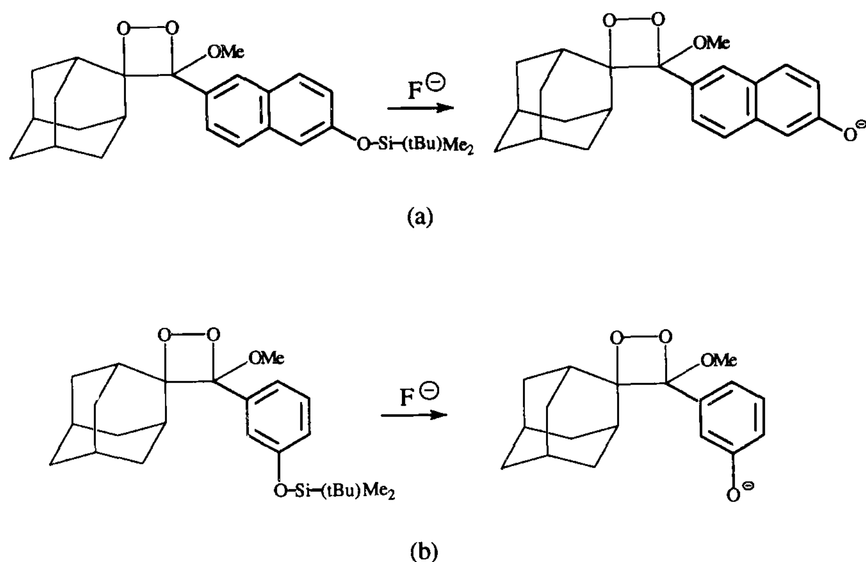


FIG. 40. Chemiluminescent decomposition of adamantyl aromatic dioxetanes by fluoride-induced cleavage of silyl ether side chains. The 2,6-substitution pattern shown in (a) has a quantum efficiency of 0.01%, whereas the 2,4-substitution pattern shown in (b) has a quantum efficiency of 45%. The data [which are taken from the work of Schaap *et al.* (S8)] can be rationalized by considering the mechanism suggested in Fig. 39.

phosphatase is reportedly 0.01 amole (B18, B23) or 0.002 amole (S7). Significant signal enhancement by BSA or CTAB (cetyl trimethylammonium bromide) has been reported (B18, B23, S9). Unfortunately, a certain degree of "vodoo science" has crept into the dioxetane field, and uncontrolled claims for a variety of proprietary signal enhancers have been advanced, e.g., Lumiphos-530 (S7, S9), Emerald (B25, B27, V5), and Sapphire (B25, B27). The nomenclature of these so-called enhancers suggests that intermolecular energy transfer to efficient fluorescers is involved, possibly in mixed dioxetane/fluorophore/detergent micelles. In fact, Schaap (S7) has described the use of a hydrophobic fluorescein derivative together with the detergent CTAB, where the mixture provides a 400-fold enhancement in chemiluminescence efficiency from the *meta*-substituted phenyl adamantyl dioxetane (Fig. 41) and alkaline phosphatase.

3.3.5. *Dioxetane Enzyme Substrates in Immunoassays*

Using alkaline phosphatase-labeled antibodies to protein antigens and the *meta*-phenyl phosphate dioxetane shown in Fig. 41 (referred to as AMPPD or Lumigen PPD), one can conduct sandwich-type immunoassays with a solid phase immobilized capture antibody and an enzyme-labeled signal antibody (B19). After the

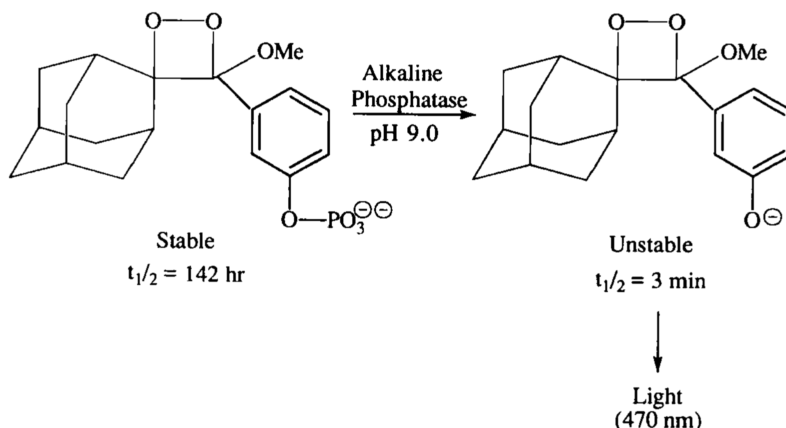


FIG. 41. Alkaline-phosphatase-triggered cleavage of the highly stable adamantyl *meta*-phenyl phosphate dioxetane, to give the unstable, light-emitting phenoxide ion. The half-life values were measured in an aqueous medium at 30°C (B18,E1).

immunoconjugation reactions, the captured enzyme labels are incubated with dioxetane substrate (>20 min) and the light intensity is measured in a suitable luminometer. Assays for human chorionic gonadotropin (β-hCG) and α-fetoprotein (AFP), which are clearly superior to the colorimetric versions, have been described (B18, B23, T9). Similarly, a high sensitivity TSH assay has been reported with a minimal detectable dose of 0.0045 μIU/ml and an excellent correlation between this and an alternative assay for 40 patient serum samples (B18, B19, B23, B27, B29). Signal generation alone took 40 min, and the total assay time was not reported in these studies. A similar methodology has been used to measure ferritin (D2) and thyroid peroxidase antibodies in patients with autoimmune thyroid disease (T5).

One major advantage of the glow-type emission obtained from dioxetane enzyme substrates is that one can easily record the emission on photographic film (in much the same way that ^{32}P emission is recorded) to obtain qualitative or semiquantitative data. This technique has been described for a dry-format membrane-based assay for human luteinizing hormone (hLH), in which Polaroid 612 film (ASA 20,000) was used to record the intensity from unknown samples and from a set of standards (B18). A dioxetane-based ELISA method for human growth hormone (somatotropin) has been recently described. The chemiluminescent assay was reported to be superior to a similar fluorescence assay, and the detection limit for hGH was found to be 5 p*M* (A9).

Although they have not been used as labels in immunoassays, dioxetane substrates for β-galactosidase have been synthesized and characterized (B18, B23, S9). Current data are sparse, but it would appear that the enzyme is less detectable

than alkaline phosphatase/dioxetane phosphate. One use for the dioxetane β-galactoside has been to quantitate the enzyme activity of the lac Z gene from *Escherichia coli.* The activity of a single transfected cell was measurable, in contrast to a fluorimetric method that required 7000 cells (J2). In another study, the superiority of the dioxetane method over a colorimetric method, employing *O*-nitrophenyl galactoside, was discussed (B6). Interestingly though, the sensitivities differed by only a factor of 2 when the assay volumes were comparable, and the high sensitivity of 0.5 μU was achieved only by reducing the assay volume to 10 μl.

One of the problems associated with all enzyme-amplified assays is that the substrates can give rise to a signal in the absence of enzymatic triggering. This background signal arises from the nonenzymic formation of 2-adamantanone and acetyl *meta*-hydroxybenzoate anion, with emission from one or both of these species. Nonenzymic breakdown is favored when the enzymatic reaction is relatively slow and the species involved are able to aggregate. In order to overcome this problem, several derivatives of the, by now familiar, adamantyl methoxy phenyl dioxetane phosphate (Fig. 41) have been synthesized. The structure of such compounds is illustrated schematically in Fig. 42, which shows that they are hydroxy- or halogeno-derivatives of the parent dioxetane, in which the substitutions are made in the adamantane ring. Such substitutions, it is claimed, reduce the background signal and facilitate the emission kinetics by reducing the half-life of the enzymatically produced phenoxy anion (B25). This improvement (which is attributed to a reduced propensity for aggregation) translates into a more sensitive assay for TSH, for which the minimal detectable dose is extrapolated to be 0.001 μIU/ml for a 60-min incubation with the dioxetane substrate (B25, B27). A

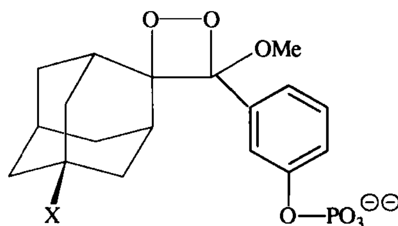


FIG. 42. Modifications to the adamantyl ring of the dioxetane alkaline phosphatase substrate shown in Fig. 41. Such modified compounds are claimed to give lower background signals in clinical assays (B25,B27).

complicating factor in interpreting both of these studies is that a proprietary enhancer was used in conjunction with the enzyme/substrate mixture, and the precision of the reported background and half-life measurements was not recorded.

3.3.6. *Dioxetane Enzyme Substrates in Nucleic Acid Detection*

Whereas Sections 3.3.2 and 3.3.4 suggest increasing potential for dioxetanes in immunoassays, the major focus in their current development and application is in nucleic acid detection. This stems from the coincidental discovery and development of stable dioxetane enzyme substrates, with the rapid growth in recombinant DNA technology, with its demands upon sequencing, DNA fingerprinting, Southern blotting, and a number of clinical assays for infectious diseases, in which DNA rather than protein is the preferred analyte. As with other types of diagnostic assay, the impetus for applying dioxetane chemistry to these endeavors has been to reduce one's reliance upon radioisotopic methods.

Most dioxetane-based gene probe assays for viral or bacterial DNA are similar to sandwich-type immunoassays. The basic strategies are outlined in Fig. 43. A solid support, which can be any of the templates used in immunoassays, e.g., coated tubes, ELISA wells, and glass beads, contains a covalently attached oligonucleotide capture probe whose sequence is complementary to a unique stretch of the target DNA sequence. After hybridization of the target to the probe, the unbound target and nontarget DNA are removed by washing. A second oligonucleotide probe containing covalently attached alkaline phosphatase (J1) is allowed to hybridize to a complementary sequence on the target and, after removal of excess labeled probe, by separation and washing, the dioxetane substrate is added and the signal detected in a luminometer. Because it is easier to synthesize and purify a biotinylated probe rather than an enzyme-labeled probe, a variation of the protocol involves an intermediate incubation with a (generic) streptavidin–alkaline phosphatase conjugate prior to treatment with the chemiluminescent substrate. Some aspects of applying dioxetanes as molecular biology probes have been recently reviewed (B7).

Infectious Diseases DNA hybridization assays that employ dioxetane substrates for alkaline phosphatase were first briefly reported in 1988, viz., herpes simplex I virus (B21) and *Chlamydia trachomatis* (C17). These two assays were followed by an assay for hepatitis B virus core antigen, HBVc, (B18, B26). Thus, 10^6 copies of HBVc-containing plasmid DNA were detected in 30 min in a dot–blot assay, whereas a colorimetric test required 10^8 copies in the same time (B18, B26). Optimization allowed 10^4–10^5 copies to be detected in 2 hr. An *in situ* hybridization assay for HSV-1 detected its presence after only 5 min (B22). A more detailed account of the *Chlamydia* assay describes how probes were prepared against two sites on an endogenous 7.4-kb plasmid and how, in a 5-hr assay,

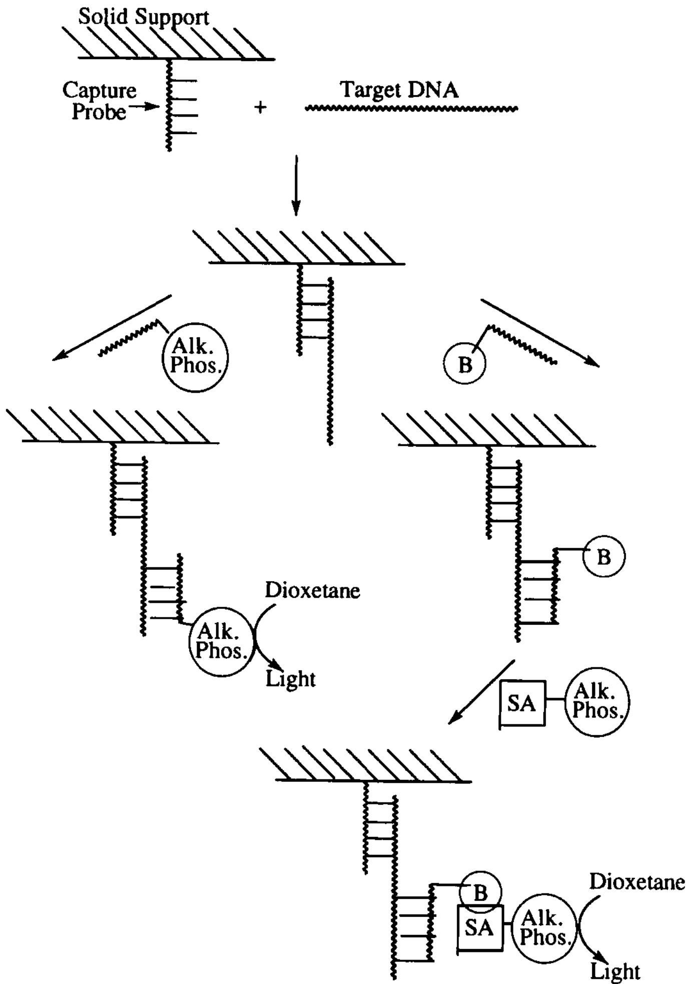


FIG. 43. General scheme for sandwich-type gene probe assays utilizing dioxetanes of the type shown in Fig. 41. Both alkaline phosphatase (Alk. Phos.) and biotin (B) are covalently attached to oligonucleotide probes. In the scheme shown on the right-hand side, the enzyme is covalently attached to streptavidin (SA), which is in turn captured by hybridizing to a biotinylated probe.

10^4 elementary bacterial bodies were detected either with a microtiter plate luminometer or Polaroid instant film (C18). A gonorrhea test has also been reported (S1). In a comparative study of colorimetric versus chemiluminescent detection of hepatitis B virus, the latter method was shown to be significantly more sensitive.

Moreover, in the same study, the dioxetane substrate was reported to have twice the sensitivity of an enhanced luminol method (U1).

DNA Sequencing There are two currently established methods for determining the nucleotide sequence of long stretches of genomc DNA. Base-specific cleavage by chemical means (M13), or by enzymatic chain termination reactions (S2), followed by isotopic labeling, allows one to electrophorese separate mixtures of so-called nested DNA fragments, where each fragment in any one mixture will terminate in the same nucleotide base. The resulting base-specific electrophoretic ladders can be detected on X-ray film, in which each lane represents fragments of increasing length terminating in the same base. The sequence can be read off the film image by starting with the smallest fragment and seeing which base extends it by one nucleotide. The process is repeated, base by base, until the largest fragment is reached, or until one can no longer visibly resolve individual fragments.

In an extension of the chemical sequencing method (C14), the electrophoretic gel bands are eluted onto suitable membranes, UV cross-linked to these membranes, and probed with ^{32}P-labeled oligonucleotides, so that the bands can be detected on X-ray film. By using biotinylated or alkaline phosphatase-labeled probes, together with the dioxetane substrate, a nonisotopic detection of sequencing gels is possible (B8). In a subsequent study, it was found that imaging of membrane-bound electrophoretic ladders required only 30 min exposure to X-ray film in the presence of dioxetane substrate, compared with 40 hr exposure with ^{32}P-labeled probes (T18). This last study also illustrated the use of dioxetanes in a multiplex sequencing protocol (C15), in which the labeled probes are melted off the membrane and different probes are allowed to hybridize to the immobilized target in a multiprobe strategy. In the most recently available report on dioxetanes used for DNA sequencing, the chloroadamantyl dioxetane shown in Fig. 42 was compared with the standard dioxetane substrate. The chloro-derivative was claimed to be superior in both band intensity (faster light emission kinetics) and resolution (M4).

Blotting Techniques Three different protocols have been described for genomic Southern blotting using the dioxetane substrate for alkaline phosphatase. The methods are illustrated schematically in Fig. 44. In Fig. 44a, the target DNA fragments (separated by electrophoresis) are UV cross-linked to a membrane and probed directly with an oligonucleotide containing a sequence complementary to part of the target DNA and a covalently attached alkaline phosphatase molecule. Detection, as before, is effected with the aid of the dioxetane substrate. In an experiment utilizing this approach, the human tissue plasminogen activator gene (single copy) was detected in 0.25 μg of genomic DNA (C10). Band intensities on Kodak XAR-5 film for 4 hr exposure to dioxetane substrate were comparable to those obtained after 1 week when the probe was labeled with ^{32}P. Depending on

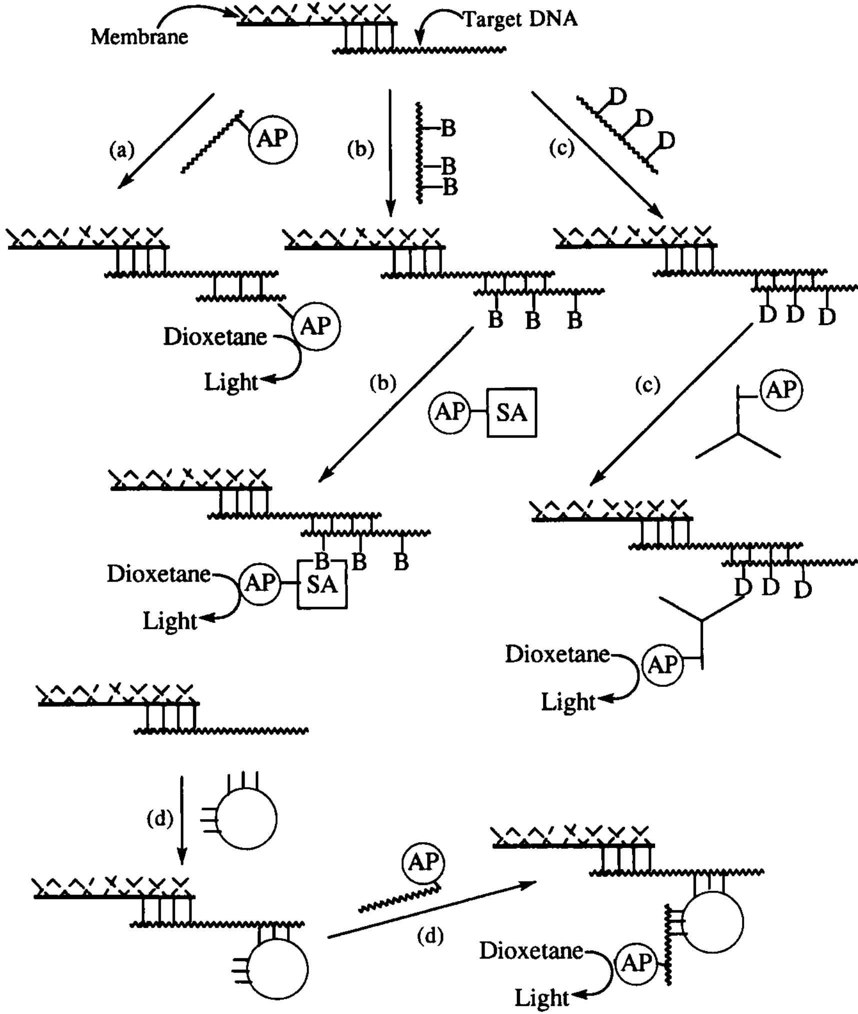


FIG. 44. Analysis of genomic DNA samples by Southern blotting techniques that utilize dioxetane enzyme substrates. Electrophoretically separated fragments of genomic DNA are UV cross-linked to a membrane support and probed with alkaline phosphatase-labeled or biotinylated probes [schemes (a) and (b)] as in Fig. 43. Scheme (c) involves a combination of probe hybridization and immunological capture of an alkaline-phosphatase-labeled digoxygenin antibody, via a target-specific digoxygenin-labeled (D) oligonucleotide. Scheme (d) taken from the work of Nguyen *et al.* (N8), employs a phagemid with hybridization sites for both the target DNA and an enzyme-labeled oligonucleotide probe. Further details for all of the above approaches are provided in the text.

the level of sensitivity required, short exposure times of 5–60 min may be sufficient (B28). Blocking nonspecific binding to the membranes is also important in determining sensitivity (B24).

One of the disadvantages of the direct method is that for each target sequence, a unique enzyme-labeled probe must be synthesized and purified. For this reason, the indirect methods shown in Figs. 44b and 44c have been developed. In Fig. 44b, the probe DNA is biotinylated by an enzymic nick-translation method (R7) or by random priming (K9), so that, after hybridization to the target, it is treated with a universal streptavidin–alkaline phosphatase conjugate, followed by the light-emitting dioxetane substrate. In this manner, picogram quantities of *mos* and *raf*-1 single-copy genes have been detected on X-ray film (P12), as well as *psp* A, a single copy gene in *Streptococcus pneumoniae* (S29). An interesting variant of this approach (Fig. 44c) involves both probe hybridizations and immunoconjugation. An enzymic method of introducing the hapten digoxygenin into probe polynucleotides has been developed, so that after hybridizing a digoxygenin-labeled probe to the genomic Southern blot, a universal label consisting of a digoxygenin antibody coupled to alkaline phosphatase is allowed to bind to the nucleotide probe. As before, final detection is on instant or X-ray film after incubation with the dioxetane substrate (H13, M8, M35, M37). Obvious advantages are the generic nature of the signal-generating molecule and the ease of synthesizing randomly labeled biotinylated or digoxygenin-containing probes. A disadvantage, though, is the lack of control of probe-specific activity, i.e., the number of biotins or digoxygenin molecules inserted per probe molecule.

A two-step method that purports to circumvent this problem has been developed, although its practicability is questionable. The method is outlined in Fig. 44d and central to it is the use of a single-stranded phagemid or M13 vector into which the probe, complementary to the immobilized target, has been inserted. The vector also contains a universal primer sequence so that the alkaline phosphatase label is again generic. The technique has been shown to work with a dioxetane substrate and, from 5 μg of hyman genomic DNA, both the single copy N-*myc* gene and the multiple copy β-actin gene have been detected (N8).

One extension of blotting techniques that is likely to be expanded considerably in the near future is DNA fingerprinting for forensic analysis, paternity testing, etc. Already, the dioxetane substrate for alkaline phosphatase has been shown to be a useful tool in this endeavor (G4).

3.4. Peroxyoxalates

The luminescent decomposition of certain oxalic acid esters (Fig. 9) in the presence of hydrogen peroxide and fluorescent sensitizers represents one of the most efficient nonbiological light-producing reactions (R1, R2). The two most commonly used esters are shown in Fig. 45. As diagnostic agents, oxalate esters

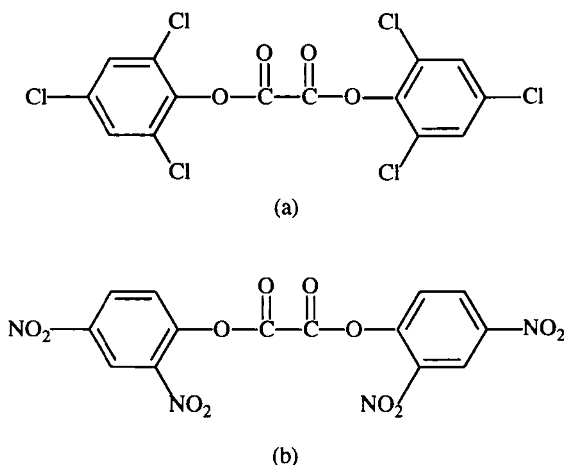


FIG. 45. The two oxalate esters most commonly used in assays involving chemiluminescent detection. (a) *bis*-(2,4,6-trichlorophenyl)oxalate (TCPO); (b) *bis*-(2,4-dinitrophenyl)oxalate (DNPO).

can be used to detect either hydrogen peroxide (G16) (cf. luminol) or the fluorescent sensitizer.

3.4.1. *Mechanism of Chemiluminescence*

A simplified mechanism for oxalate ester chemiluminescence is shown in Fig. 46, which is essentially a two-step reaction. Attack by peroxide on the ester produces an excited-state, 1,2-dioxetaneone via a peroxyoxalate intermediate, which breaks down in the presence of a suitable fluorophore to give carbon dioxide plus an excited-state fluorophore, which, in turn, emits light. The scheme depicted in Fig. 46 is an oversimplification and the true reaction mechanism continues to attract much attention (A10, C11, C11a, G8, O5).

A clear difference between peroxyoxalate chemiluminescence and that of the previous compounds we have examined is that the emission wavelength is solely determined by the fluorophore and not by the oxalate ester. Typical fluorophores are fused aromatic compounds such as pyrene, anthracene, and, especially, perylene.

3.4.2. *Analytical Uses of Oxalate Esters*

Most analytical applications of peroxyoxalates are in flow-through systems such as HPLC and industrial reactors. As such, the peroxyoxalate/sensitizer system does not fall within the realm of clinical analysis. However, since much experimental work is being done to expand our understanding of the reactions and

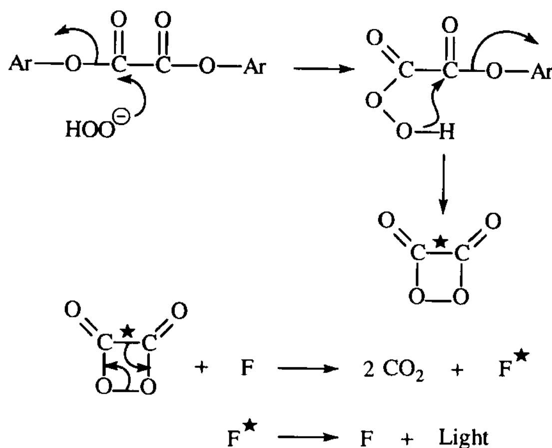


FIG. 46. Possible mechanism for chemiluminescence of oxalate esters. Attack by peroxide ions leads to a peroxyoxalate intermediate which, in turn, breaks down to an unstable excited-state dioxetaneone. In the presence of an appropriate fluorophore, energy transfer from the dioxetaneone leads to luminescence from the fluorescent sensitizer.

their analytical scope, a brief discussion of their current role in analysis is warranted. Several recent review articles include sections on peroxyoxalates as analytical tools (G13, T20), in HPLC and solid bed reactors (K35, N9), and in amino acid detection (R8).

In HPLC detection, sensitivity using peroxyoxalates is reportedly better than that for fluorescence, because of reduced light scattering and increased stability of the excitation source. Detection of some aromatic amines is in the attomole range (S30). With regard to amino acids, labeling with an appropriate fluorescent tag can be done either pre- or postcolumn, and the resulting conjugate can be detected after reversed-phase separation (B3, K13). Steroids (N10) and catecholamines (K14) can be similarly quantitated. By suitable derivatization, other classes of compound such as carbonyl compounds (M2) and carboxylic acids (G14, L10) can be detected.

Despite their high chemiluminescence efficiency, aromatic oxalic acid esters do have some disadvantages. At high levels of ester, there is some background emission (C9). They are also relatively insoluble in water, which means they are applicable only in situations in which organic solvents or water/solvent mixtures can be tolerated. A novel way of overcoming this problem is to have solid ester (e.g., TCPO) incorporated into the matrix of the immobile phase and allow it to slowly dissolve and leach out in the solvent stream, i.e., aqueous acetonitrile or aqueous methanol (P13, V8).

Unlike luminol, peroxyoxalate chemiluminescence is not affected by metal ions

in the analytical sample or medium. However, certain compounds such as inorganic ions (halide and sulfite), substituted anilines, and thiourea derivatives, have been shown to quench peroxyoxalate chemiluminescence. Gooijer and coworkers (G8, V7, V9) have sought to exploit this phenomenon by employing it as a detection method for the aforementioned quenchers. Sensitivity limits are reportedly in the nanogram range (G8).

This brief summary of peroxyoxalate chemiluminescence in no way does justice to the topic and the interested reader is urged to consult the more comprehensive accounts cited in this chapter. If and when water-soluble oxalate esters become readily available, there is no doubt that these labels will be adapted to the types of assay that have formed the main subject matter for this article, viz., immunoassays, nucleic acid and gene probe assays, and clinical chemistry assays.

4. Conclusion

In a recent brief survey of several chemiluminescent labels for nucleic acid detection, it was suggested that luminol/peroxidase or acridinium esters are appropriate for assays requiring rapid results, but that dioxetanes might be preferable for high-sensitivity assays that are not under a time constraint (P10). A similar conclusion had been reached in a more comprehensive comparison between acridinium esters and dioxetanes (N5). While the two reviews just cited were somewhat partisan in their approach, Schroeder (S19) has taken a more sanguine look at the chemiluminescent systems we have discussed in this article and has compared their efficacy in a variety of assay situations. As he correctly points out, high label detectibility does not always translate into a high assay sensitivity. Other factors such as components of the sample fluid, and nonspecific binding of the chemiluminescent label, determine the ultimate sensitivity of any assay.

Each of the labels, or enzyme/substrate systems we have examined, has its merits and its drawbacks, and it would be impossible to say that one or other is clearly superior. What one can say is that an efficient signal-generating system is a sine qua non of a good assay. Chemiluminescence has evolved from a laboratory curiosity into an analytical technology that has already shown itself able to meet the most stringent demands of the clinical analyst. The ensuing decades will bring new analytes such as disease markers (both protein and DNA) present in vanishingly low concentrations, and I have no doubt that chemiluminescence will play a major role in meeting those analytical challenges.

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ESTROGEN AND PROGESTERONE RECEPTOR PROTEINS IN PATIENTS WITH BREAST CANCER

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1. Introduction

Experiments conducted in many laboratories throughout the world have led to a recognition that steroid hormones generally affect biologic responses in target

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tissues through the mediation of high-affinity, specific binding proteins, called receptors, which are present in unique amounts in such responsive tissues. The large body of knowledge about the steroid hormone mechanism of action has been derived almost entirely from studies in which a radiolabeled steroid hormone has been used as the marker to elucidate the details of the interaction of the hormone with responsive cells.

The hormone dependency of breast cancer was first described by Sir George Beatson when he reported the dramatic beneficial effect of oophorectomy in two premenopausal women with advanced breast cancer (B2). The efficacy of oophorectomy was later studied (C1) in the treatment of premenopausal patients with advanced breast cancer. Of the 64 patients studied, a response to oophorectomy was observed in 68% of the patients with estrogen receptor protein (ERP) positive (+) tumors. There was no response in 86% of the patients with ERP negative (–) tumors. These results indicated that the ERP status in premenopausal breast cancer patients allows discrimination of those women who may benefit from oophorectomy. Huggins *et al.* showed that adrenalectomy was often effective after relapse following oophorectomy (H11, H12). Folca *et al.* first reported the uptake of labeled estrogen by human breast cancer tissue (F5). They demonstrated that when ^{3}H-hexestrol was injected into patients who were to undergo ablative adrenalectomy for breast cancer, those who responded favorably to adrenalectomy showed a correlation with higher uptake of radioactive estrogens by the tumor.

Jensen *et al.* (J2) and Terenius (T1) independently demonstrated *in vitro* the specific binding of radioactively tagged estrogens to those breast cancer specimens that had favorable clinical responsiveness. These experiments indicated that there were two general classes of breast cancer, those that show a significant accumulation of labeled estradiol *in vitro* and those that show no hormone uptake. Toft and Gorski established the usefulness of sedimentation analytical methods in the characterization of estrogen receptor from the cytosol of rat uterus (T4). Then Jensen *et al.*, using sucrose gradient analysis, showed that tumors from patients who responded to hormonal therapy contained estrogen receptor in the cytoplasm, whereas those that were unresponsive to therapy lacked the receptor (J2).

Milgrom *et al.* first noted the presence of progesterone receptors in guinea pig uterus (M15) and Terenius reported their presence in cytosol from human breast tumor tissue using [^{3}H]progesterone (T2). Horwitz and McGuire demonstrated the presence of progesterone receptors in human breast cancer tissue and showed that the synthesis of this receptor is a marker for estrogen responsiveness (H9). Thus, a tumor that binds estrogen and reacts to this binding by producing progesterone receptor protein is a truly hormone-dependent tumor and might respond to endocrine therapy.

There is little doubt that the presence of estrogen and progesterone receptors in breast cancer tissue correlates very well with the response of the patient to endocrine therapy. ERP/progesterone receptor protein (PRP) measurements have

been standard practice in the management of patients with breast cancer. In 1972, only 0.6% of United States patients had receptor assays, but, by 1981, 82% had such assays. Patients with ERP/PRP positive activity have a higher percentage of response to therapy than the women with either estrogen or progesterone positivity alone. It has been reported that 75–80% of estrogen receptor and progesterone receptor positive patients respond to therapy compared with 40 to 50% of estrogen receptor positive, progesterone receptor negative individuals and 25 to 30% of estrogen receptor negative, progesterone receptor positive patients (F1). Responses have been observed in 10% or less of estrogen receptor, progesterone receptor negative patients. The absolute concentrations of receptor protein seem to increase with age and the percentage of positive specimens is greater in postmenopausal women than in those who are premenopausal. Receptor protein is rarely found in medullary or papillary breast cancer (N3).

Hormone receptors may also be useful as prognostic indicators, particularly in node positive patients. The prognostic role in node negative patients is minimal. In node positive patients the hormone receptor positive patients seem to have a better prognosis for both disease-free and overall survival (N1). It has been observed that the measurement of the actual concentration of receptor is important in evaluation of survival (S4).

2. Mechanism of Action

The biochemical mechanism by which the reaction of estradiol and other estrogenic hormones with receptor substances elicits hormonal response has been the subject of extensive investigation. Toft and Gorski (T4) were among the first to detect estradiol receptor in cytosol from rat uterus. Shortly thereafter, Jensen (J4) employed fractionation techniques and autoradiography to demonstrate the accumulation of receptor in target cell nuclei. This, and subsequent experiments, served as the basis for a model for steroid–target cell interactions that has been accepted as dogma for almost two decades. It is believed that steroids are able to passively diffuse into target cells because of their low molecular weight. They are retained in the cytoplasm only in the presence of specific, intracytoplasmic receptor protein species by which they are bound. A hormone–receptor complex that is translocated into the nucleus is formed. The mechanism underlying translocation is unknown but is believed to be temperature dependent. Following translocation, the hormone–receptor complex is bound to specific DNA sites, after which transcription and translation occur. The end result is the formation of mRNA, new protein synthesis, and altered cell growth. Later convincing evidence indicated that ERP is not present in significant amounts within the cytoplasm of target cells, but instead is localized within the nucleus. This evidence was in two parts: immunohistochemical procedures employing monoclonal antireceptor anti-

bodies invariably showed nuclear staining of target cells (K7) and probably, during processing for biochemical assay, unbound receptor dissolves out of the nucleus and diffuses into the cytosol. At present, it is still not clear as to how steroids are retained in the cytoplasm. Perhaps the amount of receptor needed for this task is extremely minuscule and below current limits of detection. It is possible that retention of steroids in cell cytoplasm and transport of them to the nucleus may be a function of lower-affinity receptors. It does not seem reasonable to expect steroids to be retained in the cytoplasm, and to diffuse through the cell matrix and into the nucleus, in a random fashion.

In most situations, progesterone receptors are believed to be synthesized as the result of a fully functional ERP complex as an end product of estrogen-stimulated pathways in breast cancer tissue. The measurement of PRP, therefore, has also become important in predicting hormone responsiveness. Additionally, PRP is significant in predicting disease-free survival.

3. Prediction and Prognostic Usefulness of ERP/PRP

The presence of ERP/PRP in breast cancer tumors shows a high degree of concordance with response to endocrine therapy. Studies in breast cancer patients with positive nodes have (B6, B15, K9, K10) demonstrated that patients with ERP+ tumors have a longer survival rate than those with ERP− tumors. Relapse-free survival is more strongly associated with PRP+ than with ERP+ in patients without axillary node involvement (C4). This effect appears to be independent of the menopausal status of the patient (H3, A3). The likelihood of tumors containing ERP/PRP increases with the age of the patient (L2).

There is no doubt that patients who are both ERP+/PRP+ have a higher response to endocrine therapy at relapse. There are a substantial number of patients having either one or the other positive receptor status who also respond to hormonal therapy. It has been stated that ERP+ lesions are most likely to spread to bone, whereas those that are ERP− are more likely to develop visceral metastases, especially in liver or brain (K1). Most breast cancers exhibit significant steroid receptor heterogeneity at a cellular level in both positive and negative cells, but in negative cells can be shown to be highly proliferative (B1).

It has been suggested (K10) that the role of ERP in a better prognosis with respect to disease-free and overall survival is confined to subjects with positive axillary node involvement at the time of initial presentation. In node negative disease, the differences in prognoses are minimal if any. PRP status also has been associated with increased survival in node negative disease. However, recent data from patients with negative nodes who received adjuvant chemotherapy demonstrated no relationship between ERP status and disease-free survival. Therefore receptor status in node negative disease probably should not be used as either a

prognostic factor or a criterion for deciding which form of adjuvant therapy is appropriate (M2).

Although ERP+/PRP+ node positive patients have a better prognosis than ERP–/PRP– node positive patients, both groups have a high enough risk for recurrence to warrant adjuvant systemic therapy. Receptor status together with nodal status may be helpful in identifying a subset of patients with a particularly poor prognosis who may be considered for more aggressive adjuvant therapy. In this regard, cytosolic PRP is more important than cytosolic ERP as a predictor of prognosis (C4).

4. Nuclear ERP versus Cytosolic ERP/PRP Prognostic Values

Due to the failure to respond of approximately 40% of patients who were positive for cytosolic ERP and the response of 10% of patients who were negative to ERP (C4, H4), it was accepted that if the results were accompanied by PRP the predictive value would increase (D1). Another attempt to improve the predictive value of cytosolic ERP was the measurement of nuclear estrogen receptors in addition to the cytosolic ERP (L1). It was found that mixed combinations, whether cytosolic ERP+ nuclear– or cytosolic ERP– nuclear+, correlated with response rates of only 24 and 11%, respectively.

To further evaluate whether the addition of nuclear ERP has any predictive value, 95 patients were analyzed (L5) for cytosolic ERP and PRP and nuclear ERP. The incidence of cytosolic ERP+ was 74%, cytosolic PRP+ was 70%, and nuclear ERP+ was 52%. There was a trend to higher incidence of cytosolic ERP+ with increasing age, from 45% in patients less than 45 years old to 85% in patients over 70 years of age. The incidence of nuclear ERP+ was especially low among premenopausal patients, but it did not show constant correlation with age in postmenopausal women. There was 40% concordance of positivity, 14% concordance of negativity, and 46% nonconcordance in the three receptor assays, with a wide variety of other combinations.

Clinical correlation between the state of hormone receptors and disease-free and overall survival indicates a significant relationship between cytosolic ERP+ versus cytosolic ERP– patients. In the analysis of nuclear ERP patients, there was a trend toward better survival, but it was not significant compared with cytosolic ERP status. These results (G1, S5) failed to substantiate the report (L1) that nuclear ERP reinforces the accuracy of prediction obtained from cytosolic ERP and PRP measurements (L1).

The addition of quantitative nuclear ERP measurements does not offer a practical improvement to the predictive value of cytosolic receptors in the management of breast cancer patients. Therefore, clinicians should continue to rely on the experience accumulated thus far with cytosolic ERP and PRP measurements. This

is true despite studies done on frozen tissue sections and analyzed by immunocytochemical staining techniques that indicated specific staining of ERP in the nucleus. The cytosolic receptor assayed by techniques using a homogenization step may be an artifact resulting from the partition of the free nuclear receptor into the cytosol when the cell is disrupted (K7, W2).

5. Hormonal Therapy versus ERP/PRP Status

Adjuvant endocrine therapy has been correlated with ERP and PRP status. The efficacy of ERP and PRP in predicting the response of breast cancer to endocrine therapy has been well established.

In the management of disseminated breast cancer, quantitation of ERP/PRP serves two functions: it predicts which patients are likely to benefit from hormonal manipulation, and it aids in characterizing the natural history of the disease. Receptor negative breast cancer is an aggressive disease characterized by a shorter disease-free interval and a tendency to recur at visceral sites. Survival in ERP- patients is shorter than in ERP+ patients (B6, J5, K13).

The majority of patients with disseminated ERP+ breast cancer will respond to hormonal manipulation. With subsequent relapses many of these patients respond to additional hormonal therapy. It is postulated that breast cancer is a heterogenous disease composed of hormone-sensitive and hormone-resistant clones, and that measurement of the estrogen receptor protein identifies the dominant cell population. Megestrol acetate (MEG) treatment of advanced breast cancer has achieved tumor regression in sensitive patients (M9).

MEG with or without premarin (PRE) was used in the treatment of metastatic breast cancer (C5). A total of 259 postmenopausal ERP+ patients were studied. Complete or partial response was obtained in 33% of the patients receiving MEG alone and in 27% receiving PRE/MEG. The median time to progression for the patients receiving MEG alone was 50% longer than in those receiving MEG/PRE. Based upon the results of this trial it was concluded that the addition of estrogen to a MEG treatment regimen does not potentiate the therapeutic effectiveness of MEG.

The prognostic value of ERP and disease status in response to hormonal therapy and time of progression was investigated (B13) in 119 postmenopausal ERP+ women with advanced breast cancer. They were treated with MEG or tamoxifen (TAM). A total of 12 (6 MEG, 6 TAM) patients achieved a complete response, 41 (18 MEG, 23 TAM) patients exhibited a stable response. The results were correlated with ERP and PRP status (high, intermediate, or low). On the basis of data from all patients, the following correlations were found: stable response correlated with ERP status; PRP correlated with soft tissue metastasis; PRP and complete response correlated negatively with bone metastasis; and complete response corre-

lated with PRP+. With the MEG-treated patients the following correlations were found: stable response correlated with ERP; PRP correlated with soft tissue metastasis; and PRP+ correlated with complete remission. On the basis of data from TAM-treated patients, PRP correlated with soft tissue metastasis. For all patients with high PRP content and no prior chemotherapy, a longer time to progression was observed. No prognostic factors were seen in the patients treated with tamoxifen. Positive ERP/PRP patients experienced longer progression-free intervals when treated with MEG or TAM in a study (J6) with metastatic breast cancer patients. An absolute ERP relationship was shown with progression intervals.

The influence of medroxyprogesterone acetate (MPA) on ERP/PRP levels was studied in 20 postmenopausal patients with ERP/PRP+ primary breast cancers (N6). Each patient underwent needle biopsy and subsequent mastectomy. The biopsy and surgical specimens were assayed for the total ERP and PRP levels (cytosolic plus nuclear fractions) by enzyme immunoassay. Between the time of needle biopsy and that of mastectomy, 10 patients received no treatment (control group) and the other 10 patients were given MPA for 7 days. In the control group, the total ERP/PRP levels of the surgical specimens decreased by 68.2 +/− 7.3% and 60.7 +/− 8.4%, respectively, taking the receptor values of the needle biopsy specimen as 100%. In the MPA group, the total ERP/PRP levels of the surgical specimens decreased by 64.2 +/− 8.0% and 23.3 +/− 7.6%, respectively. The decrease in PRP, but not in ERP, was statistically significant between the control and the MPA groups. These results suggest that the MPA down-regulates PRP and not ERP in human breast cancer and challenges the conventional belief, extrapolated from the results on the endometrium and endometrial cancer, that MPA antagonizes endogenous estrogens by down-regulating ERP.

The predictive value of ERP/PRP levels was studied in postmenopausal women with advanced breast cancer treated with toremifene (V2). A total of 113 breast cancer patients with ERP/PRP positive status were treated with toremifene. The results indicated that ERP concentration predicts the duration of response but not the response rate to toremifene in patients with breast cancer. The PRP status did not predict the response rate or the duration of response.

6. Role of ERP/PRP in TAM Therapy

The relationship between hormone receptor status and the effect of adjuvant tamoxifen in early breast cancer remains controversial. TAM has been considered to be the hormonal agent of first choice for management of postmenopausal women with metastatic breast cancer (I1, I2). Recent studies (B4, V1) showed that response to tamoxifen was significantly related to ERP levels, but no benefit was observed among ERP negative patients. The relationship to PRP alone was of

borderline significance. Multivariate analysis indicated that most of the interaction between treatment and receptor content was explained by the interaction with ERP. PRP status appeared to modify the effect of tamoxifen on the ERP positive patients and the greatest effect was observed in patients who were positive for both receptors. However, the additional predictive information provided by the PRP assay did not help to identify patients who did not respond.

6.1. Predictive Value of ERP/PRP Concentration after Tamoxifen Therapy

Several studies (B4, B10, M3) showed that the concentration of ERP and PRP in the tumor tissue is a statistically significant predictive factor for duration of response with tamoxifen. Similarly the time to recurrence of disease correlated with quantitative ERP and PRP levels in patients treated with tamoxifen or megestrol acetate. The ERP and PRP concentrations in tumor tissue predicted the duration of responses but not the response rate in patients with advanced breast cancer. In a study of 113 patients (V2), there was no significant difference in the ERP or PRP content in responding and nonresponding patients.

6.2. Induction of ERP/PRP after Tamoxifen Therapy

An understanding of ERP and PRP status after TAM therapy is important. The duration of TAM therapy and the dose of TAM may obscure true receptor concentration. Care must be taken to stop TAM therapy for a period of time before biopsy of the tumor takes place. In a study (M10) of ERP and PRP content in the breast tumors of patients receiving TAM therapy, patients were biopsied before and after 3 weeks of TAM therapy. Therapy reduced the ERP concentration, whereas no changes were observed in PRP values. In premenopausal women plasma estradiol increased and in postmenopausal women there was a decrease in FSH. No changes were seen in progesterone, prolactin, luteinizing hormone, or testosterone levels. The effect of tamoxifen is mediated through ERP, but little is known about how it modulates breast cancer ERP. Kiang *et al.* (K6) suggested that, while patients are on tamoxifen, the conventional dextran-coated charcoal method does not yield reliable ERP data. This methodology deficiency can be circumvented by the use of an immunochemical assay. When the ERP content was measured by an enzyme immunoassay, there was a 3- to 4-fold increase in ERP content following exposure to TAM. The ERP content either was maintained or increased. It was implied that the content of ERP may be one of the mechanisms by which tamoxifen initiates its antitumor effect. Other studies (M19, N4) observed the concentration of PRP in breast tumors during TAM treatment. They found a 4.6-fold increase in the mean PRP content in postmenopausal women and a 2-fold increase in premenopausal patients after 3 to 7 days of TAM treatment.

PRP induction produced by TAM treatment reached a peak at 3 days and leveled off at Day 7. After a longer TAM treatment (14 days), the induction of PRP was no longer observed.

6.3. Combination of Tamoxifen and Hormone Therapy

Tamoxifen has been considered the hormonal agent of first choice in the management of postmenopausal women with metastatic breast cancer (I2). Fluoxymesterone (FIU) is another agent with known antitumor activity (K4). Ingle *et al.* (I3) reported the results of a randomized trial comparing TAM alone and TAM plus FIU and concluded that the combination produced higher objective response rates and a longer time to disease progression. Only ERP+ patients were entered in the study. A subset of women 65 years of age or older with higher ERP content is of special interest. In this group, survival was statistically longer for the combination approach. Estrogen receptor protein was measured by an immunocytochemical technique in fine needle aspirates before TAM treatment. It was concluded that response to tamoxifen in the elderly patients was related to the fraction of cells that were ERP+ and ERP– and patients are better treated by surgery (H5). In another study the influence of TAM and medroxyprogesterone acetate (MPA) on estrogen and progesterone receptors was studied in 48 women with operable breast cancer (N5). TAM–MPA treatment significantly lowered the PRP content regardless of menopausal status, but ERP was lowered only in the postmenopausal women.

6.4. Combination of Tamoxifen and Radiation Therapy and Chemotherapy

The effects of postoperative radiation therapy combined with adjuvant chemotherapy and tamoxifen therapy have been studied (R11). The results indicated that postoperative radiation therapy played an important role in the primary management of postmenopausal women with high-risk breast cancer and that the addition of tamoxifen may further improve the results among ERP+ patients. There was a significant improvement in status in the ERP+ patients treated with tamoxifen and radiation therapy compared with women who received chemotherapy. There was also a trend toward improved overall survival. In contrast no benefit was observed among ERP– patients. It has been reported (B7, R3) that chemotherapy and tamoxifen improved the results achieved by chemotherapy alone, particularly in postmenopausal patients; tamoxifen was particularly effective in patients with higher ERP content. Side effects were more numerous and more severe in patients receiving chemotherapy (with or without tamoxifen). These studies support the view that the choice of therapy for postmenopausal ERP+ breast cancer patients

with positive nodes is tamoxifen. In young ERP+ women tamoxifen may represent a safer alternative to chemotherapy since the addition of chemotherapy to tamoxifen did not significantly improve the effectiveness of TAM alone.

7. Chemotherapy versus ERP/PRP Status

Adjuvant chemotherapy with cyclophosphamide, methotrexate, and fluorouracil (CMF) in early breast cancer prolongs relapse-free survival and overall survival in premenopausal patients, but has only a slight effect in postmenopausal patients (B9). Because chemotherapy causes ovarian suppression (D2) and breast cancer is responsive to endocrine therapy, it is important to know if, and to what extent, the effect of chemotherapy is mediated by ovarian suppression. The efficacy of ERP/PRP assays in predicting the response of breast cancer to chemotherapy has been studied by many investigators in their search for a marker that would prepict the tumor response.

ERP status was studied in pre- and postmenopausal women with advanced breast cancer and correlated with clinical response to cytotoxic chemotherapy in a retrospective study (K5). These patients were treated with CAF (cyclophosphamide, adriamycin, 5-fluorouracil) or CMF (cyclophosphamide, methotrexate, 5-fluorouracil); CMFV (CMF, vincristine); or CAFVP or CMFVP (CAFV or CMFV, prednisone). The data suggested that ERP+ tumors responded better to cytotoxic chemotherapy than ERP− tumors. However, within the ERP+ group, the premenopausal women appear to have a better chance of responding to chemotherapy than postmenopausal ones.

The prognostic usefulness of ERP in predicting response to cytotoxic chemotherapy was studied (H7) in pre- and postmenopausal women with advanced disseminated breast cancer and in patients receiving postsurgical adjuvant therapy. In both sets of patients, the response or failure to respond to chemotherapy was independent of ERP status.

Adjuvant chemotherapy with CMF administered to premenopausal women with axillary node positive breast cancer (B3) induced permanent ovarian suppression in 47 of 77 (61%) patients. After a median observation time of 37 months, the relapse-free and overall survival times were significantly longer for patients with permanent amenorrhoea. A strongly positive correlation between CMF-induced amenorrhoea and age of the patients, as well as between age and tumor PRP status, was found. The induction of ovarian suppression predominantly occurs in patients with PRP positive tumors and may add an endocrine effect to the cytotoxic action of adjuvant chemotherapy in this particular group of older premenopausal women.

The relation between estrogen and progesterone receptor status, menstrual status, relapse-free survival, and overall survival was analyzed in 411 patients with early breast cancer randomized to receive either postoperative adjuvant chemo-

therapy with CMF or no additional treatment (control) (P1). Prolongation of time to recurrence and survival was seen predominantly in premenopausal patients; these effects were seen only with tumors positive for steroid receptors, particularly PRP. Chemotherapy led to permanent amenorrhoea in 61% of premenopausal patients. The therapeutic effects of chemotherapy were seen only with CMF-induced permanent amenorrhoea in premenopausal patients. These findings support the hypothesis that the effect of adjuvant chemotherapy in early breast cancer may be mediated by ovarian suppression.

This study also confirms the results of the first controlled trial of adjuvant CMF, namely that its effect differs in premenopausal and postmenopausal patients. Since CMF has no significant endocrine effect in postmenopausal patients (D2), the lengthening of relapse-free survival in favor of CMF for these patients is likely to be due to its direct cytotoxic effect. In contrast, the relapse-free survival for premenopausal patients shows that significant lengthening in favor of CMF is strongly associated with PRP positive tumors and induction of amenorrhoea, whereas there is no association between receptor status and CMF treatment in postmenopausal patients.

The relation of PRP status to the effects of CMF provides strong evidence of the importance of CMF-induced ovarian suppression in premenopausal patients. In a review of studies on the relation between steroid–receptor status and response to endocrine therapy in advanced breast cancer, PRP was shown to be the better predictor of response (C4). The similar but smaller effects of CMF in premenopausal patients with ERP positive tumors can be explained by the observation that about 60% of these tumors are also PRP positive. A major part of the effect of CMF in premenopausal patients with ERP negative tumors can be accounted for by the ERP negative, PRP positive subgroup (who would have a high probability of response to endocrine therapy). The possibility that CMF could have a preferential effect on PRP positive tumors is unlikely because of the lack of effect of CMF in postmenopausal patients with PRP positive tumors. Hence, CMF appears to act primarily through an endocrine effect secondary to ovarian ablation induced by chemotherapy. There is no firm evidence of synergism between endocrine therapy and chemotherapy or support for the suggestion that chemotherapy per se is more effective in premenopausal women. The possibility of a useful, but much smaller direct cytotoxic effect of CMF in premenopausal patients with receptor negative tumors cannot be excluded.

In a clinical study the change in the status and the concentration of ERP and PRP under cytotoxic chemotherapy was studied (J7). A total of 38 patients with locally advanced breast cancer were treated with cytotoxic chemotherapy, including adriamycin, cyclophosphamide, 5-fluorouracil, and prednisone. The concentrations of ERP and PRP in the tumor were studied before starting the chemotherapy and after one to nine courses of chemotherapy. This therapeutic response rate was not related to the initial level of ERP and PRP. After chemotherapy, there

was an increase in the ERP level in 14 cases out of 29 (59%) and in the PRP level in 17 out of 32 cases (53%). A therapeutic response was observed in 11 out of 17 cases when the PRP concentration was increased and in 3 out of 15 cases when the PRP concentration was either stable or decreased. These observations support the suggestion of selective initial activity of chemotherapy on lesser differentiated tumor cells.

Use of combined endocrine and cytotoxic chemotherapies is controversial, particularly for estrogen receptor positive postmenopausal breast cancer patients. In a prospective, randomized trial of 164 node positive patients (C8), those with ERP+ tumors received cytoxan, methotrexate, 5-fluorouracil, vincristine, and prednisone (CMFVP, 1 year) and tamoxifen or tamoxifen alone (3 years). Estrogen receptor negative patients received CMFVP. All patients had a modified radical mastectomy, and ERP+ premenopausal patients had a surgical oophorectomy. Among the ERP+ group, 14 of 43 premenopausal and 31 of 73 postmenopausal patients have relapsed. Comparison of CMFVPT and tamoxifen in the postmenopausal group reveals a 5-year disease-free survival among patients receiving CMFVPT of 77% compared with 53% for those receiving tamoxifen alone. Among the ERP+ premenopausal patients, 5-year disease-free survival for the CMFVPT group is 65% and for the tamoxifen group is 80%. At 5 years, 59% of ERP– patients are disease free. These results indicate that, for ERP+, node positive postmenopausal patients, tamoxifen alone may be suboptimal and consideration should be given to using combined chemoendocrine therapy.

An aggressive adriamycin containing regimen in ERP– patients with disseminated breast cancer was studied (M18). Sixty-four patients with disseminated breast cancer were treated with an aggressive chemotherapy program of prednisone, methotrexate, 5-fluorouracil, adriamycin (doxorubicin), and cyclophosphamide (PM-FAC). A response rate of 76% was seen in 44 ERP– patients, with 26% achieving complete responses. A total of 42% of 20 ERP+ patients demonstrated a response, but in none was a complete response achieved. Median response duration was 9 months for complete responders and 5 months for partial responders. The median survival for both groups of patients was 13 months. However, survival among the responding patients was less in the ERP– group (median, 14 versus 20 months). These findings suggest selective sensitivity of ERP– breast cancer to chemotherapy containing doxorubicin.

Hormonal therapy and chemotherapy versus chemotherapy alone as adjuvant treatment in breast cancer was studied in 236 patients (H8). All presented axillary node involvement and had ERP levels greater than or equal to 10 fmol/mg protein and/or PRP levels greater than or equal to 15 fmol/mg protein. They were randomized to receive either chemotherapy alone, with 9 courses of cyclophosphamide, methotrexate, and 5-fluorouracil over 6 months, or chemotherapy plus hormonal therapy, with tamoxifen for 2 years. The two groups of patients were comparable except for the mean number of axillary nodes involved (3.4 for the chemotherapy

group; 4.2 for the chemotherapy–hormonal therapy group). In premenopausal patients, amenorrhea was related to age and not to tamoxifen. A preliminary analysis with a 19-month median follow-up (8–41 months) indicated that the disease-free survival cure of patients receiving adjuvant chemotherapy and hormone therapy (8 recurrences) is significantly better than that in those receiving adjuvant chemotherapy alone (15 recurrences). The efficacy of hormonal therapy is identical regardless of age, hormonal status, histological grade, and receptor levels.

Attempts have been made to combine cytotoxic drugs such as cis-Pt (cisplatin) with a hormone receptor-binding compound. The cis-Pt analogue meso-6-PtSO was synthesized with this rationale in mind. The complexing ligand meso-6 is a derivative of the nonsteroid synthetic estrogen hexestrol and is complexed to platinum(II) as the reactive cytotoxic element. The rationale for use of this drug was that it would promote a selective antineoplastic effect by targeting the platinum complex to ER+ positive tumor cells. The water solubility of the compound facilitated application to animals and cell culture experiments (K2).

The meso-6-$PtSO_4$ has been tested for its selective antitumor effects on the ER+ and ER− MXT mammary tumors in the mouse. The Pt complex led to a marked reduction in tumor weight after a 6-week application to mice bearing the ERP+ MXT, while it displayed no tumor growth inhibition on the ERP− MXT tumor. In dimethylbenz[*a*]anthracene-induced mammary carcinomas in the Sprague–Dawley rat, tumor regression was observed as early as 7 days after beginning treatment with meso-6-$PtSO_4$. The compound was much more effective than cis-Pt or the ligand (meso-6) alone (K2). Thus, meso-6-$PtSO_4$ would appear to act selectively and with better efficacy on ERP+ mammary tumors than cis-Pt or the ligand. The action has been explained by the fact that the estrogenic ligand is selectively taken up and retained in ERP+ cells (K2). The Pt complex is brought into proximity to the DNA and interacts covalently with guanine bases. This reaction could lead to inhibition of transcription and DNA synthesis (P5). In accordance with this hypothesis meso-6-$PtSO_4$ was studied for its effects on ERP-mediated responses and for growth inhibition in cell culture (O2). Two human mammary carcinoma cell lines were used as a model system: the ERP+ MCF-7 cells and the ERP- MDA–MB. These cell lines have been used previously to screen the various Pt complexes for their selective effects on ERP+ tumor cells. The second aim of this study was to compare the *in vitro* results with previously published data obtained with this Pt complex in various animal systems. The results showed that meso-6-$PtSO_4$ displays estrogen-like properties in ERP+ human mammary tumor cells by competing with estradiol for ERP-binding sites. It reduces the number of estradiol-binding sites and increases the level of progesterone receptor. Meso-6-$PtSO_4$ showed cytotoxic activity by inhibiting proliferation of the ERP+ MCF-7 cells. This cytotoxic effect, however, did not seem to be mediated by the ERP.

The cytotoxic action of meso-6-$PtSO_4$ in cell culture appears to be due to the Pt group. While possessing all the tested estrogenic properties, the ligand showed no, or merely a marginal, growth inhibitory effect. The results show that meso-6-$PtSO_4$ possesses both estrogen-like and cis-Pt-like properties. Since growth inhibition did not correlate with ER-mediated processes, these two properties are expressed independently at the cellular level. The selective growth inhibitory effect of meso-6-PtSO *in vivo* is suggested to involve endocrinological and/or immunological factors.

8. Radiation Therapy versus ERP Status

Significant improvements in the recurrence-free survival after postsurgical radiation therapy have been reported for more than two decades (H10). The main effect of radiation therapy was the reduction of local metastatic recurrences, although there was also a significant reduction in distal metastases and a trend toward overall survival among node positive patients (H10). Conservative surgery in conjunction with breast irradiation has gained acceptance as an effective alternative to radical surgery therapy for early breast cancer. The equivalence of these treatment plans has been demonstrated by prospective clinical trials (F2). The influence of patient age on risk of recurrence was retrospectively studied (K16) in 496 stage I–II invasive ductal carcinomas treated by primary tumor excision followed by radiotherapy. With a median follow-up of 71 months, local recurrences occurred in 21% of the patients younger than 40 years old, compared with 11% of in the older patients. There was no significant difference between the EPR+ or the ERP− patients. In another study (K15) of 496 patients with infiltrating ductal carcinomas, there was a median follow-up of 71 months. Sixty (12%) patients developed recurrent cancer in the treated breast. Estrogen receptor status was not found to be a significant indicator of recurrence in these patients. In another study (E1) of 438 patients with intraductal carcinomas, estrogen receptor status was available in 58% of the cases. During a follow-up of 89 months, 23% of the patients developed distant relapse. There was no significant difference between ERP+ (29%) or ERP− (26%) patients.

9. Fine Needle Aspiration Impact on Receptor Analysis

Whereas quantitative assays for ERP and PRP have repeatedly proved their clinical utility, the increasing use of breast tumor biopsies by fine needle aspiration requires an assay that is able to identify the ERP and PRP in small tissue samples. Immunocytochemical procedures are ideal for this and in Section 11.3 a detailed explanation of this method is given. The fine needle aspiration can be used as an

adjuvant aid for decreasing the false-negative rate of mammography and/or the breast palpatory examination. A palpatory, worrisome lesion of the breast that is either mammographically benign on nonvisualized mammographically can be subjected to the fine needle aspiration for further diagnostic evaluation. Cytologic evaluation, ERP, and PRP can then be performed.

There are several major advantages in fine needle aspiration. Aspiration biopsy is a nontraumatic procedure and can be repeated easily. This is important during the course of the disease, since this procedure could be used to plan hormonal therapy in metastatic disease (B14). Also, in metastatic disease one could apply the cytochemical technique to several metastatic lesions and thus obtain information on the receptor status of multiple lesions. This technique also has an advantage over surgical biopsies because some metastatic lesions, such as pleural effusions, ascites, or liver metastases, may not be amenable to surgical biopsy. This was demonstrated (L3) by hormonal receptor determination of pleural effusions using thoracoscopic examinations.

Several investigators have utilized fine needle aspirates for hormone receptor evaluation using an immunocytochemical method (ERICA; Abbott Laboratories, North Chicago, IL) and compared the data with the quantitative values obtained by chemical methods [dextran-coated charcoal (DCC)] for both ERP and PRP. These data are shown in Table 1. There was a variation of 65 to 100% in concordance in the separate studies. The ERP immunocytochemical assay in fine needle aspirates was also compared with the immunochemical assay in biopsies with a concordance of 87% (R1). The role of the immunocytochemical assay of receptors is primarily in patients in whom surgical biopsies cannot be obtained or from whom the surgical specimen is too small.

Fine needle aspiration has limiting factors. There is a tendency for false-negative results because of relatively few cells (poor cellularity) in the tumor aspirates, for which an estimation of the proportion of positively stained cells may not be accurate. It has also been shown (R1) that stroma-rich tumors often yield

TABLE 1
CONCORDANCE DATA BETWEEN ASPIRATE ERICA AND DCC METHODS

Author	ERP (%)	PRP (%)
Reiner *et al.* (R2)	80	—
Flowers *et al.* (F4)	89	—
Massod (M4)	89	86
McClelland *et al.* (M5)	85	—
Hawkins *et al.* (H6)	65 and 63	—
Schmitt (S3)	100	—
Merle *et al.* (M14)	—	85

too few cells for a cytologic diagnosis. The same is also true for special histologic tumor types that contain abundant stroma-like tumors as seen in invasive lobular or tubular carcinomas (K12). False-negatives results were found in cases in which the lower values of the staining index are derived almost entirely from the small percentage of the cells showing staining. The assay was also found (H6) not to be sufficiently sensitive in some premenopausal women, with low levels of receptor values obtained by biochemical assay. A minimum of tumor cells, perhaps 20 (H6), is required as an adequate sample for assessment: although staining may be visible in a lesser number of cells, the small number of cells may be insufficient to take possible heterogenicity into account. Last and most importantly the immunocytochemical assay must be performed by an experienced breast cytopathologist. Nonagreement between fine needle aspiration and tissue assays may also be due to intratumoral sampling variations, aspiration of occasional normal breast ductal epithelial cells, and poor sample handling, as well as delay in freezing freshly made smears. Another possible pitfall in interpretation of the immunocytochemical assays in fine needle aspiration breast samples is nonspecific cytoplasmic reactions due to endogenous peroxidase expression by some leukocytes, histiocytes, and in particular erythrocytes. In addition, areas of fat tissue are also occasionally weakly stained (M6).

It may not be possible to obtain better correlation between the cytochemical and the biochemical methods for aspirates and excision biopsies due to the heterogeneity of breast tumors with respect to receptor concentration. Earlier studies demonstrated that cells from different areas of a tumor may show different characteristics (K12).

10. Role of ERP/PRP in α-Interferon Therapy

Interferon induces a number of genes and proteins. There is evidence in human breast cancer, both *in vitro* and *in vivo,* that hormone receptors may be among these induced proteins. We have studied (H3) *in vivo* the effect of interferon on hormone receptors in human breast cancer. Two patients with primary breast cancer and seven patients with metastatic breast cancer accessible for multiple biopsies have been studied. The initial biopsy tissue was frozen at −70°C; the patients received therapy for 14 days. A second biopsy was then performed and the ERP and PRP from first and second biopsies were determined simultaneously (to reduce intertest variability) by the DCC method. Among five patients initially ERP/PRP negative, one converted to positive (ERP/PRP 0.9/0 to 26/79 fmole/mg protein). Among four patients initially ERP positive, two have demonstrated increases in receptor levels (ERP/PRP 34/74 to 127/116 and 107/0 to 171/1.2 fmole/mg protein). One patient (ERP/PRP 107/0 fmole/mg protein) whose disease was progressing after three prior hormone trials including tamoxifen, continued to

be treated with a combination of tamoxifen and α-interferon and achieved a partial response lasting 5 months. Other investigators (B5, V3) studied the mechanism of the interaction of α-interferon and tamoxifen. The synergistic effect was most marked after 72 hr of pretreatment with interferon, when the maximum interferon-induced increase of estrogen receptor concentration is evident. The mechanism is thus probably due to an increase of the cellular receptor as a ligand for tamoxifen binding and suggests a possible role for the clinical use of interferons combined with tamoxifen.

11. Measurements of Receptors

11.1. Specimen Collection and Preparation

Hormone receptors are heat-labile proteins. Immediately after surgical removal of the specimen, the tissue should be chilled and kept at 4°C to ensure receptor stability during pathology review. At room temperature the half-life of receptors is about 2 hr. Special care must be taken to avoid direct contact with saline or formalin and direct injection of the tumor with lidocaine, since this may falsely decrease estrogen and progesterone receptor values (M11).

The tumor should be trimmed from adjacent fat and normal tissue. The specimen should be immediately examined by a pathologist to ensure that a representative sample of tumor has been chosen (M12). It is recommended that this examination be done within 5 min after the tissue is excised. The specimen should then be frozen in liquid nitrogen and stored at −70°C. Results with several human breast tumor samples examined at intervals of 2 months indicate that the estrogen receptor showed no significant loss in binding capacity over several months in tissues that were quick frozen at −86°C (W3). Studies have shown that lyophilization of cytosol followed by storage at 4°C may be a method for conveniently preserving samples for the estrogen receptor assay, but the progesterone receptor may be too unstable for this technique (B8, C2). The receptor stability is markedly influenced by pH, with maximum stability at approximately pH 7.4 to 7.8 (A2). High salt concentrations can cause changes in receptor form without a loss of binding properties (H2, P3). When using the DCC assay, low protein concentrations in the assayed nuclear or cytosol fraction may influence the estimation of binding capacity and affinity, particularly with receptor-poor tumors (P6). The general consensus is that the lowest acceptable limit of protein for most standard assays is 1 mg/ml in tumor cytosol with high receptor activity and 3 mg/ml in tumor cytosol with low receptor activity.

Miller *et al.* found that receptor integrity is promoted if 10–20 m*M* molybdate is included in the extraction buffer (M16). It was proposed that molybdate may provide protection against specific proteolytic cleavage by stabilization of the

phosphate group. In a study by Anderson *et al.* (A1) the addition of molybdate to the buffer increased both the percentage of progesterone receptor positive breast tumor samples and the absolute receptor levels. The effect of molybdate on estrogen receptor assays was less evident, although in some samples small increases in estrogen receptor levels occurred. This was later confirmed (B11) when the PRP binding capacity in a series of 75 patients who were analyzed for ERP and PRP, before and after molybdate incorporation, was found to be significantly higher. Some studies have found that buffers containing dithiothreitol also exert a stabilizing action on receptors through protection of sulfhydryl groups (B12, H2), whereas others report no effect on receptor binding sites (K3).

In our laboratory, the specimens are collected following dissection by a pathologist, trimmed of fat as necessary, weighed, minced, and homogenized with a Polytron homogenizer in 4 vol of 10 m*M* 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid (TES) and 12 m*M* thioglycerol buffer containing 250 m*M* sucrose and 20 m*M* molybdate. The homogenization is carried out in an ice bath using three 15-sec pulses with 45-sec pauses for cooling. The homogenates are then centrifuged at 105,000*g* for 1 hr at 2°C. Aliquots of the supernatant (cytosol) are analyzed for receptor protein and total protein. A variety of ultraviolet and colorimetric methods have been used for cytosol total analysis. In our laboratory we use the method of Lowry *et al.* (L6).

11.2. Biochemical Methods of Receptor Assays

Various biochemical methods have been used to detect the presence of steroid hormone receptors. The most useful methods are described.

11.2.1. *Sucrose Density-Gradient Ultracentrifugation*

Sucrose density-gradient ultracentrifugation provides data regarding the sedimentation velocity of proteins as a function of their molecular weight and density (S7, T4). Usually cytosol is first incubated with radiolabeled ligand, with or without competitor, and then layered on prepared gradients. The gradients are centrifuged (F8) and the fractionated samples are collected and counted. Standards are run in the gradient tubes for comparison with known markers. Such markers include ^{14}C-labeled bovine serum albumin (4.6 S) or human IgG (approximately 6.8 S). When molybdate was not used, most tumor cytosols displayed the presence of an 8 S estrogen receptor form, whereas some contained an additional 4 S form (W3). The receptor-bound progesterone without molybdate present sediments in the 8 S region of the gradient (H9) but with molybdate the density is increased and the absolute amounts of receptor markedly increases (S7). Since only one concentration of steroid is usually utilized, no data are obtained regarding the binding affinity of the ligand for the receptor. The method provides valuable data regarding

molecular forms of the receptor, although it is a slow, expensive way to quantitate steroid receptor binding.

11.2.2. *High-Performance Liquid Chromatography (HPLC)*

HPLC is a method for the analysis and characterization of steroid receptors based on characteristics such as molecular weight. Qualitative relationships and multiple forms of the receptor can be maintained by the rapid gel-exclusion system, and contaminants can be readily identified (P2). Advantages of using HPLC include rapid analysis time, minimal receptor modification, improved resolution, and high reproducibility. Unfortunately the HPLC assay is tedious, requiring saturation analysis for each sample and quantitative validity has not been established for this procedure. Other disadvantages associated with HPLC methods include the requirement for expensive equipment and sophisticated technical skills. Therefore, HPLC analysis of receptor proteins is currently used only in research studies.

11.2.3. *DCC Assay*

The DCC assay is dependent upon the capacity of charcoal to adsorb free hormone, whereas the addition of dextran limits the absorption of the hormone–receptor complex by the charcoal (K14). By using increasing concentrations of radiolabeled hormone and a concurrent range of unlabeled hormone competitors, titration of the receptor to a saturation end point occurs.

Specific or saturable binding can be calculated by subtracting the nonspecific or nonsaturable binding from the total binding to both specific and nonspecific sites. The values derived from these binding experiments can be used to calculate the total number of binding sites, expressed as femtomoles per milligram protein, and the K_d or dissociation constant. The K_d is a measure of the affinity of the receptor for the steroid and is approximately the concentration of free steroid at which one-half of the receptors are saturated (S2).

Several groups have demonstrated excellent quantitative correlation regarding estrogen receptor analysis between the DCC assay and sucrose density-gradient methods (A1, C2, H1), although Chester *et al.* (C3) found the DCC assay for estrogen receptor to be more sensitive than the sucrose density-gradient analysis and this difference was more pronounced when molybdate was absent.

The DCC assay is currently the most popular method for evaluating estrogen receptor and progesterone receptor because it is simple, inexpensive, sensitive, and accurate and because several samples can be processed simultaneously. This is the method that was in use in the Department of Clinical Chemistry at the Memorial Sloan–Kettering Cancer Center from 1973 through 1990. We have replaced this method with a quantitative immunochemical method (EIA) from the Abbott Laboratories (North Chicago, IL).

11.3. Cytochemical and Immunochemical Assays

Reagents for the fluorescent cytochemical assay of estrogen and progesterone assays are available in the United States and are FDA approved (Zeus Scientific, Inc). There has not been absolute correlation of these techniques with biochemical methods (P3).

Monoclonal antibodies to human estrogen receptor have also been used to develop an immunocytochemical assay (ICA) for estrogen receptor in breast cancers (K11). Frozen sections of human breast adenocarcinomas are thaw mounted on uncoated glass slides, fixed in graded ethanols, and then incubated with one or more monoclonal rat anti-estrogen receptor antibodies. Receptor-bound antibodies are visualized by the indirect immunoperoxidase method. Four different monoclonal antibodies (D75, D547, H222, and H226), each known to recognize unique determinants on the receptor molecule, detected only nuclear estrogen receptor by this ICA method, although all four antibodies bound both cytosol and nuclear forms of estrogen receptor in tissue extracts. From these studies it has been concluded that intact tissue contains only the nuclear form of the receptor, and cytosolic receptors are the result of tissue homogenization. There was a good correlation between the semiquantitative results from ICA and those from sucrose density-gradient analysis of the breast tumor cytosol. The intensity of nuclear staining was proportional to the amount of estrogen receptor determined by the sucrose density-gradient analysis and was always absent in the receptor negative tumors (P4).

In the United States a commercially available antibody "kit" for the immunocytochemical assay (Abbott ER-ICA monoclonal kit) has been approved by the FDA. This method is stated by the manufacturer to eliminate estrogen receptor contributions of normal or benign epithelium. The ICA permits ERP or PRP measurements on small specimens including fine needle aspirates. The clinical utility of ICA has been shown in a number of studies (C6, K11, M6).

The development of specific monoclonal antibodies to the estrogen and progesterone receptor protein allows detection of the receptor by enzyme immunoassay (EIA), independent of the binding labeled hormone by the receptor (G2, G3, W1). Production of monoclonal antibodies against the estrogen and the progesterone receptor proteins has permitted the development of ERP and PRP assays based on direct antigenic recognition rather than steroid binding activity (J3). By eliminating many sources of variability, these assays, which directly measure receptor protein, have the potential for improving their degree of quantitation.

Our comparisons (M13) between the EIA and the DCC methods showed the EIA to be a viable alternative to the DCC in regard to both performance and cost. The linear regressions were as follows: for ERP, $y = 5.0 \pm 1.96x$, $r = 0.928$, and $x =$ DCC; for PRP, $y = 5.0 + 3.20x$, $r = 0.934$, and $x =$ DCC. The concordance calculated for the ERP assays was 100% (75/75) and therefore the sensitivity and

specificity of the EIA compared with those of the DCC were also 100%. The concordance calculated for the PRP assays was 84% (66/78). The sensitivity of the EIA compared with that of the DCC was 90.9% (30/33) and the specificity was 80% (36/45). The EIA was found to be a faster assay for use in clinical chemistry laboratories, offering a method that can be universally applied to give a consistent cutoff from laboratory to laboratory. Other studies (M17, T3) showed similar results suggesting that the EIA yielded the more "biologically correct result."

In conclusion biochemical and immunocytochemical hormone receptor assays should complement each other until the accuracy in identification of patients with hormone-responsive carcinomas is further improved.

11.4. Possible Errors in Estrogen and Progesterone Receptor Measurements

11.4.1. *False-Negative Results*

In a few clinical situations, an assay for estrogen receptor is negative but hormonal response is seen. The major factors that may contribute to this condition are inappropriate specimen collection, improper storage, or excess manipulation during the assay procedure. The estrogen receptors are thermolabile, pH- and ionic-strength-dependent, unstable proteins. During collection the specimen must always be kept cold and transported in a petri dish over ice to its storage in a Revco at -70°C. Every tissue must be examined by a pathologist in order to obtain a well-trimmed specimen, containing only the tumor, to avoid a dilution factor. During the assay procedure, care must be taken not only to keep the sample at 0–4°C but also to prevent foaming during homogenization, which may inactivate the receptor by oxidation (S6).

Another source of error is a low protein concentration in the assayed sample. Poulsen (P6) found that with low protein, doubling the protein concentration of the cytosol proportionately increases the specific activity of the receptor. Once a critical amount is present (1–3 mg protein/ml), the receptor activity approaches a constant level.

In biochemical methods, the interference of non-receptor binders such as albumin, corticosteroid-binding globulin (CBG), and sex-hormone-binding globulin (SHBG), which are present in tumor specimens (C2), also causes problems. The assay specificity can be enhanced by the appropriate choice of labeled steroid and cold competitor. Diethylstilbestrol has affinity for receptor equal to that of unlabeled estradiol but the affinity for SHBG is far lower than that of unlabeled estradiol. Another approach is to add a steroid that binds the interfering competitor but does not bind the receptor (C2). An additional factor that can affect receptor levels in the DCC assay is the level and time of charcoal exposure. When excess charcoal concentration is used, a significant decrease in the protein content of the

cytosol occurs (P6). Prolonged exposure of the hormone–receptor complex to charcoal suspension during the DCC assay results in dissociation of the complex, with falsely reduced or false-negative assay values. Endogenous estrogen levels might be responsible for altering the receptor–estrogen complex and giving false-negative results. In order to evaluate the impact of endogenous estrogen levels on ERP measurements, we measured the estradiol receptor content together with the endogenous estrone and estradiol concentrations in human breast tissue cytosols (F3). There was no evidence for a relationship between high estrogen levels and low receptor measurement, although ERP+ tumor cytosols contained a statistically significant greater estradiol concentration than those of ERP– tumors or of normal tissue.

11.4.2. *False-Positive Results*

One of the most common explanations for unresponsiveness of apparently estrogen receptor positive tumors to hormonal therapy is tumor heterogeneity. We found that 38% of the results change when comparing the estrogen receptor proteins from the primary tissue with those from the metastatic tissue. Although there are extrinsic factors, as already mentioned, there could be intrinsic factors that alter the receptor measurement. These findings underscore the need to conduct biopsies and assay readily accessible recurrences rather than to rely on the results of prior specimens. Variation of results also occurred with metastatic tissue from some patients, obtained usually after a period of 19 months (R4).

Lippman (L4) and McGuire (M8) showed that this heterogeneity of receptor status is only one of the reasons why endocrine therapy is ineffective in approximately 40% of patients with estrogen receptor positive tumors. In addition to this problem, there is evidence that some cells have various types of receptor defects distal to the initial binding step. It was also found that some variant cells may be able to bind estrogen in the cytoplasm but not translocate the receptor to the nucleus; others are defective at the intranuclear level (N1, N2). Sato *et al.* (S1) have proposed a cytoplasmic factor in some estrogen receptor positive, endocrine-resistant tumors, which may inactivate the DNA binding site for the nuclear receptor. In some cases, nuclear translocation of receptor and binding to chromatin occur, but enhancement of transcription or translation is impaired. The accumulation of progesterone receptor in the cell, for the most part, indicates that the entire schema of events involving estrogen action is functional (M7). Cells that are estrogen receptor positive and progesterone receptor negative may harbor one or several defects in hormonal mechanism, thus possibly explaining decreased responsiveness in comparison with cells that are positive for both receptors.

In some cases, especially premenopausal patients, tumor responsiveness may potentially occur, but the hormonal alteration employed as therapy, such as oophorectomy or tamoxifen administration, does not alter ambient hormonal concentration sufficiently for an objective response to occur (M1).

11.4.3. *Reporting of Hormone Receptor Values*

In our institution, we initially (1973) determined ERP/PRP by the DCC method. With this method a tumor is positive if the specific receptor protein is equal to or greater than 6 fmole/mg protein and the dissociation constant (K_d) is equal to or greater than $5 \times 10^{-9} M$. These values are derived from cytosol binding data and require receptor values of at least 10 fmole/mg protein with an inhibition of at least 60% by unlabeled hormone. The specific ERP or PRP content (obtained by multiplying the hormone receptor value by the percent inhibition) must therefore be equal to at least 6 fmole/mg protein. Borderline results are reported when the hormone receptor is less than 10 fmole/mg protein or the percent inhibition is less than 60% but the specific ERP or PRP is 6 fmole/mg protein or greater. Although borderline values suggest a low but detectable amount of receptor binding, the value are below the limits for an accurate determination. Since 1991, we have been using a quantitative immunochemical method (EIA) (Abbott Laboratories, North Chicago, IL). The cutoff is 15 fmole/mg protein for either ERP or PRP.

11.4.4. *Quality Control*

Quality control was carried out in our laboratory by simultaneous measurements of aliquots of a preparation of rabbit pregnant and nonpregnant uteri that we prepared for the DCC method. The commercial EIA kit includes control material. In the United States and in Europe there are national programs devoted to providing quality control material and to collecting data from participants to permit laboratory to laboratory comparisons and initiation by individual laboratories of steps to improve their performance.

12. ERP/PRP Content in Sequential Breast Biopsies

According to present knowledge, hormonal control of a target cell is receptor dependent. The presence of steroid hormone receptors in breast cancer allows a prediction of the expected clinical response to endocrine manipulation. The importance of ERP/PRP in patients with breast cancer is threefold: (1) as a prognostic tool, because patients lacking steroid receptors represent a high-risk group, having earlier and more recurrences; (2) as a tool for predicting endocrine adjuvant treatment response to tamoxifen, therapy which is receptor mediated, as may be the response to combined chemohormone therapy; and (3) as a tool for selection of treatment in metastatic breast cancer, because patients with receptor positive tumors demonstrate a 60–80% remission rate.

With an understanding of the critical uses of receptors, it is important to be aware of differences in biopsies from the same patient. Levels of ERP/PRP were measured in multiple tumor and lymph node biopsies and in sequential biopsies

with and without intervening therapy (J1). In these tumor samples, an 85% concordance was found for ERP and an 86% concordance for PRP. In the simultaneous tumor and lymph node metastases, an 86% concordance was found for ERP and a 76% concordance for PRP. These results correlated with our preliminary studies (R7) with ERP. In correlations of receptor status in primary tumor and subsequent metastasis, there was a concordance of 55% for ERP, somewhat lower than our findings of 80% concordance.

The data obtained in this study suggest that two or more samples of breast carcinomas obtained at different times from one patient will have similar ERP/PRP properties only about 76–85% of the time. A somewhat greater degree of variability was found between a primary tumor and subsequent metastases if multiple metastases were evaluated at different times. It is unlikely that differences in the precision of measurement are responsible for the variations observed. When the patients had endocrine treatment between biopsies, a 57% correlation with ERP status was found between the two biopsies and a 67% correlation with chemotherapy treatment. Sequential biopsies of breast cancer tissue were studied (C7) from a total of 210 women in order to assess any change in ERP status arising spontaneously or as a result of intervening therapy. A combined assay measuring both cytosol and nuclear estrogen receptors was used for all samples. A total of 155 patients had biopsies of their primary tumors and of later recurrences; 26 had biopsies of their primary tumors and recurrences of new primary tumors in the opposite breast; and 29 had sequential biopsies of recurrent disease only. Overall only 61.2% of the primary tumors retained their original status with respect to both cytosol and nuclear estrogen receptors upon recurrence. These results were influenced by intervening therapy, however, and, if only untreated patients are considered, over 70% of their recurrences contain the same combination of cytosol and nuclear receptors found in the primary tumors. For tumors "recurring" in the opposite breast, the pattern was similar, with 69% retaining the same status as the first primary. The agent found most likely to alter ERP status was tamoxifen and, in the samples taken from patients undergoing treatment with this drug, no tumor was found to contain measurable receptor.

It has been recognized that ERP and especially PRP levels in primary breast carcinomas have a major prognostic implication. Aside from lymph node status, they are the second most important prognostic parameter. It has been suggested that during the history of breast cancer a dedifferentiation occurs. One has to be cautious to draw final conclusions from these results and from data currently available in the literature. First of all, the number of patients is small, especially of patients with sequential biopsies without intervening therapies, and our study, as well as others, is retrospective. Furthermore, usually the first of sequential receptor determinations was performed in the primary tumor, which can add to the variability not due to intervening therapy. The reported studies used different therapeutic regimens that might not result in similar receptor changes. Further-

more, some differences between two receptor determinations may be due to assay variations. Keeping these points in mind, we have drawn the following conclusions:

1. Variation in ERP levels from simultaneous tumor or tumor/lymph node biopsies can be seen in 15% of specimens. The discordance rate between tumor and lymph node metastases in PRP is 25%. This difference can partly account for the nonresponders with ERP+ or PRP+ tumors after endocrine treatment.
2. In sequential biopsies one has to expect a more frequent change from receptor+ to receptor− than the opposite. This holds true especially for PRP. Obviously important biological changes in the behavior of carcinoma cells occur over time.
3. After intervening endocrine treatment, ERP and PRP levels significantly decrease. This might reflect a selective pressure on ERP+ and PRP+ carcinoma cells. Therefore, recurrent lesions should be assayed for ERP and PRP levels if possible. However, prospective studies should clarify the prognostic importance of receptor levels in metastatic tissue as well as the role of intervening chemotherapy, especially for sequential PRP determination.

Intrinsic tumor variability is another possible explanation of the difference seen (R7). Histologic pleomorphism is a well-known characteristic of breast cancer. It is also reasonable to expect that malignant cells will not be entirely uniform physiologically. Current methods of analysis that depend upon homogenates of relatively large samples of tissue obviously obscure evaluation of any such intrinsic differences between individual cells.

Anatomic studies indicate that mammary carcinomas often arise in multiple independent foci within a breast. Variations in the chromosomal patterns of tumor cells have been seen in breast and other types of carcinomas. Most breast tumors can be classified on the basis of a dominant growth pattern upon histological examination. However, it is common to find focal variations in these features and occasionally two or more distinct types of carcinomas are found in the same tumor or in different parts of the breast. We have shown (R6) that ERP properties of mammary carcinomas may be influenced by their histological classification. In view of these observations, it would not be surprising that anatomically separate metastases might differ in their ERP properties. To some extent, therefore, variation in ERP properties is probably an intrinsic characteristic of mammary carcinomas. In addition to the intrinsic variability of breast carcinomas, consideration must be given to factors external to the tumor that might affect ERP.

Other extrinsic factors have also been considered (R7). The overall hormonal milieu influences cell growth and can apparently exert a selective influence on the proliferation of transplanted mammary carcinoma cells with specific ERP properties. This suggests that hormonal manipulation could affect ERP properties of

specimens of human metastatic carcinomas analyzed after therapy. Even if growth is not altered, exogenous estrogens could interfere with ERP measurement and it is apparent that hormones produced within the tumor tissue could have a similar effect. However, no conclusions could be drawn with regard to the patients presented in this report since there were insufficient specimens obtained after hormonal therapy. There was also no evidence that natural change in menstrual status was responsible for any variations that were observed.

It is, of course, well known that radiation therapy can affect the viability of mammary carcinomas and change their histological appearance. Review of the cases presented in this report (R7) revealed no consistent relationship between radiation therapy and variations in ERP.

13. ERP in Visceral Metastases

In our experience (M7), 54% of primary breast carcinomas contain significant amounts of estrogen receptor protein compared with 42% of metastatic deposits. By far the most common sources of the metastases submitted for assay are skin, lymph nodes, and other nonvisceral soft tissue sources. The visceral metastases are the ones in which response to treatment is generally most important clinically, yet there is little information in the literature concerning the estrogen receptor protein status of visceral metastases.

In addition, it has been known for some time that metastases to lung, brain, and liver do not usually respond to endocrine ablation. What is not known, however, is whether this phenomenon represents some inherent protective effect provided by these organs or metastases to these organs simply tend to have low estrogen receptor protein values. If the former explanation is true, ERP– visceral metastases should not respond as well as ERP positive metastases from other sites. These questions prompted a review of our experience with estrogen receptor protein (J5).

All patients were female, 33 were white, and 1 was black. In Table 2 we correlated the status of ERP with 15 patients with liver metastases. A total of 6

TABLE 2
ERP STATUS IN LIVER METASTASES

ERP status	Patients (n)	Evaluable patients who had endocrine ablation	Evaluable patients who responded[a]
Positive	4	3	3
Negative	11	9	2

[a] A χ^2 test, using Yates' correction, yielded $0.1 > p > 0.05$.

patients were premenopausal, 2 were ERP+, and 4 were ERP–. The remaining 9 were postmenopausal, 2 were ERP+, and 7 were ERP–. In the ERP+ patients, 50% responded to endocrine therapy and in the ERP– patients only 18% responded.

Eighteen patients had estrogen receptor protein determinations done on lung metastases from breast carcinomas (Table 3). Four patients were ERP+ and fourteen were ERP–. Three of the four positive results and three of the fourteen negative results were in premenopausal patients. Nine of the ten postmenopausal patients' assays were ERP–. Two patients were considered perimenopausal (within 1 year of cessation of menses) and both had ERP lung metastases. Endocrine ablation resulted in one response in the ERP+ group. One patient with positive and seven with negative results failed to respond to endocrine ablation. Two patients with positive and seven with negative results were nonevaluable. A single patient with a brain metastasis from breast carcinomas who had a biopsy and an estrogen receptor protein assay was premenopausal and had a negative estrogen receptor protein result. No endocrine therapy was initiated.

Table 4 outlines our experience with the duration of response to endocrine ablation. In the 13% of the evaluable patients with ERP– metastases who responded to this form of treatment, the average duration of response was 3.5 months. In the comparable group of valuable ERP+ responders, the average duration of response was 16.75 months. A statistically significant difference between these groups, however, was not demonstrated ($p = 0.19$, Student's t test). Although there are numerous reports dealing with hormone receptors of primary breast cancer, including receptors for hormones other than estrogen, a substantially smaller body of data has been obtained with breast cancer metastases. In particular, metastases to liver, lung, and brain have been studied only rarely. This is due in part to the magnitude of surgery needed to obtain tissue for assay and in part to the fact that castration, adrenalectomy, and hypophysectomy produce responses in only about one-third of such cases. Our data, limited to a few cases, did not achieve statistical significance. Nevertheless, biopsies of metastases to liver and lung may be useful, even though only 24% of such metastases were shown to contain significant amounts of ERP compared with 42% of those from

TABLE 3
LUNG METASTASES

ERP status	Patients (n)	Evaluable patients who had endocrine ablation	Evaluable patients who responded[a]
Positive	4	2	1
Negative	14	7	0

[a]A χ^2 test, using Yates' correction, yielded $0.5 > p > 0.25$.

TABLE 4
DURABILITY OF RESPONSE TO ENDOCRINE ABLATION

ERP[a] status	Biopsy site	Duration of response and type of surgery
Negative	Lung	No responders
Negative	Liver	3 months to BA[b] + BSO[c]; 4 months to BA + BSO
Positive	Lung	16 months to BSO
Positive	Liver	1 month to BSO; 42 months to BSO; 8 months to BA

[a]ERP, estrogen receptor protein.
[b]BA, bilateral adrenatectomy.
[c]BSO, bilateral salpingooophorectomy.

all metastatic sites (M12). A frequent major benefit of a biopsy is to resolve doubt as to the cause of a lung nodule seen on chest roentgenography or an abnormality noted on liver scan. An additional value confirms estrogen receptor protein status, which differs from that of the primary tumor in about 24% of cases (R7). If the estrogen receptor protein assay is positive, an 80% response rate to endocrine therapy is observed. This response rate is similar to that for ERP+ metastases from other sites. Conversely, if the assay is negative, one can predict that endocrine manipulation will be unlikely to provide palliation. A biopsy of liver metastases followed by estrogen receptor protein determination is useful in suggesting future management. For example, liver metastases may grow after surgical castration even though cutaneous metastases regress. In such circumstances a negative estrogen receptor protein assay of the liver metastases obtained at the time of castration will suggest prompt initiation of nonendocrine therapy instead of waiting unduly long for a response that is not likely to occur.

This study also has bearing on the uncommon situation in which metastatic carcinomas are found in the absence of known primary sites. If estrogen receptor protein is found in these metastatic deposits, an occult breast primary lesion is suggested. However, our results suggest that if estrogen receptor protein is not found in the metastases, a breast primary lesion is not ruled out, since a majority of lung and liver metastases from known breast primaries are ERP−.

14. Correlation with Epidemiology and Pathology

Several studies have been initiated to investigate how demographic and epidemiologic factors correlate with the receptor properties of carcinomas of the breast (A3, L2, O1, R9). Many of the factors analyzed are significantly related to breast cancer risk. The results obtained are of interest because, with the exception of analyses of age and menopausal status, the relationships reported here have received little attention. No significant relationship of age of menarche to ERP

status was found (Table 5) (L2); similar results were found with PRP status. Analysis of ERP/PRP was carried out in 79 premenopausal and 79 postmenopausal women with primary operable breast carcinomas. Table 6 shows the distribution of ERP/PRP according to menstrual status. There was a significant higher incidence of ERP+/PRP+ in postmenopausal women (52%) than in premenopausal women (40.5%) and a higher incidence of ERP−/PRP− in premenopausal women (30.5%) than in postmenopausal women (24%).

Significant correlation was also found between age and ERP status; the concentration of ERP increased in postmenopausal women. PRP concentration did not vary significantly with menstrual status (Table 7) (A3). No pattern was observed in the distribution and concentration of ERP/PRP throughout the phases of the menstrual cycle (Table 8), although there was a wide range of concentrations. Therefore the phase of the menstrual cycle at the time of operation does not predict the presence of ERP/PRP in human breast carcinomas (A3). The cause of menopause was not significantly related to the ERP status among these patients. ERP positive tumors were found in 61% of those considered not to have had a natural menopause (L2).

A total of 78% of the patients were mothers. Among the mothers, 49% had ERP+ tumors, 38% had ERP− tumors, and 13% had borderline carcinomas. In the group of patients who either were never pregnant or had never experienced a full pregnancy, the relative frequency of ERP positive, ERP negative, and borderline tumors was 56, 31, and 13%, respectively. These observed distributional differences between parous and nonparous women were not significant. When patients were classified as "ever pregnant" or "never pregnant," the proportions were virtually identical to those cited above. No relationship was found between parity and number of pregnancies. There was also no relationship between ERP results and mean age of first pregnancy, mean age at first birth, mean age at last preg-

TABLE 5
AGE OF MENARCHE AND ERP

Age of menarche (years)	Patients (*n*)	Tumor ERP					
		Positive		Negative		Borderline	
		No.	%	No.	%	No.	%
Under 12	155	69	44	60	39	26	17
Under 12	186	94	50	68	37	24	13
Under 13	228	116	51	84	37	28	12
Under 14	121	71	59	38	31	12	10
Under 15	49	23	47	19	39	7	14
Over 15	45	24	53	15	34	6	13

TABLE 6
DISTRIBUTION OF ESTROGEN AND PROGESTERONE RECEPTOR PROTEINS ACCORDING TO MENSTRUAL STATUS

	Receptor status[a] (ERP/PRP)			
Menstrual status	+/+	−/−	−/+	+/−
Premenopausal[b] (n = 79)	32 (40.5%)	24 (30.5%)	18 (23%)	5 (6%)
Postmenopausal (n = 79)	41 (52%)	19 (24%)	2 (2.5%)	17 (21.5%)

[a] $p < 0.001$.
[b] Four patients had two sets of receptor data on two separate days (biopsy, followed by mastectomy) and they are as follows: +/+ (Day 23) → −/+ (Day 28); −/− (Day 14) → −/− (Day 21); −/− (Day 17) → −/+ (Day 20); and −/+ (Day 1) → −/+ (Day 10).

nancy, or mean number of years between first and last pregnancies. No significant relationship was found between ERP status and history of miscarriages of stillbirth (L2). Contraceptive hormone usage did not affect the receptor status but ERP levels tended to be lower in women taking estrogen at the time of diagnosis. Similar patterns were seen whether patients had or did not have a family history of breast cancer (L2, O1).

Race was related to ERP status. Blacks, who constituted 8% of the study sample, were less likely to have ERP+ tumors (31%) than whites (53%) (L2). The distribution of ERP results did not differ significantly among the blood groups O, A, B, and AB. The frequency of positive ERP ranges from a low of 41% in patients with AB-type blood to a high of nearly 53% in patients with O-type blood (L2, R9). ERP status was also compared with major medical conditions, including diabetes mellitus, hypertension, phlebitis, thyroid disease, and intestinal polyps. No relationship was found between ERP/PRP distributions and any of the medical conditions (L2). The correlation with histopathology was investigated by Rosen *et*

TABLE 7
MEAN ERP AND PRP CONCENTRATION IN PRE- AND POSTMENOPAUSAL PATIENTS

	Mean receptor concentration (fmole/mg protein)	
Menstrual status	ERP+[a]	PRP+[b]
Premenopausal	19.54 ± 2.12[c] (n = 37)	41.32 ± 5.36 (n = 50)
Postmenopausal	53.87 ± 5.92 (n = 58)	34.06 ± 5.68 (n = 43)

[a] Cases include ERP+/PRP+ and ERP+/PRP−. $p < 0.005$.
[b] Cases include ERP+/PRP+ and ERP−/PRP+.
[c] ± SEM.

TABLE 8
DISTRIBUTION AND MEAN CONCENTRATION OF ESTROGEN AND PROGESTERONE RECEPTOR PROTEINS IN THE DIFFERENT PHASES OF THE MENSTRUAL CYCLE

	Receptor status[a]								Mean receptor concentration (fmole/mg protein)	
	+/+		−/−		−/+		+/−			
Phase (days)	No.	%	No.	%	No.	%	No.	%	ERP[b]	PRP[c]
Menstrual (28–29)	4/32	13	3/24	13	2/18	11	—	—	10.67 ± 4.46[d]	25.65 ± 30.19
Proliferative (3–7)	3/32	10	3/24	13	1/18	6	1/5	20	37.2 ± 22.09	67 ± 34.08
Follicular (8–14)	7/32	22	8/24	33	4/18	22	1/5	20	12.3 ± 6.73	39.73 ± 28.68
Luteal (15–20)	8/32	25	5/24	21	6/18	33	1/5	20	21.4 ± 10.71	50.95 ± 47.36
Secretory (21–27)	10/32	31	5/24	21	5/18	28	2/5	40	20.15 ± 10.52	32.91 ± 36.48

[a] *p* is NS.
[b] Cases include ERP+/PRP+ and ERP+/PRP−.
[c] Cases include ERP+/PRP+ and ERP−/PRP+.
[d] ± SD.

al. (R4, R6, R8) and Lesser (L2). The frequency of ERP/PRP positive tumors was highest among patients with infiltrating duct and lobular carcinomas. The lower proportion of ERP/PRP positive tumors in medullary and atypical medullary carcinomas was statistically significant ($p<0.001$). The frequency of ERP/PRP positivity in the noninvasive carcinoma group tended to be intermediate. There was a significant trend toward ERP/PRP positivity in low-grade infiltrating duct tumors compared with high-grade lesions. Also the degree of lymphocytic reaction and the lymphocyte–plasma ratio showed a high correlation with ERP/PRP+ tumors. A study (R10) was undertaken to investigate the relationship between Barr body distribution and estrogen receptor protein content of mammary carcinomas. The proportion of cells with one or more Barr bodies was determined in 105 specimens of mammary carcinomas from Guard-stained imprints. The receptor protein content of the specimen was measured by the dextran charcoal method and compared with histopathologic features of the carcinomas. Primary carcinomas with Barr bodies in more than 10% of tumor cells were more likely to have higher levels of receptor protein than those with a lower proportion of Barr body containing cells ($p<0.005$). The results obtained for primary carcinomas may explain why patients with carcinomas that have a high proportion of Barr body positive cells are more likely to respond to hormonal therapy. Furthermore, these observations, when correlated with other available data about ERP, suggest that an X chromosome is involved in the synthesis of and/or carries the locus of action for estrogen receptor protein.

15. ERP in Male Breast Cancer

Male breast cancer is a relatively uncommon disease, occurring less than 1% as often as that in females (K8). About 900 male breast cancers are diagnosed each year in the United States and 300 men die of the disease. Breast cancer represents less than 1% of all malignancies in males, and the median age at which it occurs is several years later than in females, but it has been observed in patients as young as 5 and as old as 93.

Male breast cancer patients usually present in a more advanced stage, due in part to a longer duration of symptoms in males before treatment is sought or begun. In males, breast cancer is too uncommon to allow randomized clinical trials of systemic therapy. The majority of male breast cancers are estrogen and progesterone receptor positive (35 out of 59). The use of adjuvant tamoxifen is commonly recommended. Three series using adjuvant therapy have been reported, using tamoxifen in one and chemotherapy in two. All suggest improved disease-free survival compared with historical controls. Therefore, adjuvant chemotherapy plus tamoxifen for ERP/PRP positive patients and chemotherapy for node negative patients are advised. We studied the relationship of ERP to male breast cancer

(R5). Eleven specimens of breast lesions obtained from 10 male patients were analyzed for ERP. Three patients (ages, 49, 77, and 82 years) had infiltrating duct carcinomas with no axillary metastases. ERP in each of these was positive. Eight specimens with gynecomastia, one of which was obtained from the 77-year-old patient with carcinomas in the same breast, were also analyzed. Of these, ERP was positive in a 59-year-old man who had cirrhosis of the liver; two patients with borderline ERP had hepatitis and testicular seminoma, respectively. No relationship between histopathologic features of the lesions and ERP results was found.

16. Role of ERP/PRP in Patients with Melanomas, Thyroid Cancer, or Prostate Cancer

The role of ERP/PRP in the management of other hormone-dependent cancers has been well established. Patients with melanomas are currently treated with hormonal therapy. In a preliminary evaluation (F6), the hormone binding ability of melanomas was demonstrated through the assay of ERP in cytosol preparations. This study was undertaken to verify if some melanomas are hormone dependent and to determine the usefulness of such findings in the treatment of melanomas. About 50% of the patients were positive for ERP. ERP receptors also have been observed in patients with prostatic cancer (F7).

We evaluated the ERP/PRP content in thyroid tumors (V4) and the ERP/PRP was correlated in 135 patients. There was no statistical association among receptor content, age, sex, tumor size, and classification. Metastases at presentation, seen in 50% of the patients, were unrelated to receptor content. While ERP/PRP are detectable commonly in thyroid lesions, no clear relationship to these clinical and prognostic features was found.

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ADVANCES IN HUMAN LEUKOCYTE ANTIGENS AND TRANSPLANTATION

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1. Transplantation in Historical Perspective

The idea that diseased, damaged, or worn-out tissues and organs might be replaced with healthy substitutes has long captured man's imagination. Surgeons

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in India are reputed to have performed nose reconstruction operations many centuries ago using skin flaps from elsewhere on the patient's body (D4). This procedure was certainly available during the Renaissance when Gaspare Tagliacozzi, a Bolognese anatomist and surgeon, established a reputation for his ability to rebuild a missing nose using a skin flap from the upper arm—a technique which is still practiced today under the name of the "Tagliacotian" or "Italian" method (T1).

The transplantation of skin from one part of the body to another has been performed since ancient times, particularly in the repair of injuries caused by warfare but also to make good mutilations imposed as punishments for infidelity, thievery, and other misdemeanors (D4). The first report of skin grafting in the modern medical literature is by Bünger (B26), who described a nose reconstruction using a skin graft from the patient's thigh. Skin transplants using unrelated donors were available at the end of the 19th century and were graphically described in the memoirs of Sir Winston Churchill (C13), who tells how he was used as a skin graft donor for a battle casualty in the Sudan War in 1898.

With regard to solid organ transplantation, the first half of this century saw a few isolated and ill-fated attempts at transplanting kidneys removed from cadavers but the practice did not attract significant interest until the early 1950s. A number of attempts were documented at that time but, invariably, the outcome was disappointing and the transplanted kidneys seldom functioned for longer than a few weeks, if at all. In 1952, for example, Hume and colleagues described six transplants, of which three never functioned and three initially produced urine but ceased to function within a short time (H10). The turning point came in 1956 when Merrill and co-workers in Boston described a successful renal transplant between a 24-year-old patient and his identical twin brother (M12). Although living donors had not been used previously, there was some basis for using an identical twin as a donor since it was known that skin grafts between identical twins were not rejected (B24). Nevertheless, the idea of removing a healthy organ from a living person posed a major moral and ethical dilemma for the surgeons. However, the transplant was a success and the recipient lived for several years with a functioning kidney. The successful outcome of this transplant and the absence of ill effects in the donor encouraged additional transplants using living donors. A major finding to emerge was the improved success rate when parents or siblings rather than unrelated individuals were used as donors (H11). This gave support to a prediction made by Medawar in 1957 that the possession by the donor of antigens absent in the recipient was undesirable in terms of transplant outcome (M11).

At around the same time in Paris, Dausset found that a newly discovered antigen on human leukocytes, known as MAC (after the initials of three volunteers involved in the research), strongly influenced the outcome of skin grafts from unrelated donors (D2). Recipients without the MAC antigen who were injected with leukocytes possessing the MAC antigen rapidly rejected grafts from MAC-

positive donors but retained grafts from MAC-negative donors. These findings marked the beginning of an era of prodigious research into leukocyte antigens and their role in determining the outcome of transplantation. Over 100 leukocyte antigens are now recognized, forming the most complex genetic system currently known—a system referred to as the human leukocyte antigen (HLA) system. The relationship between the HLA system and transplant outcome is now accepted without question.

The last three decades have seen tremendous advances in our knowledge of the HLA system and its role in transplantation, not only of the kidney but also of bone marrow, liver, heart, and other organs. This review describes some of those advances.

2. The Human Leukocyte Antigen System

Human leukocyte antigens are polymorphic cell-surface molecules that are intimately involved in the regulation of the immune response. The antigens are encoded by a series of closely linked genes known as the major histocompatibility complex (MHC), which is located on the short arm of chromosome 6 (B10). The complex extends over approximately 4000 kilobases or 4×10^6 nucleotides and contains three distinct subregions known as class I, class II, and class III (T19) (see Fig. 1).

Only two of these regions, class I and class II, contain genes coding for HLA antigens. The third region, class III, contains at least 36 genes coding for a diverse group of products, of which some are involved in the immune system and others have no obvious association with immunity (T19). The class III region contains, among others, genes coding for complement components C2, Bf, C4A, and C4B (C6), steroid 21-hydroxylase CyP21A and CyP21B (H5), tumor necrosis factors α and β (I1), and B-associated transcripts (BAT 1 to 5) (S16). A detailed map showing other components of the class III region is given by Trowsdale *et al.* (T19). Unlike the products of the class I and II regions, the class III gene products do not play an obvious role in determining transplant compatibility and they are not considered further in this review.

2.1. HLA Class I Genes and Their Products

The HLA class I region is the most telomeric part of the MHC and codes for at least 17 genes or gene fragments (K14). Three of these, HLA-A, HLA-B, and HLA-C, code for antigenic glycoproteins on the surface membrane of most (if not all) nucleated cells. Four other functional class I genes, now known as HLA-E, -F, -G, and -H, have also been identified (G5, G6, K15) but their status in coding for cell-surface molecules is currently unclear.

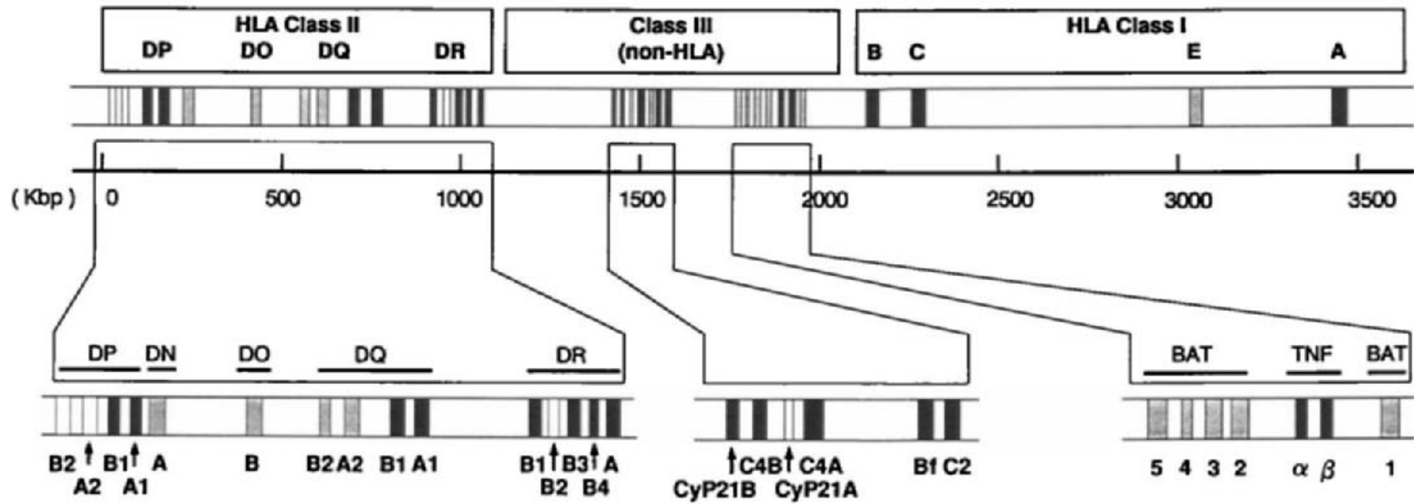

Fig. 1. Schematic diagram of the major histocompatibility complex on human chromosome 6. Fully expressed genes are shown in black; pseudogenes (which do not express a product) are shown in white; and genes of undefined or uncertain status are shaded. Distances along the chromosome are measured in units known as kilobase pairs (kbp). BAT indicates B-associated transcript; TNF, tumor necrosis factor. Adapted from Peter and Hawkins (P3) with permission, Copyright 1992, American Medical Association.

The HLA-A, -B, and -C genes are extremely polymorphic. That is to say that the locus for HLA-A may be occupied by one of several different variations (alleles) of the HLA-A gene; the locus for HLA-B may be occupied by one of several alleles of the HLA-B gene; etc. The different alleles at the individual loci are the result of subtle differences in amino acid sequence along the respective glycoprotein chains from which the HLA molecule is formed. Some of these allelic differences can be detected only by amino acid sequencing. Others reflect more substantial differences in specificity and can be defined by serological reactions using alloantisera (i.e., human sera containing antibodies against HLA antigens).

The nomenclature of the HLA system is determined by the World Health Organization (WHO) Nomenclature Committee and reflects both the serological and the nucleotide levels of classification. The 1991 report of the Nomenclature Committee listed 27 HLA-A specificities, 59 HLA-B specificities, and 10 HLA-C specificities as shown in Table 1 (B17). These are the specificities that are detectable at the serological level. Contributing to some of these individual specificities, however, can be a large number of different alleles. At least 10 different alleles code for the specificity HLA-A2, for example, and 7 code for HLA-B27. To accommodate this complexity, the Nomenclature Committee has

TABLE 1
COMPLETE LISTING OF RECOGNIZED HLA SPECIFICITIES

HLA-A	HLA-B	HLA-C	HLA-D	HLA-DR	HLA-DQ	HLA-DP
A1	B5	Cw1	Dw1	DR1	DQ1	DPw1
A2	B7	Cw2	Dw2	DR10 3	DQ2	DPw2
A203	B703	Cw3	Dw3	DR2	DQ3	DPw3
A210	B8	Cw4	Dw4	DR3	DQ4	DPw4
A3	B12	Cw5	Dw5	DR4	DQ5(1)	DPw5
A9	B13	Cw6	Dw6	DR5	DQ6(1)	DPw6
A10	B14	Cw7	Dw7	DR6	DQ7(3)	
A11	B15	Cw8	Dw8	DR7	DQ8(3)	
A19	B16	Dw9(w3)	Dw9	DR8	DQ9 (3)	
A23(9)	B17	Cw10(w3)	Dw10	DR9		
A24(9)	B18		Dw11(w7)	DR10		
A2403	B21		Dw12	DR11(5)		
A25(10)	B22		Dw13	DR12(5)		
A26(10)	B27		Dw14	DR13(6)		
A28	B35		Dw15	DR14(6)		
A29(19)	B37		Dw16	DR1403		
A30(19)	B38(16)		Dw17(w7)	DR1404		
A31(19)	B39(16)		Dw18(w6)	DR15(2)		
A32(19)	B3901		Dw19(w6)	DR16(2)		

(continued)

TABLE 1 *(Continued)*

HLA-A	HLA-B	HLA-C	HLA-D	HLA-DR	HLA-DQ	HLA-DP
A33(19)	B3902		Dw20	DR17(3)		
A34(10)	B40		Dw21	DR18(3)		
A36	B4005		Dw22			
A43	B41		Dw23	DR51		
A66(10)	B42					
A68(28)	B44(12)		Dw24	DR52		
A69(28)	B45(12)		Dw25			
A74(19)	B46		Dw26	DR53		
	B47					
	B48					
	B49(21)					
	B50(21)					
	B51(5)					
	B5102					
	B5103					
	B52(5)					
	B53					
	B54(22)					
	B55(22)					
	B56(22)					
	B57(17)					
	B58(17)					
	B59					
	B60(40)					
	B61(40)					
	B62(15)					
	B63(15)					
	B64(14)					
	B65(14)					
	B67					
	B70					
	B71(70)					
	B72(70)					
	B73					
	B75(15)					
	B76(15)					
	B77(15)					
	B7801					
	Bw4					
	Bw6					

Note. Specificities with numbers in parentheses represent "broad" specificities that include two or more subgroups. For example, the broad specificity HLA-A19 includes the subgroups A29, A30, A31, A32, A33, and A74. The number in parentheses indicates the broad specificity associated with that subgroup. Inclusion of the parenthesized number is optional when referring to a subgroup. Thus, it is not necessary to refer to A33(19); the short form A33 is acceptable. Use of the prefix "HLA" for a specificity name is optional; HLA-B46 and B46 are equally acceptable to refer to that specificity.

TABLE 2
LIST OF HLA-A, -B, AND -C ALLELES FOR WHICH UNIQUE NUCLEOTIDE SEQUENCES HAVE BEEN DETERMINED

HLA-A alleles	HLA-A specificity	HLA-B alleles	HLA-B specificity	HLA-C alleles	HLA-C specificity
A*0101	A1	B*0701	B7	Cw*0101	Cw1
A*0201	A2	B*0702	B7	Cw*0102	Cw1
A*0202	A2	B*0703	B703	Cw*0201	Cw2
A*0203	A203	B*0801	B8	Cw*02021	Cw2
A*0204	A2	B*1301	B13	Cw*02022	Cw2
A*0205	A2	B*1302	B13	Cw*0301	Cw3
A*0206	A2	B*1401	B14	Cw*0302	Cw3
A*0207	A2	B*1402	B65(14)	Cw*0401	Cw4
A*0208	A2	B*1501	B62(15)	Cw*0501	Cw5
A*0209	A2	B*1502	B75(15)	Cw*0601	Cw6
A*0210	A210	B*1503	B72(70)	Cw*0701	Cw7
A*0211	A2	B*1504	B62(15)	Cw*0702	Cw7
A*0212	A2	B*1801	B18	Cw*0801	Cw8
A*0301	A3	B*2701	B27	Cw*0802	C28
A*0302	A3	B*2702	B27	Cw*1201	—
A*1101	A11	B*2703	B27	Cw*1202	—
A*1102	A11	B*2704	B27	Cw*1301	—
A*2301	A23(9)	B*2705	B27	Cw*1401	—
A*2401	A24(9)	B*2706	B27		
A*2402	A24(9)	B*2707	B27		
A*2403	A2403	B*3501	B35		
A*2501	A25(10)	B*3502	B35		
A*2601	A26(10)	B*3503	B35		
A*2901	A29(19)	B*3504	B35		
A*2902	A29(19)	B*3505	B35		
A*3001	A30(19)	B*3506	B35		
A*3002	A30(19)	B*3701	B37		
A*31011	A31(19)	B*3801	B38(16)		
A*31012	A31(19)	B*3901	B3901		
A*3201	A32(19)	B*3902	B3902		
A*3301	A33(19)	B*4001	B60(40)		
A*3401	A34(10)	B*4002	B40		
A*3402	A34(10)	B*4003	B40		
A*3601	A36	B*4004	B40		
A*4301	A43	B*4005	B4005		
A*6601	A66(10)	B*4101	B41		
A*6602	A66(10)	B*4201	B42		
A*6801	A68(28)	B*4401	B44(12)		
A*6802	A68(28)	B*4402	B44(12)		
A*6901	A69(28)	B*4403	B44(12)		
A*7401	A74(19)	B*4501	B45(12)		
		B*4601	B46		
		B*4701	B47		

(continued)

TABLE 2 *(Continued)*

HLA-A alleles	HLA-A specificity	HLA-B alleles	HLA-B specificity	HLA-C alleles	HLA-C specificity
		B*4801	B48		
		B*4901	B49(21)		
		B*5001	B50(21)		
		B*5101	B51(5)		
		B*5102	B5102		
		B*5103	B5103		
		B*5201	B52(5)		
		B*5301	B53		
		B*5401	B54(22)		
		B*5501	B55(22)		
		B*5502	B55(22)		
		B*5601	B56(22)		
		B*5602	B56(22)		
		B*5701	B57(17)		
		B*5702	B57(17)		
		B*5801	B58(17)		
		B*7801	B7801		
		B*7901	—		

Note. Broad specificities (e.g., HLA-A9) do not appear in the table. This is because the broad specificities do not correspond to unique nucleotide sequences.

introduced a separate nomenclature for alleles (Table 2). The designation A*0212, for example, indicates that the allele codes for an HLA-A locus product; the first two numbers indicate the associated specificity (HLA-A2); and the last two numbers indicate a unique nucleotide sequence coding for that specificity.

2.1.1. *Structure of HLA Class I Genes*

HLA class I molecules exist on the cell surface in the form of glycoprotein heterodimers consisting of an α chain with a molecular weight of approximately 44 kDa bound noncovalently to a smaller nonpolymorphic protein of molecular weight 12 kDa known as β_2-microglobulin (β_2m). The gene determining β_2m is not part of the HLA complex and is coded separately on chromosome 15 (G12). Part of the heavy chain penetrates the cell membrane (Fig. 2) and the remainder is folded into three extracellular domains known as $\alpha1$, $\alpha2$, and $\alpha3$, which are analogous in structure to those of the immunoglobulin molecule. β_2m does not penetrate the cell membrane but is associated noncovalently with the $\alpha3$ domain of the heavy chain.

The three-dimensional structure of the class I molecule has been determined from radiographic crystallography studies of the HLA-A2 molecule (Fig. 3) (B13).

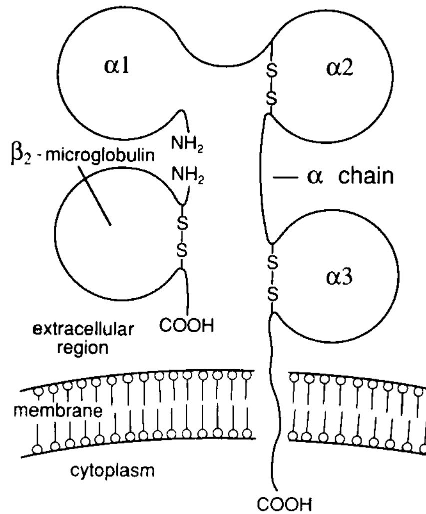

FIG. 2. HLA class I molecules consist of a glycoprotein heterodimer comprising an α chain made up of three α domains covalently associated with $β_2$-microglobulin. The molecule traverses the cell membrane at one end only. Most of the polymorphism of the molecule is located within the α1 and α2 domains, which contain the peptide binding groove (see text and Fig. 3).

The $α_1$ and $α_2$ domains each consist of four β strands and an α helix. The eight antiparallel β strands are folded into a platform that supports the two coiled α-helical loops. A large groove runs between these loops and provides a binding site for processed foreign antigens (B14). Most of the polymorphism of the molecule occurs within the α helices forming the sides of the binding site and in the β strands forming the base of the groove. This results in different class I molecules having different binding sites and restricts the number of peptide fragments with which a given molecule can bind.

2.2. HLA CLASS II GENES AND THEIR PRODUCTS

Products of what is now known as the HLA class II region were originally detected through the use of the mixed lymphocyte culture (MLC) assay (B1, B3). This test uses the principle that when lymphocytes from genetically dissimilar individuals are mixed under appropriate cell culture conditions, the lymphocytes of one individual will stimulate those of the other to reproduce and form blast cells. The finding that lymphocytes from HLA-A and HLA-B identical siblings did not stimulate each other in the MLC, whereas lymphocytes from HLA-A and HLA-B identical individuals did, suggested that the MLC reaction is related to the HLA system but is controlled by a locus separate from HLA-A and HLA-B (Y3). This

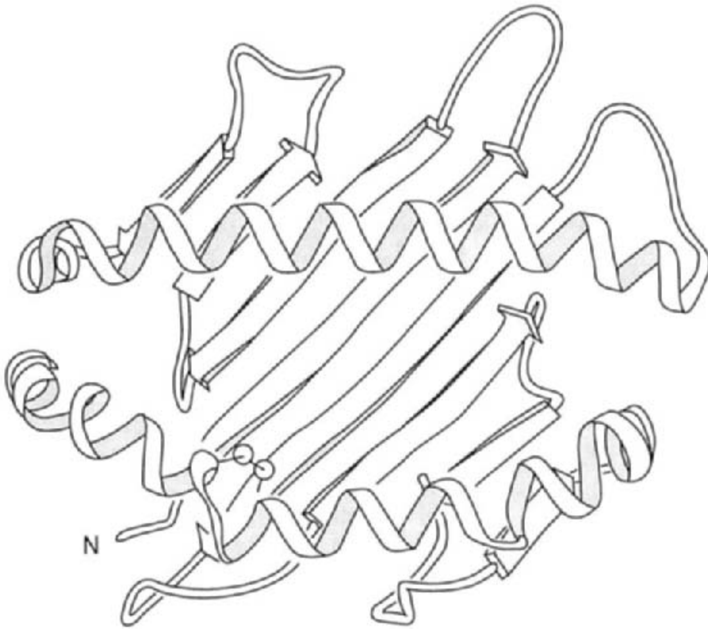

FIG. 3. Three-dimensional representation of the peptide groove of an HLA class I molecule. Reprinted by permission from *Nature* vol. 329, pp. 506–512 [Bjorkman *et al.* (B13)]; © 1985, Macmillan Magazines Ltd.

locus was later designated HLA-D (W3). It is now known that the HLA-D antigens detected in the MLC test are not the product of a single HLA-D locus but are the combined product of a group of three polymorphic subregions, HLA-DR, HLA-DQ, and HLA-DP (H2), which code for cell-surface glycoproteins and are located in what is now known as the class II region.

The HLA-DR series of serological specificities was defined at the Seventh International Histocompatibility Workshop (B16) following the finding that human serum contains antibodies that react with B cells rather than T cells (V3). The serological reaction patterns of these antibodies correlated well with the HLA-D specificities detected in the MLC test and the antigens defined in this way were designated HLA-DR (where the "DR" stands for "D-Related"). A total of 24 HLA-DR specificities are currently recognized by the WHO Nomenclature Committee (Table 1) (B17).

The HLA-DQ series had as its basis the finding, also at the Seventh International Workshop, that certain antisera reacted with subsets of cells of different HLA-DR specificities (D11, T17). Hence, for example, a particular group of antisera might react with some but not all HLA-DR2-positive cells, some but not all HLA-DR6-

positive cells, and some but not all HLA-DR8-positive cells. The reactions defined in this way were attributed to the effect of a locus termed *HLA-DQ* adjacent to the *HLA-DR* locus (W4). Nine HLA-DQ specificities are currently recognized (Table 1).

The HLA-DP specificities are detected not by serological techniques but by a cellular method known as the primed lymphocyte typing (PLT) test (S10, W1). More recently, these specificities have been detected by Southern blotting with appropriate probes and restriction enzymes, and by using monoclonal antibodies in an ELISA (B15). Six HLA-DP specificities are currently recognized (Table 1).

Recent work has shown the existence of very many other genes in the class II region in addition to those of the HLA-DR, -DQ, and -DP loci. Two loci, HLA-DN and HLA-DO, contain weakly expressed genes of uncertain function designated HLA-DNA [originally called HLA-DZα (T18) or HLA-DOα (I2)] and HLA-DOB (T16) (Fig. 1). Between HLA-DN and HLA-DO lies a fascinating cluster of novel genes that have been detected more recently (T19) but these are of little relevance to transplantation and are not considered further in this review.

2.2.1. *Structure of HLA Class II Genes*

Like the HLA class I molecules, the HLA-DR, HLA-DQ, and HLA-DP molecules consist of double-chain structures folded into domains. However, there are distinct differences between class I and class II molecules. First, unlike class I molecules, both class II chains are encoded by genes within the MHC. Second, unlike class I molecules, both chains traverse the cell membrane (Fig. 4).

The genes encoding the class II glycoprotein chains may be classified into two subtypes, designated A and B, that code for α and β chains, respectively. The HLA-DR, HLA-DQ, and HLA-DP molecules each consist of a heavy transmembrane α chain of approximately 34 kDa that is noncovalently associated with a transmembrane β chain of approximately 29 kDa (Fig. 4). Each heterodimer consists of a pair of complementary chains: DRα chains pair with DRβ chains, DQα chains pair with DQβ, and DPα chains pair with DPβ (Fig. 5).

The HLA-DR molecules contain a nonpolymorphic DRα chain coded by HLA-DRA and three or four DRβ chains, depending on the haplotype, which are coded by HLA-DRB genes. The DRB1 genes are highly polymorphic and account for much of the polymorphism within the HLA-D region. The specificities HLA-DR1 to HLA-DR18 listed in Table 1 are determined by alleles of the HLA-DRB1 gene. Over 60 DRB1 alleles are currently known (Table 3).

Not only do the DRB1 genes determine the specificities HLA-DR1 to HLA-DR18, they also combine with other DRB genes to determine additional HLA-DR specificities. When the DRB1 gene is present with a DRB3 gene, the serological specificity HLA-DR52 occurs. Combinations with a DRB4 gene result in HLA-DR53, and combinations with DRB5 (not shown in Fig. 5) result in HLA-DR51. In addition to the functional genes within the DRB region, the region contains a

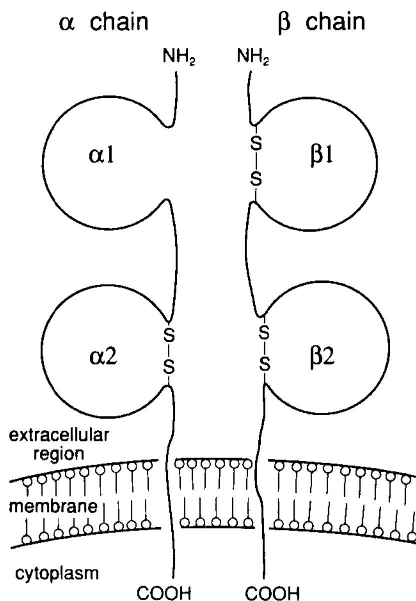

FIG. 4. HLA class II molecules consist of an α and a β chain each folded into two domains. Unlike the class I molecule (Fig. 2), both chains traverse the membrane. Most of the polymorphism of the molecule occurs within the α1 and β1 chains, which contain the peptide binding groove.

pseudogene, DRB2, containing mutations that prevent gene activation and transcription.

The HLA-DQ molecules consist of a DQα chain coded by the HLA-DQA1 gene and a DQβ chain coded by the HLA-DQB1 gene. Both HLA-DQA1 and HLA-DQB1 are highly polymorphic but the majority of the serological variability of HLA-DQ listed in Table 1 is determined by the DQβ chain. The subregion also contains the pseudogenes HLA-DQA2, HLA-DQB2, and HLA-DQB3, which are not expressed. The 1991 Nomenclature Committee Report lists 14 DQA1 alleles and 19 DQB1 alleles (B17).

HLA-DP molecules have a structure very similar to that of HLA-DQ. The HLA-DPA1 gene codes for a DPα chain and the HLA-DPB1 gene codes for a DPβ chain. Both HLA-DPA1 and HLA-DPB1 are polymorphic. Six HLA-DP specificities, HLA-DPw1 to HLA-DPw6, are currently recognized (Table 1). Analogous to HLA-DQ, the subregion also codes for pseudogenes, HLA-DPA2 and HLA-DPB2, which do not contribute to the cellularly defined specificities. A total of 8 DPA1 alleles and 38 DPB1 alleles are currently recognized (B17).

The three-dimensional structure of the class II molecule has yet to be revealed by X-ray crystallography. However, by comparing the patterns of conserved and

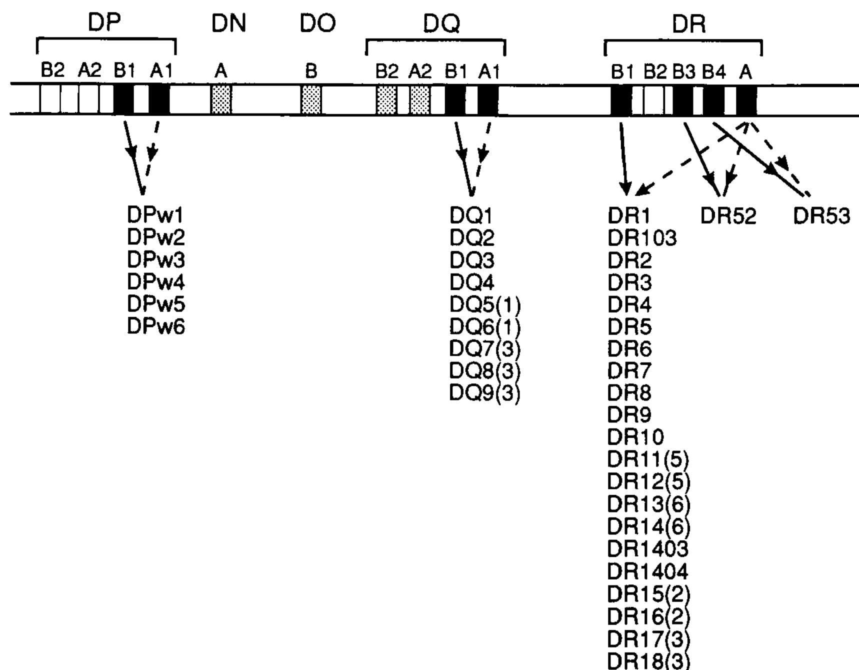

FIG. 5. HLA class II molecules consist of heterodimers coded by two genes. HLA-DP molecules are coded by a DPA1 and a DPB1 gene; HLA-DQ molecules are coded by a DQA1 and a DQB1 gene; and HLA-DR molecules are coded by a DRA gene and a DRB gene. Combinations of the DRA gene with DRB1, DRB3, and DRB4 genes result in separate classes of serological specificities (see text). The HLA-DR, -DQ, and -DP loci all contain noncoding genes (DPB2, DPA2, DQB2, DQA2, and DRB2).

polymorphic amino acid sequences in homologous domains of class I and class II molecules, Brown *et al.* were able to predict that the antigen-binding site is located in a peptide groove similar to that of the class I molecule (B25).

2.3. Inheritance of HLA Antigens

Inheritance of HLA characteristics follows a simple Mendelian pattern. The genetic loci controlling the various HLA subregions are contained within the major histocompatibility complex on chromosome 6 (Fig. 1). Each individual inherits one chromosome 6 from one parent and one from the other parent, each chromosome coding for one HLA-A gene, one HLA-B gene, one HLA-C gene, and so on. The HLA-A locus will be occupied by one of the alleles listed in Table 1 under the column headed "HLA-A", the HLA-B locus will be occupied by one of the alleles listed under "HLA-B", and so on.

TABLE 3
LIST OF RECOGNIZED HLA-DRB ALLELES AND THEIR ASSOCIATED HLA-DR SPECIFICITIES

HLA-DRB allele	HLA-DR specificity	HLA-DRB allele	HLA-DR specificity
DRB1*0101	DR1	DRB1*1304	DR13(6)
DRB1*0102	DR1	DRB1*1305	DR13(6)
DRB1*0103	DR103	DRB1*1306	DR13(6)
DRB1*1501	DR15(2)	DRB1*1401	DR14(6)
DRB1*1502	DR15(2)	DRB1*1402	DR14(6)
DRB1*1503	DR15(2)	DRB1*1403	DR1403
DRB1*1601	DR16(2)	DRB1*1404	DR1404
DRB1*1602	DR16(2)	DRB1*1405	DR14(6)
DRB1*0301	DR17(3)	DRB1*1406	DR14(6)
DRB1*0302	DR18(3)	DRB1*1407	DR14(6)
DRB1*0303	DR18(3)	DRB1*1408	DR14(6)
DRB1*0401	DR4	DRB1*1409	DR14(6)
DRB1*0402	DR4	DRB1*1410	—
DRB1*0403	DR4	DRB1*0701	DR7
DRB1*0404	DR4	DRB1*0702	DR7
DRB1*0405	DR4	DRB1*0801	DR8
DRB1*0406	DR4	DRB1*08021	DR8
DRB1*0407	DR4	DRB1*08022	DR8
DRB1*0408	DR4	DRB1*08031	DR8
DRB1*0409	DR4	DRB1*08032	DR8
DRB1*0410	DR4	DRB1*0804	DR8
DRB1*0411	DR4	DRB1*0805	DR8
DRB1*0412	DR4	DRB1*09011	DR9
DRB1*11011	DR11(5)	DRB1*09012	DR9
DRB1*11012	DR11(5)	DRB1*1001	DR10
DRB1*1102	DR11(5)		
DRB1*1103	DR11(5)	DRB3*0101	DR52
DRB1*11041	DR11(5)	DRB3*0201	DR52
DRB1*11042	DR11(5)	DRB3*0202	DR52
DRB1*1105	DR11(5)	DRB3*0301	DR52
DRB1*1201	DR12(5)		
DRB1*1202	DR12(5)		
DRB1*1301	DR13(6)	DRB4*0101	DR53
DRB1*1302	DR13(6)		
DRB1*1303	DR13(6)		
		DRB5*0101	DR51
		DRB5*0102	DR51
		DRB5*0201	DR51
		DRB5*0202	DR51

The combination of alleles inherited from a parent is described as a *haplotype,* a contraction of the term "haploid genotype" (C10). Since there are two representatives of chromosome 6 present in each person, one from each parent, an individual will possess, by definition, two HLA-A genes, two HLA-B genes, two HLA-DR genes, etc.

As shown in Fig. 6, either of the father's two haplotypes can pair with either of the mother's two haplotypes. Hence, a single mating can result in four possible haplotypic combinations in the offspring. Since the HLA region of the chromosome is only approximately two recombination units (centimorgans) in length, the frequency of recombination between the HLA loci is less than 2% and individuals almost always inherit an intact segment of the chromosome from each parent. Thus, within a given family, there is approximately a 25% chance that an individual will have identical HLA haplotypes to any one of his siblings. The chance of being identical for one haplotype is 50%, and the chance of having no haplotypes in common is 25%. In the 2% or less of meiotic divisions in which recombination occurs, the offspring inherits part of the MHC from one of the parent's chromosomes and the balance from the other chromosome of that parent (Fig. 7).

The haplotype cannot be determined directly by HLA typing as this reveals only the phenotype, i.e., the expression of the HLA antigens present rather than the alignment of genes on the chromosomes. Haplotypes can only be determined through a family study.

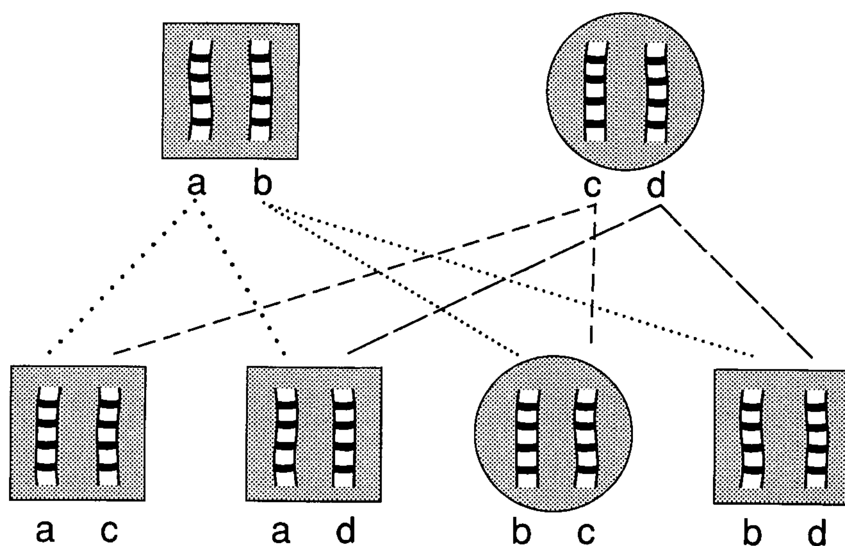

FIG. 6. Inheritance of HLA haplotypes. Either one of the father's haplotypes can combine with either one of the mother's haplotypes, resulting in four haplotypic combinations in the offspring.

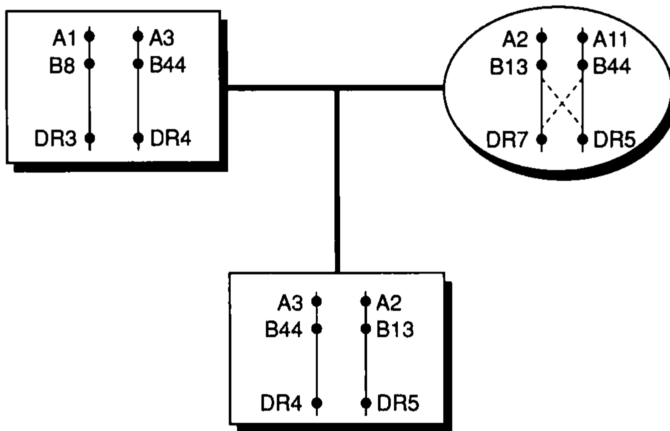

FIG. 7. Meiotic recombination affecting HLA haplotypes. The offspring has inherited an intact A3, B44, DR4 haplotype from the father and a recombinant haplotype from the mother consisting of A2 and B13 from one chromosome and DR5 from the other.

2.4. DETECTION OF HLA PRODUCTS

When leukocyte antigens were first detected in the 1950s, the assay consisted of a cumbersome agglutination technique. However, the introduction of the lymphocyte microcytotoxicity test by Terasaki and McClelland in 1964 (T6) eliminated many of the technical constraints inherent in the agglutination method and provided a more consistent and reproducible method for the demonstration of HLA products. The test has continued in use little changed since its introduction.

The principle of the test is that, in the presence of complement, a reaction between HLA antigens on lymphocytes and corresponding antibodies in serum will cause activation of complement and consequent lysis of the lymphocytes. This lysis can be detected by the addition of a supravital stain such as eosin or trypan blue. Given an antiserum of known HLA specificity (e.g., HLA-A2) and lymphocytes of unknown specificity, the incorporation of stain into the test lymphocytes following incubation with complement (usually obtained from rabbits) implies that the lymphocytes possess the antigen specificity corresponding to that of the antiserum. By repeating this technique with antisera of different specificities, the HLA phenotype of the test lymphocytes can be ascertained.

The major disadvantage of this technique is that the antisera are of human origin. Traditionally, these alloantisera have been obtained from volunteer blood donors who have developed HLA antibodies following pregnancy or blood transfusion. Antibodies of this type tend to decrease in titer or change specificity over time so that samples collected from the same donor at different times may react

quite differently. The technology of monoclonal antibody production using hybridomas, introduced by Köhler and Milstein in 1975 (K12), has partially overcome this problem by providing infinite quantities of typing reagents for some HLA class I and class II specificities. However, monoclonal antibodies to many specificities remain elusive (M2). A major reason for this is that the HLA specificities have been defined historically on the basis of serological reactions against human alloantibodies. A single alloantiserum will tend to recognize several antigen-binding sites on the HLA molecule rather than a single determinant. Monoclonal antibodies, on the other hand, recognize only small portions (epitopes) of the molecule (M2). These epitopes often occur in several HLA specificities and result in poor correlation between the reaction patterns of monoclonal antibodies and those of alloantibodies.

2.4.1. *Biochemical and Molecular Methods of Identifying HLA Gene Products*

In addition to the use of traditional serological techniques, HLA gene products can be analyzed on the basis of their biochemical characteristics. Isoelectric focusing (IEF), for example, allows HLA products to be separated on the basis of their electric charge or, more specifically, the pH at which the protein is uncharged, i.e., its isoelectric point. This technique is particularly useful for identifying variants and subtypes of HLA class I specificities. Monoclonal antibodies have been used to great effect in this technique by precipitating target molecules for the isolation of HLA antigens in a form suitable for electrophoresis (C16). A combination of the techniques of IEF and serological analysis has provided greatly improved definition of HLA class I specificities by detecting serologically indistinguishable subtypes or variants of HLA-A and HLA-B antigens and enabling the separation of cross-reactive antigens (Y2).

Analysis of class II specificities requires an extension of the technique known as two-dimensional electrophoresis. This procedure combines IEF with electrophoresis using sodium dodecyl sulfate and polyacrylamide gels. the IEF and gel electrophoresis are performed at right angles to each other so that the separations are based on both size and isoelectric point. This technique has proved to be highly efficient in detecting new class II variants (Fig. 8) and particularly in characterizing molecular variants of DR2, DR4, DR5, DR6, and DR7. The technique is one of the best typing procedures for the identification of DQa and DPa variants (C12, K11).

One of the major advances in HLA technology over the last decade was the introduction of restriction fragment length polymorphism (RFLP) analysis (Fig. 9). Genetic polymorphism within the HLA system is determined by differences in nucleotide sequences. In the RFLP technique, DNA is digested with restriction enzymes that break down the DNA molecule wherever a specific DNA sequence occurs. This breaks the molecule into multiple fragments of various lengths. Different alleles of the same gene will have different nucleotide sequences and

may generate fragments of different sizes. The fragments are separated electrophoretically on agarose gel on the basis of their size (smaller fragments have fewer negative charges). After an intermediate step to convert the DNA molecules into single strands, the DNA is transferred to a nitrocellulose filter so that an image of the agarose gel separation is obtained on the filter. This process is known as *Southern blotting,* after Edward M. Southern, who pioneered the technique (S15). The single-stranded DNA fragments are then treated with a radioactive "probe" of specific nucleotide sequence that binds, or "hybridizes," those DNA fragments with a sequence identical or homologous to that of the probe. The positions of those fragments are localized by autoradiography.

The value of the RFLP technique lies in its ability to demonstrate allelic variation within specificities that do not show variation at the serological level (K13). While the RFLP technique is valuable in determining allelic variation, it is subject to certain limitations. The technique is labor intensive and time consuming, and does not lend itself to routine application. Moreover, the procedure requires large amounts of test material. The introduction of the polymerase chain reaction (PCR) (S1) successfully overcame some of these disadvantages.

The PCR (Fig. 10) allows small segments of genomic DNA removed from crude cell lysates to be amplified in quantities sufficient for subsequent analysis. This is achieved by using a succession of incubation steps at different temperatures. Fundamental to the procedure are a pair of oligonucleotides, or "primers," that are made synthetically with nucleotide sequences complementary to those of the 3′ and 5′ ends of the gene segment of interest. One primer corresponds to the 3′ end and one to the 5′ end. The DNA sample is first heated to 94°C to convert it from

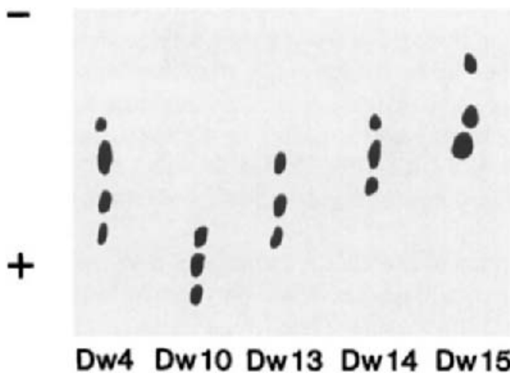

FIG. 8. Schematic representation of typical results of two-dimensional electrophoresis using polyacrylamide gels and isoelectrofocusing. The HLA class II specificity HLA-DR4 can be shown to consist of several variants. In this experiment five variants can be identified.

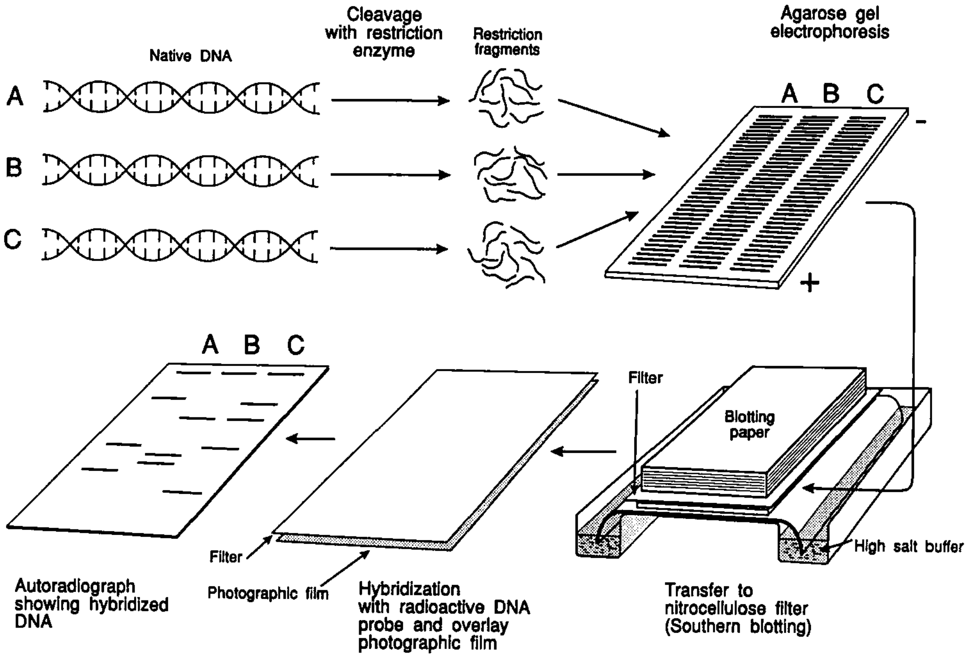

FIG. 9. Restriction fragment length polymorphism (RFLP) analysis. See text for explanation.

the double-stranded to the single-stranded form. As the sample cools to 70°C, the primers bind, or "hybridize," to the gene segment in question. This process is known as "annealing." The addition of a heat-stable polymerase, *Taq* DNA-polymerase (derived from the thermophilic bacterium *Thermus aquaticus*) facilitates the synthesis of a second DNA strand beginning at the 5′ end and moving toward the 3′ end. This completes the first cycle of the process and effectively doubles the amount of target DNA. Repeating the process over another cycle doubles the amount of DNA yet again. By repeating the process over several cycles (typically 20 to 30), the quantity of DNA becomes sufficient for further analysis.

One technique that has benefited particularly from the introduction of the polymerase chain reaction is that of HLA typing using sequence-specific oligonucleotide probes (SSOP). This technique relies on the fact that a probe of specific nucleotide sequence will bind only to an HLA molecule of complementary sequence. By testing a sample of DNA with several probes, each specific for a different allele or subtype, very precise HLA typing—to the level of a single nucleotide difference—can be achieved. Binding of the probe to its corresponding

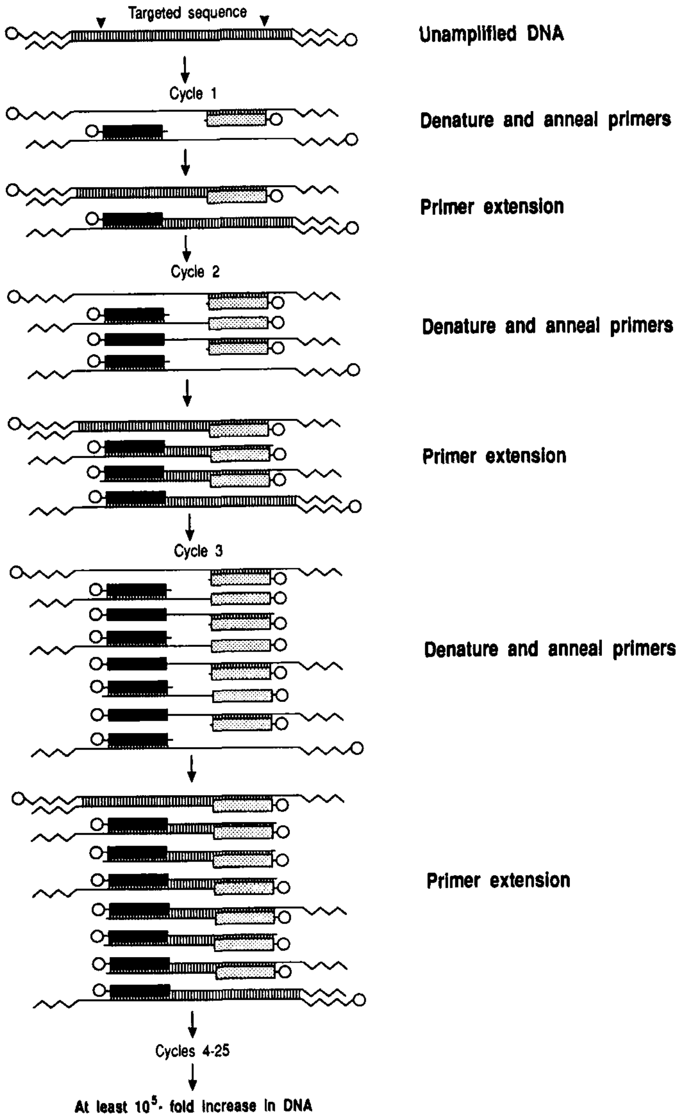

FIG. 10. The polymerase chain reaction. Target DNA is heated at 95°C and the two oligonucleotide chains become separated (denaturation). Two oligonucleotide primers (shown in black and shaded) with sequences corresponding to opposite ends of the target gene segment bind to the separate chains (annealing). In the presence of Taq polymerase and the four deoxyribonuclease triphosphates, the primers become extended along the target segment to form a complementary copy of the original DNA strand (primer extension). This process doubles the quantity of DNA. By repeating the process over a number of cycles the quantity of DNA increases exponentially (figure courtesy of the Perkin–Elmer Corporation).

sequence is visualized by incorporating a label into the probe. Originally this label was a radioisotope (e.g., ^{32}P) but nonradioactive probes such as biotin–avidin are becoming increasingly used.

3. HLA and the Immune Response

The normal immune system functions to identify foreign material entering the body and to bring about the destruction and removal of that material. The process through which this is achieved was elegantly proposed by Sir MacFarlane Burnet in his clonal selection theory of acquired immunity (B28). The central theme of this hypothesis is that an "immune surveillance" mechanism exists through which blood cells are able to differentiate normal body components ("self") from foreign material ("nonself") such as bacteria, viruses, parasites, and cancer cells. When nonself material is encountered, the host responds by generating multiple copies (or "clones") of a cell that is able to mount a specific immunological response to the foreign material.

The response to a foreign antigen is a multifaceted process involving both cell-mediated and humoral (plasma-mediated) components. Two populations of lymphocytes are involved, both originating from a common stem cell produced in the bone marrow. Stem cells that have passed through the thymus are known as *T lymphocytes* and are responsible for cell-mediated immunity. Cells that have passed through the lymphoid tissues of the gastrointestinal tract are known as *B lymphocytes* and are involved in the synthesis of circulating antibody. In avian species, there is a distinct organ, known as the bursa of Fabricius, in which stem cell processing takes place, and it is from the initial letter of "bursa" that B-lymphocytes derive their name.

T lymphocytes may be separated into two major functional subpopulations having either regulatory or effector function. Regulatory cells represent about 70% of all T lymphocytes in the peripheral circulation and are separated into "helper" cells and "suppressor" cells. The remaining T cells have an effector function and are known as "cytotoxic" cells.

3.1. Role of HLA Molecules in the Immune Response

A classic paper by Zinkernagel and Doherty in 1974 (Z1) showed that in order for T cells to recognize a foreign antigen, the antigen has to be presented by cells displaying the MHC antigens of the host. The phenomenon became known as "MHC restriction."

The recognition of a foreign antigen involves two families of cells: lymphocytes and macrophages. The antigen is first taken up and processed by a macrophage or monocyte that functions as an "antigen-presenting cell." A small peptide fragment

of the processed antigen becomes bound to the MHC molecules on the surface of the antigen-presenting cell and is expressed as a composite unit with the MHC molecule. This requirement for simultaneous recognition of antigen and MHC molecule ensures that T cells operate only when they come into intimate contact with another cell (Fig. 11).

Generally speaking, cytotoxic T cells recognize antigen in association with class I molecules, and helper T cells do so in association with class II molecules. These cells carry different antigens that are involved in the recognition process: CD4 in the case of helper cells, and CD8 in the case of cytotoxic cells. From among the vast repertoire of T cells possessed by the host, the cells recognizing the foreign structures on the antigen-presenting cells are identified. Clonal selection of these cells then takes place to produce large numbers of cells that react specifically with the foreign material.

4. The Immunological Basis of Transplant Rejection

The immunological defense mechanism that so ably protects against invading microorganisms and cancer cells also exerts its actions on foreign cells and tissues that have been intentionally transplanted for therapeutic purposes. The process

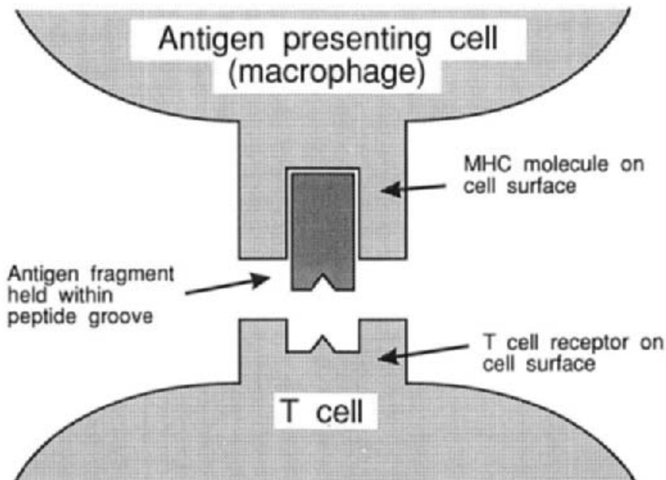

FIG. 11. Schematic diagram showing antigen presentation. Portions of foreign antigens are held within the peptide groove of the MHC molecule and are "presented" to T cells as a complex with the MHC molecule. T cells carry specific receptors on their surface that recognize this complex. The T cell cannot combine with the MHC molecule or the foreign antigen unless the two are presented as a complex.

through which a transplanted organ or tissue is destroyed by the immune system is known as "immunological rejection."

Except in a few isolated instances, such as when the recipient's immune system is grossly impaired or when the donor and recipient are identical twins, some degree of immunological rejection is a natural consequence of all transplantation.

An immunological basis for transplant rejection was described in 1938 when Peter Gorer discovered that mice possess a genetic system, later known as the H-2 system, that codes for serologically detectable alloantigens involved in determining tissue rejection (G14). However, it was Medawar's finding of accelerated rejection after repeated grafts from the primary donor in 1944 (M7) that led to his classic series of experiments and the evolution of our present ideas of transplant immunology (M8–M10).

4.1. Mechanisms of Graft Rejection

One of the most powerful factors leading to graft destruction is the presence of preexisting antibodies in the recipient to incompatible antigens on the donor tissue. Such antibodies are likely to occur following blood transfusion, in which the recipient becomes immunized by antigens expressed on the donor leukocytes and platelets; pregnancy, due to immunization of the mother by incompatible paternal antigens expressed on fetal blood cells; previous organ transplants, in which the recipient has become immunized against incompatible antigens in the previous graft; or deliberate immunization. In the present climate of AIDS and HIV infection, deliberate immunization is rare. However, it is interesting to note that deliberate immunization, particularly of laboratory staff, was commonly practiced in the early days of histocompatibility testing as a means of obtaining HLA antisera (e.g., Z2).

Destruction caused by preexisting antibodies is known as *hyperacute rejection.* The relationship between preformed antibodies and early rejection of cadaveric renal transplants was first recognized in the mid 1960s (K9, P1, W2). This type of rejection occurs very rapidly and, in the case of renal transplantation, is often seen while the patient is still on the operating table. The preformed antibodies precipitate in the walls of the small vessels of the graft, causing complement fixation and the production of neutrophil chemotactic factors. Neutrophils are attracted to the site and begin to degranulate and release cytolytic agents. Concomitant platelet aggregation results in coagulation and occlusion of the vessels leading to ischemia and eventual necrosis.

Fortunately, hyperacute rejection may be largely eliminated by cross-matching the patient's serum against the donor lymphocytes using a variation of the lymphocyte microcytotoxicity test. The patient's serum is placed in the wells of a tissue typing tray and donor lymphocytes are added. The cells are examined for lysis after an appropriate incubation period and the addition of complement. If

lysis is present it indicates that the patient's serum contains antibodies directed against the histocompatibility antigens of the donor and is a strong indication that hyperacute rejection would occur if an organ from that donor were to be transplanted into the patient.

Two other types of transplant rejection, known as *acute rejection* and *chronic rejection,* are due to cell-mediated immune mechanisms and occur at longer intervals after transplantation. These types of rejection cannot be predicted by cross-matching. The transplanted organ will often function normally for the first few days after transplant. During this time, however, the recipient's lymphocytes migrate to the graft, where they accumulate and rapidly proliferate. Monocytes are also attracted by chemotactic factors derived from the proliferating lymphocytes. The monocytes transform to macrophages, which incorporate part of the histocompatibility antigens of the graft and function as antigen-presenting cells. Damage to the graft results both from the production of cytotoxic factors during the ensuing immunological reaction and from thrombosis and ischemia caused by cellular accumulation and crowding of the small arteries.

4.2. Use of Immunosuppression to Reduce Graft Rejection

In many cases, the immunological events that lead to rejection of a transplanted organ may be abrogated by the use of immunosuppressive drugs. These drugs work on the basis that immunological rejection is a consequence of an increased production of immunologically activated cells reacting against the allograft. By preventing the production of immunologically active cells, the severity of the rejection process may be minimized. Until recently the drugs used for this purpose in renal transplantation consisted of azathioprine and prednisolone, occasionally in association with antilymphocyte globulin. These forms of immunosuppression, while effective in reducing the severity of rejection, often introduced a number of associated complications. Azathioprine is an antagonist to purine metabolism and appears to decrease nucleic acid synthesis. In this respect the drug effectively prevents the development of stimulated lymphoid cells but does so in a nonspecific manner. Hence, the drug is associated with generalized depression of hemopoietic cells and can lead to anemia, thrombocytopenia, and leukopenia with associated susceptibility to infection and malignancy. Prednisolone, while effectively depressing the inflammatory response, is associated with the well-known disadvantages of corticosteroids, including increased susceptibility to infection, Cushing's disease, hypertension, weight gain, etc.

More recently, a number of "new generation" immunosuppressants have become available without the disadvantages of the previous methods. Cyclosporin A was the first to be introduced (B18). This drug, originally isolated from a newly discovered fungal species, *Tolypocladium inflatum* Gams, is a highly lipophilic, cyclic endecapeptide (K2).

The immunosuppressive action of CyA results from its inhibitory effects on helper T-lymphocyte activation and proliferation by blocking the release from activated helper T lymphocytes of the growth factor, interleukin 2 (A8). The release of other lymphokines is also inhibited, including γ-interferon, B-cell-stimulation factor, and cytotoxic differentiation factor. Since helper T cells play an important accessory role in the allograft rejection process, their selective removal after transplant diminishes the severity of rejection. In this respect, CyA has the advantage over other types of immunosuppression that it is specific for helper T lymphocytes and does not exert an effect on other cells involved in immune responsiveness such as B lymphocytes, macrophages, and suppressor T cells. Despite the obvious benefits of CyA as an immunosuppressant, the drug has the important side effect of nephrotoxicity (K10), which has led to concerns about its long-term use. Myers *et al.* (M15) have recently reported a high incidence of renal failure in heart transplant recipients as long as 8 years after transplantation. On the positive side, however, Almond *et al.* (A3) studied renal function for up to 10 years in renal transplant recipients treated with CyA and found no evidence of progressive deterioration in renal function due to CyA nephrotoxicity.

Still more recent was the introduction of another immunosuppressive drug known as FK-506, which was first described in Japan in 1987 (K7, K8) and introduced for clinical use in 1989. The drug was discovered when a *Streptomyces* strain isolated from a soil sample from Tsukuba, Japan, was found to have powerful immunosuppressive properties (G15). Although differing considerably from CyA in structure, FK-506 acts in a similar way by inhibiting T-cell activation through interference with the production of interleukin 2 (S9).

Other immunosuppressive agents currently undergoing trials include Rapamycin, also derived from a *Streptomyces* species (D10), and RS-61443 (S13), which blocks the proliferative responses of T and B cells. These agents are still in the evaluation stage, but show exciting promise for the future as a means of minimizing both the degree of rejection following transplantation and the complications associated with prolonged immunosuppression.

Careful selection and matching of donors and recipients to reduce the genetic disparity between them is also of substantial benefit in minimizing the degree of rejection, and it is in this respect that HLA antigens play one of their most important roles in modern medicine.

5. HLA and Renal Transplantation

5.1. Role of HLA Matching in Determining Graft Outcome

Even in the middle to late 1960s, before the HLA-A and -B loci were fully identified, evidence was accumulating that improved graft outcome could be

obtained when recipients of kidney transplants from cadaveric donors were matched with their donors for the broad serological specificities known at that time (B5, M14, V2). As the HLA-A and -B antigens became better defined, most large studies from regional and national registries showed a strong correlation between HLA-A and HLA-B matching and graft survival. Dausset *et al.* (D3) showed a 70% 2-year survival of grafts identical for four HLA-A and -B antigens compared with 34% for those with one or zero identical antigens. Similar results were reported by other groups (e.g., F1, S8). Figure 12 depicts recent data from the Collaborative Transplant Study (discussed later), showing the effect of HLA-A and -B matching on the outcome of cadaveric kidney transplants in patients who have not been transplanted previously and who are treated with the immunosuppressive drug Cyclosporin A. Recipients with no mismatched antigens against their donor have the best outcome (67% graft survival at 5 years) and the survival decreases as the number of mismatched antigens increases.

Soon after the discovery that HLA matching improves transplant outcome, many centers found that matching for HLA-B antigens is more important than that for HLA-A antigens (D9, O2). Some reported no significant effect of matching for HLA-A antigens (e.g., D1) but the combined experience of most centers indicates

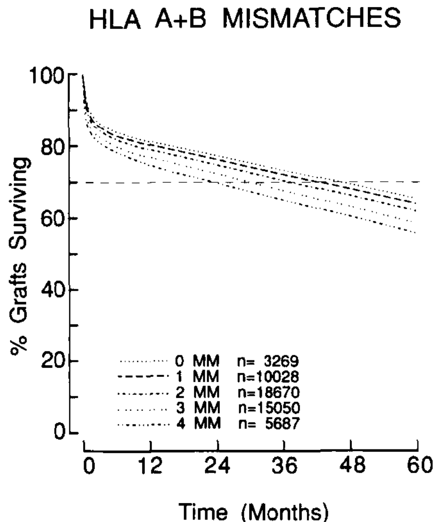

FIG. 12. Five-year graft survival in first cadaveric kidney transplant recipients according to the number of mismatched HLA-A and -B antigens between the donor and the recipient. The number of mismatched antigens (MM) and the number of recipients in each category are shown. Recipients with no mismatched antigens (0 MM) have significantly better outcomes than those with mismatches. All patients included in this analysis were treated with Cyclosporin A. From the Collaborative Transplant Study (G. Opelz, personal communication).

that HLA-A matching does play a role, albeit a weaker one than that of HLA-B. Matching for HLA-C, on the other hand, is generally acknowledged to be of little importance in determining transplant outcome (S12).

With the discovery of HLA-DR antigens in the late 1970s, many workers discovered that matching for these antigens in addition to HLA-A and -B has a more favorable effect on graft survival than matching for HLA-A and -B alone (e.g., A1, P2, T13, T14). Indeed, several reports showed that matching for HLA-DR antigens alone has a more beneficial effect than matching for HLA-A and/or HLA-B specificities (A9, G10) and some showed that matching for HLA-B and -DR is more advantageous (O4, O5). The superior role of HLA-DR matching is now generally accepted and many centers place more emphasis on HLA-DR matching than on matching for other antigens in view of the difficulty in matching donors and recipients for HLA-A, -B, and -DR antigens. However, the advantage of using a fully matched donor with no mismatched HLA-A, -B, or -DR antigens against the recipient cannot be overlooked. Particularly convincing in this respect are the results of large studies in which data are combined from multiple centers.

One such study is the Collaborative Transplant Study (CTS), which began in 1982 under the initiative of Professor Gerhard Opelz in Heidelberg, Germany. The study consists of an international, multicenter collaborative effort in which over 270 transplant centers in 40 countries send details to a central registry on all transplants performed at those centers. Baseline information is reported to the registry on tissue typing, immunosuppression, kidney preservation, etc., and the participating centers provide regular clinical updates on the outcome of the transplants. By early 1993 over 110,000 renal transplants with follow-up periods of up to 9 years had been recorded in the registry. An analysis of the effect of HLA-A, -B, and -DR antigen matching on graft outcome in over 30,000 first-time recipients of cadaveric kidney transplants in the CTS registry with at least 5 years follow-up is shown in Fig. 13. The figure shows a clear reduction in the percentage of grafts surviving at 5 years as the number of mismatched antigens increases from zero (67%) to six (48%). The correlation is statistically highly significant ($p < 0.0001$).

Because of the clear advantage of matching for six antigens, a program was started in 1987 by the United Network for Organ Sharing (UNOS) in the United States in which transplant centers maintain a nationwide register of patients requiring kidney transplantation. Whenever a donor is found with a six-antigen match with a patient on the national register, the kidney is shipped out for that patient. The 1-year graft survival for over 500 recipients of six-antigen-matched kidneys under this scheme is 89% (T3). These impressive results notwithstanding, the question of organ sharing remains controversial and is subject to vociferous debate. A rational evaluation of the issue is provided by Burdick and colleagues (B27).

In the case of living related transplantation, there is little argument that superior results are achieved when sibling donors and recipients share two haplotypes.

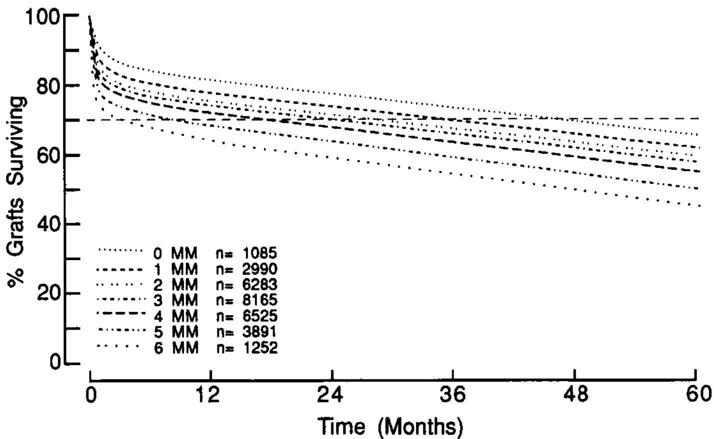

FIG. 13. Five-year graft survival in first cadaveric kidney transplant recipients according to the number of mismatched HLA-A, -B, and -DR antigens between the donor and the recipient. Numbers of mismatched antigens (MM) are shown as in Fig. 12. Recipients with no mismatched antigens (0 MM) have significantly better outcomes than those with mismatches. From the Collaborative Transplant Study (G. Opelz, personal communication).

Even when HLA matching was in its infancy, Descamps *et al.* in France reported that, of 33 renal transplants between HLA identical siblings performed between 1959 and 1972, all were functioning 1 year after transplant, and all except one retained a functioning graft for at least 4 years (D7). These successful results were confirmed in single-center studies (e.g., A6) and multicenter studies (e.g., O10). Although serious rejection can still occur when sibling donors are matched for two haplotypes (B11), sometimes resulting in loss of the graft (A6), these losses are rare and it is generally accepted that transplantation from a fully matched sibling donor is the most successful available.

5.2. Effect of Cyclosporin A on Renal Graft Outcome

The introduction of the powerful immunosuppressant Cyclosporin A (CyA) in the early 1980s had a revolutionary effect on renal transplantation.

The ability of CyA to prolong kidney graft survival was first shown by Calne and colleagues in the United Kingdom (C2, C3). Indeed, the powerful effect of CyA as an immunosuppressant led to several publications claiming that HLA matching no longer had a significant influence on graft survival (E3, T4, V1). Data

from the Scandiatransplant group showed no significant association between graft survival and closeness of HLA matching and resulted in a provisional halt to an exchange program based on HLA matching for nonsensitized recipients (L7).

These results are in marked contrast to those of the Collaborative Transplant Study. The CTS data have shown consistently that HLA matching confers significant benefits in determining graft outcome and that this benefit is amplified by the use of CyA (O5, O6, O9). Figure 14 clearly shows the effect of matching for HLA-DR antigens in patients treated with CyA and those treated without CyA. Similar results are obtained when the combined effect of matching for HLA-A and -B; HLA-B and -DR; and HLA-A, -B, and -DR is analyzed in patients treated with CyA and in those treated without. Whatever level of HLA matching is used, the use of CyA improves the outcome. The CTS results are supported by those of another large collaborative study organized by the Los Angeles group (C7).

5.3. Effect of Blood Transfusion on Renal Graft Outcome

In the first few years after renal transplantation had become an established form of therapy, blood transfusion of potential recipients was avoided as far as possible to prevent the development of antibodies against HLA antigens in the donor blood. This strategy became drastically changed after 1973 when Opelz and co-workers reported the paradoxical finding that survival of cadaveric renal allografts was

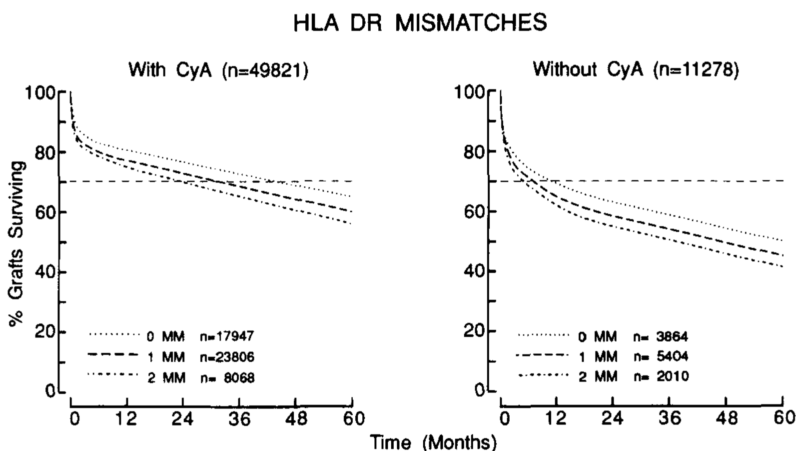

Fig. 14. Five-year graft survival in first cadaveric kidney transplant recipients according to the number of mismatched HLA-DR antigens and types of immunosuppressive therapy used. Numbers of mismatched antigens MM are shown as in Fig. 12. Patients with no mismatches have better outcomes than those with mismatches but the overall graft survival is higher in the patients treated with Cyclosporin A (CyA). From the Collaborative Transplant Study (G. Opelz, personal communication).

actually improved in patients who had received blood transfusions prior to transplant (O11). Over 400 publications in the scientific literature have since confirmed this finding.

There has been no general consensus on the actual mechanism involved but it is likely that transfusions produce a beneficial effect by inducing a state of immune unresponsiveness in the recipient. Terasaki (T5) proposed that transfusions act as a primary immunization event and that the transplant acts as a secondary stimulus. High-dose immunosuppression at the time of transplant prevents the formation of reactive cell clones and these specific clones are selectively deleted, thereby allowing the graft to remain intact. Fundamental questions still remained unanswered, however, such as the optimal number of transfusions, their timing, or the type of blood product transfused.

An additional dimension to the subject was introduced by Salvatierra *et al.*, who found that graft survival following blood transfusion in one-haplotype-mismatched recipients of living related transplants could be improved even further when the blood was obtained from the subsequent kidney donor (S4). Using a protocol of three donor-specific transfusions given at bi-weekly intervals prior to transplant, graft survival in one-haplotype-mismatched donor-recipient pairs was found to be similar to that obtained with HLA-identical sibling donors (S3). The practice of giving donor-specific transfusions carries with it the danger that the recipient will form antibodies against incompatible donor antigens and preclude subsequent transplantation. Salvatierra's group reported that 30% of patients receiving donor-specific transfusions developed antibodies against the donor (S2). However, some workers subsequently found that this high sensitization rate could be reduced by administering the transfusions together with the immunosuppressive drug azathioprine (A5, E1, G8).

The significant improvements in transplant outcome following the introduction of CyA in the early 1980s resulted in heated debates on the continued efficacy of pretransplant blood transfusions. A multicenter trial of the drug in Europe showed no evidence for a beneficial effect of pretransplant blood transfusions in patients treated with CyA, whereas transfused patients treated with other immunosuppressants showed better graft survival (E3). Several single-center studies have also shown that the transfusion effect is absent or even deleterious in recipients treated with CyA (A2, G1, H3, K1), leading Groth (G16) to conclude that there is no need to give blood transfusions as pretreatment for renal transplantation in the Cyclosporin era. However, two large collaborative studies (C9, O5) have continued to show a favorable effect of pretransplant transfusion on graft survival in patients treated with CyA, although the beneficial effect appears to be declining (O7). Pretransplant blood transfusion is not without risk, particularly that of sensitization and the transmission of infection, and many centers decided to discontinue the practice of elective transfusions and rely on the beneficial effects of CyA therapy alone. The experience gained from the "blood transfusion effect," however, has

taught us much about the way in which the immune system can be manipulated to improve graft survival.

5.4. The Role of Cross-matching

One of the most, if not *the* most, important tests performed by the HLA laboratory is the complement-dependent cytotoxicity cross-match to detect the presence of preformed antibodies in the recipient's serum against HLA antigens on the donor lymphocytes. The existence of such antibodies in the recipient almost always suggests that rapid rejection will occur if an organ from that donor is transplanted into that recipient. The role of preformed antibodies in determining the outcome of cadaveric renal transplantation was recognized in the mid 1960s when a strong association was found between a positive cross-match and a rapid failure of transplanted kidneys (K9, P1, W2). Since then, a positive cross-match has been considered a strong contraindication to transplantation. However, the approach to cross-matching has changed dramatically in recent years.

Not all antibodies detected by the complement-dependent cross-match are involved in transplant rejection. In particular, autoantibodies (especially in patients with systemic lupus erythematosus) and antibodies against B cells rather than T cells are of little relevance to transplantation (E2, T15). Since these antibodies are usually of the IgM class (L3), a number of techniques have been developed to determine whether the antibodies detected in the cross-match are IgM or IgG. Typically these techniques employ reducing agents such as dithiothreitol (DTT) or dithioerythritol (DTE) to block the reactivity of IgM antibodies by breaking the disulfide bonds of the IgM molecule (O1, O3). A positive cross-match before treatment of the serum with DTT or DTE and a negative cross-match afterwards provide strong evidence that the antibody is of the IgM class (B4, C11).

New techniques have also been introduced to increase the sensitivity of the standard cross-match and allow the identification of antibodies that are poor activators of complement. These techniques include extending the incubation times of the standard cross-match (T7) and the addition of an anti-human globulin step (F4, J3). The most sensitive assay, however, is a cross-match using flow cytometry, which does not involve complement fixation (G3).

The flow cytometry cross-match is similar in principle to the traditional serological method. Donor cells are first incubated with the recipient's serum. Instead of adding complement, however, a fluorochrome-labeled anti-human immunoglobulin is added. If a reaction occurs between antibodies in the patient's serum and antigens on the donor cells, the bound antibody will combine with the fluorescent anti-immunoglobulin and will be detected by the flow cytometer. Several workers have reported a higher incidence of graft loss in patients with a positive flow cytometry cross-match but negative cytotoxicity cross-match (G2, L2, M1) and the technique is becoming increasingly used in the evaluation of prospective renal

transplant recipients. By demonstrating the existence of weak antibodies that are not detectable using serological crossmatch methods, flow cytometry effectively identifies the recipient at special risk and provides an effective means of monitoring the patient's immune status after transplant (P5). Currently, flow cytometry is confined mainly to the evaluation of renal transplant recipients, but the technique also shows promise for cardiac transplantation (S11).

The introduction of more sensitive cross-match techniques has raised questions as to the level and types of antibodies that are relevant to clinical transplantation, and the optimal cross-match procedures necessary to improve graft survival (K6). These questions have been compounded by factors such as whether the recipient is undergoing a first or subsequent transplant (K5). In addition, events in recent years have completely changed the approach to the nature of the sera that should be used for cross-matching.

Most laboratories regularly screen for HLA antibodies in the sera of patients awaiting renal transplant, especially after blood transfusions. Until recently, most laboratories routinely cross-matched all sera found to possess antibodies whenever a potential donor was being considered. This was based on the traditional dogma that an individual maintains a permanent immunological memory of an immunizing event. Hence, if a transplant recipient has previously been immunized against a particular HLA specificity, a transplant from a donor possessing the same specificity would be expected to elicit a rapid anamnestic response leading to rejection of the graft. Cross-matching all previous sera found to possess antibodies, even if those antibodies had subsequently disappeared, was generally regarded as an appropriate precaution against graft rejection.

This approach changed in the 1980s when the University of Toronto transplant group reported that they had successfully transplanted cadaveric kidneys to a group of patients from whom serum collected previously showed a positive cross-match but in whom current sera gave a negative cross-match (C5). Other workers were soon able to corroborate these findings (G9, N2, S6). Various explanations have been proposed for this unexpected departure from classical understanding but the subject is still open to debate (G9). Of major benefit from the finding, however, have been the many highly sensitized patients who have received transplants when previously they would have been excluded on the basis of a positive historical cross-match.

6. HLA and Bone Marrow Transplantation

Allogeneic bone marrow transplantation was first attempted in the 1960s, usually as a last resort for immunodeficiency syndromes when other forms of treatment were unsuccessful. The results were singularly disappointing. In 1970, Bortin (B19) reviewed 203 bone marrow transplants reported in the scientific

literature until 1968 and showed that only a few presented clear evidence of engraftment leading to improvement or cure. However, the finding in 1968 that various immunodeficiency syndromes could be treated successfully using bone marrow transplanted from an HLA-identical or closely matched donor led to a resurgence of interest in this form of therapy (B2, D6, G4).

During the next 10 years, bone marrow transplantation became established as a viable treatment for severe combined immunodeficiency (B20) but benefited only a small minority of patients with acute leukemia (T11). In 1979, however, Thomas and colleagues in Seattle showed that the outcome of bone marrow transplantation for patients with acute nonlymphoblastic leukemia could be dramatically improved when the transplant was performed during the first remission rather than later in the course of the disease (T10). Another report in the same year, showed that severe aplastic anemia could also be successfully treated with bone marrow transplantation (C4). Since then, the results have improved progressively to the extent that bone marrow transplantation from an HLA haplotype-identical sibling is now considered the best chance of cure for many conditions, including immunodeficiency syndromes (F2), aplastic anemia (L4), and hematological malignant disorders, particularly chronic myelogenous leukemia (G11, T12). Other disorders that have been successfully treated with bone marrow transplantation include Fanconi's anemia (D5, H8), β-thalassemia (L6), and congenital cytopenias (e.g., Wiskott–Aldrich syndrome) (O13).

Leukemia, however, was the most common indication for bone marrow transplantation in the 1980s and accounted for 73% of all bone marrow transplants reported to the International Bone Marrow Transplant Registry (B21, B22). Data collected by the registry between 1980 and 1989 show a steady decline in transplant-related mortality from 48 to 36% over the decade and progressively improving leukemia-free survival—from 34% to 45% (B23). The last decade has also seen increased use of bone marrow transplantation in children following the introduction of the cytotoxic drugs busulphan and cyclophosphamide for preconditioning (S7), which avoids the adverse effects of total body irradiation on the growth and development of young patients (S5).

6.1. Significance of Graft versus Host Disease in Bone Marrow Transplantation

One of the major obstacles to successful bone marrow transplantation in children and adults is the problem of graft versus host disease (GVHD). This potentially life-threatening complication of bone marrow transplantation occurs when the suppressed immune system of the recipient (the host) is unable to recognize and mount an immune response against foreign histocompatibility antigens on the donor tissue (the graft). Instead of the grafted tissue being rejected by

the recipient's immune system, the graft is able to mount its own immune response against the histoincompatible recipient (B12).

Following extensive research into GVHD, Billingham identified three essential requirements for the condition to occur (B12):

1. the graft must contain immunologically competent cells,
2. the host must have transplantation antigens sufficiently different from those of the graft for the graft to be recognized as antigenically foreign, and
3. the host must be unable to mount an effective immunological reaction against the graft.

Since a major component of the conditioning regime for a bone marrow transplant recipient is complete inactivation of the recipient's immune system by total body irradiation or cytotoxic drugs, a major precondition for GVHD is satisfied by the bone marrow transplant recipient.

GVHD may be acute or chronic. Between 20 and 50% of patients receiving marrow transplants from HLA-identical siblings develop significant acute GVHD (S20). Patients may demonstrate various manifestations, ranging from a mild, transient, and self-limiting skin rash that does not require therapy to a severe necrotizing skin rash with severe gut and/or liver involvement that may be fatal. The intensity of disease is usually graded on a scale of I to IV developed by Thomas *et al.* (T9), in which the most severe disease is Grade IV.

Chronic GVHD may or may not follow the acute form of the disease. Sullivan and co-workers (S22) reported that chronic GVHD developed in 30% of recipients of allogeneic bone marrow transplantation who survived longer than 150 days. Clinically, chronic GVHD resembles an overlap of several collagen–vascular diseases with involvement of the skin, liver, eyes, mouth, upper respiratory tract, and esophagus (J2).

Since the treatment of severe GVHD is often unsuccessful, the most appropriate strategy is primary prevention of the condition. For this reason, most bone marrow transplantation to date has used HLA-identical siblings as donors. In Western countries, however, only about 30% of patients have an HLA-matched sibling (O12). In Asia the figure is slightly higher (H4). However, between 60 and 70% of patients who might otherwise benefit from a bone marrow transplant are deprived of the opportunity because no HLA-matched sibling donor is available. To overcome this problem, many centers have turned their attention to the use of HLA-matched unrelated bone marrow donors and partially matched family members.

6.2. The Use of Partially HLA-Matched Relatives as Donors

Pioneering work in the use of partially HLA-matched family members as bone marrow donors was reported in 1979 by the Seattle group (C14), who initially

reported transplants in 12 patients with acute leukemia and 9 with aplastic anemia in whom the donor was a sibling or parent with at least one matched haplotype. The successful results of this study led to a larger study of 105 patients who received transplants from partially HLA-matched family members. A comparison with 728 patients transplanted from genotypically identical siblings showed that as the HLA disparity between the donor and recipient increased, the risk of developing significant acute GVHD increased and the disease occurred appreciably earlier (B8).

In a later study of partially HLA-matched marrow transplant recipients, the Seattle group showed a 9% incidence of graft failure when the donor and recipient were mismatched for one HLA antigen and a 21% incidence when they were mismatched for two antigens (A4). These figures compared with a 2% incidence in recipients who had received grafts from matched siblings.

On the basis of these findings it may be concluded that transplantation from a partially matched relative has a distinct role as a treatment option when a fully matched relative is not available. But what is the solution for patients who have neither an HLA-identical sibling donor nor another relative with a one antigen mismatch? Many centers have begun to look outside the patients' immediate families for the answer.

6.3. The Use of Unrelated Bone Marrow Donors

The first bone marrow transplants using unrelated donors in modern times appear to be by Mathé *et al.* (M3), who treated survivors of a nuclear reactor accident in Yugoslavia. O'Reilly and colleagues (O14) subsequently used the technique to reconstitute the marrow of a patient with severe combined immunodeficiency. The first successful transplant for acute leukemia using HLA-matched marrow from an unrelated donor was reported by Hansen and colleagues in 1980 (H1). Successful results may also be obtained when unrelated donors are used for the transplantation of patients with chronic myelogenous leukemia (B6), although the results are less encouraging when the transplant is performed in the accelerated or blastic phase of the disease.

Bone marrow transplantation from an unrelated donor introduces a number of important considerations that do not apply to the related donor. Within a family there is a one in four chance that two siblings will inherit the same pair of HLA haplotypes from their parents. HLA haplotype identity is determined by typing the patient's siblings and, where possible, parents for at least their HLA-A, -B, and -DR antigens. In most cases, the haplotype configuration can be established from the family study and allows the haplotype-identical siblings, if any, to be identified. If two siblings are shown to be haplotype identical on the basis of HLA-A, -B, and -DR typing and a family study, then, by definition, they will be identical at all the intervening loci of the major histocompatibility complex,

including loci that were not tested. If one of these siblings acts as a bone marrow donor for the other, the risk of the patient developing GVHD is minimized because they are not mismatched for their major transplantation antigens.

When two *unrelated* individuals are shown to have identical HLA-A, -B, and -DR antigens, the most that can be said is that they are *phenotypically* identical. Their haplotypes are not derived from the same parents and may be quite dissimilar (see Fig. 15). Even if the haplotype configuration is the same in the donor and recipient, i.e., each haplotype of the donor codes for the same combination of HLA-A, -B, and -DR antigens as do the haplotypes of the recipient (and this could be established with certainty only by testing the relatives of the donor), there is no guarantee that the donor and recipient will be identical at all the intervening loci or for HLA-DQ or -DP. Moreover, serological typing for the HLA-A, -B, and -DR antigens is unable to demonstrate a number of allelic variations that are detectable by other techniques. HLA-B27, for example, is known to have at least six variants at the amino acid level (L5). Disparity between a donor and a recipient for a single antigenic variant differing by only one amino acid has been implicated in the rejection of a marrow graft (F3).

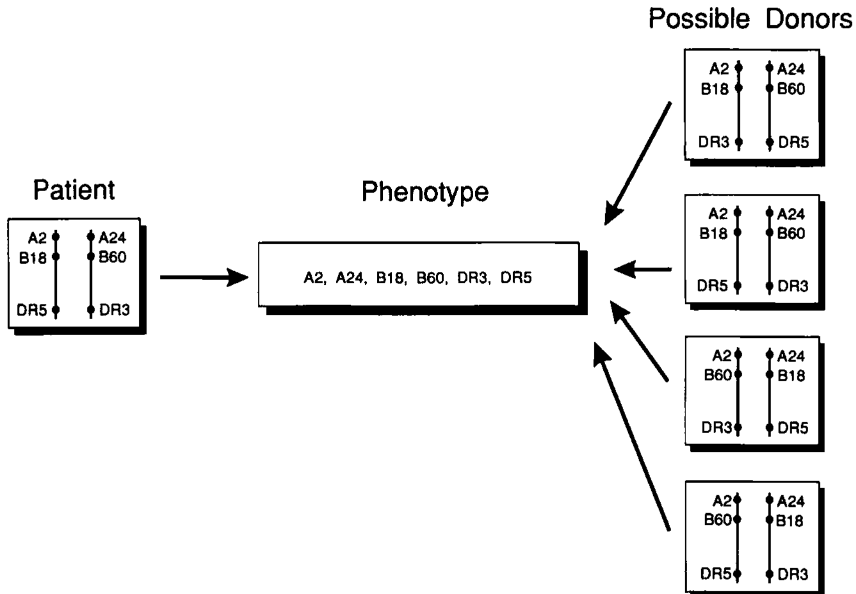

FIG. 15. An identical HLA phenotype in a patient and a potential donor does not imply that the patient and donor have identical haplotypes. In the example shown, donors of four possible haplotypic configurations could produce phenotypes corresponding to that of the patient.

Furthermore, patients receiving bone marrow transplants from unrelated individuals are more likely to be mismatched at minor histocompatibility loci than are their counterparts receiving grafts from HLA-identical siblings. The relative contribution of these loci to the development of GVHD and graft failure is not known. However, a number of reports have shown that severe GVHD is a major complication of unrelated marrow transplantation and occurs in 50% or more of recipients (A7, B9, G7, H9, M6). Despite this high incidence of GVHD in patients receiving marrow from unrelated donors, the Seattle group have shown that the probability of relapse-free survival is similar to that of patients receiving grafts from HLA-identical siblings (B9). A study in the United Kingdom, on the other hand, showed a higher incidence of graft failure and poorer actuarial survival in recipients of bone marrow from unrelated donors than that in recipients of bone marrow transplanted from HLA-identical siblings (H7). The conclusion from Seattle, nevertheless, is that marrow transplantation using matched unrelated donors should be considered in most cases where a matched sibling would have been used if such a sibling were available (B9).

Stimulated by both the success of transplants from unrelated donors and the urgent need for more, a number of unrelated bone marrow donor registries have been set up around the world in recent years. The first such registry was established in London in the early 1970s, when, following an intensive media campaign, a large number of volunteers came forward as potential bone marrow donors for a child with Wiskott–Aldrich syndrome. Unfortunately, the child died before a suitable donor could be found but the donor panel remained in operation for other patients requiring bone marrow transplantation (J1).

More recently, additional panels have been established or are being planned in Scandinavia (R4), the United States (M4, M5, N1), Canada (P4), Japan (H6), Hong Kong (B. R. Hawkins, unpublished), and Australia (R. L. Dawkins, personal communication). For maximum efficiency, these registries operate on a collaborative basis, whereby other registries will be searched if the patient's local registry is unable to find a matched donor. Currently, the HLA types of nearly a million volunteer donors are stored in registries worldwide.

A major consideration in setting up a registry is the overall probability that a donor can be found for a given patient. Estimates of such probabilities have been made both on the basis of theoretical calculations (S14) and on the success of actual searches for donors using existing registries. Probabilities based on computer-simulated searches for North American Caucasian patients show a 45% chance of finding an HLA-A-, -B-, and -DR-matched donor in a panel of 100,000 and a 32% chance of finding a donor who is also negative for mixed lymphocyte culture (B7).

These estimates, however, apply only when the potential recipient is of the same ethnic origin as the potential donors on the registry. Since the majority of potential donors on existing registries are Caucasian, the expectation of finding a matched

donor is significantly reduced if the potential recipient is of an ethnic origin in which the HLA antigen distribution is very different from that of Caucasians (H4, S21). Much effort is currently being put into the establishment of registries of non-Caucasian donors to overcome this difficulty.

Even when the potential recipient is of the same ethnic origin as the majority of donors on the registry, the probability of finding a match can extend over a broad spectrum. A potential recipient with a common Caucasian phenotype such as HLA-A1, -A2, -B7, -B8, -DR2, and -DR3 could probably find a match from as few as 500 Caucasian potential donors and would have virtually a 100% chance from a registry of 5000. Conversely, there undoubtedly exist patients with very rare phenotypes who would have difficulty finding a match in a registry of 1,000,000. Taking into consideration the effect of factors such as haplotype recombinations (Fig. 7) and racially mixed parentage (H4) (Fig. 16), it is the unfortunate reality that some potential recipients will not be able to find donors (B7).

7. Transplantation of Other Organs

Improvements in surgical technique and immunosuppression over the last two decades have greatly increased the scope of clinical transplantation beyond that of kidney and bone marrow. Heart transplantation was first performed at Stanford

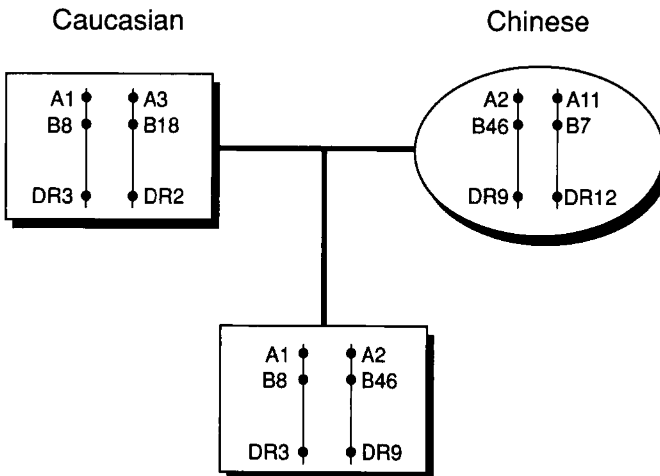

FIG. 16. Effect of racial mixing in producing rare HLA phenotypes. The Caucasian father has common Caucasian haplotypes. The Chinese mother has common Chinese haplotypes. The offspring, however, has inherited a phenotype that is extremely difficult to match in an unrelated donor.

University in 1968 (S19), but the results were originally disappointing, with only 29% of patients surviving 6 months or more. However, improvements in the early detection of rejection (C8) and the successful use of Cyclosporin A as an immunosuppressant (R3) transformed the procedure into the treatment of choice for many patients with idiopathic cardiomyopathies and ischemic disorders. As of early 1993, over 12,000 heart transplants had been reported to the Collaborative Transplant Study from 107 transplant centers throughout the world. Typical patient survival rates in a single center are 74% at 1 year and 42% at 5 years (L1).

Prospective HLA matching of heart donors and recipients is impractical because of the scarcity of suitable donors and the urgency with which some patients require a transplant. As a result, the question of whether HLA matching is of value in heart transplantation has been difficult to answer definitively. The first reported retrospective study showed little influence of class I matching in 204 recipients of cardiac transplants but showed at a marginally significant probability ($p = 0.05$) that grafts with one mismatched HLA-DR antigen had a better outcome than those with two HLA-DR mismatches (Y1). Another group reporting at around the same time studied 142 cardiac transplants with available HLA class I data and 70 with class II data and claimed a beneficial effect of class I matching and to a lesser extent class II matching (R1). A preliminary analysis of data submitted to the Collaborative Transplant Study had the benefit of more donor–recipient pairs with good HLA matches than would be possible in a single-center study (O8). The analysis of 73 transplants with less than 2 HLA-B and -DR mismatches compared with 1340 with 2 or more mismatches showed a clear but statistically nonsignificant increase in survival at 12 months in the better matched grafts. Similarly, transplants with no HLA-DR matches had better outcome than those with one or two mismatches but the data were not statistically significant (O8). Clearly, more data are required before the role of HLA matching in heart transplantation can be determined with certainty.

Liver transplantation is another example in which prospective HLA matching is impractical because of the urgency of the procedure. Human orthotopic (i.e., "in the right place") liver transplantation was first performed in 1963 (S18) and, with the introduction of Cyclosporin A (S17), has become increasingly successful for patients with certain liver diseases (e.g., cirrhosis and some malignancies) that were previously fatal within a few months. Between 1968 and the end of 1988, 4972 transplants had been recorded in the European Liver Transplant Registry and 4126 in the UNOS registry in Pittsburgh (G13).

Unlike in some experimental animals (C1, K3), there appears to be no immunological privilege associated with liver transplantation in humans. Acute rejection is therefore a major problem. Curiously, antibody-mediated hyperacute rejection is of less concern and is not an inevitable consequence of transplantation with a positive cross-match or an ABO-incompatible liver (I3). This does not imply, however, that ABO incompatibility and positive cross-matches are not

important. Most liver transplants reported to the UNOS Liver Transplant Registry are performed between donors and recipients who are ABO-compatible (D8), and there is evidence that patients with positive cytotoxicity cross-matches have a poorer outcome (T2).

As in the case of heart transplantation, retrospective HLA matching of liver donors and recipients shows that well-matched grafts are generally associated with a lower risk of rejection but not necessarily with improved graft survival. A definitive evaluation of the benefits, if any, of HLA matching in liver transplantation will require substantially more data than are available at present.

A third form of transplantation that has increased in usage in recent years is that of the pancreas. Unlike liver and heart transplantation, which are life saving, pancreas transplantation is currently performed to improve life rather than save it. The procedure is intended to restore normal glucose metabolism and prevent the secondary sequelae of diabetes, particularly nephropathy, neuropathy, and retinopathy. The transplant may consist of the entire organ, a segmental graft, or separated islets of Langerhans. Pancreas transplantation is usually performed in patients who already have diabetic complications and is often performed in association with a renal transplant.

HLA matching, at least for HLA-DR, appears to play a role in pancreas transplantation. Over 2700 transplants were reported to the International Pancreas Transplant Registry between 1966 and 1990, representing the vast majority of all pancreas transplants performed throughout the world (S23). Grafts mismatched for zero HLA-DR antigens have significantly better survival than those mismatched for one or two antigens (S23), although a similar effect of HLA-A and -B mismatching is not apparent (S24). There are, however, many variables to consider, such as the type and duration of preservation, recipient condition, surgical factors, type of immunosuppression, etc. As with heart and liver transplantation, considerably more data will be required before the relevance of HLA matching in pancreas transplantation is fully known.

8. Future Developments in HLA and Transplantation

Renal transplantation as a curative therapy for end-stage renal disease evolved in the 1950s and 1960s in parallel with the development of tissue typing. The finding that the outcome of cadaveric kidney transplantation is related to the number of HLA antigens in common between the donor and the recipient firmly established the importance of the major histocompatibility complex in clinical transplantation. While this important role is generally accepted, the role of traditional HLA matching in transplantation has become open to question recently (K4, K16, M13).

Traditionally, donor–recipient matching has been performed on the basis of serological typing. However, it is well known that some kidney transplant recipients achieve excellent long-term graft function in the face of five- or six-antigen mismatches against their donors. Similar results are seen with heart transplant recipients in whom HLA matching is not normally used in the selection process and who would be expected on probability alone to be poorly matched against their donors. Even in the case of six-antigen-matched renal transplants, the matched loci are normally restricted to HLA-A, -B, and -DR since few centers assign kidneys on the basis of HLA-DQ and -DP. Given that some donors who are "well matched" by conventional criteria have poor graft outcome and some who are "poorly matched" have good outcome, the question naturally arises whether the conventional matching criteria are adequate. The introduction of DNA technology in recent years provides evidence to suggest that these criteria are not adequate.

First, and perhaps most importantly, what is detected and matched using conventional serological techniques does not represent a biological reality. Serological tests rely on the recognition of intact HLA molecules by alloantibodies. When a transplanted organ is evaluated by the host immune system, however, it is epitopes on the HLA molecule that function as immunogens and that are the target of the immune response (M2, R2). These epitopes are not recognized in isolation, but in conjunction with the host's own MHC antigens. Second, the allelic variability in HLA class I and class II molecules shown by biochemical and DNA techniques greatly exceeds that detected by serological methods. As described in Sections 2.1 and 2.2.1, at least 10 different alleles code for the serological specificity HLA-A2, for example, and 13 code for HLA-DR6. For the most part, these allelic variations cannot be detected serologically and it is often impossible to claim "identity" between donor and recipient for these specificities on the basis of serological testing alone.

Against this background, it is clear that some degree of mismatching is inevitable between an unrelated donor and a recipient, even when they show a six-antigen match at the serological level. Conversely, the successful outcome of some grafts that are regarded as poor matches on the basis of serological testing suggests that there is some level of mismatching which is acceptable for transplantation purposes. Terasaki's group in Los Angeles has developed this argument and is attempting to identify the important epitopes of the various HLA specificities in terms of their amino acid sequences and their relation to renal graft outcome (T8). If it can be shown conclusively that mismatches for certain epitopes are of less importance than others in determining graft outcome, we could perhaps look forward to a time when organs are allocated to recipients on the basis of "intelligent mismatches" rather than on the number of serologically defined antigens in common as is performed at present.

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GLUTATHIONE *S*-TRANSFERASES: BIOMEDICAL APPLICATIONS

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1. Introduction

The glutathione *S*-transferases (GST) (EC.2.5.1.18) are ubiquitous enzymes found in bacteria, yeast, nematodes, insects, fish, birds, and mammals. They constitute a complex supergene family that collectively metabolizes chemotherapeutic drugs, carcinogens, environmental pollutants, and a broad spectrum of other harmful foreign compounds (xenobiotics). Because these enzymes play a pivotal protective role against xenobiotics, variations in their levels of expression can have profound effects on susceptibility to chemical insult. Overexpression of GST by tumors is associated with resistance to anticancer agents and represents an important mechanism of acquired drug resistance. The induction of hepatic GST by antioxidants, or other anticarcinogenic xenobiotics, enables experimental animals to tolerate exposure to carcinogens. Conversely, the absence of particular isoenzymes in humans, a result of genetic polymorphism, has been linked to increased risk of carcinogenesis in certain individuals; such GST represent susceptibility markers.

Glutathione *S*-transferases are abundant enzymes of modest size. The fact that GST are present at high levels in the liver ensures that their concentration in plasma provides an exceptionally sensitive index of hepatocellular damage. The expression of certain isoenzymes is limited to particular tissues and their measure-

ment in plasma should reflect damage to specific organs. Besides the utility of these enzymes as markers of active tissue damage, a unique GST is expressed in many tumors and this isoenzyme represents a useful marker of neoplastic transformation.

Although measurement of these enzymes has important applications in the fields of hepatology, oncology, pharmacology, and toxicology, it should be recognized that much basic research into the genetics, the structure, and the regulation of GST remains to be undertaken. Undoubtedly, additional isoenzymes have yet to be isolated and further functions of GST will be discovered. The aim of this article is to provide a broad understanding of the biochemical and molecular properties of GST as well as provide an overview of the value of GST measurements. Areas of future research and development that are worthy of investigation will be discussed.

2. The Multiple Activities and Pseudonyms of GST

Whereas the function of GST is considered primarily to be one of detoxifying foreign compounds, these enzymes possess activities other than catalyzing the formation of glutathione–xenobiotic conjugates. For example, GST exhibit peroxidase activity toward organic hydroperoxides and serve to combat oxidative stress. Certain isoenzymes possess ketosteroid isomerase activity, catalyzing the conversion of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids. Besides their catalytic properties, the GST can bind numerous nonsubstrate ligands. Most ligands are hydrophobic and are bound noncovalently by GST. However, a number of reactive metabolites formed from carcinogens, such as *N,N*-dimethyl-4-aminoazobenzene, are bound covalently by GST; the major binding protein was designated ligandin. The binding properties of the transferases have led to the suggestion that GST may act as intracellular transport proteins or receptors, and in this context it is noteworthy that the non-histone protein BA, isolated from chromatin-associated nuclear proteins, has been identified as a GST. A consequence of the multifunctional properties of the transferases is that they have appeared in the scientific literature under several guises, namely, selenium-independent glutathione peroxidase, ketosteroid isomerase, ligandin, and non-histone protein BA.

3. Historical Perspective

A chronological listing of the major advances in research into the glutathione *S*-transferases is summarized in Table 1.

TABLE 1
HISTORICAL OVERVIEW OF GST RESEARCH

1961	Demonstration of GST activity
1973	First purification schemes for GST
1974	Ligandin (Y protein) identified as a GST
1976	Selenium-independent glutathione peroxidase is a GST
1977	Use of SDS–PAGE to identify Ya, Yb, and Yc GST subunits
1979	Ligandin activity attributed to the Ya-type subunit
1981	Proof that distinct GST subunits can hybridize, forming heterodimers
	Demonstration of polymorphic expression of GST in humans
1982	Isolation of a unique microsomal GST
1984	First full-length cDNAs encoding GST described
	Expression of pi-class GST in hepatic preneoplastic nodules
1985	Overexpression of GST in drug-resistant cells lines
	Alpha-, mu-, and pi-class GST families defined
1986	Association between absence of mu-class GST and susceptibility to lung cancer
1988	Identification of a bacterial GST responsible for resistance to the antibiotic fosfomycin
1990	Identification of novel cis-acting elements in flanking regions of GST genes that respond to xenobiotics
1991	Theta-class GST characterized
	X-ray crystallography of GST

3.1. ENZYMOLOGY OF GST

The GST were originally studied because of their involvement in the mercapturic acid biosynthetic pathway. The conjugation between reduced glutathione (GSH) and xenobiotics represents the first of four steps leading to the formation of mercapturic acids; subsequent steps involve (1) the removal of the γ-glutamyl moiety from the glutathione conjugate by γ-glutamyltransferase, (2) the enzymic removal of glycine from the cysteinyl glycine conjugate, and (3) the N-acetylation of the cysteine conjugate by an acetyl-CoA-linked acetylase. The final *N*-acetylcysteine thioethers, or mercapturic acids, produced by the enzymes that catalyze this pathway are water soluble and readily excreted from the body.

Reports of urinary mercapturic acids first appeared in the late 19th century and arose from the work of two independent research groups that were both investigating the metabolism of monohalogenobenzenes; the metabolism of bromobenzene (see Fig. 1) was being studied by Baumann and Preusse (B9) and the metabolism of chlorobenzene was being studied by Jaffe (J1). However, the source of the N-acetylated cysteine moiety within mercapturic acids remained controversial for 80 years until Bray *et al.* (B41) demonstrated conclusively that it was derived from reduced glutathione (GSH). The existence of an enzyme(s) in rat liver cytosol responsible for catalyzing the conjugation of GSH with foreign compounds was

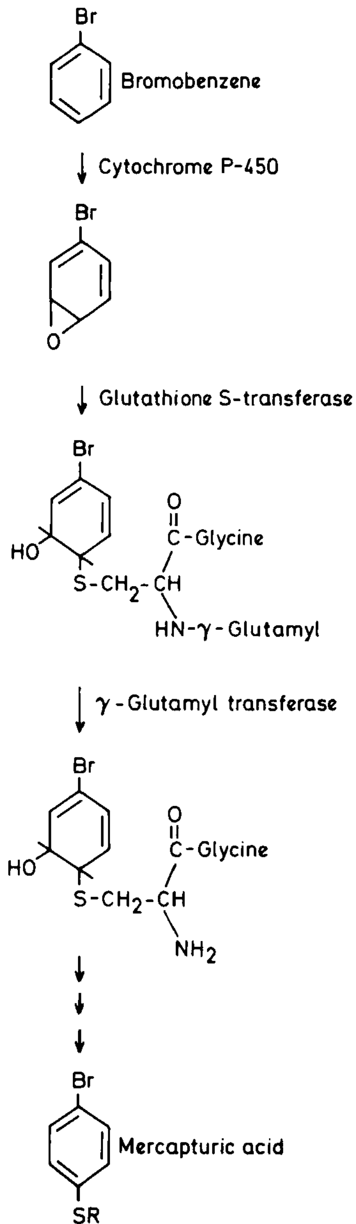


FIG. 1. Contribution of GST to the metabolism of bromobenzene.

reported first in 1961 (B36, C17). In these original reports of GST, the conjugation of GSH with 1,2-dichloro-4-nitrobenzene as well as with bromosulfophthalein was described. However, by the end of the 1960s at least 50 GST substrates had been identified (B40). At this stage it had become apparent that GST activity was represented by a number of distinct isoenzymes and attempts to classify the enzymes by the chemical structure of the electrophilic substrates resulted in the use of such terms as aryl transferase, alkyl transferase, aralkyl transferase, alkene transferase, and epoxide transferase (B40). A number of the model substrates that have been used to measure GST activity are shown in Fig. 2.

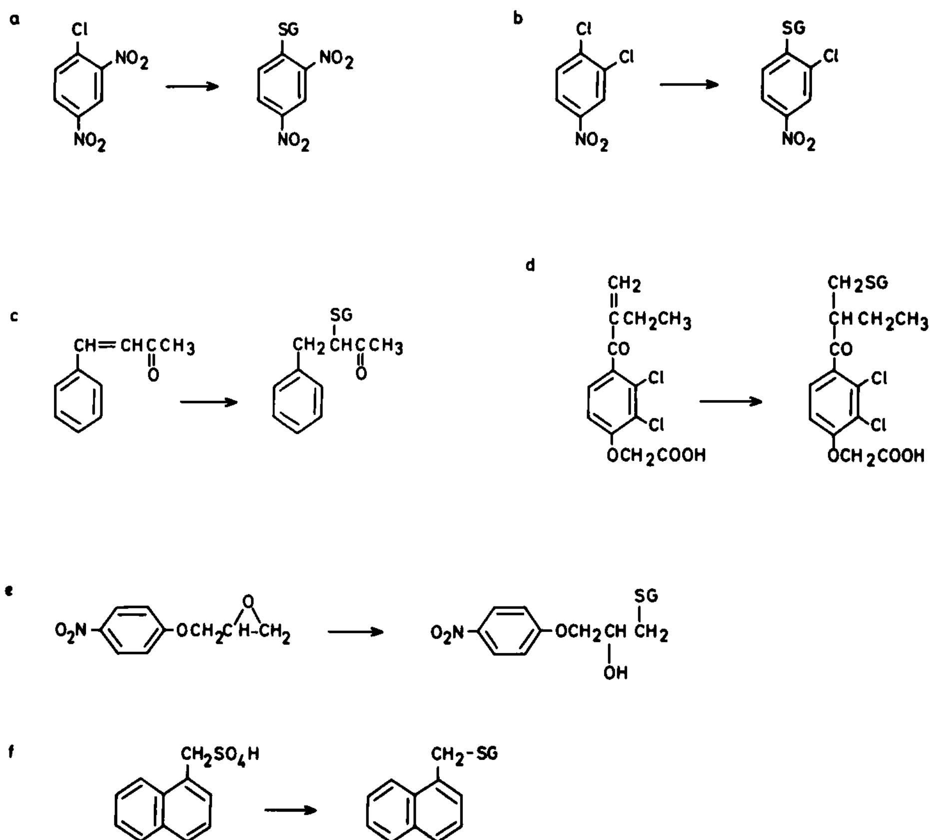


FIG. 2. Metabolism of model substrates by GST. The following reactions are catalyzed by GST: (a) 1-chloro-2,4-dinitrobenzene, (b) 1,2-dichloro-4-nitrobenzene, (c) *trans*-4-phenyl-3-buten-2-one, (d) ethacrynic acid, (e) 1,2-epoxy-3-(*p*-nitrophenoxy)propane, and (f) menaphthyl sulfate.

The inadequacy of nomenclatures based on substrate specificity became apparent when homogeneous preparations of the individual separate isoenzymes were analyzed and shown to exhibit overlapping activities toward many of the substrates. Much of our knowledge of the properties of GST has stemmed from studies of these enzymes in the rat. The first purification schemes for rat GST were reported by workers in Jakoby's laboratory (F12, H5) and the first purification of human GST by Kamisaka *et al.* (K1) resulted from a collaboration between the laboratories of Jakoby and Arias; at this stage the designations given to separate GST were based on their chromatographic properties (i.e., rat GST E,D,C,B,A and AA were designated by their elution order from CM–cellulose); however, more recent nomenclatures are based on the subunit compositions of the individual isoenzymes.

The purification scheme reported by Habig *et al.* (H4, H5) was of particular significance because it provided a systematic method of defining GST and enabled the multifunctional properties of these enzymes to be examined in more detail. In addition to their ability to form glutathione conjugates (see Fig. 2), GST were shown to be able to catalyze diverse reactions, including thiolysis, the reduction of organic nitrates, the isomeration of a ketosteroid, and the reduction of organic hydroperoxides (see Fig. 3). Marked differences in the specificity of the various isoenzymes were noted by Habig *et al.* (H4, H5) and, indeed, with the benefit of hindsight, the data of these workers show the existence of several catalytically distinct families of enzyme. For example, the GST that were found to utilize DCNB and/or tPBO were inactive with cumene hydroperoxide (and vice versa). Also, the enzyme with the highest activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane was inactive with DCNB, tPBO, or 1-chloro-2,4-dinitrobenzene (CDNB). Having developed a systematic purification scheme for the transferases, Jakoby and his colleagues were in a position to compare individual GST isoenzymes (and derived antisera) with proteins that were considered likely to represent GST that had been prepared by other laboratories. Hence, following the demonstration by Kaplowitz *et al.* (K3) that the organic anion-binding protein ligandin was a GST, Habig *et al.* (H3) reported that ligandin activity is attributable to a specific isoenzyme, namely GST B. Similarly, those GST that possess selenium-independent glutathione peroxidase activity (P13) and Δ^5-3-ketosteroid isomerase activity (B24) were identified by their chromatographic behavior (the reactions are shown in Fig. 3). Although these early pioneering purification studies were of considerable merit, the fact that certain GST homodimers were not recovered subsequently produced considerable confusion in the literature. For example, the homodimer with the greatest ligandin-type activity and the highest Δ^5-3-ketosteroid isomerase activity was not recovered by Habig *et al.* (H4, H5) and these activities were attributed solely to the heterodimer GST B. Failure to recover all the GST homodimers from rat liver also served to obscure the basis for the overlapping substrate specificities of the isoenzymes.

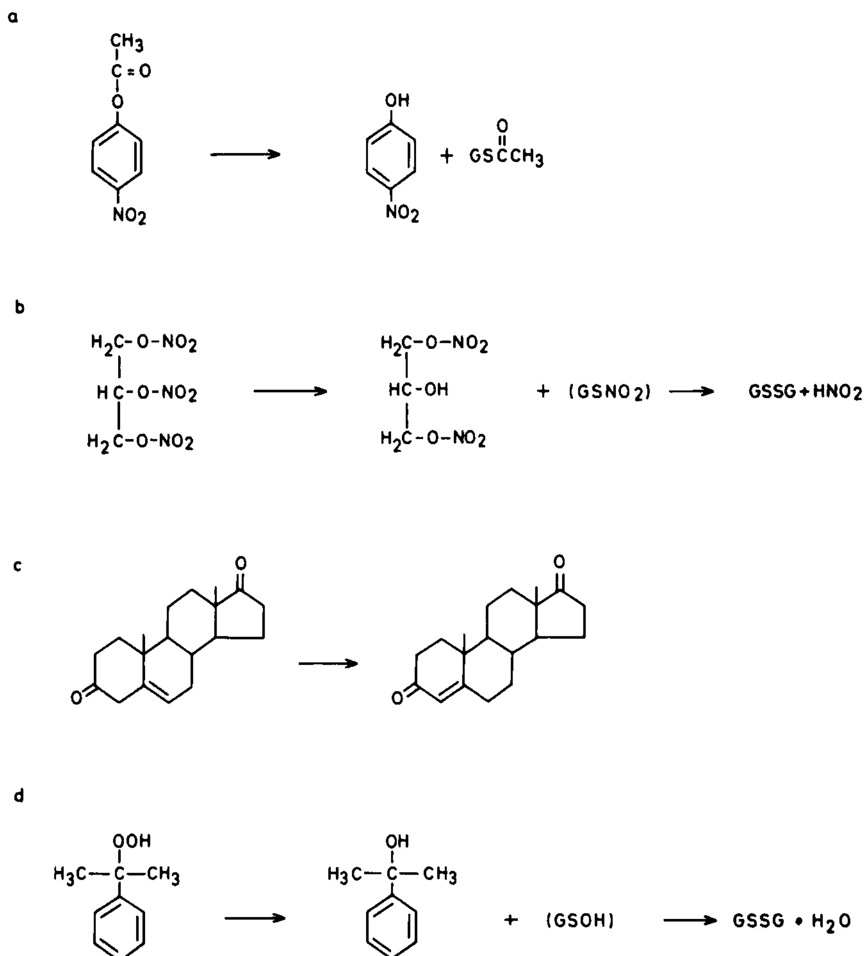


FIG. 3. Various types of reaction catalyzed by GST. Besides the formation of thioethers, GST catalyze thiolysis, denitrozation, isomerization, and peroxidase-type reactions (see text). As examples of such reactions, the metabolism of *p*-nitrophenyl acetate, nitroglycerine, Δ^5-androstene-3,17-dione, and cumene hydroperoxide are shown in a, b, c, and d, respectively.

3.2. STRUCTURE OF GST

The GST are dimeric and the two subunits that each enzyme comprises function independently. Consequently, knowledge of the subunit composition of GST is fundamental to understanding the reason for their overlapping activities. Early work on purified GST focused on enzymology, not on the structural relationship

among the GST isoenzymes (A22, H5, P1). Surprisingly, analyses of different rat ligandin preparations by SDS–PAGE (B5, C5, H28, L11) gave the most important insight into subunit heterogeneity among GST isoenzymes. Using SDS–PAGE, Bass *et al.* (B5) identified three electrophoretically distinct bands, the Ya, Yb, and Yc polypeptides, in eluate from Sephadex gel-filtration columns of rat hepatic cytosol containing proteins of 45–55 kDa [i.e., the Y fraction; see Levi *et al.* (L5) for the definition of the Y designation]. Although not pure, the Y fraction is enriched with GST activity and subsequent analysis of individual GST isoenzymes by SDS–PAGE showed that they comprise dimeric combinations of Ya-/Yc-type subunits or Yb-type subunits (H28, H29, S10). These electrophoretic data provided a structural basis for dividing GST into distinct families. Moreover, SDS–PAGE showed that individual isoenzymes within a given GST family may share a common subunit and that the overlapping activities of distinct isoenzymes is often due to the presence of a specific subunit common to two, or more, GST. For example, ligandin and Δ^5-3-ketosteroid isomerase activities can be attributed to the Ya-type subunit (H30, M1, O4).

The first demonstration that GST heterodimers do indeed represent hybrid enzymes came from the reversible denaturation analysis of rat GST YaYc described by Hayes *et al.* (H30), in which the subunits were dissociated with urea and, following reassociation of the subunits, by dialysis, the products (namely YaYa, YaYc, and YcYc) were separately recovered by ion-exchange chromatography. Confirmation of this result was provided by Kitahara and Sato (K11), who used guanidinium chloride to dissociate rat YaYc. Subunit hybridization is not a phenomenon limited to the rat; Stockman *et al.* (S35) have shown that human GST subunits can also form hybrids. It is now recognized that hybridization among a limited number of GST subunits accounts for the large number of GST isoenzymes that are found in most mammalian species. For example, hybridization among Yb_1, Yb_2, and Yn subunits in rat liver leads to the formation of Yb_1Yb_1, Yb_1Yb_2, Yb_2Yb_2, Yb_1Yn, and Yb_2Yn (H13, H14). Boyer *et al.* (B38) have shown that only subunits within the same gene family can hybridize.

Following the recognition that the Ya-/Yc-type and the Yb-type subunits in rat liver represent two separate families of GST, reports also began to appear of an abundant extrahepatic GST comprising subunits of a type distinct from those of the normal liver isoenzymes (G14, G15, H14, H26, T14). This enzyme, which represents a third family of cytosolic GST, has been extensively studied because, although it is not found in normal liver, it is expressed in rat liver tumors (J7, K12, M15, S8, S9). Since this enzyme is found in placenta, it has been called GST P and the subunits that it comprises have been designated Yp by Kitahara *et al.* (K12); the Yp subunit has also been called Yf as it migrates faster during SDS–PAGE than Ya (H14). The Ya, Yb, Yc, and Yf bands are indicated in the SDS–PAGE gel shown in Fig. 4.

Throughout the 1980s, many reports of additional cytosolic GST appeared in

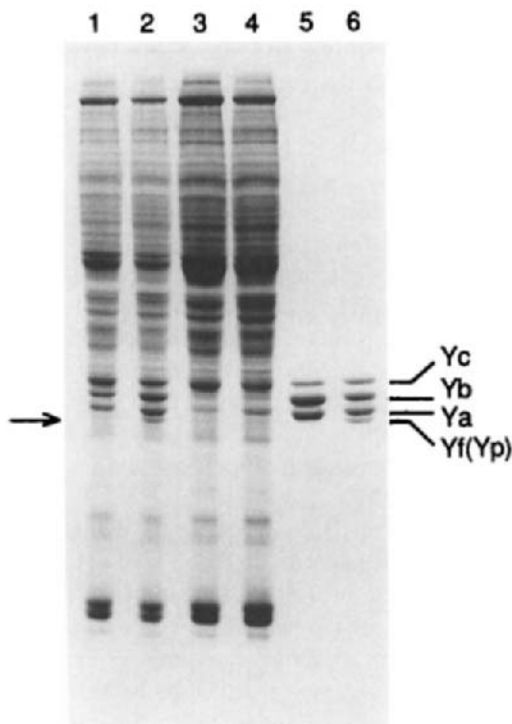


FIG. 4. Identification of GST subunits in rat liver cytosol. SDS–PAGE was performed in a 12% polyacrylamide resolving gel. Samples were run from the cathode (top) to the anode (bottom) and the gel was stained with Coomassie brilliant blue. The fractions are as follows: (1) liver cytosol from normal rats, (2) liver cytosol from nodule bearing rats that had been fed aflatoxin B_1 (2 ppm) for approx 16 weeks, (3) hepatic cytosol from normal rats that did not bind the glutathione–Sepharose affinity matrix, (4) hepatic cytosol from aflatoxin B_1-treated rats that was not retained by the glutathione–Sepharose affinity gel, (5) affinity-purified GST from normal rat liver, and (6) affinity-purified GST from rat livers that contained aflatoxin-induced preneoplastic nodules. The positions of the Ya, Yb, Yc, and Yf subunits are shown. The horizontal arrow adjacent to track 1 shows the position where the Yf (or Yp) would migrate if present. These data are taken from Hayes *et al.* (H23).

the literature, studies that were facilitated by the availability of good affinity matrices for the purification of these enzymes (G15, M5, S24). However, one of the more surprising findings was the isolation of a unique GST, present at high levels in the endoplasmic reticulum and the outer mitochondrial membrane, that is distinct from the cytosolic forms (M23). This enzyme, the microsomal GST, is unusual in that its activity is activated 15-fold upon treatment with the thiol modifying agent *N*-ethylmaleimide (M20).

3.3. Genetics and Regulation of GST

Molecular cloning of GST represented a major step forward in the understanding of the structure and genetic diversity of these enzymes. Much of the original work in this area was undertaken by the laboratories of Pickett and of Tu, who between them provided definitive proof that the Ya and Yc subunits are members of a gene family that is separate from that encoding Yb subunits (L1, L2, P8, T7). The third gene family of GST was identified by Muramatsu and his colleagues, who undertook cloning of the cDNA encoding Yp (S46). Mannervik *et al.* (M2), recognizing that these three families of GST identified in the rat were also represented in the human and the mouse, proposed that the designations alpha, mu, and pi class be adopted as a unifying system of nomenclature for the GST gene families of all mammalian species; hence the Ya/Yc, the Yb, and the Yp (or Yf) subunits represent alpha-, mu-, and pi-class GST, respectively. More recently, a fourth cytosolic family of GST has been identified, called theta (H8, H46, M17), and a cDNA encoding one of its members has been described by Ogura *et al.* (O2). Structural analysis of the microsomal GST demonstrated that this enzyme is genetically distinct and shares no sequence homology with the four cytosolic GST gene families (D3, M22). Present evidence suggests that the cytosolic enzymes have a common evolutionary origin and that the ancestral gene, from which they diverged, was possibly more closely related to the theta-class genes than to the alpha-, mu-, and pi-class genes. By contrast, the microsomal GST appears to have arisen through a distinct evolutionary route. For information, the designation of the different rat GST subunit types of the various gene families is presented in Table 2.

TABLE 2
Rat Cytosolic GST Subunits and Gene Family Designations

Y subunit or other designation	Apparent M_r (by SDS–PAGE)	Family
Ya	25,500	Alpha
Yc	27,500	Alpha
Yk	25,000	Alpha
Yb_1	26,300	Mu
Yb_2	26,300	Mu
Yn	26,000	Mu
Yo	26,500	Mu
Yf (or Yp)	24,800	Pi
Yrs (GST M)	26,000	Theta
GST E	—	Theta

Source. Data taken from Hayes (H15), Hiratsuka *et al.* (H46), and Meyer *et al.* (M17).

The regulation of GST is complex; they are subject to developmental control, their expression is tissue specific, and they are inducible by many drugs (B23, H26, L8, M12, S40, S44). The mechanisms responsible for the induction of GST by treatment with foreign compounds currently represent an active area of research that is likely to have important implications for other inducible drug-metabolizing and GSH-dependent enzymes. Pickett and his colleagues have identified two cis-acting elements in the 5′ flanking region of the rat Ya subunit gene. One of these elements responds to planar aromatic compounds, such as dioxin, and is identical to the XRE found in the cytochrome P450 1A1 gene (R14), whereas the other element, designated the ARE, responds to phenolic antioxidants (R13); the ARE has also been identified in the NAD(P)H:quinone reductase gene (F3) and is present in the γ-glutamyl transpeptidase gene (T. H. Rushmore and J. D. Hayes, unpublished observation). Friling *et al.* (F16, F17), studying induction of the murine Ya subunit gene by xenobiotics, have identified an element that responds to electrophiles (the EpRE). Interestingly, this element comprises two adjacent, nonidentical, 9-base-pair motifs, both of which are related to the ARE and the TRE (this element was originally identified as being responsive to the tumor promotor phorbol 12-*O*-tetradecanoate 13-acetate). Regulation of the rat pi-class GST gene has been studied by Muramatsu and his colleagues, who have shown that its derepression during hepatocarcinogenesis is mediated by an enhancer, called GPE1, which consists of two TRE-like elements (O5, S1). The Yp subunit gene also contains several negative elements, approximately 400 bp upstream from the cap site, that appear to regulate basal expression of this GST (I2).

Little is known about the regulation of the mu-class, the theta-class, or the microsomal GST at the molecular level and it is clear that much more work must be undertaken before we have a complete understanding of the mechanisms responsible for the control of expression of these enzymes.

3.4. Molecular Enzymology

The catalytic mechanism(s) of GST is of particular interest because of its multifunctional nature. In its native conformation each GST subunit contains a specific GSH-binding site adjacent to a nonspecific hydrophobic ligand-binding site (J2, M1, M4). Early speculation that the GSH-binding site serves to allow ionization of GSH to the thiolate ion (GS) and that catalysis merely represents a proximity effect may be a little simplistic. The publication of X-ray crystallographic data for a pi-class GST by Reinemer *et al.* (R5) has allowed identification of amino acid residues involved in binding GSH; three contact residues, namely, tyrosine 7, glutamine 62, and aspartate 96, have been identified. The work of Reinemer *et al.* (R5) has paved the way for work to be undertaken to assess, through site-directed mutagenesis, the contribution of contact residues to catalysis. Studies into the residues responsible for determining the specificity

of binding electrophilic substrates and nonsubstrate ligands promise to yield interesting data.

4. Properties of Glutathione *S*-Transferases

4.1. Catalysis

The glutathione *S*-transferases catalyze numerous reactions in which the glutathione thiolate anion (GS^-) serves as a nucleophile (A20, G11, J2). Thus the fundamental catalytic action of GST is to facilitate the formation or stabilization of GS^-, which can, in turn, attack electrophilic carbon, nitrogen, sulfur, or oxygen atoms contained in any xenobiotic. Literally hundreds of different compounds exist that contain a carbon atom sufficiently electrophilic to be able to react with GS^- and form thioether conjugates. Thioether formation has been widely studied since the earliest convenient spectrophotmetric assays represented this type of reaction; for example, the conjugation of GSH with 1-chloro-2,4-dinitrobenzene, 1,2-dichloro-4-nitrobenzene, *trans*-4-phenyl-3-buten-2-one, ethacrynic acid, 1,2-epoxy-3-(*p*-nitrophenoxy)propane, and menaphthyl sulfate are shown in Fig. 2. Besides reaction with an electrophilic carbon atom, GST also catalyze the reaction of GSH with the electrophilic sulfur atom in organic thiocyanates to form an asymmetric disulfide and HCN (H1). The GSH-dependent reduction of organic nitrate esters (Fig. 3) is an example of a GST-catalyzed reaction between GS^- and an electrophilic nitrogen atom; this activity was originally called glutathione organic nitrate ester reductase but is in fact catalyzed by GST (H1). It has been argued that the ability of GST to reduce organic hydroperoxides, such as cumene hydroperoxide (Fig. 3), is an example of an interaction between GS^- and an electrophilic oxygen.

GST catalyze the isomerization of at least two types of physiologically important compounds. First, there is the isomerization of Δ^5-3-ketosteroids to α-β-unsaturated Δ^4-3-ketosteroids (Fig. 3). Second, there is the isomerization of maleylacetone to fumarylacetone (K4). Both of these reactions are thought to involve the formation of a short-lived glutathione conjugate that rearranges to yield the isomer and GSH. Thiolysis of *p*-nitrophenyl acetate (Fig. 3), with the formation of *p*-nitrophenol and the thiol ester *S*-acetylglutathione, is also catalyzed by GST (K4).

4.2. Metabolism of Carcinogens by GST

Because GST can catalyze the addition of GSH to the epoxide group in model substrates such as 1,2-epoxy-3-(*p*-nitrophenoxy)propane (F12), it is reasonable to suppose that these enzymes can detoxify epoxide-containing metabolites of many genotoxic carcinogens (C6). One of the most important naturally occurring hepa-

tocarcinogens is aflatoxin B_1, produced by the mold *Aspergillus flavus.* The ultimate carcinogenic metabolite formed from aflatoxin B_1 is the 8,9-epoxide. Recent studies (B43, H19, H21, Q1, R1) have provided evidence that the detoxification of aflatoxin B_1-8,9-epoxide by GST (Fig. 5) is a critical factor in determining the outcome of exposure to the mycotoxin aflatoxin B_1; failure of rodents to express the GST responsible for catalyzing this reaction is associated with increased susceptibility to liver cancer (H20).

The polycyclic aromatic hydrocarbons (PAH) are common environmental pollutants that have been implicated as etiological factors in human chemical carcinogenesis (I3, I4). One such PAH that has been studied extensively is benzo[*a*]-pyrene (G3). The metabolic activation of this compound to various reactive intermediates, including epoxides, phenols, and quinones, is performed by the cytochrome-P450-containing mixed function oxidase (MFO) system. Subsequent metabolic steps may involve the hydration of epoxides to dihydrodiols, mediated

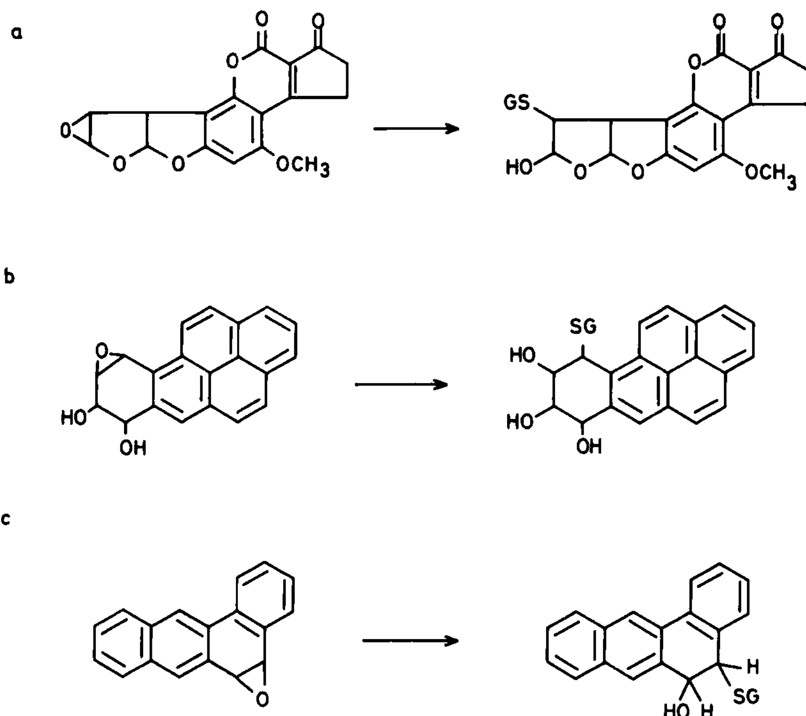


FIG. 5. Detoxification of epoxide-containing carcinogens by GST. The following carcinogens are GST substrates: (a) aflatoxin B_1-8,9-epoxide, (b) anti-benzo[*a*]pyrene-7,8-diol-9,10-oxide, and (c) benz[*a*]anthracene-5,6-epoxide.

by the enzyme epoxide hydrolase, which may be followed by further oxygenation of these compounds to form diol epoxides. Several of the reactive intermediates arising during the metabolism of benzo[*a*]pyrene have been shown to be conjugated with GSH. A human hepatic GST has been shown to exhibit high specific activity toward benzo[*a*]pyrene 4,5-oxide (W2, W3). This compound and other K-region epoxides yield positive results in bacterial mutagenicity tests, although they have not been directly implicated in carcinogenesis (C15). The "Bay region" epoxides, on the other hand, are responsible for the carcinogenic effect of PAH (J8). Glutathione conjugation has been shown to be an important protection mechanism against the DNA binding of the Bay region diol epoxide benzo[*a*]pyrene-7,8-diol-9,10-oxide (BPDE) (H38). The GST that have high activity toward this metabolite include mu- and pi-class enzymes (J9, R8). It is of interest that the nucleophilic attack of GSH on both K-region and Bay region epoxides occurs stereoselectively. For instance the conjugation of GSH with BPDE catalyzed by rat mu-class GST Yb_2Yb_2 is selective toward the biologically most active (+)-enantiomer (R9). Benz[*a*]anthracene is another polycyclic aromatic hydrocarbon that is detoxified by GST following its metabolism to a reactive K-region, 5,6-epoxide (see Fig. 5) (H43).

Although the formation of epoxide metabolites of polycyclic aromatic hydrocarbons is regarded as a major metabolic pathway yielding carcinogenic compounds, the existence of an alternative activation mechanism, involving the formation of reactive sulfate esters, has recently been demonstrated (O7, W6). The bioactivation of benz[*a*]anthracene and chrysene, PAH that are regarded as weak carcinogens, involves methylation followed by enzymic oxidation at the methyl carbon, mediated by microsomal monooxygenases, to yield the corresponding hydroxymethylarenes. These potent carcinogens may then be further activated by hydroxysteroid sulfotransferase to the corresponding reactive sulfate esters (O7, W7). The metabolically formed sulfate esters react with purine bases on both calf thymus and preweanling rat DNA (S47, W5). GSH conjugation, mediated by GST present in rat hepatic cytosol, has been shown to inactivate both 5-hydroxymethylchrysene sulfate and 7,12-dihyroxymethylbenz[*a*]anthracene sulfate and inhibit the formation of DNA adducts (O6, W7). Hiratsuka *et al.* (H46) have purified an enzyme from rat liver that can detoxify sulfate esters formed from the arylmethanols 7-hydroxymethyl-12-methylbenz[*a*]anthracene (W8), 7,12-dihydroxymethylbenze[*a*]anthracene (W6), and 5-hydroxymethylchrysene (O6); these reactions are shown in Fig. 6. The carcinogens *N,N*-dimethyl-4-aminoazobenzene and 2-acetylaminofluorene can also form GSH conjugates (H43).

4.3. Detoxification of Drugs by GST

Besides the metabolism of carcinogens, GST detoxify a wide spectrum of other xenobiotics, many of which we encounter as herbicides, pesticides, or chemothera-

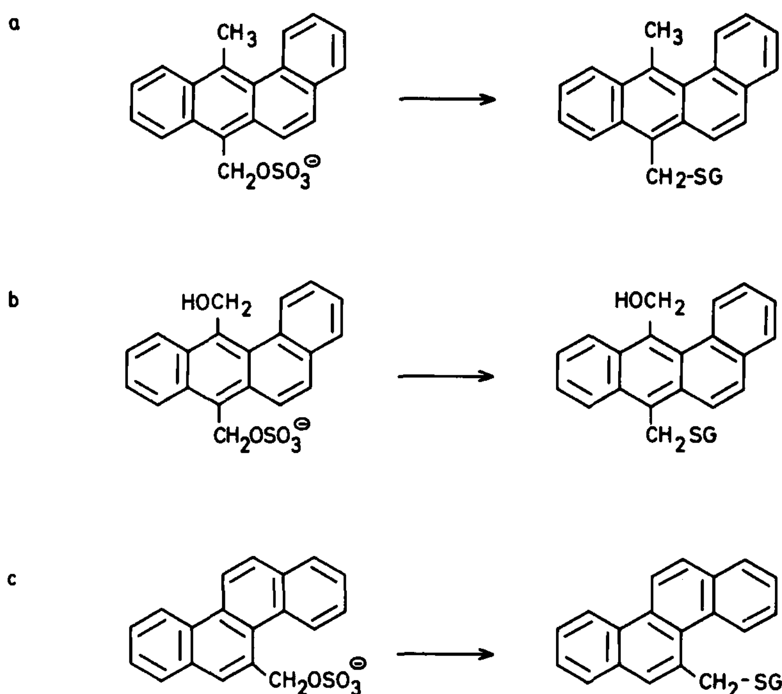


FIG. 6. Metabolism of reactive sulfate esters by GST. GST catalyze the conjugation of (a) 7-hydroxymethylbenz[*a*]anthracene sulfate, (b) 7,12-dihydroxymethylbenz[*a*]anthracene sulfate, and (c) 5-hydromethylchrysene sulfate with GSH (H46).

peutic drugs. The thiocarbamate and *S*-triazine herbicides are substrates for GST, as are organophosphorous insecticides and halogenated hydrocarbon insecticides [for a review, see Hayes and Wolf (H31)]. Among the drugs that serve as GST substrates, paracetamol, a commonly used analgesic, is perhaps the most widely known. Paracetamol is metabolized to *N*-acetyl-benzoquinone imine, which reacts readily with GSH to form 3-(glutathione-*S*-yl) paracetamol (Fig. 7); this reaction can occur spontaneously but is also effectively catalyzed by GST (C16). Nitroglycerin, which is used to treat patients with angina pectoris and congestive cardiac failure because of its vasodilating effects, is metabolized by GST (H2, H41, T10) to form glyceryl dinitrate and nitrite (see Fig. 3). Also, as the pioneering study of Coomes and Stakelum (C17) demonstrated, the organic dye bromosulfophthalein, which is used as a test of hepatic transport, is a GST substrate (Fig. 7). The narcotic drug α-bromoisovalerylurea is another substrate (Fig. 7) that has been used to study

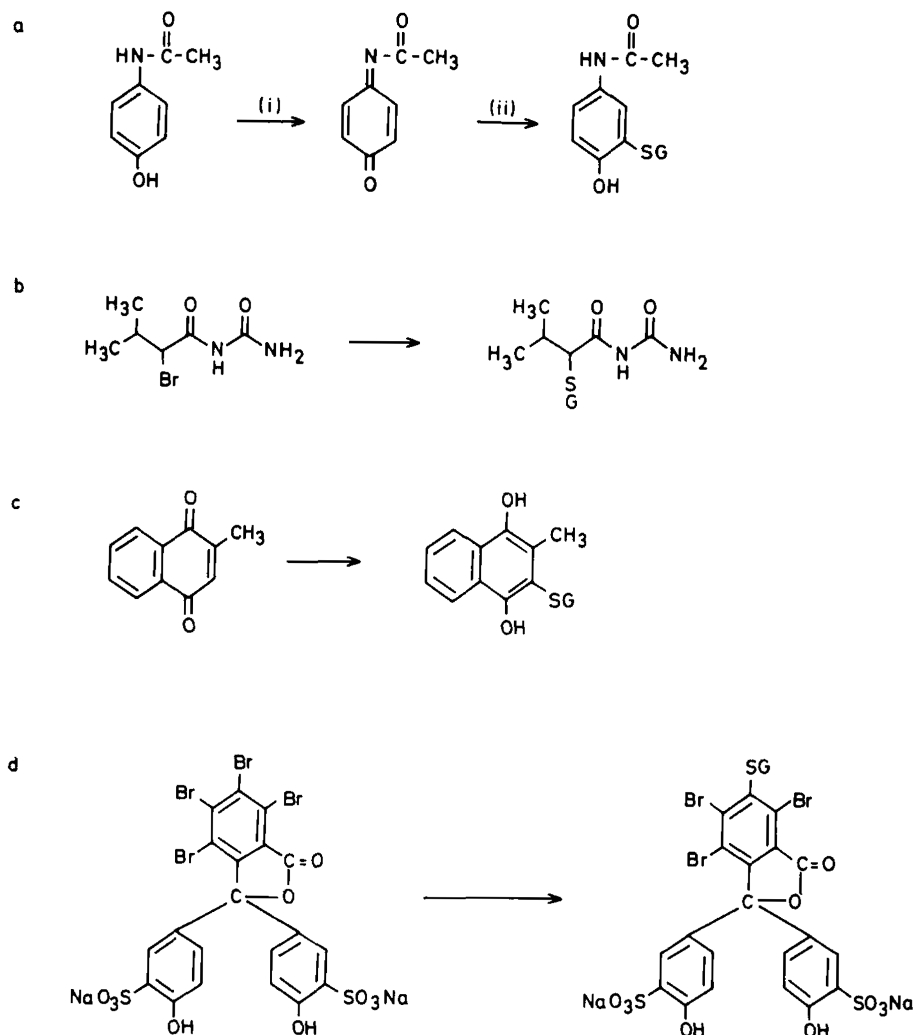


FIG. 7. Reactions between drugs and GSH that involve GST. The reactions between GSH and (a) paracetamol, (b) α-bromoisovaleryl urea, (c) menadione, and (d) bromosulfophthalein have been shown to be catalyzed by GST.

GST activity *in vivo* (T6). Menadione, vitamin K_3, is also conjugated with GSH to form thiodiol (Fig. 7), as well as thiodione and menadiol (F11).

Several anticancer drugs that act as alkylating agents have been shown to be detoxified by GST. The nitrogen mustards melphalan and chlorambucil have both

been shown to form GSH conjugates (B35, C11, D10, D11); the formation of the monochloro monoglutathionyl conjugate of melphalan is shown in Fig. 8. Denitrozation of the nitrosourea BCNU is catalyzed by GST. As shown in Fig. 8, the GSH-dependent deactivation of BCNU may occur via two reactions, namely, (1) the direct denitrozation of the nitrosourea through the formation of *S*-nitroso glutathione (W10) and (2) indirect denitrozation proceeding through GSH conjugation to one of the chloro-bearing carbon atoms with subsequent loss of the nitroso group (T1). The fact that GST efficiently catalyzes the conjugation of GSH with acrolein (B26) (see Fig. 8) suggests that these enzymes can protect against the cytotoxic effects of cyclophosphamide since acrolein is a major metabolite of this nitrogen mustard (A8).

4.4. Endogenous GST Substrates

Peroxidized lipid and DNA, both of which are produced during oxidative stress, serve as substrates for GST (Fig. 9). Hence, it has been proposed that GST have

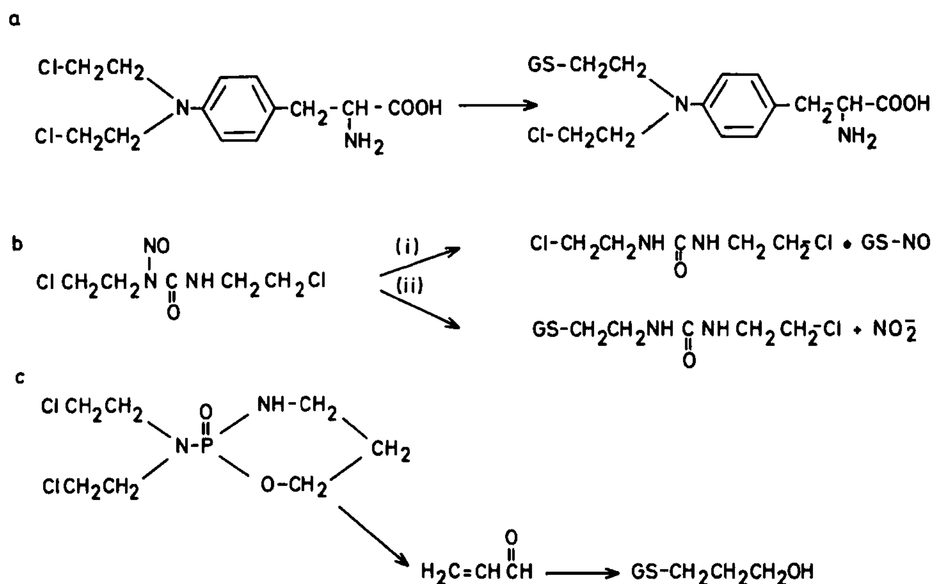


FIG. 8. Metabolism of anticancer drugs by GST. The GST-catalyzed formation of a glutathione conjugate with melphalan is shown in a. The denitrozation of BCNU (b) can occur via either (i) a direct reaction or (ii) an indirect reaction. Acrolein, a cytotoxic metabolite of cyclophosphamide, is also a GST substrate (c).

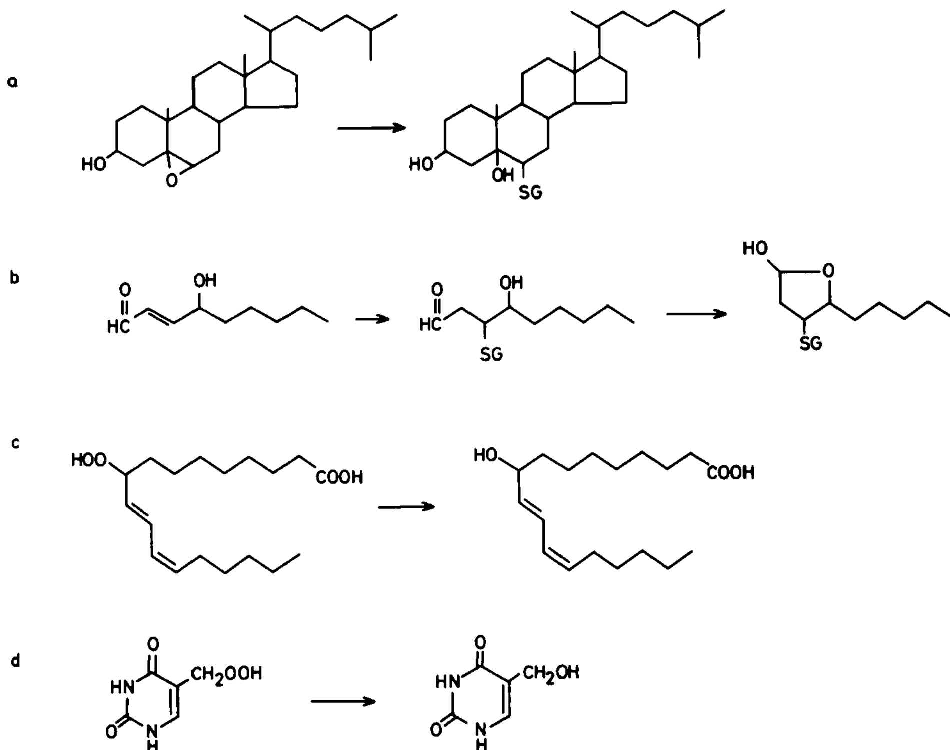


FIG. 9. GST substrates that represent components of oxidized lipid and DNA. Cholesterol-5,6-oxide (a) and 4-hydroxynon-2-enal (b) are detoxified by GST through thioether formation, whereas 9-hydroperoxy-linoleic acid (c) and 5-hydroperoxymethyl-uracil (d) are reduced by GST peroxidase activity.

evolved in response to the toxic products of oxygen metabolism (K9, M1). Reactive oxygen species, such as superoxide anion, hydrogen peroxide, and the hydroxyl radical, are continuously produced during normal aerobic metabolism. The peroxidation of lipid biomembranes occurs following the attack of free radicals upon the polyunsaturated fatty acyl moieties. In the presence of oxygen this can lead to chain propagation reactions involving lipid peroxy radicals and hydroperoxides. The peroxidase activity of GST, along with that of GPx, contributes to the reduction of peroxidized lipid and therefore helps combat oxidative damage to lipid. Some workers have demonstrated that the consecutive action of the enzyme phospholipase A_2, which releases fatty acyl hydroperoxides from peroxidized phospholipids, and certain GST, which reduce the free fatty acid hydroperoxides released by phospholipase A_2, is sufficient for the inhibition, *in vitro,* of microsomal lipid peroxidation (T2). Ketterer *et al.* (K9) have subsequently reported

that a number of purified rat GST have activity toward hydroperoxides of linoleic acid and arachiodonic acid, two polyunsaturated fatty acids that are abundant in biological membranes.

If the process of lipid peroxidation continues unimpeded, the consequences include the release of toxic breakdown products and the eventual destruction of the lipid component of biological membranes (S28). Such breakdown products include the aldehydes, malondialdehyde, 2-alkenals, and 4-hydroxyalkenals. A number of mammalian GST isoenzymes are highly efficient in the detoxification of these compounds (D1). Indeed, 4-hydroxynonenal is one of the best GST substrates identified to date, and with one of the rat GST isoenzymes the K_{cat}/K_m value obtained indicates that catalysis proceeds relatively close to the diffusion-controlled limit. Cholesterol-5,6-epoxide is a further example of a by-product of lipid peroxidation, and the conjugation of GSH to this weakly mutagenic compound is catalyzed by certain GST (M18).

DNA is also susceptible to free radical attack during oxidative stress. The participation of GST isoenzymes in the detoxification and repair of the potentially mutagenic radical damage to DNA has been studied by Ketterer and his colleagues. Both thymine hydroperoxides and DNA peroxidized by ionizing radiation in the presence of oxygen have been shown to serve as substrates for rat GST (T3, T4). However, these workers reported that the specificity of rat GST isoenzymes toward the peroxidized DNA differs from that toward the free thymine hydroperoxide, 5-hydroxymethyl uracil. From their data, Ketterer *et al.* (K7, K9) propose that GST act in concert with DNA glycosylase to repair oxidized DNA.

In addition to their protective functions, the GST are known to participate in the biosynthesis of biologically active molecules, including leukotrienes and prostaglandins. Prostaglandin A_1, for example, has been shown to be conjugated with GSH by homogeneous rat and human GST (C1) and 15-keto prostaglandins also form conjugates with GSH (C7). The hydroxy endoperoxide prostaglandin (PG) H_2 may undergo three different GSH-dependent conversions to form biologically active compounds (Fig. 10); PGH_2 can be reduced to $PGH_{2\alpha}$ or isomerized to PGD_2 or PGE_2. All three reactions are catalyzed by rat cytosolic GST *in vitro* (B45, C10).

The biologically active cysteine-containing leukotrienes C_4, D_4, and E_4 mediate anaphalacttic and allergic reactions through their smooth muscle-stimulating and edema-inducing properties. Leukotriene C_4 is formed from leukotriene A_4 following conjugation of GSH to an epoxy group in the parent compound (B1) (Fig. 10). The GST responsible for leukotriene C_4 synthase activity has recently been purified (N2, N3) and it is now certain that the reaction is catalyzed by a unique membrane-bound enzyme that is distinct from the microsomal GST characterized by Morgenstern and his colleagues (S29, S30).

Several other specific compounds arising during metabolism have been identified as substrates for GST, such as reactive intermediates of estradiol metab-

olism (E4, M9). In this instance, the formation, *in vivo* and *in vitro,* of GSH conjugates of certain steroids has implicated reactive intermediates of estradiol-17β (J6) and 2-hydroxyestradiol-17β (E3) as possible substrates for GST (Fig. 10). Quinones also represent a group of reactive compounds that may be detoxified by GST since dopaquinone, which is an intermediate in the melanin biosynthetic pathway, has been found to be conjugated with GSH in humans suffering from malignant melanoma (A6).

Recently, Odell *et al.* (O1) described the presence of bilirubin–GSH conjugates in the bile of Gunn rats, a strain possessing unconjugated hyperbilirubinemia. These rats are deficient in the microsomal bilirubin UDP–glucuronosyl transferase, and Odell *et al.* (O1) have found that this strain, although it cannot form bilirubin glucoronides, readily conjugates GSH with bilirubin (see Fig. 11). Like the Gunn rat, the type 1 genetic disorder Crigler–Najjar syndrome also involves the absence of UDP–glucuronosyl transferase and it is possible that in this disease GSH conjugation represents an important excreting pathway for bilirubin. The rat GST responsible for catalyzing the conjugation of GSH with bilirubin has not been identified. However, in the EHB strain of rats, which exhibit hereditary hyperbilirubinuria, the alpha-class Yc_2 subunit is markedly overexpressed (I1), possibly suggesting that this GST may be involved in bilirubin metabolism.

4.5. Catalysis of Adverse Reactions by GST

There are a small number of GST substrates that either are not detoxified by conjugation with GSH or become more toxic when they are conjugated with GSH. Although these toxification reactions are relatively rare, their importance should not be overlooked. Three types of toxic GSH conjugate have been identified (A13, V2). For example, conjugates such as 2-bromoethylglutathione (Fig. 12) are directly toxic through spontaneous formation of reactive episulfonium ions and therefore do not require the involvement of other enzymes in the toxification process. Alternatively, the GSH conjugates of compounds such as trichloroethene or hexachlorobutadiene require further metabolism by γ-glutamyltranspeptidase and cysteine conjugate β-lyase before their potential toxicity is realized. Last, certain conjugates, such as that formed from benzyl isothiocyanate (Fig. 12), are unstable and as the electrophilic species is readily regenerated the conjugate can be regarded only as a "temporary storage form" of the toxic compound.

One of the best studied examples of GST involvement in the biosynthesis of toxic conjugates is the metabolism of the potent nephrotoxin hexachloro-1,3-butadiene (HCBD). This compound has no adverse effects in the liver but its toxicity has been shown to arise following the initial hepatic GST-catalyzed direct conjugation of HCBD with GSH. The product of this reaction, pentachlorobutadienyl-glutathione, is subsequently metabolized by γ-glutamyl transpeptidase and

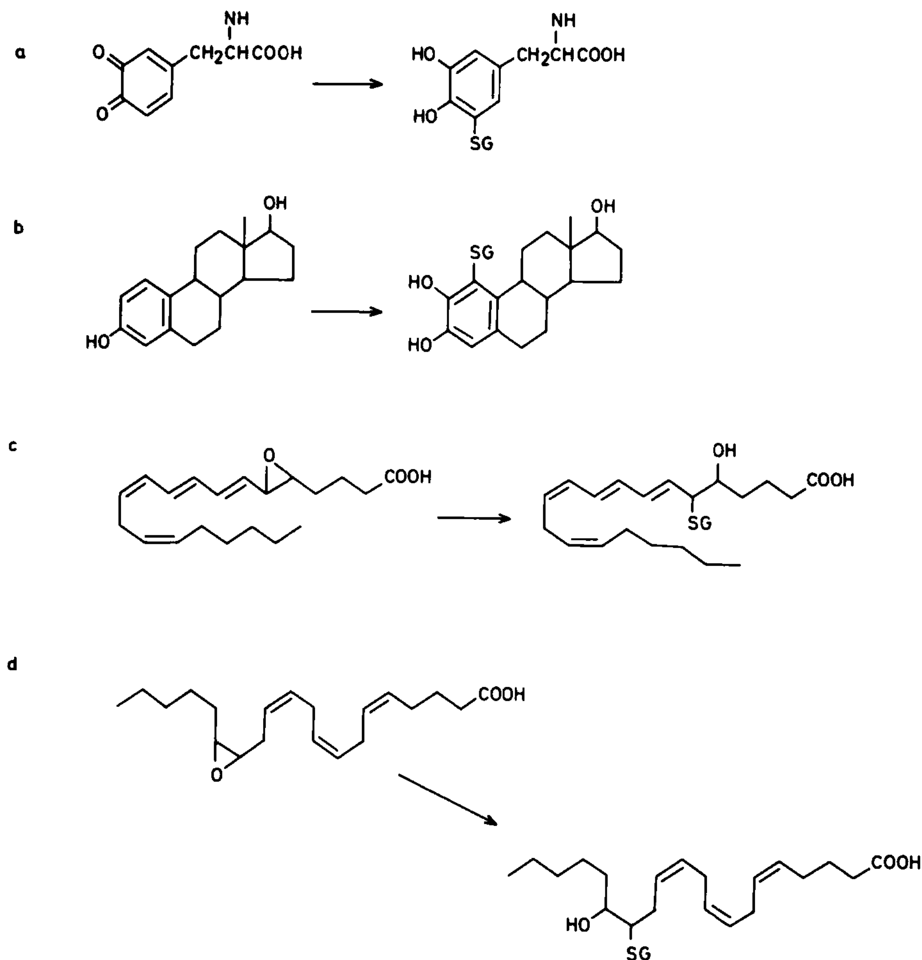


FIG. 10. Endogenous GST substrates. The following "normal" products of cellular metabolism serve as GST substrates: (a) dopaquinone, (b) estradiol-17β, (c) leukotriene A_4, (d) 14,15-epoxy-5,8,11-eicosatrienoic acid, (e) prostaglandin A_1, and (f) prostaglandin H_2.

cysteinyl glycinase in the kidney to form the cysteinyl conjugate. Metabolism of pentachlorobutadienyl-cysteine by renal cysteine conjugate β-lyase results in the formation of a thiol that is either directly or indirectly toxic (G10, J10, W11). McLellan *et al.* (M13) have shown that the human microsomal GST is responsible for the conjugation of HCBD with GSH and, interestingly, Anders *et al.* (A13) have reported that the microsomal GST, rather than cytosolic GST, is responsible for the bioactivation of a number of other haloalkanes.

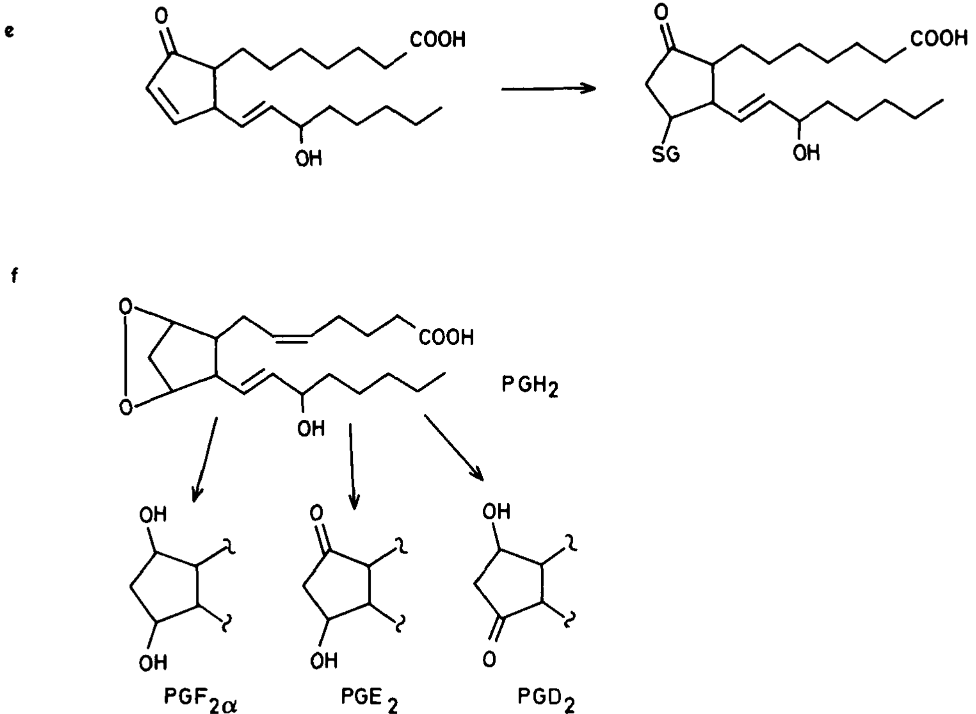


FIG. 10. *Continued.*

4.6. Binding Functions of the GST

The GST are able to bind, covalently and noncovalently, numerous hydrophobic compounds that are not substrates. It has therefore been proposed that GST may serve to sequester/store such compounds or, alternatively, they may be involved in the transport of ligands, either across cells or between subcellular compartments/organelles. Although many of the early binding studies were performed using preparations of ligandin, it has been shown subsequently that most soluble GST display a high affinity for a broad range of nonsubstrate ligands (K5). The physiological importance of this binding activity has been the subject of considerable debate but it should be noted that, as the intrahepatic concentration of GST is approximately 0.2 m*M*, a high level of diffusable lipophilic binding sites that are likely to alter significantly the mobility of hydrophobic molecules within hepatocytes and other cells are available.

It is reasonable to speculate that highly lipophilic xenobiotics of limited solubility require carrier proteins to facilitate their mobilization, metabolism, and

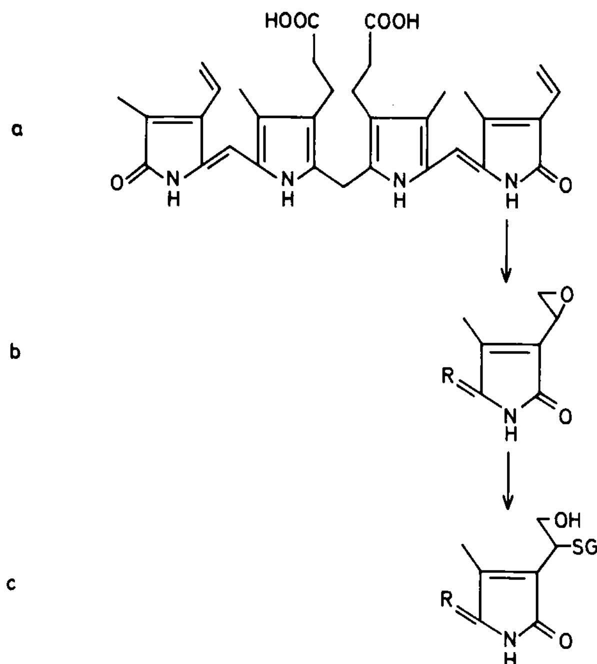


FIG. 11. Formation of bilirubin–glutathione conjugates. This mechanism for conjugation of bilirubin and GSH was proposed by Odell *et al.* (O1).

ultimate excretion from cells. GST are good candidates for the role of carrier protein (H45) and it is of particular interest that many nonsubstrate ligands for GST are indeed substrates for cytochrome P450 (T9). An endogenous ligand for GST is heme and it has been proposed that GST mediate the intracellular transport of heme from mitochondria to apoenzymes (H57).

The hypothesis that GST (particularly ligandin) influence hepatic and renal uptake of organic anions is based not only on studies of their binding capabilities, but also on certain physiological observations. Levi *et al.* (L5) showed that animal tissues that do not preferentially extract bilirubin or bromosulfophthalein from plasma do not contain ligandin (i.e., Ya subunits). This group of workers has demonstrated that newly born animals, which have low hepatic levels of ligandin, also exhibit impaired organic-anion uptake by the liver (L6). Fleischner *et al.* (F14) reported that an increase in hepatic concentrations of ligandin, achieved by treating rats with phenobarbital, was accompanied by an increased rate of clearance of exogenous organic anions from plasma. Bile acids represent another group

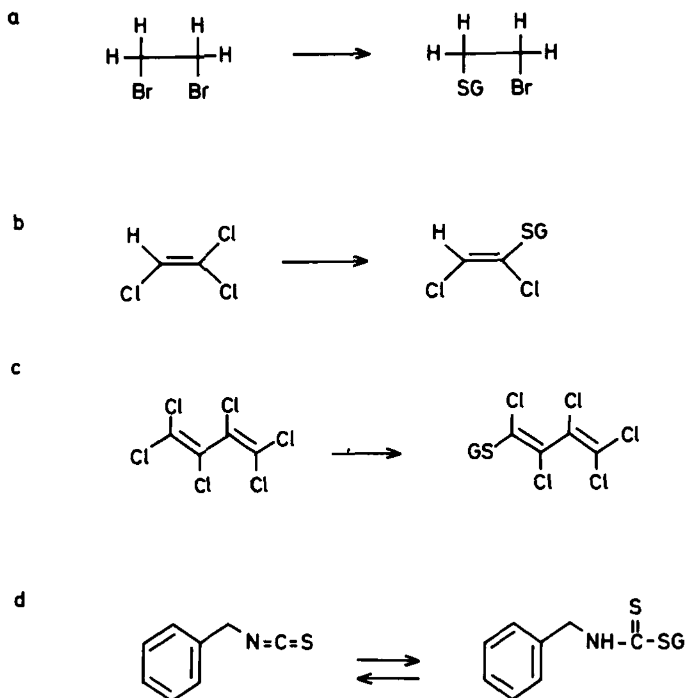


FIG. 12. Toxication reactions catalyzed by GST. Examples of compounds that are not detoxified by conjugation with GSH include (a) ethylene dibromide, (b) trichloroethene, (c) hexachloro-1,3-butadiene, and (d) benzyl isothiocyanate.

of organic anions that are bound noncovalently by GST (H28, H29) but, with the possible exception of lithocholic acid, it appears probable that they cross the hepatocyte for excretion into bile by free diffusion rather than by a carrier-mediated mechanism (S39). GST also bind noncovalently steroid hormones, thyroid hormones, and neurotransmitters (A1).

The ability of GST to bind covalently reactive xenobiotic metabolites has been recognized for many years. Azo-dye carcinogen metabolites have been shown to combine *in vivo* specifically with three soluble proteins of rat liver, and the original description of ligandin was as one of these azo-dye carcinogen-binding proteins (K8). The binding of azo-dye carcinogens to ligandin is covalent, and it has been shown that it is the Ya subunit that selectively binds this type of compound (O4). Ligandin has also been shown to bind, covalently, activated metabolites of 3-methylcholanthrene (K6). The covalent binding of activated

polycyclic aromatic hydrocarbons to cytosolic proteins has also been extensively investigated in the mouse and has led to the identification of the *h*-protein, which is present in mouse liver, skin, and lung (S4). A common identity between the *h*-protein and a protein in mouse liver cytosol that is equivalent to ligandin has been surmised as both proteins have similar physical properties. Subsequent studies demonstrated that a protein, immunologically related to rat ligandin, is present in mouse liver but this protein appeared to be distinct from the *h*-protein (S5, S6). The *h*-protein was found to bind 3-methylcholanthrene to a greater extent than "mouse ligandin." Interestingly, low levels of GST activity were associated with highly purified preparations of the *h*-protein (S6), and, although such activity may represent impurities in the preparation, the possibility remains that the *h*-protein is a GST.

In the rat, the Ya-/Yc-containing GST are not solely responsible for covalent binding of toxic compounds, as it has been reported that Yb_1 and/or Yb_2 subunits in rat liver bind ethacrynic acid covalently *in vivo* (Y1). Furthermore, Morgenstern and his colleagues have implicated microsomal GST in the covalent binding of metabolites of benzo[*a*]pyrene, *trans*-stilbene oxide, and phenol (M25).

As discussed above, the physiological significance of covalent binding of electrophiles to GST is unclear, but it has been speculated that, in addition to participating in detoxification by catalyzing conjugation reactions with GSH, GST may play a sacrificial role in the detoxification of reactive metabolites. Therefore, together with their contribution to mercapturic acid synthesis, their capacity for noncovalent binding of organic anions, and the covalent binding of reactive zenobiotics, the GST function in three separate ways to aid the detoxification of noxious chemicals (J2).

5. Glutathione *S*-Transferase Isoenzymes

5.1. Families of GST

In all mammalian species the GST are represented by a large number of isoenzymes. Historically, the human GST were originally subdivided, on the basis of their charge, into the basic, neutral, and acidic forms (G13, J2, M8, W2). However, a comparison of the catalytic properties, immunochemical cross-reactivities, and N-terminal amino acid sequences of the basic, neutral, and acidic enzymes with the major cytosolic GST from the rat, mouse, and cow demonstrated the existence of three distinct families of GST in all the mammalian species studied. *Mannervik et al.* (M2), therefore, proposed a species-independent classification that has now been generally adopted. As described earlier (Table 2), the three evolutionary classes of GST identified were designated alpha, mu, and

pi, and these represent the human basic, neutral, and acidic GST, respectively. More recently, three cytosolic rat and two human GST isoenzymes have been described (H8, H46, H59, M17) that are catalytically and structurally distinct from alpha-, mu-, and pi-class GST. These appear to constitute an additional family, designated theta-class GST. The alpha-, mu-, pi-, and theta-class GST share some homology and hence appear to have arisen from a common ancestral gene. A summary of the approximate homology of different GST within specific classes and between classes is shown in Table 3.

In addition to the four families of mammalian cytosolic GST, membrane-bound forms of the enzyme exist. Amino acid sequencing and cDNA cloning have demonstrated that the *N*-ethylmaleimide-activatable microsomal GST shares no homology with the cytosolic enzymes (D3) and therefore appears to be an example of convergent evolution. Recent evidence suggests that leukotriene C_4 synthase

TABLE 3
SEQUENCE HOMOLOGY BETWEEN GST TO DEFINE GENE FAMILIES

		Percentage homology within and between different GST families				
Family	No. of amino acid residues	Alpha[a]	Mu[b]	Pi[c]	Theta[d]	Microsomal[e]
Alpha	221–222	58–95				
Mu	217–220	<25	68–95			
Pi	209	<28	<26	82–98		
Theta	239–244	<15	<10	<5	55	
Microsomal	154	NS	NS	NS	NS	77

Note. The amino acid sequences of individual rat and human GST, obtained directly from automated Edman degradation or deduced from cDNA clones, have been compared. Mammalian GST isoenzymes that are members of the same gene family share at least 55% sequence identity with members of the same family. By contrast, mammalian GST that are members of distinct gene families possess less than 30% identity. The microsomal GST share no significant homology with the cytosolic enzymes. NS, not significant.

[a] Data for alpha-class GST are from Lai *et al.* (L2), Pickett *et al.* (P8), Telakowski-Hopkins *et al.* (T7), Board and Webb (B33), Rhoads *et al.* (R7), Alin *et al.* (A10), and Hayes *et al.* (H19).

[b] Data for mu-class GST are from Lai *et al.* (L1), Abramovitz and Listowsky (A2), DeJong *et al.* (D2), Lai *et al.* (L3), Seidegard *et al.* (S16), Kispert *et al.* (K10). Campbell *et al.* (C2), and Vorachek *et al.* (V6).

[c] Data for pi-class GST are from Suguoka *et al.* (S46), Kano *et al.* (K2), Ahmed *et al.* (A7), and Bora *et al.* (B37).

[d] Data for theta-class GST are from Hiratsuka *et al.* (H46), Harris *et al.* (H8), Meyer *et al.* (M17), Ogura *et al.*, (O2), and Pemble and Taylor (P6).

[e] Data for microsomal GST are from DeJong *et al.* (D3).

represents another family of GST but confirmation of this hypothesis by molecular cloning is eagerly awaited.

5.2. Human GST Nomenclature

Several systems of nomenclature have been adopted by workers in different laboratories for the classification of the human GST isoenzymes. Kamisaka *et al.* (K1) originally described five basic (alpha-class) forms of GST in human liver, which were assigned Greek alphabetical symbols (α, β, γ, δ, and ε). Following the identification of neutral (mu-class) GST in liver and acidic (pi-class) GST in placenta, the additional forms were also designed by the Greek symbols μ, ψ, ϕ, and π (G13, H16, S26, S36, W3).

Further structural studies on the hepatic basic GST established the subunit composition of these enzymes (S35). This paved the way for a system of nomenclature based upon the quaternary structure of the proteins, to be adopted for the human alpha-class GST. This system describes three enzymes formed by the combination of two distinct subunits called B_1 and B_2 (H22, S35, S37). The homodimeric enzyme GST B_1B_1 represents GST ε, and the heterodimer B_1B_2 is equivalent to GST δ. However, the relationship of B_2B_2 to the enzymes described by Kamisaka *et al.* (K1) is less clear; this homodimeric enzyme is probably equivalent to GST α, β, and γ, three forms that may have arisen following the autoxidation of single gene product (H22).

The human GST have also been named according to their p*I* value. Del Boccio *et al.* (D4) have described a very basic enzyme, called GST 9.9, purified from human skin, that has an N-terminal amino acid sequence identical to that of the rat alpha-class enzyme GST Yc_1Yc_1.

Other workers, who have taken a genetic approach to the classification of the human GST, have numbered the enzymes according to their gene loci, using evidence obtained from zymogram analysis (B30, S41). This approach has given rise to the designation GST 1, GST 2, and GST 3, which are the loci encoding enzymes of the mu-class, alpha-class, and pi-class GST, respectively. Similar investigations into a number of different human tissues indicated the existence of at least three more gene loci, termed GST 4, GST 5, and GST 6 (L4, S49). Later reports have shown that GST 4 and GST 6 encode enzymes that belong to the mu evolutionary class (B32, S50).

Recently, Mannervik *et al.* (M3) have proposed a unifying nomenclature for GST (Table 4) in which each genetically distinct subunit has its own designation and is classified by gene family. Most importantly, the designations given to individual isoenzymes in this system reflect their subunit composition, and allelic variants, encoded at the same gene locus, are distinguished by letters. However, in this review we will refer to the alpha-class A1-1, A1-2, and A2-2 dimers as

TABLE 4
HUMAN GST ISOENZYME NOMENCLATURE

1992 nomenclature[a]	Class	Previous subunit-based designation	Greek letter nomenclature	Previous genetic designation
A1–1	Alpha	B_1B_1	ε	GST 2, type 1
A1–2	Alpha	B_1B_2	δ	GST 2, types 1–2
A2–2	Alpha	B_2B_2	α,β,γ	GST 2, type 2
M1a–1a	Mu	$N_1^aN_1^a$	μ	GST 1, type 2
M1a–1b	Mu	$N_1^aN_1^b$	—	GST 1, types 1–2
M1b–1b	Mu	$N_1^bN_1^b$	ψ	GST 1, type 1
M1a–2	Mu	$N_1^aN_2$	—	—
M1b–2	Mu	$N_1^bN_2$	—	—
M2–2	Mu	N_2N_2	—	GST 4
M2–3	Mu	N_2N_3	—	—
M3–3	Mu	N_3N_3	φ	GST 5
P1–1	Pi	YfYf	π	GST 3
T1*–1*	Theta	T_1T_1	θ	—
T2*–2*	Theta	T_2T_2	—	—
Microsomal	Microsomal	—	—	—

[a]Based on designations proposed by Mannervik *et al.* (M3).

B_1B_1, B_1B_2, and B_2B_2 because most of the clinical biochemistry RIA-based research about GST uses the B_1 and B_2 designations.

5.3. HUMAN ALPHA-CLASS GST

The highest levels of alpha-class GST are found in liver; however, they are also present in significant amounts in testis, kidney, and adrenal glands (A3, M14, S27, T5).

A number of workers have purified the hepatic alpha-class GST and shown that they are represented by multiple isoenzymes. Kamisaka *et al.* (K1) originally observed five basic forms of GST (α–ε), which had different p*I* values but were indistinguishable by other criteria. It was, therefore, proposed that these enzymes are charge isomers formed as a result of deamination *in vivo* of a single gene product. Subsequent studies have shown, however, that the basic forms of GST present in human liver constitute two immunochemically and catalytically distinct

subunits (S33, S35, S37). These subunits may combine to form two homodimeric enzymes, which have been designated GST B_1B_1 and GST B_2B_2, and one heterodimeric enzyme, called B_1B_2 (S35, S37). In contrast to these reports, Vander Jagt *et al.* (V3) have described the isolation of 13 alpha-class GST from a number of different liver specimens. However, it is likely that these do not represent separate isoenzymes but are multiple forms generated by the purification strategy (H22).

Molecular cloning studies, which allowed the investigation of human alpha-class GST at a nucleic acid level, have been undertaken. Tu and his colleagues described two separate full-length cDNA clones, called GTH_1 and GTH_2, which both encode human alpha-class GST subunits (R7, T15). Independently, Board and Webb (B33) cloned a cDNA that encoded the same GST subunit as the cDNA clone GHT_1 described by Tu and Qian (T15). An analysis of the deduced amino acid sequences from GTH_1 and GTH_2 indicates that they encode two highly homologous subunits that differ in only 11 amino acids. A direct molecular relationship between the human alpha-class cDNA clones described in the literature and the basic isoenzymes that may be purified from human liver was established following protein sequencing experiments. Hayes *et al.* (H22) showed that the amino acid sequences obtained from CNBr-derived peptides from GST subunits B_1 and B_2 were virtually identical to the deduced amino acid sequences of the cDNA clones GTH_1 and GTH_2 (R7), respectively.

At the present time it is clear that there are at least two alpha-class GST subunits that are the products of two separate gene loci (B31, H22). The chromosomal location of the genes that encode these human alpha-class enzymes has been investigated using *in situ* hybridization techniques. Separate studies have indicated that the genes are clustered on the short arm of chromosome 6 at band p12 (B33, C9).

The possibility that there are additional human alpha-class GST has also been the subject of investigation. One group of workers has reported the existence of an alpha-class isoenzyme that is distinct from the hepatic GST. Del Boccio *et al.* (D4) have purified a basic GST from human skin that has a close relationship to the rat GST Yc_1Yc_1. It is also of interest that the polymorphic expression of a similar GST has been observed in human prostate (D9). At a nucleic acid level, Southern blotting experiments, using a cDNA encoding the GST B_1 subunit, have revealed hybridization patterns that indicate that there may be multiple alpha-class genes in the human genome (B33).

5.4. Human Mu-Class GST

A neutral GST that was distinct from the five basic forms (GST α–ε) purified earlier by Kamisaka *et al.* (K1) was first identified in human liver in 1980. Warholm *et al.* (W3, W4) showed that the characteristic features of this isoenzyme were its high activity toward the substrate *trans*-4-phenyl-3-buten-2-one and its

near-neutral isoelectric point of 6.0–6.5. Another noteworthy feature of this enzyme is that it was found to be present only in a limited number of liver specimens. This new form, called GST μ, was found to exhibit physical properties that distinguish it from both hepatic GST α–ε and a placental form called GST π, indicating a unique genetic origin (W2, W3). GST μ was also found to be highly efficient at catalyzing the conjugation of glutathione with the epoxides benzo[*a*]pyrene-4,5-oxide and styrene-7,8-oxide.

A number of isoenzymes that are related to GST μ have since been identified. A hepatic enzyme, called GST ψ, has been purified in several laboratories (H27, S26), and it has been shown that this homodimeric protein has an N-terminal amino acid sequence identical to that of GST μ (A11, H16). The expression of this additional mu-class form in human liver is also subject to variation (H63). GST μ and GST ψ are homodimers and it is now generally accepted that these two enzymes represent allelic variants encoded at the GST 1 locus identified by Board (B30) and Strange *et al.* (S41). Less information is available on the heterodimeric enzyme formed by the combination of "μ-type" and "ψ-type" subunits (H16, V4). The existence of this isoenzyme has been demonstrated by means of starch–gel electrophoresis and chromatofocusing (F2, S41); however, so far, it has not been fully characterized. A third hepatic mu-class enzyme, named GST ϕ, was identified by Stockman and Hayes (S36). This isoenzyme, which was present in only 1 of 20 livers examined, was shown to be immunologically related to GST μ and GST ψ but could be distinguished from these forms by its lower isoelectric point and the fact that, unlike GST μ and GST ψ, it has a blocked N terminus (P. K. Stockman and J. D. Hayes, unpublished information).

In an early investigation into the genetic basis of the expression of multiple GST isoenzymes in man, Board (B30) used zymogram analysis to examine the enzyme levels in liver cytosol extracts from a large number of individuals. This electrophoretic study showed that the products of the mu-class GST 1 locus were represented by three activity bands that were subject to phenotypic variation. The most acidic of these enzymes was termed GST 1 type 1 and the least acidic was termed GST 1 type 2. These workers proposed that the isoenzyme with an intermediate mobility was a heterodimeric protein formed by the combination of GST 1 type 1 and type 2 subunits. This type of analysis also revealed that a large number of individuals did not express any of the GST 1 isoenzymes. A statistical analysis of the data obtained indicated that the different phenotypes observed were attributable to different combinations of three autosomal alleles, including a null, at a single gene locus.

Other workers subsequently confirmed this genetic model and extended this type of investigation to include the analysis of GST activity in tissues other than liver (L4, S41). These studies showed that the GST 1 phenotype was a constant individual characteristic that was observed in all of the tissues that had detectable levels of these enzymes. The enzymes that correspond to GST 1 type 1 and GST

1 type 2 have been purified from human liver and are called ψ and μ, respectively (H16, S26, W2). GST ψ and μ have isoelectric points of 5.5 and 6.1, respectively, but are catalytically indistinguishable. Reversible denaturation experiments have shown GST ψ and μ to represent homodimers, comprising subunits that can hybridize. The cloning of hepatic mu-class GST has resulted in the isolation of two cDNAs that encode proteins that differ only at amino acid residue 173 (D2, S16). The clone isolated by DeJong *et al.* (D2) encodes an enzyme with lysine at residue 173, whereas the clone described by Seidegard *et al.* (S16) encodes a GST containing asparagine at residue 173. It is believed that the GST with lysine 173 represents μ and the enzyme with asparagine 173 represents ψ. Widersten *et al.* (W9) have proposed that these two cDNAs represent the allelic variants GST 1 types 1 and 2 because their noncoding regions are closely similar.

Several extrahepatic GST, which were initially identified using starch–gel electrophoresis, were purified by Board and co-workers (S49). An enzyme, called GST 4, was isolated from human skeletal muscle and shown to be immunologically related to GST μ and to have N-terminal amino acid sequence homology with GST μ (B32). Two other forms, termed GST 5 and GST 6, were purified from brain by this group of workers (S49). GST 5 was shown to be a homodimeric enzyme that is structurally similar to the products of the GST 1 locus. GST 6, on the other hand, was an immunochemically distinct protein that contained two dissimilar subunits. The cDNA encoding GST 4 has been cloned by Vorachek *et al.* (V6) and that encoding GST 5 has been cloned by Campbell *et al.* (C2). The relationship between GST 6 and other GST awaits clarification but Suzuki *et al.* (S50) have shown that over the first 24 N-terminal amino acids GST 6 is identical to GST 4.

To date, therefore, three types of mu-class subunits, encoded at separate gene loci, have been identified. Hussey *et al.* (H62) have referred to these as N_1 (N_1^a, or N_1^b), N_2, and N_3 but in the revised nomenclature of Mannervik *et al.* (M3), which uses a single letter prefix to indicate the gene family, these subunits are called M1 (M1a or M1b), M2, and M3, respectively. Hence, at the GST 1 loci the two allelic variants M1a and M1b are encoded, whereas at GST 4 and 5 the M2 and M3 subunits are encoded. Hussey *et al.* (H62) have shown that all three subunit types can hybridize; in human liver M1a-1a and M1b-1b have been characterized, whereas in human skeletal muscle M1-2, M2-2, and M2-3 have been isolated. The immunochemical relationships between the mu-class enzymes and other GST are shown in Table 5.

It is likely that additional mu-class GST exist. For example, not only is the relationship between GST 6, described by Suzuki *et al.* (S49), and the M1, M2, and M3 designations uncertain but Tsuchida *et al.* (T12) have isolated several mu-class GST from human heart and aorta that are not included in the nomenclature of Mannervik *et al.* (M3). Southern blotting analysis has revealed a high level of complexity in the genomic organization of human mu-class genes, suggesting that in humans this family comprises at least five genes.

TABLE 5
IMMUNOCHEMICAL RELATIONSHIPS BETWEEN HUMAN GST

Subunit	Cross-reactivity with antisera against GST						
	A1–1 (B_1B_1)	Ma1–1a ($N_1^aN_1^a$)	M2–2 (N_2N_2)	M3–3 (N_3N_3)	P1–1 (Yf Yf)	Rat GST E	Microsomal
A1 (B_1)	++++	—	—	—	—	—	—
A2 (B_2)	++++	—	—	—	—	—	—
M1a (N_1^a)	—	++++	++++	—	—	—	—
M1b (N_1^b	—	++++	++++	—	—	—	—
M2 (N_2)	—	++	++++	—	—	—	—
M3 (N_3)	—	+	—	++++	—	—	—
P1	—	—	—	—	++++	—	—
T1*	—	—	—	—	—	ND	ND
T2*	—	—	—	—	—	++++	ND
Microsomal	—	—	—	—	—	ND	++++

Note. Cross-reactivity was determined by Western blotting. The GST designations included in parentheses represent previous names used in our laboratory. ND, not determined.

5.5. HUMAN PI-CLASS GST

In humans, the pi-class isoenzyme is widely distributed and represents the most thoroughly characterized extrahepatic GST. Pi-class GST have been purified from a number of sources, including placenta (G13, K17, P2) and erythrocytes (M8). Although these enzymes are now commonly referred to as GST π, the forms isolated from placenta, lung, and erythrocytes were originally named GST π, GST λ, and GST ρ, respectively.

Some controversy exists in the literature regarding the possible existence of more than one pi-class GST. The acidic isoenzymes isolated from erythrocytes, lung, and placenta have been shown to share immunological identity and have the same subunit M_r value and isoelectric point (A19, H52, K16). However, using nondenaturing starch–gel electrophoresis, both Laisney *et al.* (L4) and Suzuki *et al.* (S49) have shown that an acidic isoenzyme present in erythrocytes has a mobility different from that of the GST 3 enzyme observed in other tissues. Other workers have described two pi-class isoenzymes present in skeletal muscle tissue that have minor differences in their isoelectric point (S25).

At the nucleic acid level, a number of investigators have isolated cDNA clones encoding human pi-class GST (B34, K2, M29). A human cDNA that encodes fatty acid ethyl ester synthase (FAEES-III) has been isolated by Bora *et al.* (B37) and

found to share extensive identity with pi-class GST. Out of the 210 amino acids encoded by these cDNAs, only four differences were noted between the predicted amino acid sequence of FAEES-III and that of GST π; at positions 32, 39, 52, and 194 FAEES-III contains glycine, cysteine, glutamic acid, and valine, whereas GST π contains glutamic acid, tryptophan, glutamine, and leucine, respectively. It is interesting to note that the side chain at one of these positions, namely residue 39, has been shown to line the glutathione binding site (R5), and the tryptophan-to-cysteine substitution noted by Bora *et al.* (B37) may be responsible for the change in substrate specificity of GST π. It is therefore unclear whether the cDNA reported by Bora *et al.* (B37) encodes a functional GST.

The genomic GST π gene has been cloned and characterized by several groups of workers (C19, M26, M27). The location of this gene was originally mapped to chromosome 11 by zymogram analysis of the GST isoenzymes expressed in somatic cell hybrids (L4, S48). Subsequently, *in situ* hybridization experiments confirmed these earlier results and assigned the GST 3 locus to chromosome 11q13 (B34, M29). The report published by Board *et al.* (B34), however, also contained evidence for the existence of a second human pi-class gene (or pseudogene) in region q13–14 of chromosome 12.

5.6. Theta-Class GST

The existence of a fourth class of cytosolic GST has recently been recognized following the purification of several novel rat GST. Hiratsuka *et al.* (H46) identified at least three hepatic isoenzymes, which were shown to possess catalytic activity toward reactive sulfate esters. These investigators purified one of these forms, designated GST YrsYrs, and demonstrated that it has catalytic properties and an N-terminal amino acid sequence that distinguish it from alpha-, mu-, and pi-class GST. In a subsequent study, Meyer *et al.* (M17) purified two enzymes from rat liver that are structurally related to GST YrsYrs. These two forms, designated GST 5–5 and GST 12–12, were previously isolated in a preparation called GST E (F12, M16). Evidence suggests that YrsYrs and 12–12 are identical proteins. A third rat theta-class GST, called 13–13, has been purified from mitochondria (H8). The isolation of cDNA clones encoding YrsYrs and 5–5 has demonstrated unequivocally that these enzymes represent a separate family of cytosolic GST (O2, P6).

It is not known how many theta-class GST exist in the human. A single theta-class transferase, GST θ, which is active toward 1,2-epoxy-3-(*p*-nitrophenoxy)propane, has been isolated by Meyer *et al.* (M17) from human liver. More recently, a second human theta-class enzyme, which is active toward 1-menaphthyl sulfate, has been purified (H59). In accordance with the nomenclature guidelines proposed by Mannervik *et al.* (M3), the enzyme that is active toward 1,2-epoxy-3-(*p*-nitrophenoxy)propane has been designated GST T1-1* and that is

active toward 1-menaphthyl sulfate has been designated GST T2–2* (H59). SDS–PAGE analysis of GST T2–2* and its comparison with human alpha-, mu-, and pi-class GST are shown in Fig. 13. Substrates that can be used to facilitate the identification of GST isoenzymes are listed in Table 6.

5.7. Microsomal GST

The presence of a unique membrane-bound GST was first proposed based on studies of the metabolism of α-hexachlorocyclohexane by subcellular fractions of rat liver (K18, K19). Proof of the existence of such an enzyme was hampered by the fact that cytosolic GST interact strongly with rat liver microsomes and are not readily removed by washing, sonication, or treatment with high salt concentrations. Although the cytosolic GST can "mask" the existence of microsomal GST,

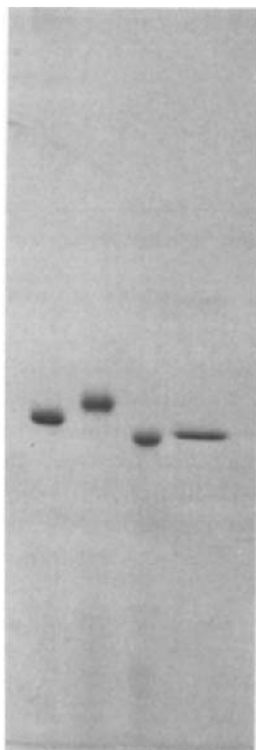


Fig. 13. SDS–PAGE of human cytosolic GST. Purified GST samples, run from the cathode (top) to the anode (bottom), were loaded from left to right as follows: (1) alpha-class GST, (2) mu-class GST, (3) pi-class GST, and (4) theta-class GST. These data are from Hussey and Hayes (H59).

TABLE 6
PHYSICAL AND CATALYTIC PROPERTIES OF GST SUBUNITS

Subunit	Class	Subunit M_r (by SDS–PAGE)	Isoelectric point (of homodimer)	Useful substrates for identification
A1 (B_1)	Alpha	25,900	8.9	Δ^5-Androstene-3,17-dione: Cumene hydroperoxide
A2 (B_2)	Alpha	25,900	8.4	Cumene hydroperoxide
M1a (N_1^a)	Mu	26,700	6.1	*trans*-4-Phenyl-3-buten-2-one: *trans*-Stilbene oxide
M1b (N_1^b)	Mu	26,600	5.5	*trans*-4-Phenyl-3-buten-2-one: *trans*-Stilbene oxide
M2 (N_2)	Mu	26,000	5.3	1,2-Dichloro-4-nitrobenzene: 4-Hydroxynon-2-enal
M3 (N_3)	Mu	26,300	5.0	4-Hydroxynon-2-enal
Pi (Yf)	Pi	24,700	4.7	Ethacrynic acid: Acrolein
T1*	Theta	ND	ND	1,2-Epoxy-3-(*p*-nitrophenoxy)propane
T2*	Theta	25,100	ND	Menaphthyl sulfate
Microsomal	Microsomal	17,300	ND	Hexachlorobuta-1,3-diene: Cumene hydroperoxide

Note. The GST designations included in parentheses represent previous names used in our laboratory. ND, not determined.

the recognition that the latter enzyme is activated by treatment with *N*-ethylmaleimide, whereas cytosolic GST are inactivated by this agent, was a key observation that facilitated the purification of the microsomal enzyme (M21). Purification of a unique membrane-bound GST in rat liver was achieved following solubilization with Triton X-100 and sequential chromatography on hydroxyapatite and CM–Sepharose (M23). Later, Morgenstern and DePierre (M20) also isolated the microsomal GST from rat liver in its unactivated form and showed that the specific activity of the purified enzyme toward 1-chloro-2,4-dinitrobenzene could be increased 15-fold after reaction with *N*-ethylmaleimide. This activation was associated with the modification of a single residue, cysteine 49. Limited proteolysis with trypsin can also activate the enzyme (M24) as can thiol–disulfide interchange (A15, A16, M10), heat (A17), and radiation (B39).

The microsomal GST from human liver was first purified by McLellan *et al.* (M13), who demonstrated that the activity of this enzyme toward 1-chloro-2,4-dinitrobenzene and cumene hydroperoxide could be increased by treatment with *N*-ethylmaleimide. These workers also showed that the human microsomal GST

catalyzed the conjugation of hexachlorobutadiene with GSH, whereas the cytosolic enzymes were essentially inactive with this nephrotoxin. The human and rat microsomal GST were found to comigrate during SDS–PAGE (Fig. 14) and were immunochemically identical.

The molecular cloning of rat and human microsomal GST, described by DeJong *et al.* (D3), has demonstrated the absence of homology between these enzymes and the cytosolic GST. Although it is clear that microsomal GST and the membrane-bound leukotriene C_4 synthase are distinct enzymes (A14, N2, S29), their exact molecular relationship is at present unclear; it is unlikely that they are members of the same gene family (N3).

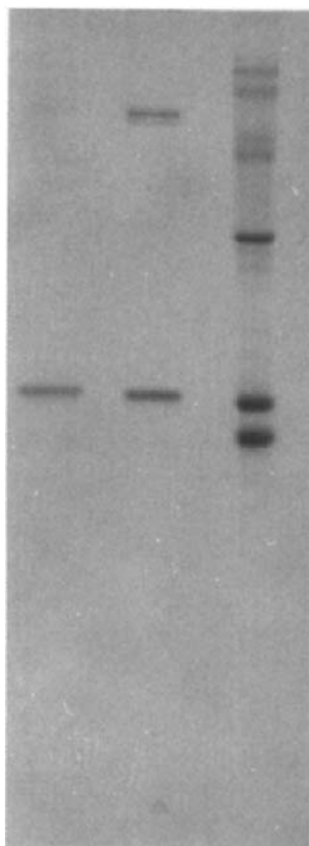


FIG. 14. SDS–PAGE of microsomal GST. The samples were loaded from left to right as follows: (1) human microsomal GST; (2) rat microsomal GST; and (3) a mixture of ovotransferrin (M_r 76,000), albumin (M_r 66,000), chymotrypsinogen A (M_r 27,500), myoglobin (M_r 17,000), and cytochrome c (M_r 12,300). Data obtained from McLellan *et al.* (M13).

6. Biomedical Applications of GST

Whereas the GST were studied originally because of their involvement in mercapturic acid biosynthesis, these enzymes are also currently of interest to researchers working in fields other than toxicology. Some of the research areas in which GST are of importance include tumor markers, inherited cancer susceptibility, drug resistance, biotechnology, and clinical biochemistry.

6.1. Tumor Marker

The demonstration that in rat the pi-class GST P, which is essentially absent from normal liver, is expressed at high levels in hepatic preneoplastic lesions has illicited substantial interest in this enzyme as a potential marker of hepatocellular carcinogenesis (M7, S7). Pi-class GST in different species are regulated by different mechanisms and therefore this family cannot be utilized as tumor markers in all species. In the rat, GST P is not significantly induced by drugs such as phenobarbital and 3-methylcholanthrene and it is therefore thought that its expression in the liver mirrors neoplastic transformation and/or reflects a stress response (H31, H32). In practice, it has proved to be a valuable marker of experimental hepatocarcinogenesis, being used to help identify genotoxic compounds. However, pi-class GST cannot be used for this purpose in the mouse as it is constitutively expressed in murine liver; interestingly, pi-class GST YfYf displays sex-specific regulation in mouse liver (H12, M11) and, besides being inducible by the antioxidant butylated hydroxyanisole (H24, M12), it is regulated by interferon (A5). In humans, GST π is not expressed in hepatocytes but is found in moderate levels in the biliary epithelium (H36). However, GST π is not readily detected in human hepatocellular carcinoma, but it is expressed in hepatocytes in severe alcoholic liver disease (H11). Although not detected in hepatocellular carcinoma, GST π is expressed in human cholangiocarcinoma.

As GST π is present in erythrocytes and platelets, it has not provided a reliable serum/plasma test for neoplasia in humans (please see following text). However, GST π can provide the pathologist with a useful immunohistochemical marker of neoplastic transformation in certain tissues. For example, Kodate *et al.* (K14) found that a high proportion of differentiated colonic adenocarcinomas and undifferentiated colonic carcinomas expresses GST π. This enzyme is also found, using immunohistochemical techniques, in dysplastic and neoplastic human uterine cervix (S23). Several research groups have found GST π to be of value as an immunohistochemical marker of lung carcinoma (C4, E2). Cowan and his colleagues have also found that GST π expression correlates with estrogen receptor status in breast tissue (M28, M29) and the levels of this enzyme can provide a prognostic index in breast cancer.

The pi-class enzyme is not the only GST that can serve as a tumor marker. A

unique rat alpha-class GST subunit, Yc_2, which is not found in normal adult rat liver but is expressed in aflatoxin B_1-induced preneoplastic nodules and hepatomas, has recently been identified (H19). Immunohistochemistry of human hepatocellular carcinoma and cholangiocarcinoma specimens have been found to give highly variable staining for alpha-class GST (H35). In a survey of various tumor types, Campbell *et al.* (C3) found alpha-class GST in approximately 33% of specimens but did not obtain positive results in tumors when the benign tissue was negative. Unfortunately, much remains to be learned about the human alpha-class GST. Although preliminary studies are disappointing, it is too early to make dogmatic statements about the immunohistochemical applications of this enzyme family in humans.

6.2. GST and Drug Resistance

The over-expression of GST can confer resistance to carcinogens and chemotherapeutic drugs. Induction of Yc_2 in rat liver by the chemoprotective antioxidant ethoxyquin is associated with resistance to aflatoxin B_1 (H19). Agents that protect against carcinogenesis induce most GST and it is likely that the chemoprotection afforded by the antioxidant butylated hydroxyanisole against benzo[*a*]pyrene is through induction of mu-class GST (B23, P5). Similarly, in the plant kingdom, treatment of maize with the herbicide safeners *N,N*-diallyl-2,2-dichloroacetamine and 1,8-naphthalic anhydride results in induction of GST and resistance to phytotoxicity by herbicides such as thiocarbamates, chloroacetanilides, and *S*-triazines (H31).

The experimental data suggesting that GST overexpression represents a mechanism of drug resistance are strongly supported by the fact that a prokaryotic GST, which catalyzes the conjugation of GSH with the epoxide-containing antibiotic fosfomycin (Fig. 15), has been obtained from clinical isolates resistant to this antibiotic (A18). The fosfomycin-metabolizing GST is plasmid encoded and it is thought that the gene encoding this enzyme probably originated in *Streptomyces* and evolved as an intrinsic resistance mechanism in fosfomycin-producing microorganisms. Just as the induction of GST in normal tissue produces resistance to carcinogens, so can the overexpression of GST in tumors produce resistance in the neoplasm to certain anticancer drugs. Substantial levels of GST have been observed in human and animal tumors (C4, D6, D7, H51, L7, M19, S9, S18). Cell lines selected for resistance to nitrogen mustards have been shown to possess elevated levels of GST (B44, R10, W1). Furthermore, transfection of cDNAs encoding GST into drug sensitive eukaryotic cells has also been shown to confer resistance to alkylating agents (B27, M6, P14).

GST-mediated drug resistance can be overcome by treatment with chemicals that are (1) inhibitors of GST activity, (2) GST substrates, or (3) agents that deplete intracellular GSH. The inhibitors piriprost, indomethacin, and gossypol (Fig. 16)

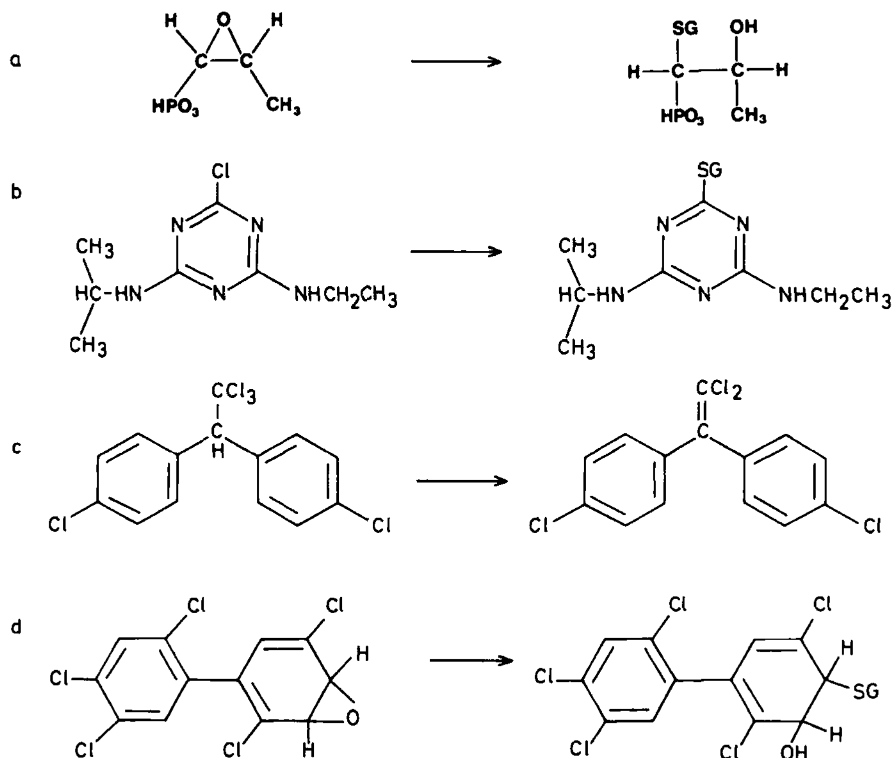


FIG. 15. Examples of an antibiotic and various environmental pollutants that are detoxified by GST. Reactions a–d show the GST-catalyzed detoxification of fosfomycin, atrazine, DDT, and a polychlorinated biphenyl, respectively.

(B25, H6, T8) have been used as antitumor agents. Similarly, the GST substrate ethacrynic acid has been of value in enhancing the cytotoxicity of anticancer drugs in resistant cell lines (T8). Buthionine sulfoximine, an inhibitor of GSH synthesis (Fig. 16) (G12) has been found to be able to resensitize resistant cells to anticancer drugs (B27, S34).

6.3. Association between Polymorphic Expression of GST and Susceptibility to Toxic Chemicals

Failure to express GST can have a pronounced effect on the sensitivity of cells to chemical insult. In humans, one of the mu-class GST genes, GST M1, is absent in approximately 45% of the population (B29, B30, S41). A characteristic feature of the enzyme encoded at this locus is its high conjugating activity toward certain

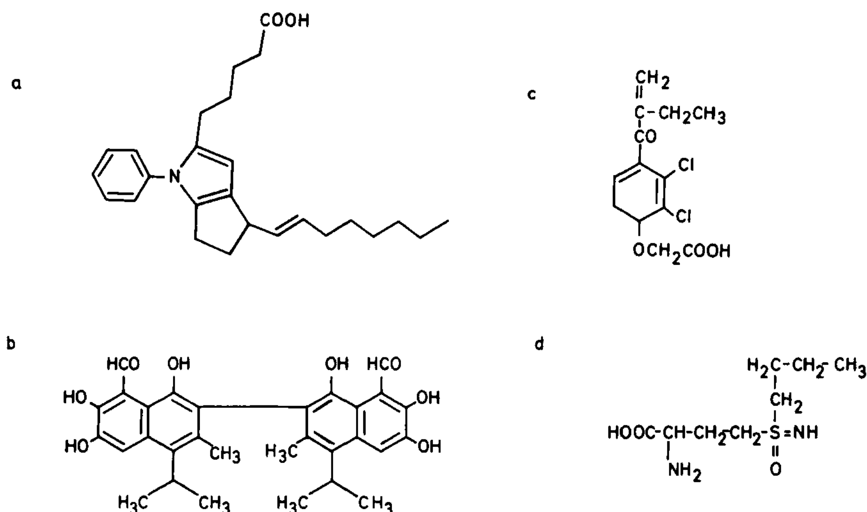


FIG. 16. Agents that have been employed to circumvent GST-mediated drug resistance. Piripost (a), gossypol (b), ethacrynic acid (c), and buthionine sulfoximine (d) have been employed to resensitize resistant cells to various alkylating agents.

mutagenic epoxides, including benzo[*a*]pyrene-4,5-oxide and styrene-7,8-oxide. This has led to the suggestion that individuals with the null allele at this locus, and therefore deficient for GST μ/ψ, are more susceptible to the toxic effects of various xenobiotics.

Various research groups have quantitated the expression of mu-class GST using several different methods of analysis, including starch–gel electrophoresis (B30, L4, S41) and specific radioimmunoassay (H60). A wide variation in the frequency of the nulled phenotype has been reported both within and between different racial groups (Table 7). One method that has been introduced for the detection of certain mu-class enzymes is the measurement of GST activity toward *trans*-stilbene oxide in mononuclear leukocytes (S12, S13). Seidegard and co-workers used this activity assay to assess the degree of protection afforded by the expression of mu-class GST against the chemical carcinogens present in cigarette smoke. In two separate studies, these workers demonstrated significant differences between the distribution of *trans*-stilbene oxide activity in populations of control smokers and that in populations of lung cancer patients (S14, S15). These investigators concluded that the expression of mu-class GST may be used as a marker for susceptibility of an individual to lung cancer. Recently, Strange *et al.* (S45) used a starch–gel zymogram approach to compare the frequency of the mu-class GST null allele in controls with that in a group of patients with adenocarcinoma of the

TABLE 7
THE FREQUENCY OF THE MU-CLASS GST NULL PHENOTYPE

Population	No.	Mu-class GST Null phenotype (%)	Study
Caucasian	23	40	Warholm *et al.* (W4)
Caucasian	40	65	Board (B30)
Caucasian	49	41	Strange *et al.* (S41)
Caucasian	56	43	Laisney *et al.* (L4)
Caucasian	248	54	Seidegard and Pero (S13)
Caucasian	42	45	Hussey *et al.* (H60)
Chinese	96	58	Board (B30)
Indian	43	35	Board (B30)
Japanese	168	49	Harada *et al.* (H7)

stomach or colon. In support of the findings of Seidegard *et al.* (S14, S15), these workers also observed an increase in the frequency of the null phenotype in patients with carcinoma. However, there are several reports in the literature that contain contradictory evidence about the expression of mu-class GST in patients with cancer. Peters *et al.* (P7) used an immunochemical detection method for the measurement of GST in mononuclear blood cells and demonstrated that there were no significant differences in the distribution of mu-class enzymes in patients with breast or colon cancer compared with that in controls.

The polymorphism associated with human mu-class GST has also been investigated at a genomic level using Southern blotting analysis. Seidegard *et al.* (S16) showed that the null phenotype correlated with the absence of a hybridization to a cDNA probe encoding a hepatic mu-class GST. One group of workers used a genotyping assay, which involves this kind of analysis, to investigate the association between mu-class GST polymorphism and the susceptibility of an individual to lung cancer (Z1). In contrast to the findings of Seidegard *et al.* (S14, S15), these workers saw similar distributions of the null allele in both a control population and a large group of lung cancer patients.

The data obtained from the investigations mentioned above are summarized in Table 8. The differences observed among the findings of these studies means that the consequences of the mu-class GST polymorphism remain ill defined.

In the context of a null GST phenotype sensitizing cells to chemicals, it is interesting to note that a Mississippi in-bred strain of maize that displays a marked increase in sensitivity to atrazine has been found to lack the GST responsible for the detoxification of this herbicide (S22). Similarly, the absence of GST in the CSMA housefly has been linked to sensitivity to tetrachlorvinphos (O8). These data therefore suggest that, throughout nature, failure to express GST may result in increased sensitivity to a variety of toxic chemicals.

TABLE 8
THE FREQUENCY OF THE MU-CLASS GST NULL PHENOTYPE IN CARCINOMA PATIENTS

Population	No.	Mu-class GST Null phenotype (%)	Study
Control smokers	192	42	Seidegard *et al.* (S14, S15)
Lung cancer patients	191	62	
Controls	49	41	Strange *et al.* (S41, S45)
Adenocarcinoma patients	45	67	
Controls	64	38	Peters *et al.* (P7)
Carcinoma patients	106	31	
Control smokers	225	42	Zhong *et al.* (Z1)
Lung cancer patients	228	43	

7. The Quantitation of GST in Biological Fluids

The quantity of an enzyme in plasma, serum, or urine is usually determined by measuring catalytic activity but it is also permissible to determine the mass of the enzyme by direct immunological methods, such as radioimmunoassay. There are advantages and disadvantages to both of these approaches. Activity measurements may be modified by the presence of activators and inhibitors in blood or urine and it may be difficult to distinguish between isoenzymes. The measurement of enzyme activity is, however, generally simple, rapid, and convenient to perform and can be readily automated. The immunoassay methods for mass measurements are in general unaffected by the presence of endogenous substances that modify the catalytic activity of enzymes and it is usually possible to distinguish the various isoenzymes with a high degree of specificity. The main disadvantage of immunological methods is the fact that these methods are often slow and expensive and do not readily lend themselves to simple automation (B10).

7.1. Activity Measurements of the GST

For the quantitation of the GST both activity and immunoassay approaches have been tried. The substrate that is most commonly employed is CDNB as it is a substrate for the majority of the GST isoenzymes in humans, the rat, and other species (C13). There are considerable differences in the specific activity of the various subunits to CDNB and therefore interpretation of such measurements is difficult if more than one isoenzyme is present. In the rat, 1,2-dichloro-4-nitrobenzene and bromosulfophthalein have been used but these substrates are active only with the rat Yb2 subunit and essentially display no activity with the human GST (B36).

At present the use of activity measurements to quantitate plasma, serum, or urinary levels of the GST are inadequate. With CDNB it is difficult to obtain sufficient sensitivity to allow the measurement of the levels in normal subjects (A4). In addition many drugs and endogenous substances may inhibit the activity to values that lie within the reference range. For example bile salts and bilirubin inhibit GST activity (H17) and since both of these nonsubstrate ligands are increased in liver disease their accumulation in plasma could theoretically suppress GST activity to within the reference range. An important problem with GST activity measurements concerns the ubiquitous nature of the GST since poor organ specificity will result unless specific isoenzymes are measured. For example, platelets, erythrocytes, and white cells contain high levels of the isoenzyme and these cells may release their GST into plasma prior to separation of the blood sample (G4, H52, L12, M8, R11, S43). With the substrates that are available to date, the activity measurements are inadequate for clinical use.

7.2. Immunoassay Measurements of the GST

A number of immunoassay approaches have been used for the GST. Western blotting provides a semiquantitative method for studying the GST (H37) but it may be necessary to concentrate the protein by, for example, affinity chromatography prior to blotting. Blotting methods are too insensitive for use in plasma but they are suitable for semiquantitative measurement of GST in tissue.

Radioimmunoassay (RIA) is the method most commonly used for the measurement of plasma and urinary concentrations of GST. The first RIA described for the GST was for rat ligandin (GST YaYa) (B6) and in 1978 the first assay for human ligandin was described (T13). The assay for human ligandin lacked sensitivity as did the RIA for human ligandin described in 1983 by Sherman *et al.* (H18, S20). In 1984 we described the first RIA methods that could differentiate the B1 and B2 subunits with a high degree of sensitivity and specificity as shown in Table 9 (B16). These assays could detect both the upper and the lower reference limits for plasma measurements. When animals were immunized with the homodimer B_1B_1, or B_2B_2, the cross-reactivity that the antisera had with the heterodimer B_1B_2 was quite variable. Because the proportion of the B_1B_2 heterodimer in liver can vary markedly (H63), it is important to choose antisera that will react only with one of the subunits in the heterodimer. The antisera we used for our subsequent clinical studies on plasma B_1 and B_2 subunit measurements in plasma or serum exhibited approximately 50% cross-reactivity with the B_1B_2 heterodimer and thus it was possible to measure total B_1 or B_2 subunits rather than the total subunits in the homodimer and a variable proportion of the same subunit in the homodimer.

We have also described a specific RIA for the pi-class enzymes that is sensitive enough to detect plasma levels as shown in Table 9 (H52). Other assays for human pi-class GST have also been described using both polyclonal and monoclonal

TABLE 9
DETAILS OF SOME METHODS DESCRIBED FOR THE MEASUREMENT OF PLASMA OR SERUM GST CONCENTRATIONS IN HUMANS

Method	GST measured	Class	Reference range (μg/liter)	Year and reference
RIA	Ligandin	Alpha	<5.3	1978 (S13)
RIA	B_1B_1	Alpha	0.5–2.6	1983 (H18)
RIA	Ligandin	Alpha	<12	1983 (S20)
RIA	B_1B_1	Alpha	1.2–4.0	1984 (B16)
RIA	B_2B_2	Alpha	0.6–1.8	1984 (B16)
EIA	Cationic	Alpha	0–2.3	1984 (H44)
EIA	Anionic	Alpha	0–1500	1984 (H44)
RIA	Mu	Mu	ND	1987 (H60)
RIA	Pi	Pi	<10	1988 (H52)
EIA	Pi	Pi	<30	1989 (N4)

Note. **ND**, not detectable in normal serum; **RIA**, radioimmunoassay; and **EIA**, enzyme immunoassay.

antibodies (H28, H48, N4, T11). For the Mu-class enzymes no RIA has been described that has sufficient sensitivity to detect levels in normal plasma and for this reason whole blood or lymphocyte measurements are employed for the study of the polymorphic expression of mu-class GST (H60, Z1); lymphocytes have high levels of the mu-class GST (S11, S12).

There are also now available commercial enzyme-linked assays for the measurement of mu-class subunits in whole blood and also the quantitation of total alpha-class subunits in plasma (Biotrin International, Dublin). These assays have the advantage of speed over the RIA methods since the results can be obtained in less than a day but few studies have been reported using these methods and their reliability and validity remain to be established.

7.3. Reference Values

The reference values found for the GST in biological fluids will vary depending on the various subunits that are detected by the antisera. In general, however, the GST are found in plasma at micromolar concentrations. Examples are given in Table 9.

8. Developmental Expression of GST in Human Tissues

The expression of the GST isoenzymes shows clear time- and tissue-specific expression during fetal, neonatal, and infant life. These changes are of interest

because of the putative importance of these enzymes in the metabolism of both endogenous and exogenous compounds. A number of techniques have been employed to study the developmental changes in the GST, including starch–gel electrophoresis, column chromatography, immunohistochemistry, and radioimmunoassay, and this has allowed quantitation of the various classes and subclasses of the GST together with a clear identification of the morphological development (B19, C18, F2, H39, H40, H47, S40, S44). Radioimmunoassay data are summarized in Table 10 [data are taken from (A1, B19, S44)].

8.1. Liver

8.1.1. *Alpha-Class GST*

The B_1 subunit represents the major form of the total GST mass in the liver throughout development, with this subunit comprising approximately 80–90% of

TABLE 10
DEVELOPMENTAL CHANGES IN GST EXPRESSION IN LIVER, KIDNEY, AND LUNG

	In utero ontogeny group (10–40 weeks postmenstrual)			Postnatal group (40–145 weeks postmenstrual)				Adult group	
	Liver	Kidney	Lung	Liver	Kidney	Lung	Liver	Kidney	Lung
GST B_1									
Mean	9.0	0.042	0.035	14.3	0.17	0.050	12.8	5.75	0.09
SD	3.1	0.050	0.032	4.7	0.17	0.060	5.5	4.3	0.03
N	22	8	14	20	14	10	20	6	6
GST B_2									
Mean	0.93	0.030	0.008	2.3	0.36	0.016	3.7	2.79	0.06
SD	0.76	0.045	0.005	2.3	0.59	0.020	2.1	1.5	0.04
N	22	8	14	19	14	10	20	6	6
GST μ									
Mean	0.099	0.32	0.14	0.43	0.13	0.06	0.46	0.06	0.05
SD	0.054	0.15	0.13	0.33	0.09	0.05	0.27	0.02	0.06
N	10	4	8	11	10	5	11	3	3
GST π									
Mean	0.53	0.70	0.59	0.21	0.58	0.16	0.008	0.61	0.94
SD	0.27	0.30	0.44	0.22	0.35	0.09	0.013	0.23	0.50
N	22	8	14	20	12	10	20	6	6

Note. GST levels were determined by radioimmunoassay and expressed as mg/g cytosolic protein.

the cytosolic GST pool in the fetus and the adult. The expression of the B_1 subunit appears to be relatively stable during fetal development but in the period between term and 85 weeks postnatal the mass of the B_1 subunit is approximately 50% greater on average than the levels found during *in utero* ontogeny. Adult levels of the B_1 subunit are not significantly different from those found in postterm infants.

The B_2 subunit is expressed at considerably lower levels than the B_1 subunit during development. During *in utero* development the average hepatic concentration of the B_2 subunit is only approximately 10% of that of the B_1 subunit. Although there is apparently no significant change in the expression of B_2 between 10 and 40 weeks gestation, the expression of the B_2 subunit is significantly higher in the postnatal period than during *in utero* ontogeny and the levels of B_2 in the adult are significantly higher than the levels found in the postnatal period. This continued increase in expression of the B_2 subunit into adult life results in the B_2 subunit comprising approximately 25% of the total hepatic alpha-class subunits in the adult.

Immunohistochemistry of the alpha-class enzymes has shown that the tissue distribution is similar in both fetal and adult livers. Most staining is found in hepatocytes but not all hepatocytes appear to express the alpha-class enzymes. Hematopoietic and Kupffer cells, biliary epithelia, and the biliary canaliculi remain negative through development for alpha-class GST but the occasional positive cell is found in the large bile ducts of both the fetus and the adult (H40, S44).

8.1.2. *Mu-Class GST*

The marked polymorphism of the mu-class GST was discussed earlier and using radioimmunoassay Strange *et al.* have shown that mu-class GST is expressed in only approximately 50% of livers during all stages of development (S44). In livers that express the mu isoenzyme no change in expression occurs between 10 and 40 weeks gestation but as with the B_1 and B_2 subunits these levels are significantly lower than those found in postnatal infants and adults. Using immunohistochemistry, Strange *et al.* found that it was not possible to detect the levels of mu-class GST in any liver during fetal development but after delivery the enzyme was found to be confined to the hepatocytes in individuals with phenotypes other than GST 1–0 (S42). In contrast to these immunochemical findings Harrison (H9) has reported that the expression of mu-class GST is not related to gestational age.

8.1.3. *Pi-Class GST*

The level of the pi-class GST has been shown, using radioimmunoassay, to fall significantly during the period of 10 to 40 weeks gestation from levels as high as 1.0 μg/mg cytosolic protein at 10 weeks to a mean level of approximately 0.3 μg/mg cytosolic protein at delivery (S44). However, during this time the total mass of pi-class GST in the liver increases as a consequence of the marked increase in liver weight during fetal development. Between 41 and 145 weeks there appears

to be no further significant change in hepatic pi-class GST expression but adult levels are significantly lower than those found post-delivery.

Using immunohistochemistry Strange *et al.* have shown that, up to 24 weeks, hepatocytes stain positive for pi-class GST; however, in some samples both positively and negatively stained hepatocytes were found to be intermixed. The epithelia of the developing bile ducts were also strongly positive, whereas hematopoietic cells were negative. After 24 weeks, staining of hepatocytes for pi-class GST became progressively weaker whereas the bile duct epithelia remained strongly positive. In the adult the epithelia of both large and minor bile ducts were strongly positive, whereas hepatocytes, Kupffer cells, and bile canaliculi were usually negative (S42). Harrison has described a similar decrease in pi-class GST expression during development using immunohistochemistry (H9).

8.2. Kidney

The expression of the B_1 and B_2 subunits shows a pattern during development similar to that found in the liver, with lower levels being found in the period up to 42 weeks gestation than those found in the period between term and 110 weeks postnatal. Adult levels of both GST B_1 and GST B_2 are, however, significantly higher than levels found *in utero* or in postnatal groups. No significant change in the expression of mu- or pi-class GST occurs between 10 and 110 weeks postmenstrual (B9).

Immunohistochemistry has shown that before 20 weeks the developing collecting tubules and the cuboidal cells of the primitive Bowman's capsule stain for alpha-class GST. As the nephron elongates the entire length becomes positive for alpha-class GST but after 35 weeks the adult pattern is observed, with only the proximal tubule staining positive. The expression of pi-class GST was similar to that of alpha-class GST up to 35 weeks; thereafter the isoenzyme was down-regulated in the proximal tubule and Bowman's capsule but was strongly expressed in the distal and collecting tubules. Mu-class GST isoenzymes were expressed along the entire tubule during development (S42). Harrison has reported a similar pattern of developmental expression, and pi-class GST was also found in all medullary tubules in the adult (H9, H10).

8.3. Lung

The expression of the GST has been studied both in fetal tissue and in lung organ culture (B19, C18). No quantitative changes in the expression of B_1, B_2, or mu-type subunits have been detected during the first 100 weeks of development using radioimmunoassay but, as in the liver, the expression of pi-class GST fell

progressively during *in utero* ontogeny. The pi-class isoenzyme remained quantitatively the most predominant GST at all stages of development, including that of the adult (B19).

Immunohistochemistry has shown that, at between 12 and 18 weeks, epithelial cells of the large intrapulmonary airways and their distal branches stain strongly positive for pi-class GST. However, with the appearance of type I and type II pneumatocytes at 24 to 27 weeks, these differential cells are normally negative for pi-class GST, as are the epithelial lining cells of distal airways. Most epithelial cells of the major intrapulmonary airways remain strongly positive for both pi-class GST and alpha-class GST throughout development (F18, S42).

In summary, it is apparent that the controls exerted on GST expression during development are not identical for each GST class. In the liver, alpha-class GST predominates and the expression of B_1 and mu-class GST is relatively stable in the postnatal period, whereas there is a continued increase in the expression of B_2. There is also a down-regulation of pi-class GST during the first half of gestation but the expression of the isoenzyme is not completely switched off. In kidney, pi-class GST is the major GST isoenzyme until adulthood when alpha-class GST becomes the major form. Whether this is a sudden switch at puberty or over a more prolonged period is not known. In lung, pi-class GST is the major GST at all stages of development but its expression decreases *in utero* in a pattern similar to that observed in liver. The fact that lung and liver are of endodermal embryonic origin may explain the similarity in the changes observed in pi-class GST expression during gestation. The function of the mu-class GST is unclear but the fact that in early gestation the prevalence of the nulled phenotype is similar to that found at birth, i.e., approximately 50%, suggests that its presence is not of central importance to development.

9. Plasma Alpha-Class GST Measurements in Liver Disease

9.1. Theoretical Advantages of Plasma GST Measurements for Detecting Hepatocellular Damage

The standard battery of biochemical tests used to assess liver function usually includes the measurement of the activity in plasma of one of the aminotransferases [either aspartate aminotransferase (AST) or alanine aminotransferase (ALT)]. Such measurements are performed to assess the integrity of the hepatocyte membrane. The measurement of AST provides poor organ specificity due to the ubiquitous nature of the enzyme and both ALT and AST are relatively poor at detecting damage that is occurring to the centrilobular hepatocytes. The inadequacy of the aminotransferases at detecting centrilobular liver damage may be

related to the lobular distribution of the enzymes. The aminotransferases are mainly distributed within the periportal hepatocytes as are alkaline phosphatase and γ-glutamyltransferase and as such these enzymes provide satisfactory markers to detect damage to this zone of the liver but they are poor at detecting alcohol- and drug-related liver damage that may occur in centrilobular hepatocytes; indeed frank centrilobular necrosis may occur without any abnormalities being evident in the plasma aminotransferases (R2).

The alpha-class GST in humans are found in very high concentration in hepatic cytosol and are distributed evenly throughout the entire liver lobule (H40). Theoretically, plasma levels of GST B_1 and B_2 subunits should provide a sensitive and reliable indicator of damage to any part of the liver. The very high cytosolic concentrations of the B_1 and B_2 subunits in liver together with their low molecular weight and cytosolic location also mean that these subunits will be released in quantity when only a minor impairment in hepatocellular integrity occurs. The B_1 and B_2 subunits appear to have a very short plasma half-life (less than 1 hr); thus, when active hepatocellular damage has ceased, plasma levels will rapidly fall to reference values, whereas enzymes with relatively long plasma half-lives (e.g., ALT and AST) may remain elevated (B6, B7, B13). Overall it can be concluded that, on theoretical grounds, plasma B_1 and B_2 measurements should provide a more sensitive and specific indicator of impaired hepatocellular damage across the liver lobule than aminotransferase measurements.

9.2. Plasma GST Measurements for the Detection of Acute Liver Damage

Two early studies in rat and humans demonstrated that plasma or serum measurements of the alpha-class GST may provide a useful indicator of liver damage following an acute hepatic insult. Tsuru *et al.* showed in 1978 using radioimmunoassay that in humans plasma ligandin concentrations were elevated in a number of situations that produced an acute or chronic insult to the liver (T13). The rat has proved to be a good model to study the sensitivity of plasma GST measurements as a means of detecting drug-induced liver damage. Bass *et al.* in 1978 showed that, when the hepatotoxin carbon tetrachloride was administered to rats, plasma GST increased at a greater rate and to higher levels than plasma AST activity (B7). These workers also found in the rat that the plasma half-life of GST mass was much shorter than the half-life of AST activity. Few other studies were published concerning the use of plasma GST measurements until the early 1980s but these subsequent studies have demonstrated that not only do plasma alpha-class GST measurements have a role in detecting significant liver disease but also these measurements are useful in the investigation of the mechanisms involved in drug toxicity (B17).

9.2.1. *Paracetamol (Acetaminophen) Poisoning*

Paracetamol is a toxic agent because it is metabolized by the liver to a potent electrophilic arylating metabolite that binds to numerous macromolecules within the cell and impairs their function. When ingested in small amounts the toxic metabolite can be inactivated within the liver by conjugation with glutathione but, if the amount of paracetamol ingested is large, glutathione stores may be insufficient for complete detoxification with the result that irreversible liver damage ensues.

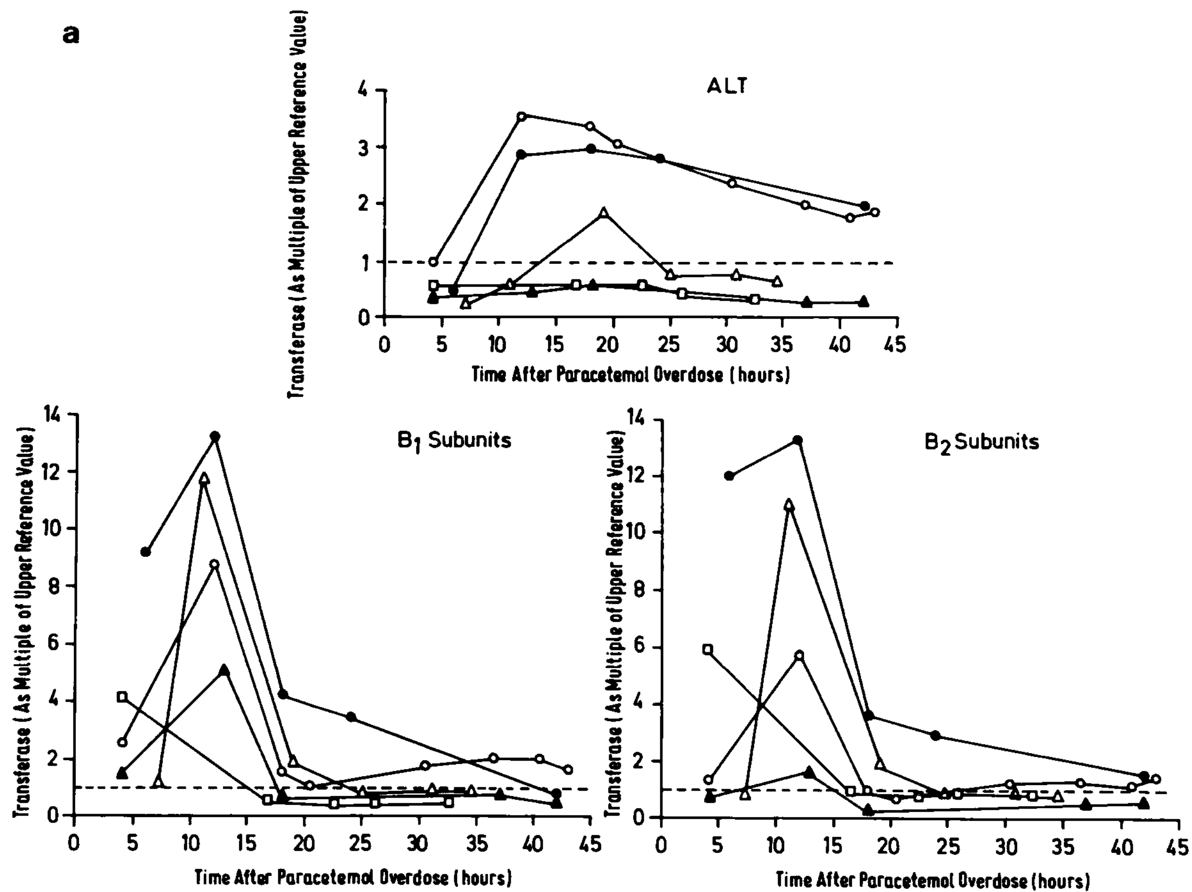
Patients who have ingested excessive quantities of paracetamol may be effectively treated with either *N*-acetylcysteine (NAC) or methionine since both agents can replenish hepatic glutathione stores and thus prevent hepatotoxicity (P11, P12, V1). These agents are effective only if administered within 12 hr of poisoning, and because there is a slight risk from administration of NAC or methionine they are given only to patients in whom the plasma paracetamol lies above a treatment line joining semilog plots of 200 μg/ml at 4 hr and 30 μg/ml at 15 hr.

Paracetamol poisoning provides a useful human model to compare the effectiveness of plasma GST with aminotransferase measurements because in such patients there is often a wide spectrum of the severity of liver damage encountered. In addition, damage to the liver initially occurs in the centrilobular hepatocytes, where phase I drug metabolizing enzymes produce the toxic metabolite (J3).

We used our specific RIA methods for B_1 and B_2 subunits to investigate the time course of hepatocellular damage in 19 patients admitted with paracetamol overdose (B12, B14). Seven patients had plasma paracetamol concentrations that were below the treatment line and four of these patients had marginal elevations (approximately one and a half times the upper reference limit) of B_1 upon admission within 4 hr of the overdose. Only one of the seven patients subsequently showed a significant rise in plasma GST B_1 to nine times the upper reference limit but in this patient and in each of the other seven patients no abnormalities in ALT were found at any time during their hospitalization.

Seven of the nineteen patients were treated with NAC and each had an elevated or equivocal plasma B_1 concentration upon admission within 6 hr of overdose. The plasma B_1 levels continued to increase despite NAC administration in each patient but 13 hr after the overdose peak plasma B_1 concentrations had been reached, with levels of up to 20 times the upper reference limit being found. Thereafter, B_1 concentrations fell until, more than 24 hr after taking the overdose, normal B_1 concentrations were found in all but two patients. In contrast, to B_1, ALT activates were normal in all patients upon admission and never rose to values in excess of four times the upper reference limit. Examples of the enzyme profiles obtained in these patients are shown in Fig. 17.

Five patients presented more than 10 hr after the overdose and in each of these


a
ALT
Transferase (As Multiple of Upper Reference Value)
Time After Paracetemol Overdose (hours)
B1 Subunits
B2 Subunits
0
1
2
3
4
5
10
15
20
25
30
35
40
45
6
8
12
14

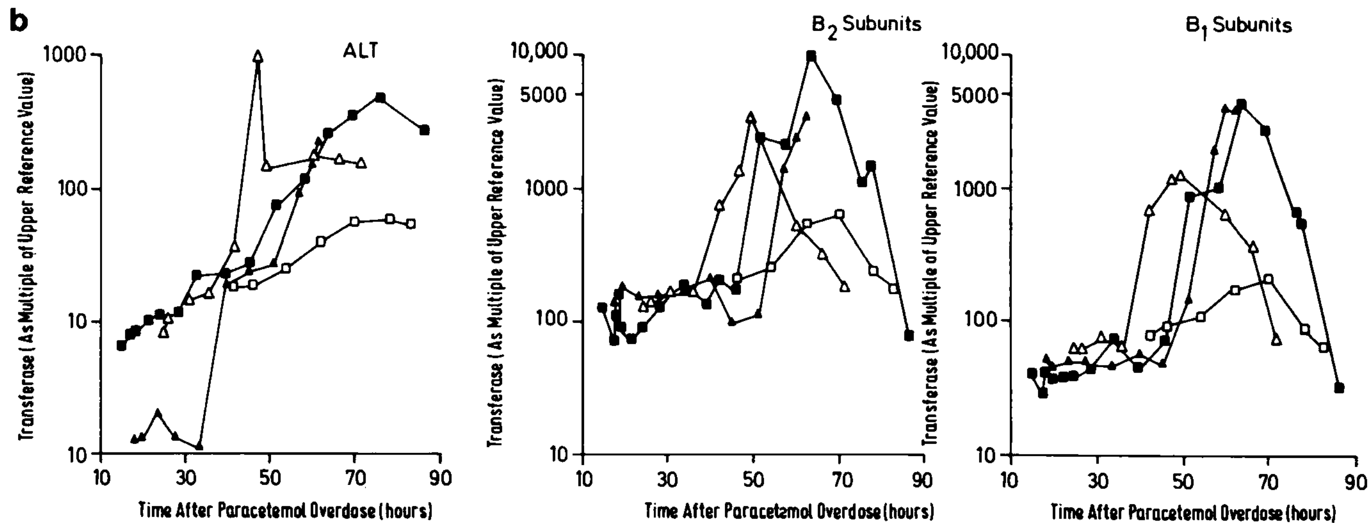


FIG. 17. Plasma GST B_1 and B_2 subunits and ALT activities in patients with paracetamol poisoning. The upper (a) figure shows enzyme levels in patients treated successfully with *N*-acetylcysteine following the admission (first) blood sample and the lower (b) figure the enzyme levels in patients admitted too late after the overdose for effective treatment with *N*-acetylcysteine. All data are expressed as multiples of the upper reference limit.

patients plasma GST B_1 was in excess of 50 times the upper reference limit upon admission; abnormalities in ALT did not exceed 10 times the upper reference limit at this time. Forty hours after the overdose there was a rapid rise in GST B_1 to values in excess of 9000 times the upper reference limit, whereas abnormalities in ALT did not exceed 1000 times the upper reference limit (for examples of the profiles obtained, see Fig. 17). When plasma B_2 concentrations were measured in the same patients, results were essentially the same as those found for B_1 (Fig. 17).

The sensitivity of GST measurements and their short plasma half-lives allowed us to identify early and late phases of drug toxicity, which could not be easily recognized using ALT measurements (Fig. 18). Abnormalities in B_1 and B_2 were detectable within 4 hr of poisoning and these rose progressively until after 10 to 12 hr plateau levels were reached at approximately 100 times the upper reference limit. If NAC was given during the first 10 hr, then the rise in GST could be arrested but, if plateau levels had already been reached, NAC appeared to have no effect and a second and severe stage of toxicity was then apparent at 38–45 hr, characterized by a large transient peak in plasma B_1 and B_2. This late peak was probably the result of cellular necrosis.

In a subsequent study we found that patients in whom the concentration of B_1 exceeded 10 μg/liter (two and a half times the upper reference limit) upon admission subsequently went on to develop moderate liver damage despite treatment with NAC. In contrast, ALT or F protein (another sensitive marker of hepatocellular damage) (F15) was not as efficient as B_1 for predicting the clinical outcome of the patients (B15).

Although NAC is well tolerated, cysteine, its major metabolite, has been shown to cause damage to isolated hepatocytes grown under aerobic conditions (V5). In our early study on paracetamol poisoning we observed an increase in plasma B_1 within 30 min of commencing NAC treatment in two patients with paracetamol poisoning (B12). It was not clear at the time, however, if this rise in plasma B_1 represented NAC-induced hepatotoxicity or if it was caused by a coincidental rise produced from paracetamol toxicity. In a later study, however, using 10 patients we found that overall there was a significant decrease in plasma B_1 within 4 hr of starting treatment with NAC and we could find no evidence that NAC was hepatotoxic with the doses used for treatment (B13).

9.2.2. *Acute Viral Hepatitis*

Plasma GST concentrations are elevated in most patients with viral hepatitis. Sherman and his colleagues using sequential blood sampling found that AST was elevated in each of 68 patients with viral hepatitis upon admission but in 1 patient normal concentrations of GST were found (S20). Because of the short half-life of

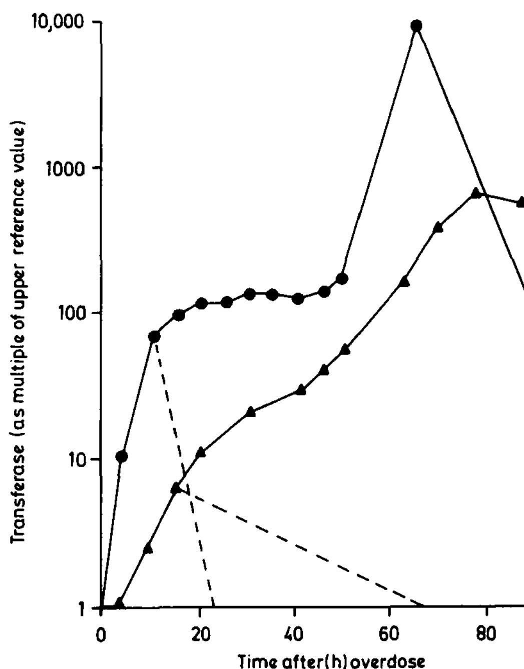


FIG. 18. Schematic diagram of the plasma profile of GST B_1 (●) and ALT (▲) following poisoning with paracetamol. The dashed lines represent the profiles if patients are treated with *N*-acetylcysteine within 12-hr of the overdose. All data are expressed as multiples of the upper reference limit.

GST, a marked and rapid fall in GST was observed such that 5 weeks after diagnosis all 48 patients with non-B hepatitis had normal plasma GST levels, whereas 32 of the 48 still exhibited elevated AST. In patients with hepatitis B a similar effect was seen such that 8 weeks after diagnosis all 20 patients had normal plasma GST, whereas AST was still elevated in 4 patients (Fig. 19). It was concluded that the short half-life of GST in plasma weakened its use as a diagnostic test in acute viral hepatitis but it was suggested that GST might provide a useful indicator of the progress of the active disease since the disappearance of GST from plasma correlated well with clinical recovery. It is thus possible that plasma GST measurements may provide a useful additional test and prognostic indicator in patients being investigated for suspected viral hepatitis. The GST measurement would provide a good indicator of the degree of ongoing active liver damage, whereas a measure of AST or ALT activity would reflect previous as well as ongoing liver damage.

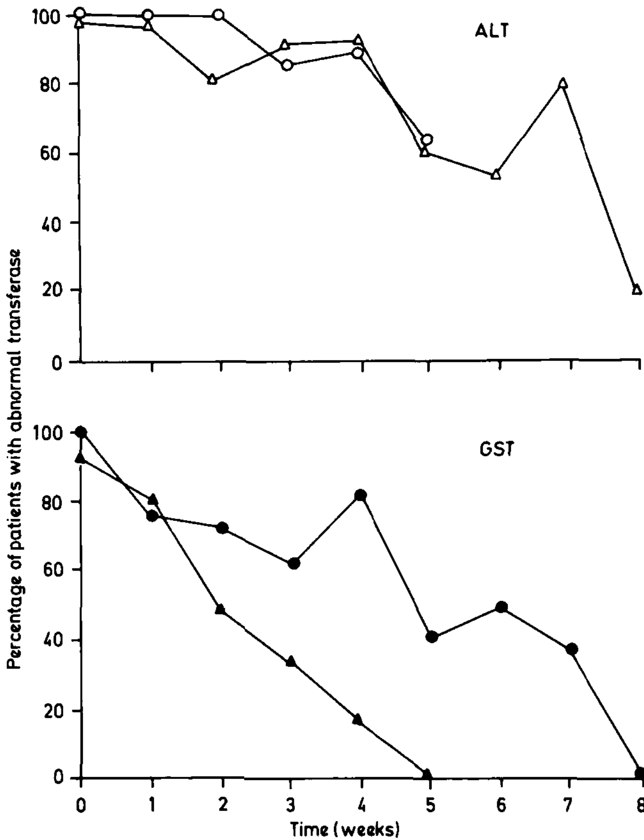


FIG. 19. The percentage of patients with viral hepatitis in whom serum AST and GST levels were elevated at various stages of the disease. GST (▲) and AST (○) levels in 48 patients with non-B hepatitis. GST (●) and AST (Δ) levels in 20 patients with hepatitis B.

9.2.3. *Acute Alcohol Ingestion*

The mechanism of hepatocellular damage by alcohol and the reasons why there are marked interindividual variations in the susceptibility to alcohol-related liver damage are poorly understood. Ethanol may be metabolized to acetaldehyde by cytosolic alcohol dehydrogenase or it can be oxidized by the microsomal ethanol oxidase system. The metabolites from ethanol metabolism can have direct toxic effects on the cell or they may lead to a reduction in membrane fluidity or increased free radical damage potentiated by a reduction in hepatic glutathione (L9, L10, R12).

A test that could identify individuals at particular risk from alcohol-induced

liver damage would prove useful and plasma B_1 measurements may provide such a marker because of their centrilobular location; centrilobular cells are most susceptible to ethanol-induced damage. The standard enzyme measurements are ineffective for this purpose as aminotransferase levels may be normal when there is significant liver damage and γ-glutamyltransferase (GGT) may be induced when there is no significant abnormality in histology (S32).

In a study of the effects of acute alcohol ingestion in plasma B_1, we gave 80 g of ethanol, as vodka, over a 30-min period to six normal male volunteers who drank infrequently and nine male heavy drinkers who consumed between 720 and 2000 g of alcohol per week. None of the subjects had clinical evidence of liver disease (H33).

In each of the normal drinkers and in all but one of the heavy drinkers a normal basal B_1 concentration was found. As a group there was no significant change in plasma B_1 after alcohol ingestion in the controls but in the heavy drinkers there was a significant increase in plasma B_1 that was maximal after 60 min (Fig. 20). However, in two of the control subjects plasma B_1 also rose to an abnormal level after 60 min and, conversely, two of the heavy drinkers showed no significant rise in B_1 postalcohol.

These data show biochemical evidence that heavy alcohol consumption can produce significant deterioration in the integrity of the hepatocyte since in the majority of heavy drinkers a rise in plasma GST occurred after the alcohol load. The study also suggested that there was considerable interindividual variation in the plasma GST response to alcohol even in moderate drinkers, suggesting that the test might be a useful predictor of susceptibility to alcohol-induced liver damage. Further detailed studies are required to clarify if this is the case and a comparison of the responses in males and females would be of interest.

9.2.4. *Hypoglycemia*

Hypoglycemia is a common side effect of treatment with insulin, and, in insulin-treated diabetics admitted with severe hypoglycemic attacks, pronounced abnormalities in plasma ALT and AST activities have been reported (G5, S31). In contrast, when prospective studies using controlled insulin hypoglycemia have been performed few abnormalities in the activities of AST and ALT have been found when there was no evidence of preexisting liver disease; nevertheless, abnormalities in liver function tests are common in diabetic patients (S2, P3).

We have investigated the effects of insulin-induced hypoglycemia on plasma B_1 concentration in normal subjects and diabetic patients (H55) (Fig. 21). In 5 of the 6 nondiabetic male volunteers subjected to insulin-induced hypoglycemia there was a transient significant increase in plasma B_1, which reached a maximum 3 hr after hypoglycemia. In patients with type I diabetes there also occurred a significant increase in plasma B_1, 3 hr after hypoglycemia and the average increase in B_1 was not significantly different from that found in the control group. In both

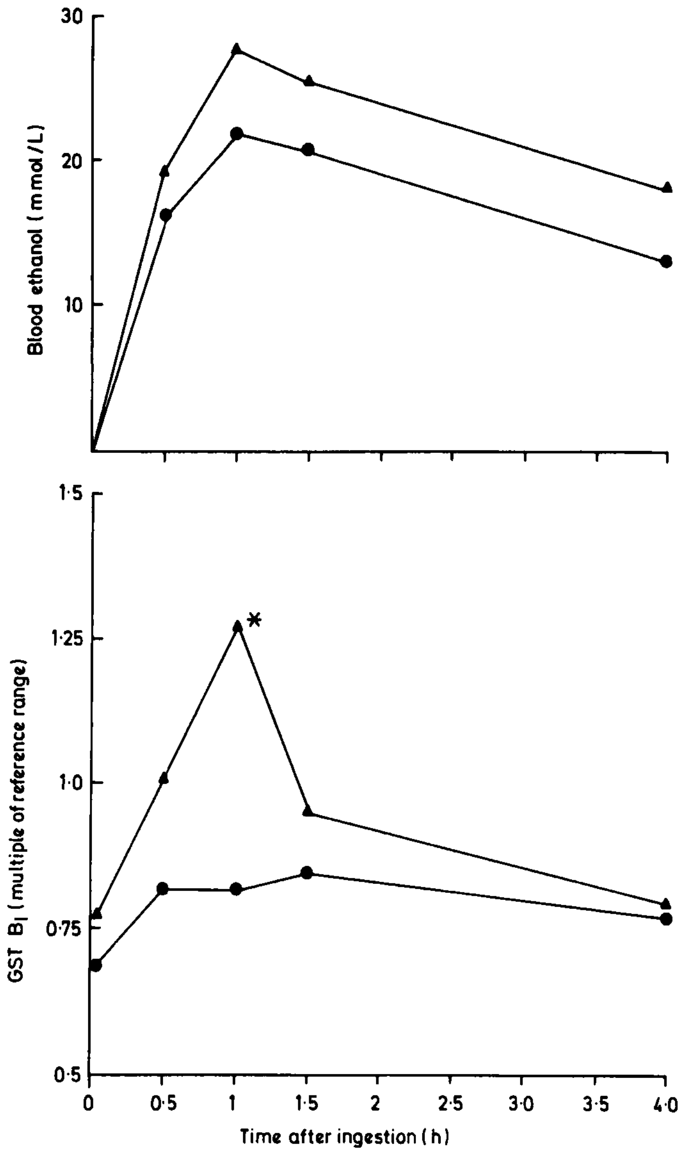


FIG. 20. The effect of alcohol ingestion on mean plasma ethanol and GST B_1 concentrations in nine heavy drinkers (▲) and five volunteers with moderate alcohol intake (●). The asterisk signifies a $p<0.05$ increase from the basal level.

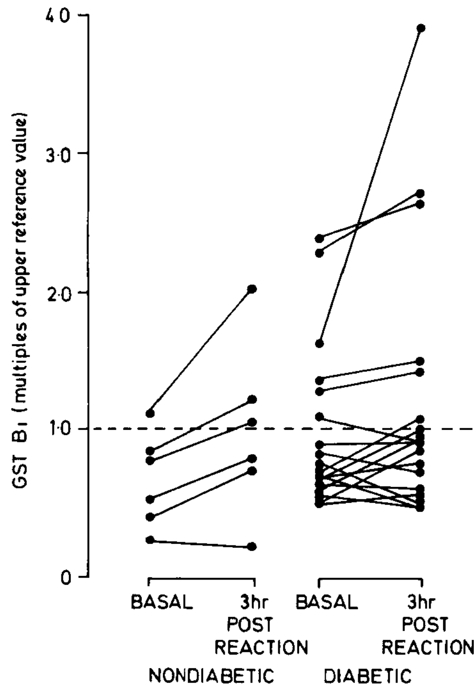


FIG. 21. The effect of insulin-induced hypoglycemia on plasma GST B_1 concentrations in diabetic patients and nondiabetic volunteers. Values are expressed as multiples of the upper reference limit.

groups no significant changes occurred in ALT, AST, or GGT, all of which remained within their reference intervals. Abnormal B_1 levels were found 3 hr after hypoglycemia in 3 of the 6 control subjects and in 6 of the 18 diabetic patients.

We also measured plasma B_1 concentrations in nine diabetic patients admitted as emergencies with hypoglycemic comas of 0.7 to 8 hr duration. Four of the nine diabetics had elevated B_1 upon admission but the abnormalities were relatively minor, with mean levels being 4.7 ± 0.9 μg/liter (reference range, 1.2–4.0 μg/liter).

These studies show that abnormalities in hepatocellular permeability can occur in hypoglycemia and the fact that no changes in ALT and AST were observed suggests that mainly centrilobular hepatocytes are affected. The cause of the altered hepatocellular permeability in hypoglycemia is unclear but possibilities include reduced hepatic blood flow (H42) and diminished cellular ATP generation since insulin inhibits hepatic glycogenolysis and gluconeogenesis, thus rapidly depleting hepatocytes of glucose.

9.2.5. *Birth Asphyxia*

It is common practice to assess hepatic function in neonates who have suffered transient asphyxia at birth by measuring the plasma activities of ALT or AST. We have investigated the changes in plasma B_1 and B_2 subunits that occur in babies following a normal delivery and 14 infants who suffered transient asphyxia during birth; results were compared with the changes found in ALT (B21).

9.2.5.1. Control Group. The control group comprised samples taken from 62 infants who had had blood taken as part of the investigation of a number of conditions not involving the liver. The plasma B_1 concentrations in samples obtained within 24 hr of delivery were significantly higher than the levels found in samples taken between 24 and 174 hr after delivery. In the first 24 hr, B_1 concentrations were as high as 100 μmole/liter; these values compare with an upper reference limit of 4.0 μg/liter found in the adult. The high B_1 values found in the plasma of babies following a normal delivery were not due to increased expression of the subunit in the liver. In samples taken between 80 and 174 hr after delivery all but one baby had B_1 below 17 μg/liter and we have found that in young children (greater than 1 year of age) the levels of B_1 are not significantly different from adult values. These data would thus suggest that following a normal delivery there is a transient impairment in hepatocellular integrity and since we were unable to demonstrate any abnormality in ALT levels during this early period the data suggest that the source of the elevated B_1 is the centrilobular hepatocytes. From a diagnostic view it is clear that, as with many parameters in the newborn, appropriate reference ranges must be applied.

9.2.5.2. Birth Asphyxia Group. The plasma concentration of B_1 was significantly higher in the first 6 hr following delivery when compared with values measured at 24 hr but no change in ALT occurred during this initial 24-hr period. When appropriate reference ranges were applied, 11 of the 14 infants had abnormal B_1 concentrations, whereas only 7 had abnormal ALT activities. In the 24- to 72-hr postpartum period there was a significant decrease in B_1 concentrations compared with values found at 12 hr, such that abnormal B_1 levels were found in only 6 patients. In contrast, ALT abnormalities were recorded most frequently in the 24- to 48-hr period, with 10 of the 14 infants showing elevated activities. The measurement of the B_2 subunit proved unreliable for detecting any abnormality (B21) (Fig. 22). This is probably due to the fact that this subunit is only poorly expressed in the liver of term infants. These results show that plasma B_1 can provide an earlier and more sensitive indicator of liver damage in the first 24 hr after birth asphyxia.

9.2.6. *Anesthesia with Halothane, Enflurane, or Isoflurane*

Hepatic injury has been associated with the use of hologenated anesthetic agents for many years (B42, N1, P10). Halothane was first introduced in 1956 after being

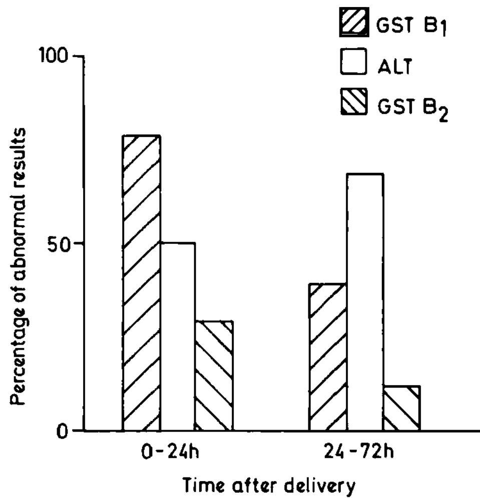


FIG. 22. The percentage of babies with abnormal GST B_1, GST B_2, and ALT levels with 24 and 72 hr of birth asphyxia. Fourteen babies were studied.

developed for use as a safe, nonflammable anesthetic agent but it has become apparent that 20% of patients who receive the anesthetic may exhibit biochemical evidence of liver damage, with modest abnormalities in their plasma aminotransferases being apparent in the first or second postoperative week; this is known as type I hepatic damage. Fulminant hepatic failure after exposure to halothane (type II hepatic damage) may also occur in the first or second postoperative week in up to 1 in 7000 patients and has a 20–50% mortality (F1, N1). The mechanism by which halothane produces these toxic effects is unclear but using animal models it has been shown that hypoxia and hepatic microsomal enzyme induction are factors that are necessary to cause consistent liver damage following exposure to halothane (J5). Halothane is normally metabolized by hepatic mixed function oxidases and, in hypoxia, halothane can be metabolized by a reductive pathway to hepatotoxic electrophilic agents that can bind covalently to macromolecules in the liver and cause necrosis (C14). In animals, halothane-induced hepatic damage can be diminished or prevented by cimetidine, which inhibits the reductive pathway, and this is put forward as evidence in support of a metabolic cause for the toxicity (P9, W13). The observation that enflurane and isoflurane appear to be associated with a lower incidence of postanesthetic hepatic damage is also consistent with the theory that halothane toxicity results from biotransformation to toxic metabolites and/or effects on hepatic blood flow (E1, S38). Halothane is metabolized to a greater extent (20% of inhaled dose) than enflurane (2%) or

isoflurane (0.2%) (B28). However, halothane is also known to decrease hepatic blood flow, whereas isoflurane and enflurane have little or no effect.

When plasma B_1 and B_2 concentrations were measured in a group of 20 patients who received halothane for minor urological surgery, 16 showed a small and transient rise in B_1 and B_2 between 1 and 3 hr after anesthesia (H61). It was also noted that in 2 patients there occurred a secondary and larger increase in B_1 and B_2 24 hr after anesthesia. No significant change in ALT occurred in these patients. This early study demonstrated that plasma GST levels could provide a sensitive and simple method of investigating the changes in hepatocellular integrity that occur following anesthesia. It was proposed that the rise in plasma B_1 and B_2 that occurred within 3 hr was due to a direct effect of halothane on reducing hepatic blood flow, whereas the secondary rise observed after 24 hr resulted from metabolism of halothane to toxic metabolites.

The changes in GST observed post-halothane anesthesia are small and it is possible that they may reflect circadian changes in the enzyme levels. To study this we measured plasma B_1 concentrations in 30 healthy volunteers after an overnight fast and subsequently at 3, 6, and 24 hr, i.e., the sampling intervals used for the study on the effects of halothane on GST. We could detect no significant increase in B_1 subunits over this time period.

We also thought it possible that the increase in B_1 observed 3 hr post-halothane anesthesia resulted from decreased clearance of B_1 via the reticuloendothelial system. To examine this the activity of plasma amylase was measured in samples from 10 patients who exhibited a significant rise in B_1 3 hr after receiving halothane. In each of the 10 patients, plasma amylase was significantly lower 3 hr after anesthesia when compared with preoperative values. This decrease in plasma amylase probably results from hemodilutional changes and confirms that clearance of plasma enzymes does not appear to be decreased 3 hr post-halothane anesthesia.

The effects of halothane on plasma B_1 concentrations have been confirmed by subsequent studies using short and long exposures to the anesthetic (A12, H58, M30, R3). It has also been shown that isoflurane has little or no effects on plasma B_1, whereas enflurane has an effect intermediate between halothane and isoflurane (Fig. 23) (A12, H58). In one study, 50% of patients receiving halothane had an abnormal B_1, whereas only 20 and 11% of patients receiving enflurane and isoflurane, respectively, had an abnormality (H58). These results are consistent with the view that the 3-hr rise in GST B_1 post-halothane anesthesia is caused by reduced hepatic blood flow as enflurane and isoflurane have much less of an effect on hepatic blood flow than does halothane. In addition, the results are also consistent with the 24-hr rise resulting from biotransformation; enflurane and isoflurane are metabolized to a far lesser extent than halothane.

A number of studies have been performed in an attempt to clarify the cause of the changes in B_1 3 and 24 hr post-halothane anesthesia. Gelman and Van Dyke have proposed that the hepatotoxic effects of volatile anesthetics may result from

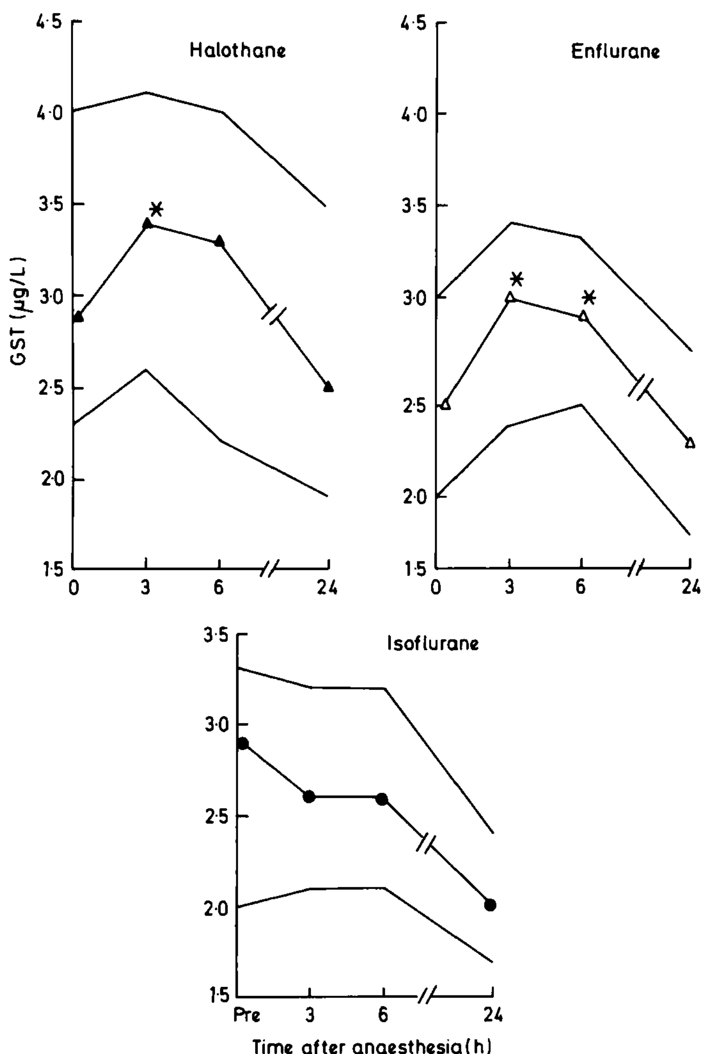


FIG. 23. The effect of halothane (▲) (22 patients), enflurane (Δ) (30 patients), and isoflurane (●) (18 patients) anesthesia on plasma GST B_1 levels. The median and interquantile ranges are shown. The asterisk signifies a $p<0.05$ increase from the basal levels.

a disruption of the mechanisms that maintain cellular calcium homeostasis, and halothane has been shown to produce excessive accumulation of cytosolic calcium (G4). The calcium antagonist nicardipine reduces significantly the extent of liver

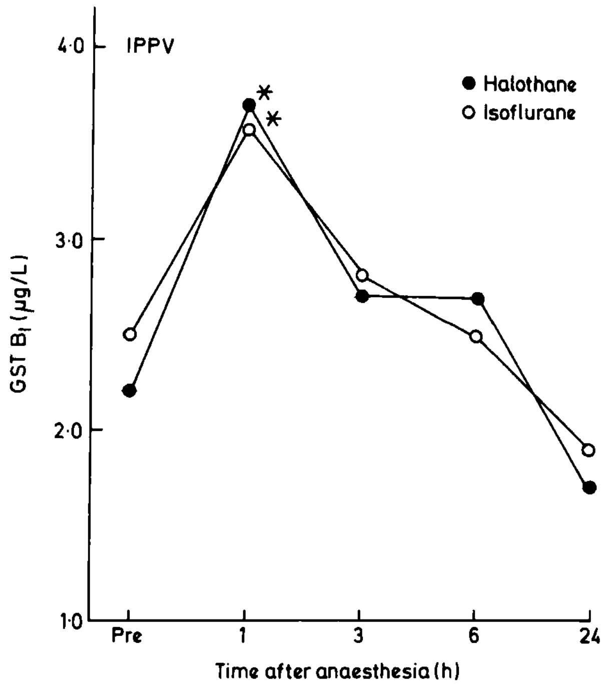
damage in rats after administration of carbon tetrachloride (G1) and this prompted us to study the effects of nicardipine on the plasma B_1 profile after halothane exposure (R3). Patients were allocated to one of two groups to receive, 1 hr before halothane anesthesia, an intravenous infusion of either saline (placebo group) or nicardipine at a rate to achieve a steady-state plasma concentration of 120 ng/ml after 1 hr of infusion. Plasma B_1 increased significantly 3 and 6 hr after anesthesia in both the placebo and the nicardipine group but in males the 3-hr rise in B_1 in the nicardipine group was significantly greater than that in patients receiving the placebo. Thus nicardipine infusion at this dose was unable to reduce the 3-hr rise in plasma B_1 but it should be noted that the body-weight-related doses of nicardipine used to prevent hepatotoxicity in the rat model are 25 to 50 times greater than the total dose given to our patients.

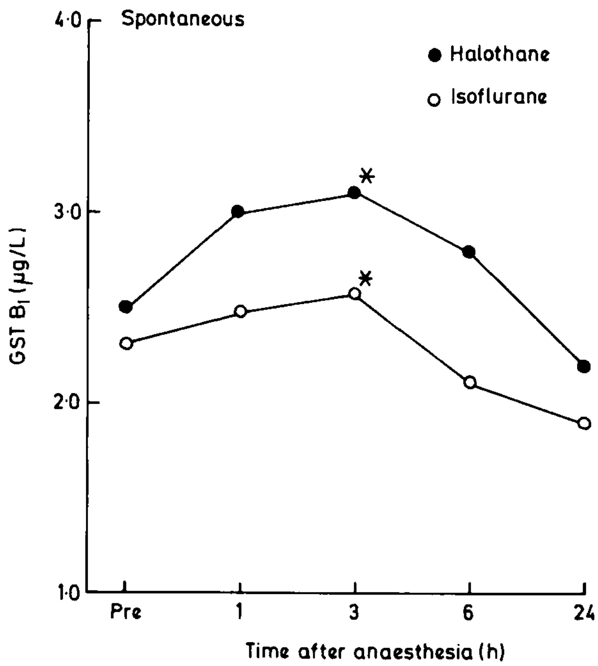
Cimetidine has been shown to impair metabolism of drugs by the mixed function oxidase system by binding to cytochromes P450 and P448 (B8, D5). In animal models cimetidine reduces halothane metabolism and lessens the severity of hepatic injury (P9, W13). It has not been possible to demonstrate any effect of cimetidine administration on the plasma B_1 profile obtained in humans post-halothane anesthesia (R4). However, as with nicardipine the doses of cimetidine used were far lower than the doses used in animal models.

Volatile anesthetic agents depress oxygen availability to the liver, partly as a result of decreased hepatic blood flow, and it is possible that these changes may be exacerbated by surgery, leading to global or regional hepatic hypoxia. Indeed, we have found that there is marked elevation in plasma B_1 in patients admitted to an intensive care unit after major surgery (H56). Anesthetics have different effects on hepatic arterial blood flow, with blood flow being preserved with isoflurane and decreased with halothane. The mode of ventilation can also alter hepatic blood flow, positive pressure ventilation is associated with a reduction and redistribution of cardiac output, and hepatic blood supply becomes reduced (G2).

In the studies mentioned previously all patients were allowed to breathe spontaneously. The effects of positive pressure ventilation on the plasma B_1 profile have been investigated by comparing the effects of halothane with those of isoflurane on plasma B_1 when patients were allowed to breathe spontaneously or were ventilated. It was found that significant increases in B_1 occurred at 1 hr in all four groups and the rise at 1 hr was significantly greater in the patients who were ventilated compared with that in the patients receiving the corresponding anesthetic but who were allowed to breathe spontaneously (Fig. 24). We have also

FIG. 24. The effects of intermittent positive pressure ventilation (IPPV) on plasma GST B_1 concentrations in patients receiving halothane or isoflurane anesthesia. A total of 60 patients were studied: 28 were ventilated (14 halothane, 14 isoflurane) and 32 breathed spontaneously (14 halothane, 18 isoflurane). An asterisk signifies a $p<0.05$ increase from the basal levels.


IPPV
● Halothane
○ Isoflurane
4·0
3·0
2·0
1·0
GST B_1 (μg/L)
Pre
1
3
6
24
Time after anaesthesia (h)


Spontaneous
● Halothane
○ Isoflurane
4·0
3·0
2·0
1·0
GST B_1 (μg/L)
Pre
1
3
6
24
Time after anaesthesia (h)

observed in children a similar effect, whereby positive pressure ventilation can exacerbate the 3-hr rise in plasma B_1 (G. J. Beckett and A. F. Howie, unpublished observations).

Overall, these studies would support the view that the early transient hepatic dysfunction characterized by a rise in plasma B_1 3 hr postanesthesia results from alterations in hepatic blood flow and not chemical toxicity. To date, however, no clinical value for the measurement of the B_1 subunit as a predictor of patients who will subsequently develop clinical hepatic dysfunction has been described.

9.3. Plasma GST Measurements for the Detection of Chronic Liver Disease

9.3.1. *Autoimmune Chronic Active Hepatitis*

One of the earliest documented advantages of GST measurements relates to the observation that all patients with chronic active hepatitis had elevated plasma ligandin concentration. Sherman *et al.* also noted that in 16 patients with the disease serum ligandin levels correlated closely with activity of the disease, whereas no such correlation was found using AST (S20).

The conventional approach to the management of autoimmune chronic active hepatitis is to use a steroid such as prednisolone either alone or in conjunction with azothioprine. The drugs are usually given at doses that produce biochemical remission of the disease and when this is achieved a liver biopsy is often performed to exclude any ongoing active liver damage. Although these treatment regimens have proved effective at improving survival, many patients receiving this treatment still develop cirrhosis, suggesting that there are still significant levels of active disease despite the fact that none can be detected using conventional biochemistry (S32, W14).

The measurement of B_1 subunits in serum or plasma may provide a useful alternative or adjunct to the conventional approach of biochemical monitoring in these patients during treatment (H34). In a study of 22 patients receiving prednisolone with or without azothioprine it was found that B_1 levels were elevated in 17 or 26 samples, whereas AST activity was found to be elevated in only 6. It was also found that of 20 samples with normal AST activity, B_1 concentrations were elevated in 11 but no patient was found to have elevated AST activity in the presence of normal B_1 concentration. When B_1 levels were compared with GGT activities it was found that GGT was increased in 16, and 11 of these also exhibited elevated B_1 concentrations. In 7 samples increased B_1 with normal GGT was found. A significant correlation was found between B_1 and GGT ($r = 0.69$) but a good correlation between B_1 and AST was observed only if samples with B_1 concentration more than twice the upper reference value were excluded. Abnormal

AST was found only when B_1 levels exceeded twice the upper reference limit (Fig. 25).

The majority of patients in this study were considered to be in remission but the findings of an elevated B_1 concentration in many of these patients provides evidence that active disease is still present. In support of this view Korman *et al.* have shown that patients with the disease who are in histological and biochemical remission frequently have elevated fasting plasma bile salt concentrations (K15). Patients with elevated plasma bile salts quickly relapsed if treatment was withdrawn.

It is tempting to suggest from our data that, if the aim of immunosuppressive

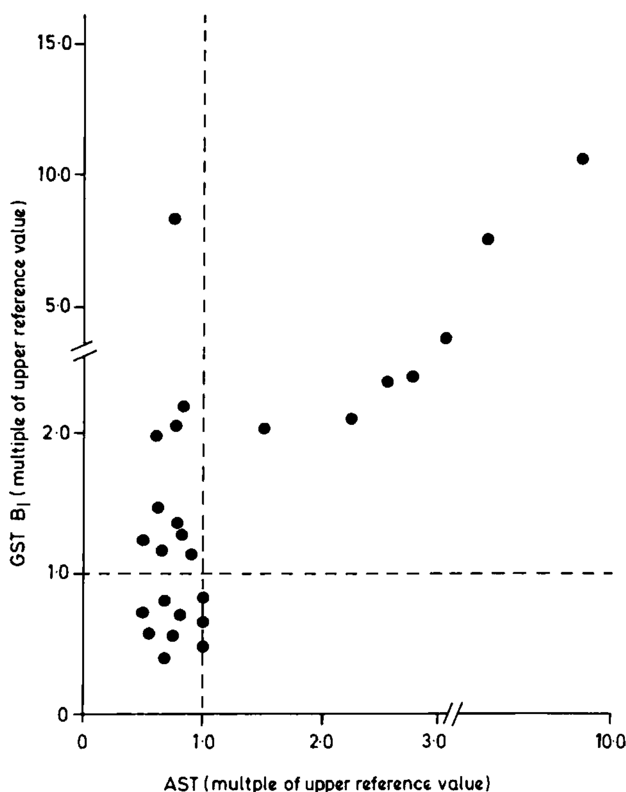


Fig. 25. A comparison of plasma asparate aminotransferase (AST) and GST B_1 levels in patients with autoimmune chronic active hepatitis treated with prednisolone and azothioprine. The enzyme levels are expressed as multiples of the upper reference limit. The dashed lines represent the upper reference limit.

therapy became the normalization of B_1 rather than AST, then improved survival might occur. This is an important question that has not yet been addressed.

9.3.2. *Alcoholic Cirrhosis*

There are a number of conflicting reports concerning the levels of plasma or serum GST measurements in patients with cirrhosis. One study reported that serum ligandin concentrations were elevated in over 50% of patients with the disease, with mean levels being increased more than three times the upper reference limit (T13). Another study using an enzyme immunoassay has produced a similar finding (H44). In complete contrast to these studies Sherman *et al.* using a radioimmunoassay for human ligandin found normal plasma concentrations in each of 20 patients with cirrhosis (S20). This finding is surprising since all patients in Sherman's study had an elevated plasma AST but the discrepancy may be related to the fact that Sherman used an upper reference limit of 12 μg/liter, when we have found using our assay that the upper reference limit is 4.0 μg/liter for B_1 (B16). Adachi *et al.* measured the activity of GST with CDNB as a substrate and also reported normal plasma levels in all patients with cirrhosis (A4).

The reasons for the discrepancy among the above studies may lie in the GST subunits that are being measured since each of the assays were developed before the discovery of immunologically distinct B_1 and B_2 subunits. In none of the studies is it clear what subunit or combination of subunits is being measured. Another problem in interpreting the data from previous studies lies in the fact that the type of cirrhosis studied was not clearly defined and it is possible that the biochemistry may vary with cirrhosis of different etiologies.

We have used our assays for B_1 and B_2 to quantitate the levels of these subunits in sera from 79 patients with biopsy-proven alcoholic cirrhosis (B18). Our results showed that abnormalities in AST were not always associated with corresponding abnormalities in B_1 or B_2, although there were equal numbers of patients (28) who exhibited elevated levels of AST or B_1. The measurement of B_2 was the poorest of the three measurements at identifying abnormality (only 17 patients had elevated B_2) and all but 3 of the patients with increased serum B_2 also had elevated concentrations of B_1, showing that the measurement of B_2 is unlikely to be of any diagnostic value in this condition. Our results would suggest that, although a proportion of patients with alcoholic cirrhosis have elevated plasma GST B_1 levels, the correlation between plasma aminotransferases and the GST is poor in patients with this disease compared with those with acute liver damage. Why should this be?

The presence of elevated plasma B_1 in the presence of normal AST could be explained by the lobular distribution of the enzymes since AST is mainly in periportal cells and alcohol damages centrilobular cells that are rich in B_1 but not AST (R2). The explanation for the converse finding of normal B_1 in the presence of a raised AST is less clear. This pattern of results could occur if cellular necrosis

occurred intermittently and because of its short half-life B_1 would be removed from plasma prior to AST. A more likely possibility has become apparent from the immunohistochemistry of the GST in alcoholic liver disease. Harrison has shown that in alcoholic cirrhosis hepatocytes begin to express pi-class GST in a manner similar to that of the fetus (H11). Although it has not been quantitated, it is possible that, in cirrhosis, GST expression may be similar to that of the fetus; i.e., there is reduced expression of B_1 and B_2 and increased expression of pi-class GST, and thus B_1 and B_2 subunit measurements in plasma could lack sensitivity for detecting liver damage.

From a clinical point of view if B_1 and AST were measured in our patients an abnormality in one of the serum markers would have been detected in 38 of the 54 patients. If AST alone had been used, an abnormality would have been detected only in 28.

9.4. Thyroid Hormones and the Liver

9.4.1. *Hyperthyroidism*

Before the introduction of effective treatment for thyrotoxicosis, hepatobiliary complications were commonly associated with the disease. Liver biopsy samples taken from hyperthyroid patients often showed morphological changes such as fatty change, glycogen depletion, and cirrhosis but a direct detrimental effect of thyroid hormones on the liver was thought unlikely; rather it was thought that hepatic abnormalities resulted from other factors such as altered cardiac output, infection, hypoxia, and malnutrition (K13, S19).

Prolonged hyperthyroidism is now an uncommon occurrence and consequently severe hepatobiliary dysfunction resulting from thyrotoxicosis is rare. There is much biochemical evidence, however, to suggest that hyperthyroidism may still result in subclinical hepatic dysfunction. In patients with hyperthyroidism elevations in the activity of plasma aminotransferase, γ-glutamyltransferase, and liver-derived alkaline phosphatase are often found. Hepatic anion transport of bilirubin and sulfobromophthalein is also impaired in the disease (A21). Animal studies have also suggested that high levels of thyroid hormone may predispose the animal to hepatic damage following an hepatic insult. Rats given thyroid hormones show liver damage following exposure to halothane and such animals also show more marked liver damage than rats not given exogenous thyroxine when exposed to other halogenated hydrocarbons (W12).

We have shown that plasma B_1 levels may be elevated in over 50% of patients with thyrotoxicosis (B22). In addition, when patients are rendered euthyroid by ^{131}I-thyroid ablation, plasma B_1 levels return to within reference limits. In contrast, we found that thyrotoxic patients that were rendered euthyroid with carbimazole had B_1 concentrations that fell significantly from pretreatment levels but not to

values that were within the reference range. In some patients there was a transient rise in B_1 in the first 2 weeks of commencing therapy before B_1 levels began to fall. In one patient, we measured a marked rise in B_1 after carbimazole treatment was commenced and this coincided with a concomitant systemic sensitivity reaction (B22). These data would suggest that carbimazole may produce transient subclinical liver dysfunction in some patients; however, this effect seems minimal since few serious hepatobiliary problems with carbimazole have been reported.

9.4.2. *Thyroxine Replacement Therapy*

There is little evidence to suggest that hypothyroidism can lead to liver damage; however, patients with hypothyroidism will receive lifelong treatment with oral T_4. When administered by this oral route a large bolus dose of T_4 (usually 100 or 150 μg) will be presented to the liver; this compares unfavorably with the physiological situation, in which the thyroid secretes 90 μg of T_4 over 24 hr into the systemic circulation. It is possible therefore that in T_4 replacement the liver may have some degree of tissue hyperthyroidism.

In an initial study we showed that plasma B_1 concentrations rose in each of eight hypothyroid patients following T_4 replacement for periods of between 3 and 9 months, and in four patients an elevated plasma B_1 was found post-treatment. In general, patients with elevated plasma-free T_4 concentrations had elevated plasma B_1 concentrations (B22). Using a rat model we excluded the possibility that T_4 produces induction of B_1, since in rats given T_4 or T_3 in their drinking water the hepatic levels of each of the GST classes decreases and plasma GST concentration increased (B11, B20). With the introduction of sensitive TSH methods (S17) it was found that many patients treated with T_4 were being given doses that resulted in a suppression of thyrotroph function in that TSH concentrations were below the detection limit of the assays (G8). However, since there was no evidence to suggest that patients receiving T_4 at doses of up to 200 μg/day had exhibited any clinical problems, it was argued that this suppression of thyrotroph function may simply reflect an increased sensitivity of the pituitary to plasma T_4 (P4). We, therefore, undertook a study to determine if the pituitary was representative of the liver with regard to thyroid status (G8).

We found that, in patients who had been receiving the same dose of thyroxine for at least 6 months, abnormalities in plasma B_1, ALT, and GGT were more commonly found in patients who had an undetectable TSH than in patients with a normal or raised plasma TSH (G8). If the dose of T_4 was reduced in the patients with undetectable TSH to a dose that normalized plasma TSH, the plasma levels of ALT and B_1 fell to within the reference range in practically all patients (G9). These data led us to suggest that the aim of thyroxine replacement therapy should be to normalize plasma TSH as it appeared that the thyrotrophic response to exogenous T_4 was representative of the thyroid status of other tissues in the body.

The changes that occur in plasma B_1 in the early weeks following commencement of T_4 therapy have also been studied (G7). Hepatocellular integrity in patients with spontaneous hypothyroidism appeared to be impaired, as characterized by a significant increase in B_1, when doses of 100 μg/day of T_4 were given. In contrast, in patients in whom hypothyroidism had resulted from ^{131}I-thyroid ablation, T_4 doses as high as 200 μg/day had no significant effect on plasma B_1 levels (Fig. 26). In the former group of patients hypothyroidism is likely to have been of long duration, whereas in patients with previous hyperthyroidism, it is unlikely to have been present for more than a few months, because of the regular clinical follow-up that these patients receive. These results suggested that the liver from a patient with prolonged hypothyroidism may be particularly prone to the effects of T_4 when treatment is commenced but we postulated that with prolonged treatment tolerance could occur.

We investigated this postulate by correlating TSH and B_1 in patients receiving a fixed dose of T_4 for over 1 year. In contrast with our previous findings (G8), few abnormalities in B_1 were found even when TSH was undetectable. When the data from our previous study (G8) were reanalyzed, excluding patients on T_4 for less than 1 year, no correlation was found between TSH and B_1. These results would support the view of hepatic tolerance to T_4 developing after 1 year of treatment on a fixed dose of T_4.

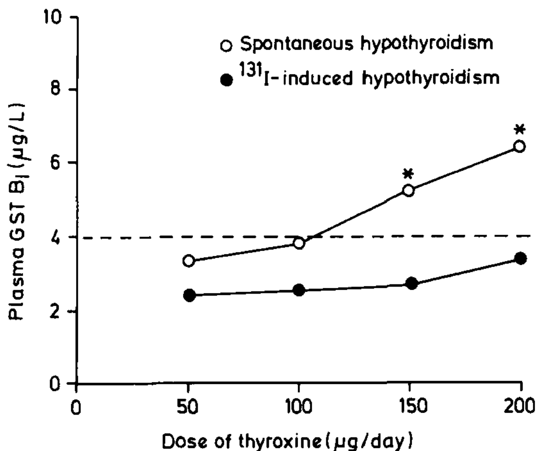


FIG. 26. Mean plasma GST B_1 responses to patients with spontaneous (9 patients) or ^{131}I-induced hypothyroidism (10 patients) following treatment with increasing doses of thyroxine. Patients were kept on the same dose of thyroxine for 4 weeks before the dose was increased. The upper limit of the reference range is denoted by the dashed line. The asterisk signifies a $p<0.05$ difference between GST B_1 levels in the basal sample.

10. GST Measurements in Malignancy

10.1. Plasma/Serum Measurement

The pi-class GST is expressed in a wide range of malignant tumors and levels of this isoenzyme are, in general, higher than the levels found in corresponding normal tissue (C12, D6–D8, E2, H41, H54, T11). A number of reports, particularly from Japan, have suggested that serum pi-class GST levels measured by immunoassay may be increased in a wide range of gastrointestinal and hematological malignancies and thus the measurement of serum pi-class GST concentration might provide a useful tumor marker. Niitsu *et al.*, using an immunoradiometric assay, reported that elevated serum pi-class GST concentrations were found in 38 of 62 patients with gastric cancer, 8 of 15 patients with esophageal cancer, 20 of 26 patients with colonic cancer, 5 of 12 patients with pancreatic cancer, and 7 of 10 patients with colonic cancer (N4). Normal levels of pi-class GST were found in sera from patients with benign gastrointestinal disease. These workers also reported that in two patients with gastric cancer, pi-class GST levels fell to within normal limits following gastrectomy. Tsuchida *et al.*, using an ELISA method, reported elevated serum pi-class GST concentrations in 10 of 23 patients with gastric cancer, 26 of 43 patients with esophageal cancer, 3 of 9 patients with biliary carcinoma, and 3 of 9 patients with colonic cancer. Abnormal levels were also found in a small percentage of patients with hematological malignancy (T11).

To investigate the levels of pi-class GST in serum we developed a radioimmunoassay using antisera raised to pi-class GST purified from placenta (H52). Using sera from patients with a wide range of malignancies we found that there was considerable overlap between normal subjects and patients with malignancy. We subsequently found that during the clotting process platelets released large amounts of pi-class GST into serum. At room temperature serum pi-class GST levels rose from a mean of less than 10 μg/liter in serum separated immediately to 30 μg/liter 1 hr after blood sampling (H52). These large changes in serum pi-class GST levels during the clotting process led us to conclude that serum was an inappropriate matrix for measuring pi-class GST as a marker of malignant disease. Studies using plasma suggested that this may be a suitable matrix for pi-class GST measurements but in order to minimize contamination of platelet-derived pi-class GST we found that samples must be taken in cooled tubes containing EDTA and platelet stabilizers. It was also important to keep the samples on ice prior to centrifugation in a cooled centrifuge at a minimum force of 2500 *g* for at least 30 min. Under these conditions whole blood could be kept for 2 hr prior to separation (H52).

Using the above sampling procedure the concentration of pi-class GST in plasma was usually <10 μg/liter in normal subjects. When we used plasma from

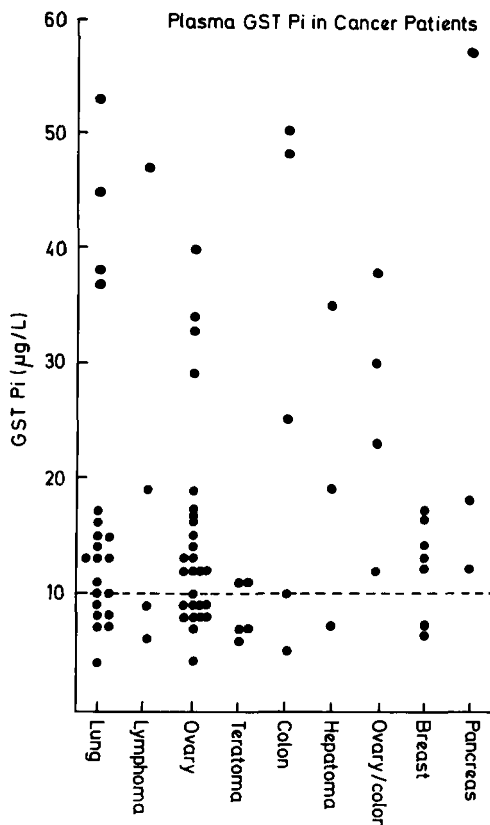


FIG. 27. Plasma GST π concentrations in patients with various malignancies. The dashed lines denote the upper limit of the reference range.

patients with various malignancies, using the sampling technique described above, we were able to demonstrate elevated pi-class GST levels in a number of malignancies including lung, colon, and ovary (Fig. 27). In a previous study of patients with cancer of the bronchus we found that 19 of 29 patients had elevated plasma pi-class GST concentrations and 8 of 9 patients with adenocarcinoma of the lung had elevated concentrations (H50). In small cell cancers other markers are more reliable (J4).

In practice, although plasma pi-class GST concentrations are increased in a wide range of malignancies, we have found that it is difficult to obtain samples that are collected under appropriate conditions and thus the possibility of generating false-positive results is extremely high. Although not studied formally, it is also likely that the predictive value of pi-class GST measurements will be low since

pi-class GST is found in many normal tissues at levels similar to those in tumors. Raised pi-class GST levels have been reported, for example, in patients with benign liver disease (N4, T11).

Ohmi and Arias have investigated the plasma levels of alpha-class GST in hepatocellular carcinoma in the rat and humans (O3). They found that serum ligandin concentrations rose progressively following the transplantation of a ligandin-containing rat hepatocellular carcinoma in rats or athymic mice. The mean serum ligandin concentrations within 4 months exceeded normal serum levels by a factor of 10. When serum ligandin concentrations were studied in humans it was reported that levels were increased in 11 of 15 patients with primary hepatocellular carcinoma. Normal levels were found in 19 of 22 patients with primary carcinomas without hepatic involvement.

It is unclear what mechanisms might lead to elevated serum ligandin since hepatomas express considerably reduced levels of the alpha-class GST. Ohmi and Arias suggested that hepatoma cells might readily release their ligandin into peripheral circulation but this suggestion has remained largely unsubstantiated (O3).

10.2. Measurements in Bronchoalveolar Lavage Fluid

The measurement of alpha- and pi-class GST levels in bronchoalveolar lavage fluid obtained from patients undergoing bronchoscopy for suspected malignancy may have some diagnostic importance. Using immunoassays we found that lavage fluid contained B_1, B_2, and pi subunits and this was confirmed by purification of the proteins with affinity chromatography followed by identification with SDS–PAGE (H49).

In human lung obtained within 8 hr of death the pi-class GST isoenzyme is present at concentrations that are approximately 10-fold greater than those of either the B_1 or the B_2. In contrast, in bronchoalveolar lavage fluid the B_1 and B_2 were present at concentrations that were in excess of those of pi-class GST. We have suggested that the predominance of the alpha-class GST in lavage fluid is related to the relative stability of this GST class and the instability of the pi-class GST isoenzyme. We have found that in postmortem lung tissue, obtained more than 24 hr after death, pi-class GST levels in the tissue were reduced to approximately 3% of the levels found in fresh lung tissue. No change was found in B_1 or B_2 subunit content. We believe, therefore, that the GST found within the lavage fluid arises from leakage of intracellular GST but that, within the environment of the alveoli, the pi-class GST isoenzyme degrades, leaving the GST B_1 and GST B_2 subunits as the major forms (H49).

In patients with benign lesions of the lung the concentrations of B_1, B_2, and pi subunits in lavage fluid taken from a normal area of lung were not significantly different compared with the concentrations found in the area of diseased lung. In

patients with bronchogenic cancer, however, concentrations of B_1, B_2, and pi subunits in fluid from the area containing the malignancy were significantly higher compared with those in fluid from a normal area taken from the same lung.

10.3. GST in Human Bile

Bile contains a large number of diverse proteins, some resulting from leakage of hepatocellular protein and others derived from plasma (A9, R6). In certain cases, the liver may secrete specific proteins into bile to bind and thus prevent the reabsorption of potential toxins and thus facilitate fecal excretion of these substances (S3). As an example, copper is excreted principally by the biliary route, bound to a carrier protein that prevents intestinal absorption of the biliary copper (G6). The GST can bind a wide range of substances and we have studied the GST content of human bile using material obtained from intact gallbladders following removal at cholecystectomy.

Using both radioimmunoassay and affinity chromatography techniques we found that all bile samples contain B_1, B_2, and pi subunits and in 9 of 18 samples mu-class GST was also detected (H53). Although there were large interindividual variations found in GST concentration, the mean concentration of pi-class GST was on average approximately three times greater than that of the B_1, B_2, or mu subunits, which were expressed in a similar concentration. In liver cytosol pi-class GST comprises only an extremely (<1%) small percentage of the total GST pool, with the B_1 and B_2 subunits predominating. The high ratio of pi- to alpha-class GST in bile suggests that, although biliary alpha-class GST may originate from hepatocytes, biliary pi-class GST could not arise from simple leakage from these hepatocytes but rather must arise from the biliary epithelia, which immunohistochemically appear to express high levels of the isoenzyme.

The high concentrations of pi-class GST found in bile also suggested that the enzyme might be actively secreted from biliary epithelial cells and such an efflux might be beneficial to the cell in that it provides a mechanism whereby toxins could combine with GST and be actively excreted along with the enzyme. There appears to be no role for GST in bile per se since in the presence of bile salt concentrations similar to those found in bile, pi- or alpha-class GST have no catalytic activity (H17, H25, H53). In addition, it appears unlikely that GST have a physiological role to bind substance excreted from the liver as GST concentrations at least 1000 times those found in bile would be required to bind bile salts alone.

The concentrations of GST in normal bile have been compared with the concentrations of the enzymes found in bile obtained using endoscopic retrograde cholangiopancreatography from two patients with cholangiocarcinoma and one patient with pancreatic cancer (H53). The concentrations of B_1 and B_2 were not significantly different in the cancer patients when compared with concentrations

in normal bile but the pi-class GST concentrations were increased more than 10-fold in bile obtained from the two patients with cholangiocarcinoma. This increase in pi-class GST could arise from excretion of the GST directly from the tumor, necrosis of normal biliary epithelial tissue, or blood passing into bile as a result of vascular damage (erythrocytes contain high concentrations of pi-class GST). Larger studies are required but these preliminary results suggest that biliary measurements of pi-class GST may be useful in the differential diagnoses of pancreatic carcinoma and cholangiocarcinoma.

11. The Glutathione *S*-Transferases and the Kidney

Glutathione *S*-transferase of the alpha-class comprises up to 2% of soluble protein in rat kidney, and early immunofluorescent studies localized ligandin to the proximal tubule, with no staining occurring in the glomeruli or interstition (F13, F14). A similar distribution of the enzyme has been found using microdissection techniques in the rabbit (F10). Campbell *et al.* using immunohistochemistry found that, in human kidney, ligandin was largely confined to the proximal convoluted tubule and the thick segments of the loop of Henle (C3) and this has been confirmed in recent studies in adult kidney (H10, S42).

Kidney also expresses high concentrations of the pi-class GST, this isoenzyme being largely confined to the distal tubules in human kidney and to Bowman's capsules in the glomerulus (H10, S42). Harrison has shown that the expression of both mu-class and microsomal GST is generally weak and variable (H9).

11.1. Urinary GST Measurements as a Marker of Renal Damage

11.1.1. *Animal Studies*

The high level of expression of the alpha-class GST has led a number of workers to investigate the use of urinary GST measurements as a marker of renal tubular cell necrosis (B4, F5–F9). Ligandin was detected using immunological and enzymic techniques in the urine of rats treated with mercuric chloride but not after poisoning with potassium dichromate (B4, F7). These data are consistent with the known nephrotoxic qualities of these agents in that mercuric chloride produces selective necrosis of the pars recta of the proximal tubule, an area rich in ligandin, whereas potassium dichromate affects the convoluted portion of the proximal tubule, which immunohistochemically expressed much lower amounts of the enzyme.

Using activity measurements with CDNB as a substrate Feinfeld *et al.* showed that ligandinuria was present 12 hr after mercuric chloride administration, and using a sensitive radioimmunoassay (F7) Bass *et al.* demonstrated a significant increase in urinary ligandin excretion within 6 hr of mercuric chloride injection,

which preceded the occurrence of uremia (B4). These workers also showed that serum ligandin concentrations were raised 12 hr after mercuric chloride administration, whereas renal tissue concentrations fell to 30% of control values by 24 hr (B4). Administration of potassium dichromate also produced ligandinuria when measured by radioimmunoassay but the concentration of urinary ligandin was normal up to 24 hr. Thereafter the ligandinuria was at least a magnitude lower than that detected in mercuric-chloride-treated animals. These different time courses of ligandinuria correlated well with the sequence of damage, assessed histologically, to the pars recta and pars convuluta of the proximal tubule, respectively. Further studies have demonstrated ligandinuria in rats treated with gentamicin and cisplatin as assessed by GST activity (F6, F8).

In normal rats urinary ligandin has been reported as undetectable using immunodiffusion and as assessed by GST activity with CDNB as a substrate (F7). Using a sensitive radioimmunoassay, however, Bass *et al.* could detect ligandin in all urine samples obtained from normal rats; values ranged from 1.0 to 189 μg/liter compared with values as high as 80,000 μg/liter in mercuric-chloride-treated animals. Ligandin in normal urine could arise from leakage from renal or bladder epithelial cells or from excretion of ligandin arising from glomerular filtration of plasma ligandin (B4).

11.1.2. *Human Studies*

11.1.2.1. Normal Subjects. It has been reported, using immunodiffusion or activity measurements with CDNB as a substrate, that in normal subjects ligandin is undetectable in the urine (B2, F7). In contrast, using a radioimmunoassay method Sherman *et al.* were able to identify ligandin in urine at a mean concentration of 24.7 μg/liter, a concentration that was equivalent to a urinary excretion rate of approximately 1.5 μg/hr (S21). Backman *et al.*, using a radioimmunoassay for human "basic" GST with a detection limit of 1 μg/liter, were unable to detect the enzyme in the urine of most patients with stable renal function (B2). It is our experience using immunoassay, that B_1 and pi subunits are detectable in the urine of practically all normal subjects.

Feinfeld *et al.*, using immunodiffusion and GST activity measurements, were unable to detect ligandin in the urine of 12 patients with chronic renal disease and in the urine of 12 patients with acute renal disease not involving tubular necrosis (F7). Ligandinuria was detected in 2 patients with acute tubular necrosis in whom the urine was obtained within 24 hr of the precipitating event. Using these assays the same workers were also able to demonstrate ligandinuria in 6 of 17 patients following renal arteriography.

In a follow-up study Feinfeld *et al.* (F9) used an upper limit of normal for urinary ligandin excretion of >5 μg/liter. They found that patients who received 34 g or more of iodine in radiocontrast media developed elevated urinary ligandin

outputs within 36 hr of the radiocontrast media being administered. Values had returned to normal after 48 hr.

11.1.2.2. Urinary GST Measurements and Renal Transplantation. Measurement of GST in a renal perfusate has been used to predict cadaver kidney function prior to transplantation (C8) but there has been more interest in using GST measurements to monitor rejection following transplantation. The use of cyclosporin A to prevent renal transplant rejection can produce a problem of differential diagnoses since cyclosporin toxicity can mimic acute rejection. Cyclosporin is reported to be toxic to the renal proximal tubular epithelium and Backman *et al.* have used radioimmunoassay for the alpha-class GST to try and differentiate among patients with stable renal transplants, patients with transplant rejection, and patients with cyclosporin toxicity (B3). In their study 28 patients were considered to have stable renal function, with a serum creatinine that was below 200 μmole/liter, and were clinically free from significant cyclosporin-induced toxicity. A total of 38 patients had morphological changes on renal biopsy, suggestive of graft rejection, or there was a strong clinical suspicion to support the diagnosis. In 11 patients morphological examination showed no signs of acute rejection, cyclosporin toxicity was assumed, and the dosage of the drug reduced. A diagnosis of cyclosporin toxicity was also made in 16 patients without renal biopsy on the grounds of improved renal function after reducing the dosage of cyclosporin.

Patients with stable renal function generally had undetectable (<1.0 μg/liter) alpha-class GST in their urine but in six low but detectable concentrations were found. The concentration of alpha-class GST in the urine of patients with acute rejection was significantly lower than the concentration found in patients with cyclosporin rejection but there was considerable overlap between groups. It would seem from the data that urinary GST measurements cannot provide clear discrimination between these two situations.

12. Conclusions

It can be seen that the multiple forms of the GST have many roles in protecting the cell from a wide range of toxic insults. This is beneficial to the normal tissue but it is an unfortunate occurrence in malignancy since the GST may provide a mechanism to protect the cell from cytotoxic agents during chemotherapy. The measurement of the alpha-class GST in serum or plasma has proved to probably be the most sensitive biochemical test available to monitor the effects of acute hepatic insults but the role of pi-class GST measurements is as yet unclear.

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