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PREFACE

This volume is the initial effort arising out of the application of a new philosophical approach being adopted in preparing *Advances in Clinical Chemistry*.

Progress in clinical chemistry as a profession and as a science has been achieved through international contributions. The amount of new information being produced is staggering. Although the world grows smaller because of communications technology, there is still some benefit to geographic proximity. The best of all ideals is to take advantage of communication while enhancing its value through creation of local scientific "tactile sense" networks. As a response to the perception of these circumstances, the editorial philosophy of *Advances in Clinical Chemistry* has been changed.

An International Editorial Board has been assembled, which will be responsible for identifying the leading edges of technology, new information relative to the science of health and disease, as well as improvements in current practices of the profession. Each of the editors brings a complementary area of expertise and a unique perspective. The geographical distribution of the Editorial Board, which will be changed from time to time, will provide for diversified subject matter and will result in contributions from scientists throughout the world. The Editorial Board will identify areas of interest, and these will be coordinated by the Executive Editor.

Areas of concern to the readers of *Advances in Clinical Chemistry* are of concern to the editors. Therefore, we welcome suggestions and comments. Our goal is to be a viable vehicle valuable to our readership.

The five articles comprising this volume were contributed by international scientists and edited by a Board of International Editors. A scan of the table of contents indicates the wide ranging subject matter presented. The fields of instrumentation analysis, metabolism, nutritional biochemistry, and immunology in its several phases have been presented. Two basic approaches for presentation have been used. The first approach is an in depth consideration of a specific subject, which is exemplified by the article on the clinical chemistry of vitamin B_{12}. The second approach is an overview of an extensive field of active research as represented by the article on immune complexes. All of the articles comprising this volume reflect permutations on these approaches.

The contributors to Volume 24 and the editors have worked diligently and strenuously to provide a product from the above-mentioned philosophy. To all of them, I extend my gratitude.

HERBERT E. SPIEGEL

IMMUNE COMPLEXES IN MAN: DETECTION AND CLINICAL SIGNIFICANCE

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1. Introduction

Von Pirquet, as early as 1911, suggested that serum sickness was a consequence of the toxic effects of antigen–antibody interactions (V2). This prediction was confirmed in the 1950s and 1960s with the establishment of animal models of acute and chronic serum sickness (D6, D7, D9, G5–G7). These models allowed, for the first time, a dissection of the factors involved in the formation and fate of immune complexes, the timing, distribution, clearance, and deposition of immune complexes, and the mechanisms by which immune complexes elicit an inflammatory response. All these factors, which lead to immune complex disease and tissue damage, have counterparts in the normal host responses that are presumably beneficial and designed to eliminate or neutralize antigen. Thus, the concept has emerged that immune complex disease is best understood as an untoward or deleterious aberration of the normal humoral immune response. Moreover, immune complexes can be viewed, quite separate from their inflammatory properties, as potential regulators of cellular and humoral immune responses.

In the last decade, numerous new techniques have been developed for the detection and quantitation of immune complex material in tissue and biological fluids. In many respects, clinical studies using these techniques have substantiated the primary pathogenic role of immune complexes in a variety of human diseases. At the same time, these studies have reaffirmed the notion that immune complexes occur frequently in the course of an immune response or as an epiphenomenon unrelated to pathogenesis, and that, despite the presence of immune complexes, typical clinical manifestations of immune complex disease may be an unusual event.

Given these facts alone, it was clear from the beginning that to establish the usefulness of immune complex assays in clinical medicine might be difficult and would require long-term observations on defined groups of patients. If one also takes into account the inherent deficiencies of the testing methodologies and the enormous accumulated literature in which virtually any disease having an inflammatory component has been associated with immune complexes, it is not surprising that an initial enthusiasm when these tests were first introduced has been replaced by a certain amount of disenchantment when they failed to live up to their clinical potential (A2, A3, B3, K1, S13, W13).

In the following review, we will discuss in some detail the principles and performance of the most commonly used tests for detecting immune complexes and summarize studies in those diseases for which considerable clinical experience with these tests has accumulated. Emphasis is on the clinical relevance of this technology. We consider a test clinically relevant if

it (1) defines or clarifies the pathology or etiology of a disease, (2) is of diagnostic usefulness, (3) is of value for monitoring disease activity and therapy, (4) provides prognostic information with respect to disease outcome or response to therapy, or (5) defines subsets of patients with certain disease manifestations.

With respect to studies in animal models which dissect the mechanisms of formation, localization, and fate of immune complexes, the mechanisms by which they elicit an inflammatory response, and their biologic activities, we provide little more than summary information required to understand the concept of immune complex disease and the principles of the tests. For more detailed information, the reader is referred to several excellent reviews (C14, H2, T11, U1, W12).

2. The Concept of Immune Complex Disease

An immune complex is simply the noncovalent combination of antigen with its respective, specific antibody. Antibody can react with antigen that is fixed or localized in tissue or with antigen that is released or present in the circulation. (This review is primarily concerned with diseases and tests related to circulating immune complexes.) Once formed in the circulation, the complex may or may not fix complement and is rapidly eliminated from the circulation by the mononuclear phagocytic system (reticuloendothelial system). The mononuclear phagocytic system is composed of fixed and wandering cells, all derived from the bone marrow monocyte, which, by virtue of receptors for immunoglobulin or complement, bind, phagocytose, and dispose of or sequester immune complexes. Under normal circumstances, this process does not lead to pathologic consequences and, indeed, may be viewed as a major host defense against the invasion of foreign antigens (Fig. 1). It is only in unusual circumstances that immune complexes persist as soluble complexes in the circulation, escape mononuclear phagocytic clearance, and deposit in endothelial or vascular structures where they elicit an inflammatory response—the hallmark of immune complex disease.

The factors which determine whether an immune complex will remain soluble in the circulation, escape mononuclear phagocytic clearance, and cause tissue damage are a subject that is central and a major theme in the investigation and understanding of immune complex disease. The level of circulating immune complexes is determined by the rate of formation, by the rate of clearance, and, most importantly, by the nature of the complex formed (Table 1). Although multiple factors appear to contribute, these factors can be conceptually integrated. In general, complexes that remain soluble and escape clearance tend to be small-sized or small lattice-struc-

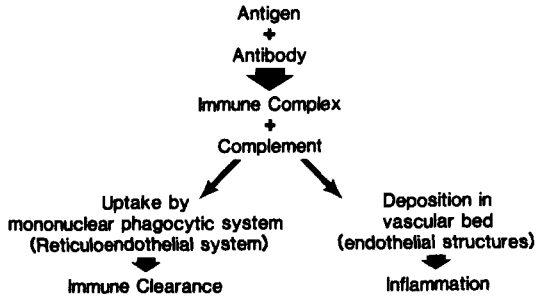

FIG. 1. Schematic of immune complex formation, elimination, and deposition.

TABLE 1
FACTORS DETERMINING IMMUNE COMPLEX LEVELS

Properties of the complex
Nature of the antigen
Quantity available for reaction
Number of determinants (epitopes) per molecule
Size
Nature of the antibody
Quantity
Affinity
Class (isotype)
Valence
Complement-fixing properties
Reactivity with cellular receptors
Degree of lattice formation
Size of the complex
Solubility properties
Complement-fixing properties
Clearance and distribution properties
Rate of formation
Antigen availability
Antibody synthetic rate
Rate of clearance
Degree of lattice formation
Nature of the antigen
Ability of the complex to react with complement
Ability of the complex to react with cellular receptors
State of the mononuclear phagocytic system

tured complexes. Hence, antigens that are small or paucivalent with respect to antibody-binding sites are likely to form small lattice complexes (A18, A20, C10, H2, H18, L17, S8, T11, W11). Low-valence (determined by antibody class) or low-affinity antibodies are more likely to form smaller complexes (A10, C14, H2, P3, T11). Finally, complexes that are formed in marked antigen excess tend to be smaller (C10, H2, L17, T11).

In the presence of a reasonable antibody response, antigen availability determines the rate of formation of immune complexes. A case in point is the rabbit model of chronic serum sickness in which repeated injections of antigen are used to maintain a state of antigen excess. The dose and duration of antigen exposure determine the amount and the size of complexes formed, and, thus, their clearance, distribution, deposition, and phlogogenic properties (C14, D6, H2). Antigen availability also has theoretical implications with respect to disease associated with endogenous versus exogenous antigens. Endogenous antigens (such as DNA) which are associated with autoimmune disease are present, at least potentially, in marked antigen excess—a feature favoring circulating immune complex formation. This is one explanation why autoimmune diseases, in particular, tend to be associated with immune complexes and manifestations of immune complex disease.

Features of immune complexes that favor rapid uptake and clearance by the mononuclear phagocytic system are the opposite of those features that favor solubility and persistence in the circulation. Thus, large lattice complexes or complexes formed in antibody excess fix complement more efficiently and, by virtue of multipoint attachment, are more likely to bind to Fc and complement receptors of the mononuclear phagocytic system (A18, A20, F9, F10, H1, H2, M5). Some antigens such as DNA are cleared rapidly from the circulation regardless of whether an antibody response has occurred (G10, I7). The state of the mononuclear phagocytic system is also an important determinant of clearance rates. It may be affected by illness (A15) or drugs (A16), or it may become "saturated" by large quantities of complexes that ordinarily would be rapidly cleared (F9, F10, H1). Genetic factors also play a role in mononuclear phagocytic system function, as has been demonstrated in persons with the *DR3* and/or *B8* alleles of the major histocompatibility locus, alleles that are associated with systemic lupus erythematosus and other autoimmune diseases (F9, L6, S30), and which may be related to a numerical deficiency of C3b receptors (W18, W24).

The deposition of immune complexes along vascular basement membranes and the ensuing inflammatory response are the hallmark of immune complex disease. The distribution of deposition by and large determines the clinical features of the disease. In the human, factors governing the localization of immune complexes are incompletely understood, though it is presumed that blood flow physiology plays a role. Areas of relatively high-

pressure blood flow with transcapillary filtration functions such as the glomerulus, choroid plexus, and uveal tract are particularly prone to trap immune complexes. Sites of turbulent flow as occurs at arterial bifurcations are also subject to immune complex deposition. The prominence of lesions in the skin and synovia is probably related to the size of these organs (the surface area of synovia and bursa is estimated to be four times that of the skin), to their high degree of blood flow per unit mass, and to their ready accessibility to clinical examination. In the rabbit, immune complex-induced release of vasoactive amines and increased vascular permeability are absolute prerequisites for immune complex deposition (C13, C14, K12, W16). In the human, similar mechanisms of vasoactive amine release have been demonstrated (B7, I5, J7, O4), but whether this is required for immune complex deposition is not known.

Once deposited, there are multiple mechanisms by which an immune complex initiates an inflammatory reaction (Fig. 2). Foremost among these is activation of the complement system. Immune complexes can activate the classical complement pathway as well as, indirectly or directly, the alternative complement pathway. The biologic activities of complement activation which are relevant to tissue inflammation include the generation of anaphylatoxins C5a and C3a (H29) and chemotactic peptide C5a (H29, T6), direct and indirect membrane lysis by the terminal complement components C56789 (T17), leukocytosis by C3e (G8), macrophage activation by Bb (G12), immune complex solubilization by C3b (C21), and immune adherence, the binding and activation of cells bearing complement receptors.

In the literature there is controversy as to how small an immune complex can be and still fix complement (H2, M5). However, it is generally agreed that the larger the complex, the more efficient complement fixation will be and that bound complexes are more efficient than fluid-phase complexes (M23). As mentioned, smaller complexes tend to escape mononuclear phagocytic system clearance but may be relatively inefficient activators of the complement system, whereas large complexes are efficient activators but are readily cleared. Therefore, it is likely that the most phlogistic complexes tend to be somewhere between these extremes. While complement activation is of paramount importance in immune complex-mediated disease, it is not an absolute requirement for all manifestations of the disease. In the rabbit model of chronic serum sickness, rabbits depleted of complement do not develop necrotizing arteritis but will develop glomerulonephritis (A10, H5).

Immune complexes may also evoke an inflammatory response and tissue damage through direct interaction with cells having Fc or complement receptors. Thus, neutrophils and macrophages may react by attempting to phagocytose tissue-bound complexes, releasing granules or lysosomal en-

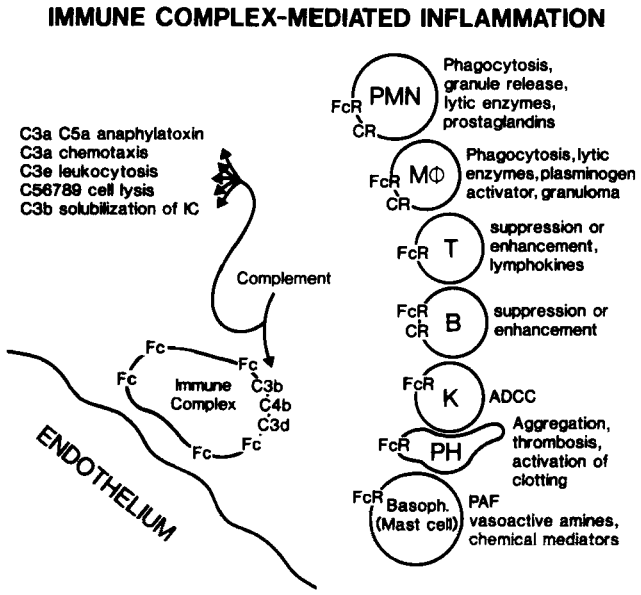

FIG. 2. Mechanisms by which immune complex deposition elicits an inflammatory reaction.

zymes that cause tissue damage (C12, S10). Human platelets have Fc receptors and may react by aggregation, release of vasoactive amines, thrombosis, and subsequent activation of the clotting protein and kinin cascades (B6). Basophils have high-affinity receptors for IgE (as well as separate receptors for IgG and C3b) and may react by releasing mediators of anaphylaxis which increase vascular permeability and the influx of inflammatory cells (H29, I5, J7, S31). The consequences of interaction of immune complexes with lymphoid cells comprise a vast and controversial literature (C14, T11, W11). Depending on the system, one can find evidence for enhancement, suppression, blockade, or activation of specific immune functions. It is presumed that interference with multiple lymphoid cell functions by immune complexes may also play a role in some human diseases, though this is less apparent than their inflammatory effects.

3. Criteria for Determing Whether a Disease Is Caused by Immune Complexes

Evidence that immune complexes are of primary pathogenic importance in human diseases is almost always indirect. The following criteria are those

most generally used. In the absence of being able to ethically fulfill Koch's postulates for cause and effect, the more criteria that can be satisfied, the more likely it is that the disease under study is mediated by immune complexes.

3.1. Demonstration of Antigen and Specific Antibody in Tissue Lesions

This has been demonstrated only rarely. Both DNA and anti-DNA have been demonstrated in glomerular deposits from patients with systemic lupus erythematosus (A6, K13, K15, W19). The inciting antigen usually is not known and, when suspected, can be difficult to demonstrate. Elution of immunoglobulin deposits from tissue in quantities sufficient to demonstrate specific antibody activity is also difficult. Often the detection of antigen (if it is known) and/or immunoglobulin and complement components is taken as prima facie evidence for immunopathogenesis. The use of proper controls is essential to exclude nonspecific deposition of these proteins. It should be remembered that the failure to find immunoglobulin deposits does not exclude the possibility of an immune complex-mediated reaction. Cochrane *et al.* demonstrated that immunoreactants are removed by phagocytosis within 24 to 48 hours in the Arthus reaction (C15). Similar findings have been noted in comparison of acute with chronic vascular lesions in vasculitis (B14, C14, C19, S3).

3.2. Complement Activation

In experimental serum sickness, a fall in serum complement level occurs at the time immune complexes form and inflammatory lesions develop (D6). However, levels of complement do not always reflect activation or consumption by immune complexes. The rate of synthesis of complement proteins may be sufficient to replace the amount being consumed, and several of the complement components are so-called acute-phase reactants, i.e., their levels rise with inflammation. Thus, activation may occur despite normal or even elevated levels in the serum. Turnover studies provide more direct evidence of complement utilization but are technically cumbersome (K4). A simpler approach is the detection of split products of complement components, which provides direct evidence of complement activation, or the examination of effusions for evidence of complement depletion (H31, N7, P7).

The detection of complement components in association with immunoglobulins in tissue supports the possibility that immune complexes have activated complement. Biesecker *et al.* describe a neoantigen formed when

the terminal complement components, C56789, have been activated and assembled into the membrane attack complex (MAC) (B8). Immunologic reagents specific for this neoantigen have been used to provide evidence that complement found in tissue has been activated. For instance, immunoglobulin and complement are found in the epidermal–dermal junction of patients with systemic lupus erythematosus (SLE) and discoid lupus. In discoid lupus, deposits are found only in areas where skin lesions are found. In systemic lupus, deposits are found in both involved and uninvolved areas. The MAC neoantigen is found in both disorders but only in areas of skin involvement (B8).

3.3. Impaired Immune Clearance

As larger amounts of complexes are infused into experimental animals, the rate of clearance slows and vascular lesions appear, presumably as a result of overload (B9, H1, H2). Similar phenomena may occur in man, and impaired clearance has been demonstrated in several diseases associated with manifestations of immune complex deposition including systemic lupus erythematosus (F9, H8, K9, L19, P2), primary biliary cirrhosis (G19), Sjögren's syndrome (H9), and dermatitis herpetiformis (L6). Impaired clearance may be a result of circulatory overload by immune complexes, or a primary defect in mononuclear phagocytic system function may contribute or predispose to immune complex deposition (A15, A16, H1). However, impaired clearance, as currently measured, is neither a prerequisite nor a consistent consequence of immune complex disease.

3.4. Animal Models of Disease

Animal models of disease, whether naturally occurring or artificially induced, provide valuable insights into human disease and allow rigidly controlled studies that are not possible in humans. Probably the most intensively studied model of an immune complex disease is the spontaneous lupus-like disease that occurs in certain inbred strains of mice, especially the (NZB × NZW) F_1 hybrid. Its study has provided insights with respect to genetic predisposition (H25), the role of endogenous retroviruses (D8), the influence of sex hormones (R9), the identification of T suppressor cell abnormalities (T3), and the propensity to form certain autoantibodies, especially to double-stranded DNA (S36) and to the RNA–protein complex, Sm (E2). Though animal studies are not the subject of this review, we emphasize that the entire concept and framework by which we view immune complex disease in humans are based on initial observations in animals.

3.5. Transfer of Disease with Complexes or Antibody

Formal proof of pathogenesis comes from producing the disease, in another host, with the causative material. Humans cannot be used, but accidents of nature do occur. Several examples of neonatal disease due to placental transfer of maternal antibody have been described: hemolytic disease (erythroblastosis fetalis) from anti-Rh antibodies (L9); neonatal myasthenia from antiacetylcholine receptor antibodies (S15); neonatal pemphigus (M19); congenital heart block associated with anti-SS-A/Ro antibodies (F8, S16) and skin lesions (J1) in infants of mothers with systemic lupus. In these examples, the host provides endogenous antoantigen for reaction with transferred antibody. Technically, these are examples of organ-specific autoimmune disease in which complexes form *in situ* in the target organ rather than examples of disease caused by preformed complexes containing antigen that is irrelevant to the tissue being damaged. Passive transfer of preformed complexes to animals results in experiments that are difficult to interpret because of critical dose requirements and the host response to heterologous proteins.

3.6. Demonstration of Complexes in Blood, Effusions, or Other Biological Fluids

In human diseases, it has been difficult to demonstrate antigen bound to its specific antibody in biological materials since the responsible antigens are rarely known. Because of this problem, a number of assays for immune complexes which are not antigen specific and, therefore, are readily applicable to a wide variety of diseases have been developed. These are discussed in greater detail below.

4. Principles of Detecting Immune Complexes

Indirect or antigen-nonspecific methods for detecting circulating immune complexes generally rely on properties of complexed immunoglobulin that distinguish it from free immunoglobulin. These include physical properties such as size or solubility, and binding properties with soluble or cellular receptors for immunoglobulin or complement (Table 2). Receptors which have been used in assays for immune complexes include Fc binding receptors (such as the first component of complement, C1q, monoclonal rheumatoid factors, staphylococcal protein A, and cellular Fc receptors) and receptors which bind complement components (such as bovine conglutinin,

TABLE 2
ANTIGEN-NONSPECIFIC METHODS FOR DETECTING IMMUNE COMPLEXES

Principle	Examples
Physical properties	
Size	Ultracentrifugation, gel filtration, ultrafiltration, electrophoresis
Solubility	Cryoprecipitation, precipitation in polyethylene glycol (PEG)
Reactivity with serologic receptors for Ig or C	
Fc receptors	Monoclonal rheumatoid factor assay, C1q binding and deviation tests, complement consumption tests
Complement receptors	Bovine conglutinin assay, monoclonal anti-C1q assay, anti-C3 assays
Reactivity with cellular receptors for Ig or C	
Fc receptors	Platelet aggregation test (PAT), staphylococci binding assay, L1210 cell assay, macrophage inhibition assay, inhibition of antibody-dependent cellular cytotoxicity, rosette inhibition assay
Complement receptors	Raji assay, human erythrocyte assay, rosette inhibition assay

heterologous anti-C3 or anti-C1q antibodies, and cellular receptors for complement components or their split products).

These receptors appear to bind complexed immunoglobulin much more avidly than free immunoglobulin, and the reason for this is of considerable theoretical and practical immunologic interest. One suggestion is that receptors recognize a conformational change within antibody when combined with antigen. Conformational changes do occur (B17, J4, J5, M14, S7), but there is little experimental support that this is either necessary or sufficient for receptor binding (J4, M14). The alternative hypothesis is that aggregation of antibody molecules, which occurs after binding multivalent antigens, permits multipoint attachment of the complex to receptors, and this accounts for why complexes bind to receptors with apparently greater avidity than monomeric immunoglobulin or free complement components. The latter hypothesis predicts that the receptor binding site itself does recognize and bind monomeric immunoglobulin or free complement components. Considerable experimental support for the latter hypothesis has been obtained in several systems including binding studies of C1q (A21, H19, H28, M2, M24), rheu-

matoid factors (C9, M14, S37), and cellular Fc and complement receptors (A19, S17, S31, T8, T12). Conceptually, the binding of complexed immunoglobulin (or complement) should be favored over monomeric immunoglobulin (or complement) by considering the interaction with receptors as simple equilibrium processes:

$$[\text{monomer}] + [\text{receptor}] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [\text{monomer–receptor}] \tag{1}$$

$$[\text{oligomer}] + [\text{receptor}] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [\text{oligomer–receptor}] \tag{2}$$

where k_1 and k_2 are the forward and reverse (association and dissociation) rate constants, respectively. The equilibrium constant is

$$K = \frac{[\text{monomer–receptor}]}{[\text{monomer}]\,[\text{receptor}]} = \frac{k_1}{k_2} \tag{3}$$

which is related to the individual bond energy (ΔF) as follows:

$$\Delta F = -RT \ln K \tag{4}$$

where R is the gas constant and T is the temperature.

If oligomer contains n monomer molecules, each of which is capable of binding to the receptor with binding energy (ΔF) equivalent to that of free monomer, the net binding energy of oligomer would be additive ($n \times \Delta F$), but the effect on the equilibrium constant would be exponential (K^n), i.e., at equilibrium, oligomer binding is markedly favored.

For most receptor–ligand interactions the forward rate of reaction is diffusion limited and therefore concentration dependent. It is the reverse rate of reaction, the rate at which ligand is released from receptor, that is the major determinant of K. This is illustrated in Fig. 3 in which monomeric and aggregated IgG binding to the Fc receptor of staphylococci protein A are compared. There is little difference in the rate of uptake; the major difference is in the rate of release. The concept can be likened to two men, each randomly opening and closing his fists. One man holds two dumbbells; the other is holding a barbell. The barbell is likely to be retained much longer than the dumbbells.

In reality, the total bond energy of oligomer binding may be somewhat less than the product of monomer binding energy and the number of binding sites ($n\Delta F$) because of spatial, steric, or strain limitations imposed on binding sites by the oligomeric structure. Therefore, the actual difference in K and binding energy observed might not be as dramatic as predicted but should still be substantial. Also, the concept applies to equilibrium conditions. The kinetics of the binding reactions and competitive interactions between

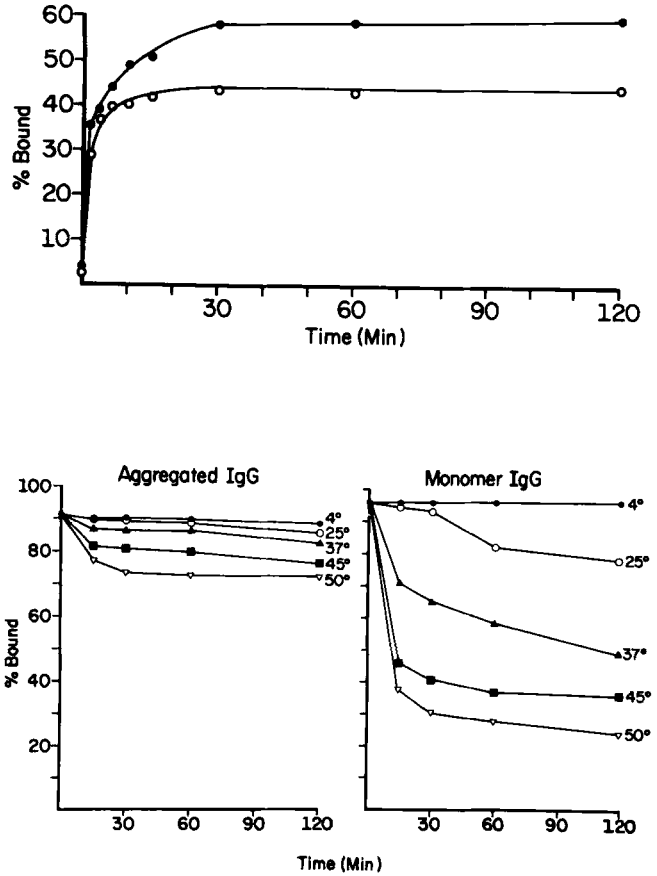

FIG. 3. Kinetics of reaction of aggregated and monomeric IgG with Fc receptors of staphylococci. Upper panel: ^{125}I-labeled monomeric and aggregated IgG were added to staphylococci (at twice their saturating concentrations) and uptake was measured. Lower panel: staphylococci were saturated with ^{125}I-labeled monomeric IgG (right) or aggregated IgG (left) and the rate of release of label was determined. Rebinding of label was minimized by adding a large excess of unlabeled IgG. (Reproduced with permission, J. S. McDougal, P. B. Redecha, R. D. Inman, and C. L. Christian, *J. Clin. Invest.* 63, 627–636, 1979, by copyright permission of the American Society for Clinical Investigation.)

oligomer and monomer could be such that equilibrium takes too long to reach. Thus, the feasibility of using any immunoglobulin or complement receptor in an immune complex assay depends on whether the affinity properties of the receptor can be manipulated by test conditions to result in preferential binding or activation by immune complexes. Such conditions have been found for a number of receptors.

The preceding construct is a very useful concept for approaching immune complex assays. It allows a physicochemical appreciation of the manipulations by which immune complexes are preferentially detected. More importantly, it predicts that nonspecifically aggregated immunoglobulin may be detected by these assays and helps in understanding differences between the tests in sensitivity, specificity, types of complexes detected, and individual idiosyncrasies.

5. Methods for Detecting Immune Complexes

It is not possible or useful to describe all of the techniques that have been developed. The following groups are selected because they are representative of the different principles used in detecting immune complexes (Table 2), because they have been found to be reasonably reproducible in multiple laboratories, and because a fairly large clinical experience has been accumulated with them. Inherent in all the antigen-nonspecific techniques is the potential of the tests, without exception, for detecting nonspecifically aggregated Ig. Moreover, as will be discussed, the tests have individual characteristics which determine their sensitivity, specificity, complexity, types of immune complexes which they detect, and materials other than immune complexes or aggregated Ig which may interfere with the assays.

5.1. Tests Based on Physical Properties of Immune Complexes

Cryoprecipitation, which utilizes the relative cold insolubility of some immune complexes, is one of the oldest techniques. Certain hydrodynamic properties related to the tertiary structure of the particular immunoglobulins in the complex account for this solubility property (G16, M17). Three types of immunoglobulin-containing cryoglobulins have been described: Type I cryoglobulins contain a single molecular species, a monoclonal paraprotein; Type II cryoglobulins contain a monoclonal rheumatoid factor associated with polyclonal IgG; Type III contain polyclonal antibodies associated with antigen (B16). Type I and II cryoglobulins are associated with lymphoid malignancies. Type III cryoglobulins are associated mostly with rheumatic diseases, inevitably contain polymeric immunoglobulins (IgM or IgA) in addition to IgG, and very often contain polyclonal rheumatoid factors.

To perform the test it is best to allow a freshly drawn blood specimen to clot at 37°C. The serum is then held at 0–4°C for 48–72 hours (a Wintrobe tube is convenient). After centrifugation the volume of cryoprecipitate is measured (cryocrit). After washing the cryoprecipitate in the cold, it is dis-

solved in 37°C buffer for protein and immunoglobulin quantitation. The last step is essential to confirm that one is indeed dealing with a reversible cold-insoluble precipitate that contains immunoglobulin, because not all cold-insoluble precipitates are composed of immunoglobulin (e.g., cryofibrinogen, fibrin debris).

It requires relatively large amounts of cryoprecipitable immune complexes to register a positive result in this test (i.e., cryocrit of greater than 0.5%). Its major drawback, however, is that many, if not most, immune complexes do not have the property of cold insolubility. However, the method has been valuable, at least for the study of those complexes with this property. The method lends itself very conveniently to the isolation and characterization of antibody and antigen in immune complex material (provided the technique is controlled for nonspecific trapping of serum components) (B2, B3, F1, L13, M13, W9, W20). Despite its poor sensitivity, the test for cryoglobulins, more than any other immune complex assay, is, in a sense, a diagnostic test. The demonstration of cryoglobulins is required to make a diagnosis of lymphoma or myeloma-associated cryoglobulinemia or essential cryoglobulinemia (Meltzer–Franklin syndrome; M15).

A more widely used method is precipitation of immune complexes with polyethylene glycol (PEG). PEG is an uncharged linear polymer which in solution differentially precipitates proteins in relation to their aggregate molecular size or conformation. If protein concentration is held constant, there is an inverse linear relationship between the concentration of PEG and the logarithm of the aggregate molecular weight of the protein or protein complex that is precipitated [with some exceptions, e.g., α_2-macroglobulin and lipoproteins (D5, Z5)]. Thus, PEG is used to detect complexes of IgG that precipitate at concentrations of PEG (usually 3.5 or 4.0%) that do not precipitate monomeric IgG. Like the cryoglobulin assay, it is wise to confirm that the precipitate contains IgG (noncomplexed IgM, IgA, or C1q may be found in 3.5–4.0% PEG precipitates from normal sera) (D5, M11, Z5) or that the precipitate fixes complement (H13). Often, however, a turbidometric measurement (S12) or an increased protein concentration in the precipitate is taken as presumptive evidence for the presence of immune complexes. The amount of IgG precipitated from sera at a given concentration of PEG is affected by the dilution of serum used, the pH, and, to a lesser extent, the ionic strength and amount of IgG initially present (D5, Z5). All but the last are easily controlled. Hypergammaglobulinemic sera (IgG concentration above 2.5 g/dl) are common in autoimmune diseases and some 7 S IgG will be precipitated, even at 3.5% PEG. Thus, the principal disadvantages of the method are its lack of specificity for immune complexes and the influence of IgG concentration on the test. On the other hand, it is a very simple test to perform, results agree fairly well with more specific tests such as C1q bind-

ing, and PEG precipitation is often used as a first step for isolating immune complexes (B3, D5).

5.2. Tests Based on Reactivity with Serologic Receptors for Immunoglobulin

C1q, the first component of complement, reacts with the C_{H^2} domain of IgG1, IgG2, and IgG3 subclasses and with IgM (A21, M2, M23–M25, Y1). The reaction is weak and reversible but, as discussed, markedly favored by aggregation of the reactants. C1q has been put to use in a wide variety of assays for immune complexes. The C1q binding assay developed by Nydegger *et al.* (N6) as modified by Zubler *et al.* (Z3) is based on the original observation of Agnello *et al.* (A7) that purified C1q forms precipitin lines with immune complexes or heat-aggregated IgG in agar gel. It is the most widely used assay based on C1q binding. In this assay, serum is first incubated with EDTA to chelate calcium and prevent the insertion of C1q into the C1qrs complex. Radiolabeled C1q and PEG (final concentration, 2.5%) are then added. Under these conditions, C1q bound to macromolecular complexes is precipitated, whereas free C1q remains soluble. A disadvantage of this assay is its relative insensitivity to small amounts of complexes (<50 μg/ml), particularly complexes of intermediate size (11–19 S), relative to other assays (G1, L2, M10, M11, Z3). More sensitive variants of the C1q binding assay have been devised. The C1q deviation tests measure inhibition of C1q binding to a particulate substrate such as antibody-coated erythrocytes (S28), IgG-coated latex particles (L21), or IgG-coupled Sepharose beads (G1). Inhibition of radiolabeled aggregated-IgG binding to solid-phase C1q has also been used (S39). Solid-phase C1q assays in which direct binding of immune complexes to C1q is detected with anti-Ig reagents (A8, F2, H16) have an advantage over fluid-phase, deviation, and inhibition assays in that they are immunoglobulin specific by virtue of the anti-Ig probe used in the assays.

With all the assays based on C1q binding, it is important to remember that the types of complexes detected are restricted by the specificity of C1q for IgM and the IgG1, IgG2, and IgG3 subclasses. The tests may also be affected by the C1q concentration in the test sample (G1, H16, L21, M10, R8, Z3). More importantly, a number of nonimmunoglobulin substances that are C1q reactive may occur in sera and interfere with the results. Polyanions such as DNA, heparin, and bacterial endotoxins bind C1q (A7, G1, L2, L21, S28, S39, T5, W26, Z3). C-Reactive protein reacts with C1q through a calcium-dependent bond, a problem usually circum-

vented by calcium chelation with EDTA (G1, Z3). Claims that polyanions do not cause false positives in certain tests may be quantitative or relative rather than absolute advantages and these substances may still interfere with the tests by inhibiting immune complex detection causing false negatives (L2, M10, T5).

Human rheumatoid factors are low-affinity antibodies (usually of the IgM class) that react with human IgG, and, like C1q, they have been used for the detection of immune complexes containing IgG. The reaction of IgM rheumatoid factors with monomeric IgG is weak and reversible. Because of multivalency the reaction with complexed IgG is much stronger. There is, however, considerable variation between rheumatoid factors in their differential reactivity with monomeric and aggregated IgG. The successful use of a rheumatoid factor for the detection of immune complexes depends to a great extent on the rheumatoid factor selected. The more consistent assays use monoclonal IgM paraproteins with rheumatoid factor activity from patients with Waldenström's macroglobulinemia. These are preselected for more efficient reactivity with aggregated IgG than with monomeric IgG. This selection is made by comparison of precipitation with limiting amounts of aggregated and monomeric IgG and by comparison of the amount of monomeric IgG required to inhibit precipitation of aggregated IgG.

Winchester *et al.* first used a monoclonal rheumatoid factor to detect immune complexes using double diffusion in agar (W23). Since then, much more sensitive assays based on the inhibition of monoclonal rheumatoid factor binding to aggregated IgG (L22), IgG-coated Sepharose (G1) and IgG-coated latex beads (L21) have been devised. These assays are very sensitive to small amounts of immune complexes and are much better than the C1q binding assays in detecting smaller (11–19 S) sized complexes (G1, L2, L21, L22, M10). They are independent of the complement-fixing properties of the complexes but do not detect complexes other than those containing IgG. The tests may be affected by the concentration of monomeric IgG in the test sample, a problem that can be circumvented (or equalized) by running all test specimens at the same IgG concentration (L22, M10).

Other antiglobulin assays using polyclonal rheumatoid factors or low-affinity antisera raised in animals have been devised as well but are not widely used. One such assay has particular theoretical appeal. Kano *et al.* described an antiglobulin assay using a reagent which binds IgG only after IgG has combined with antigen. The reagent is nonreactive with monomeric or aggregated IgG, and presumably recognizes a conformational change in IgG that results from antigen binding (K3). The reagent is very rare and difficult to produce, and the assay did not perform particularly well in a comparative evaluation (L2).

5.3. Tests Based on Reactivity with Serologic Receptors for Complement

Conglutinin is a naturally occurring protein found in the sera of cattle (L22). Conglutinin forms a calcium-dependent bond with C3 split products. Solid-phase assays have been developed in which test serum is incubated with conglutinin-coated plastic tubes. Immune complexes containing the appropriate C3 split product bind and are detected with a radiolabeled (C5, E1) or enzyme-linked (T13) anti-immunoglobulin reagent. The assays are relatively easy to perform and are quite sensitive for small quantities of complexes although there is marked preferential reactivity with larger size complexes (>19 S). They have the advantage of being specific for immunoglobulin-containing complexes which have activated complement. This may also be a disadvantage since the C3 split product detected may be relatively short lived making it likely that the conglutinin assays detect only a portion of complement-fixing complexes. A similar assay in which conglutinin is replaced by anti-C3 antibodies has been developed. Short-lived C3 intermediates are less of a problem with this assay (P5). A commercial test adaptation of the anti-C3 assay using a monoclonal anti-C3 reagent has been developed as well (Ortho Pharmaceutical).

A murine monoclonal antibody has been developed that is specific for conformational changes that occur in C1q after binding to immune complexes. It does not bind native C1q (G14, H19). This antibody (or a similar one) has been used in a solid-phase, enzyme-linked assay for the detection of immune complexes (R3). We have evaluated this test and the monoclonal anti-C3 test with a large panel of specially prepared specimens. We previously used this panel to evaluate five other assays for immune complexes (M10). The monoclonal anti-C3 and anti-C1q assays performed as well as, if not better than, the other assays we evaluated.

5.4. Tests Based on Reactivity with Cellular Receptors for Immunoglobulin

Human platelets have receptors for the Fc portion of IgG which have high avidity for aggregated IgG. Immune complex-induced platelet aggregation forms the basis for one of the earliest tests for immune complexes (M29, P4). The method is quite sensitive to small quantities of immune complexes, but, like other bioassays, has the disadvantage that fresh, metabolically active platelets are required and day-to-day reproducibility can be a major problem. Spontaneous aggregation or poor aggregation is not an unusual problem, and substances other than immune complexes (antiplatelet antibody, enzymes, coagulant proteins) may induce aggregation (M13, P4).

The cell wall of most strains of staphylococci contain a protein, termed "A," which reacts with the Fc portion of IgG1, IgG2, IgG4, and some preparations of IgA and IgM (G17, H15, K16). Like C1q and rheumatoid factors, protein A binds to aggregates with greater avidity than monomeric IgG. An assay has been devised which is performed under conditions in which complexed or aggregated IgG is preferentially bound and detected on formalin-fixed staphylococci cells (M11). The assay is sensitive to small amounts of complexes including intermediate-size complexes (11–19 S) (M10, M11), but, because of receptor specificity, does not detect all classes of immunoglobulin in immune complexes. Unlike other tests that rely on cell receptor interactions, active cellular processes are not involved and viable intact cells are not required.

The binding of immune complexes to Fc receptors on cells may either induce or block the expected biologic function of the cells. A number of bioassays for detecting immune complexes are based on these phenomona. The prototype assay, already mentioned, is the platelet aggregation assay. Other assays include the inhibition of antibody-dependent cellular cytotoxicity (ADCC) (J6); the inhibition of binding of antibody-coated or antibody plus complement-coated erythrocytes (rosette inhibition assays) (M20, S26); the direct binding to continuous B cell lines (L1210 assay) (P15); neutrophil phagocytosis (S35) or inhibition of neutrophil or macrophage phagocytosis (O2); peroxidase release from eosinophils (T2); and histamine release from mast cells (B4). All of these models tell us much about some of the effects of immune complexes on cells, and some are exquisitely sensitive to small amounts of immune complex material. However, the techniques themselves are difficult to employ for routine clinical measurement of immune complexes because they are often cumbersome and require indicator cells with intact and uniform biologic functions, which are difficult to obtain. The latter problem makes comparison of results from batch to batch difficult. When compared to other tests that use more stable reagents, these types of assays have not compared favorably (L2), and none has really found wide use in clinical studies.

5.5. Tests Based on Reactivity with Cellular Receptors for Complement

Foremost among these assays is the Raji cell assay developed by Theofilopoulos and co-workers (T15). The Raji cell line, derived from a patient with Burkitt's lymphoma, lacks surface immunoglobulin, has Fc receptors that bind monomeric IgG only weakly, and possesses numerous receptors for C1q, C3b/C4b, C3d, and other complement components (G23, S4, S27, S28, T8, T12). Immune complexes which have fixed complement bind to

both C3 (C3b and C3d) (T8, T12) and C1q (G23) receptors. Binding is detected with a radiolabeled anti-IgG reagent. This assay is very sensitive to small amounts of immune complex material, is reproducible and relatively easy to perform, and has been widely used in clinical studies. A disadvantage of the technique is that autoantibodies to lymphocyte surface antigens or nuclear materials that are often present in systemic lupus erythematosus will also produce elevated values (A13, H24, W14), although the frequency with which this occurs has been debated (D3, L2, T13, T15, W26).

Tsuda *et al.* introduced the use of human red cells in place of Raji cells for the detection of immune complexes (T22). Human erythrocytes have "immune adherence" receptors for C3b/C4b, but do not have C1q or C3d receptors like the Raji cell (C22, G9, M23, N3, R5). This assay is appealing because tissue culture facilities are not required, and there is no problem with antilymphocyte antibodies. Unfortunately, antierythrocyte antibodies, which are common in many systemic autoimmune diseases, may interfere (isoagglutinins are not a problem if group "O," Rh-negative erythrocytes are used). During complement activation, C3b is rapidly cleaved in serum by C3b inactivator to fragments which no longer react with the immune adherence receptor (M23, N2). Because the Raji cell has several different kinds of complement receptors, it is probable that some complexes which have fixed complement would be detected by the Raji assay but not by the human erythrocyte assay. A parallel comparison of the two assays has not been done. It is best to use the same red cell donor for this assay because of variability in receptor density among individuals (W24).

6. Comparison of Immune Complex Assays

The ultimate validity of an immune complex assay depends on how it performs with clinical material, and there is a great deal of controversy regarding the relative merits of some immune complex assays. Some diseases may be associated with certain types of complexes which are detected by some tests but not by others. Since the tests differ in their complement dependence or independence and in the class or subclass of Ig that they detect, many of the discrepancies between assays can be directly attributed to this—a qualitative restraint imposed by the specificity and receptor principle used in a particular assay. Moreover, even with assays of similar principle, discordant results are not uncommon. Much of this variation is due to the performance characteristics of the assays themselves with respect to reproducibility, sensitivity, specificity, the size, quantity, and conformation of immune complexes detected, and the role of interfering substances. Per-

formance characteristics are best evaluated by running of the tests in parallel on the same samples and referring the results to a common standard.

Relatively few studies of this sort have been done (F12, G22, H3, L2, R8, S21, W26). The most extensive comparative study was carried out under the auspices of the World Health Organization (WHO) and reported in 1978 (L2). Eighteen methods were evaluated. The same samples were analyzed by all collaborators, and their positivity or negativity was determined centrally by reference to a panel of coded normal sera. This is the usual means for determining the cutoff value between a positive and a negative test result. The "normal range" is defined by some arbitrary or statistical manipulation (90th or 95th percentile, mean plus 2 standard deviations) of results with normal sera. Since specificity (the percentage of normal sera with normal results) is preselected, it is meaningless to compare specificity between tests. However, given the same definition of specificity determined from the same panel of normal sera, it is very useful to compare tests with respect to sensitivity (percentage of abnormal sera with abnormal results), and this is what the WHO study did. With respect to test performance, sensitivity–specificity results are influenced considerably by test reproducibility and by whether small quantities of immune complex material register a value outside the normal range.

In the WHO study, there were considerable differences between the tests in reproducibility, especially between-run variation. With the exception of the Raji assay, the bioassays which require viable, intact cells were much less reproducible than assays that use more stable and uniform reagents. Even the latter tests do not approach the precision one would demand from, say, a clinical chemistry test.

Tests such as immune complex assays register some "background" activity with normal sera (which by definition may be assigned a value of zero), have a range of assay readouts, and measure an analyte for which there is no truly relevant standard. For tests such as these, two conceptually similar but operationally different ways of expressing results have been used. They can be expressed either as standard deviations from the mean of a panel of normal sera included in each run or by reference to a positive standard or standard curve included in each run. Since the same raw data are used to derive both sets of units, the advantage of one over the other depends on the operational consistency of the panel of normal sera relative to the positive standard as well as the performance characteristics of each assay in the normal versus abnormal range. We found similar reproducibility with both methods for five immune complex assays (M10). However, the assays we examined tend to have less intrinsic variation than most (E1, L2, L22, M10, M11, T15, Z3), and the standard (chromatographed, heat-aggregated, al-

bumin-stabilized IgG) is very consistent from run to run (K6, M10). It is conceivable that under less favorable conditions of reproducibility or with different standards that some advantage of one method over the other would be found for certain tests.

In the WHO study the ability of the tests to detect limiting quantities of heat-aggregated IgG and aggregates of "small," "medium," and "large" size was examined (L2). In general, the radioassays using stable reagents and the Raji assay performed better than the other bioassays, agglutination, or rosette-inhibition assays (L2). However, the collaborators reported quantitative results by reference to their own particular standard. Therefore, it is not possible to determine whether some of the quantitative differences between the tests relate to the particular standard used or to the assay per se. This is best evaluated by referring the results to a common standard.

The ability to detect limiting amounts of immune complex material, complexes of different sizes, or the antigen–antibody ratio has been examined. Usually this has been done, in isolation, in the original description of the assays, but in some instances has also been compared to other tests using a common standard (E2, G1, G22, H3, L21, L22, M10, M11, N6, P5, R8, S21, T14, T15, W26, Z3). We performed a comparison of sensitivity for small quantities of various-sized immune complex material using five assays (M10). Results were referred to a common standard and were generally consistent with other individual studies of these tests. Four of the tests had been shown to be quite sensitive for small quantities of immune complex material (<20 μg/ml), namely, the bovine conglutinin assay (BCA), the monoclonal rheumatoid factor (MRF) radioassay, the Raji assay, and the staphylococci binding assay (SBA) (E2, H3, L2, L22, M11, S21, T15). The fifth, the fluid-phase C1q binding assay (C1qBA), is generally acknowledged to be less sensitive (50–100 μg/ml) (L2, M11, T11, Z3). Heat-aggregated IgG was fractionated into different molecular weight ranges to determine the size of complex detected and the minimum quantity of each required to register a positive test result (M10). With equal concentrations of aggregates, higher assay readouts were obtained with large-sized aggregates than with smaller aggregates in all the tests. With heterogeneous aggregates or aggregates greater than 19 S in size, all tests except the C1qBA were capable of discriminating as little as 10–25 μg/ml of this material in normal human serum. The C1qBA detected 50 μg/ml. There were, however, marked differences in the amount of material required to discriminate intermediate-sized (11–19 S) aggregates. The C1q and complement-detecting assays, BCA and Raji, showed the most dramatic drop in sensitivity in this range whereas the Fc-receptor assays, MRF radioassay and SBA, were more sensitive for this material. These findings are consistent with most studies that have examined this (E1, M11, N6, T14, T15, Z3) and are also consistent with observations that com-

plexes less than 19 S in size are not efficient activators of complement (G1, L21, M5). However, despite differences in sensitivity, all assays were indeed capable of detecting intermediate-sized complexes.

The results with fractionated aggregates are also illustrated and confirmed for true immune complexes in Fig. 4. Declining assay readouts were obtained with increasing antigen excess, i.e., progressively smaller immune complexes. This figure also underscores why there is no truly relevant quan-

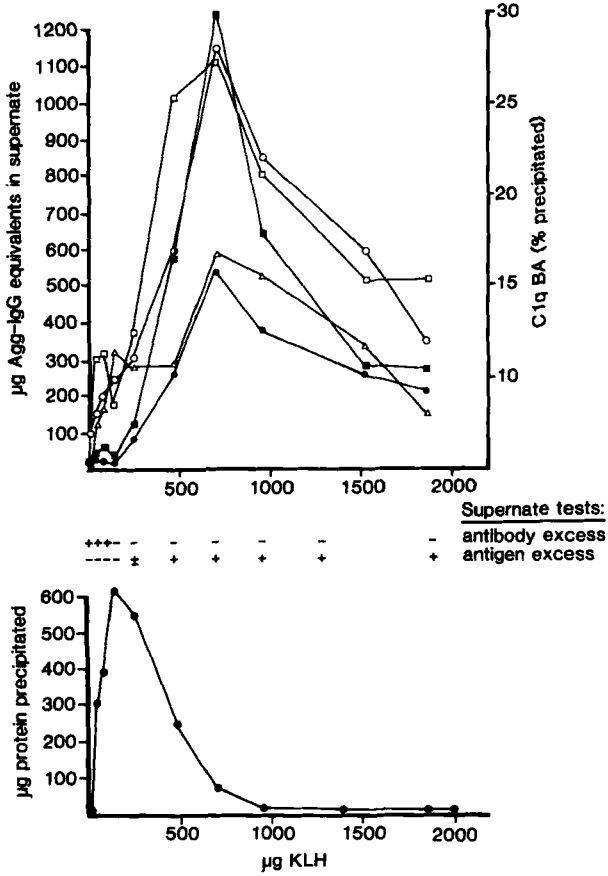

FIG. 4. Detection of immune complexes in supernates from a precipitin curve. Lower panel: total protein precipitated by increasing amounts of keyhole limpet hemocyanin (KLH) added to a human anti-KLH serum. Upper panel: supernates were analyzed for immune complexes by the conglutinin (●), monoclonal rheumatoid factor (△), Raji (□), and staphylococci binding (■) assay, and results expressed as μg/ml aggregated IgG equivalents per ml. C1q binding assay (○) results are expressed as percent C1q precipitated. (Reproduced with permission, J. S. McDougal, M. Hubbard, P. I. Strobel, and F. C. McDuffie, *J. Lab. Clin. Med.* **100**, 705–719, 1982.)

titative standard for these tests. In marked antigen excess (the point beyond equivalence at which no precipitation occurs), as antigen is increased the amount of Ig in the complexes is constant; individual complexes become progressively smaller but the total amount of antigen involved in complex formation (and therefore the total mass of all complexes in the sample) increases. Yet all assays give declining readouts.

The specificity of the receptor in any given assay necessarily imposes absolute limitations on the types of immune complexes detected. Other limitations such as the size, quantity, and conformation of the immune complexes, we feel, are largely related to quantitative rather than qualitative differences in the tests. The influence of interfering substances (endogenous and exogenous) conforms to predictions based on knowledge of the receptor principle of each test. Polyanions such as DNA, heparin, and endotoxin do influence C1q binding assays. Much of the controversy on this point (L2, N6, R8, S21, T5, W26, Z3) can be accounted for by the fact that a polyanionic preparation that does not cause false positives may still be capable of causing false negatives or enhanced estimates of immune complex material (M10, T5). Those tests that detect immune complexes which have fixed complement are dependent not only on the amount of immune complex material but also on the amount of complement available for reaction with the complex (E1, G22, M10, T14, T15). Although we (M10) and others (Z3) have found little influence of the amount of endogenous C1q on the fluid-phase C1qBA, this effect may be more pronounced with more sensitive C1q binding assays (G1, H16, L21, R8). Conversely, the binding of complement to the C_H2 domain of IgG may sterically interfere with Fc-receptor binding to the C_H3 domain. Although some steric hindrance occurs, it is difficult to demonstrate or is only a minor influence on Fc binding by monoclonal rheumatoid factor or staphylococci protein A (M10), but may be a greater influence in binding to lymphoid or macrophage Fc receptors (K2, S5, T12, T14). Although interference by rheumatoid factors has been considered a drawback of some immune complex assays relative to others (T11), none of the assays we examined are free of this influence (M10). IgM rheumatoid factors often exist in sera as 22 S or greater complexes of IgM and IgG and, as such, can be considered true immune complexes (F11, N5, S11). Reported noninterference by rheumatoid factors in some tests might be considered an advantage and at the same time an indictment of the sensitivity of the test. Theoretical considerations of the receptor principle involved in these tests—especially the tests dependent on Fc detection—predict some influence of the relative monomeric IgG concentration. Some assays involve manipulations designed to minimize or abolish this problem at least with respect to the range of IgG concentrations usually found in clinical specimens (L22, M10, M11). In other tests, the influence is small but is an accepted possible explanation for a positive test result (B3, D5, Z5).

If certain types of complexes predominate in some diseases and if the performance characteristics and receptor specificity of the assays are considered, it is not unexpected that different sensitivities would be obtained. In the WHO study, the Raji cell assay gave the highest proportion of positive results in systemic lupus (L2) and this is compatible with other studies (M10, T15). Conversely, the rheumatoid factor assays have a low percentage of positivity in systemic lupus but were quite high in rheumatoid arthritis and comparable to other assays in other diseases (G1, G22, H3, L2, L22, M10). The C1q binding assay measured the highest levels in rheumatoid arthritis in the WHO (L2) and other studies (G22, H3, M8, N7, Z4), perhaps because of binding to IgM rheumatoid factors. A surprising result was the lower than expected percentage of positive results in patients with chronic glomerulonephritis. Furthermore, there was little correlation between the presence of immune deposits in glomeruli as detected by immunofluorescence and serum levels of immune complexes by any test (L2). In patients with vasculitis, about one-half had positive tests by each method but there was very little correlation among any of the methods. In two correlative studies of several immune complex (IC) assays in rheumatoid arthritis, a large range of correlations between assays was found ($r = +0.06$ to $+0.74$) (G22, M8). In general, methods based on similar principles are correlated more closely

TABLE 3
CORRELATION AND CONCORDANCE BETWEEN IC ASSAYS TESTED ON PATIENT SERA[a]

Two IC assays compared		Receptor principle similar or different[b]	Correlation coefficient	Concordance in results[c]	Number of sera tested	p value
C1qBA	SBA	Similar	0.60	72	1891	<0.0001
BCA	Raji	Similar	0.54	77	398	<0.0001
MRF	SBA	Similar	0.49	70	294	<0.0001
BCA	SBA	Different	0.49	57	446	<0.0001
C1qBA	MRF	Similar	0.42	66	300	<0.0001
MRF	Raji	Different	0.35	56	296	<0.0001
Raji	SBA	Different	0.34	66	1552	<0.0001
BCA	MRF	Different	0.30	66	242	<0.0001
C1qBA	Raji	Different	0.26	66	1556	<0.0001
BCA	C1qBA	Different	0.25	63	448	<0.0001

[a] Reproduced with permission, J. S. McDougal, M. Hubbard, P. I. Strobel, and F. C. McDuffie, *J. Lab. Clin. Med.* **100**, 705–719 (1982). IC = immune complex.

[b] IC assays are divided into two groups on the basis of receptor principle. Assays which make use of a receptor for the Fc portion of immunoglobulin in IC are the C1qBA, SBA, and the MRF assay. Assays which make use of a receptor for complement components fixed to IC are the BCA and the Raji assay.

[c] Percentage of sera in which both tests agreed as to positivity or negativity.

than those based on different principles. This is illustrated in Table 3 where test results on a wide variety of clinical specimens (mostly rheumatic disease, renal disease, malignancy, and infectious disease) are correlated.

7. Diseases Associated with Immune Complexes

Immune complex-like material has been found in sera of patients with so many diseases that it is unlikely that all of these positive results represent true antigen–antibody complexes, or, if they do, that the complexes bear any great relevance to the pathogenesis of the diseases. Soltis and Hasz (S29) have shown that spontaneous aggregation of IgG (resulting in positive C1q binding) can occur in serum at 37°C, a phenomenon markedly enhanced if the albumin concentration is low. *In vitro*, activation of neutrophil enzyme systems can cause monomeric IgG to aggregate, suggesting that inflammation per se can generate immune complex assay reactants (J3).

We selected various diseases for discussion. Rather than a litany, the list is somewhat eclectic. We summarize findings in those diseases in which considerable clinical experience with laboratory tests has accumulated and in which immune complex testing has been shown to bear some clinical relevance to the disease (as defined in the introduction). Our emphasis is on those diseases in which immune complexes appear to have a definite pathologic role, the antigen–antibody system has been well defined, or testing for immune complexes is of some value in clinical management.

7.1. Systemic Autoimmune Diseases

7.1.1. *Rheumatoid Arthritis*

Rheumatoid factors of the IgM and IgG classes have been shown to form immune complexes in serum or joint fluid either by self-association (K17, M4, M26, P13, S11, W21) or by reaction with native IgG (C4, K17, M4, N5, S11, W21, W22), and these appear to be the predominant immune complex material in rheumatoid arthritis (C4, G1, K17, M4, M26, N5, S11, W21, W22). The primary cause of rheumatoid factor production in rheumatoid arthritis is unknown. However, rheumatoid factors are known to be present in other diseases associated with chronic antigenic stimulation (C14, M14) and can be induced *in vitro* by stimulation with antigens, autologous aggregated IgG, anti-idiotype reagents, and polyclonal B cell activators such as lipopolysaccharide and Epstein–Barr virus (C4, D11, F6, F7, G11, I6, P10, S24). Rheumatoid factors, including IgG rheumatoid factors which form self-associating intermediate-sized (11–19 S) complexes, play a major role in

perpetuating, if not initiating, chronic inflammation in rheumatoid arthritis. They are found in abundance in synovial fluid (C4, M4, M26, P12, W21, W22), have been shown to be produced by cells within the synovium (B12, G11, M27), are capable of complement fixation (B8, G9, M21, W11, Z5), can activate phagocytic cells (H33, K10, M26), and may thereby elicit inflammatory erosive disease in joints and extra-articular vascular lesions. Depending on the test and patient selection, IgM rheumatoid factors are found in 80–95% of rheumatoid arthritis patients (C4, M4). IgM rheumatoid factor detection is, therefore, a useful adjunct to the diagnosis of rheumatoid arthritis, but it has not been particularly useful either as a measure of disease activity or as a prognostic indicator in the management of rheumatoid arthritis (C4, M4, M7). Serum levels of IgG rheumatoid factor have been associated with more severe disease in most (A9, C3, J2, M8, P11, P12, T10) but not all (W10) studies. However, high levels ordinarily are found only in association with high levels of IgM rheumatoid factor.

Although most of these immune complex materials appear to be combinations of IgG–IgG rheumatoid factor or IgG–IgM rheumatoid factor, other antigen–antibody systems may be important as well. The majority of patients with seropositive rheumatoid arthritis have antibodies to nuclear antigens present in Epstein–Barr virus-infected human cell lines (A11, C6, N4, T4). Indeed, some rheumatoid factors appear to have dual specificity for both autologous IgG and these nuclear antigens (A13). Other, as yet unknown, types of complexes may be important as well (L3).

Depending on the test, 75 to 95% of seropositive rheumatoid arthritis patients have immune complex assay reactants in their sera. In general, the highest levels and percent of positivity are found with tests that detect IgM-containing complexes such as C1q binding assays and tests that are sensitive for intermediate-sized (11–19 S) complexes such as MRF assays and the SBA (A14, C5, F5, G2, G22, H3, H16, H32, L2, L7, L22, M8, N7, R1, Z3, Z4). The best correlations with articular disease activity are found with these tests as well (C5, G22, H3, H16, M8, N7, Z4), and the levels of immune complex assay reactants tend to be higher and complement activation more intense in synovial fluids (H30, M11, N7, Z4). The latter group of tests are especially well correlated with extra-articular disease manifestations such as Sjögrens syndrome, pleuropulmonary disease, Felty's syndrome, and vasculitis (A10, C5, F5, G22, H3, H32, L7, L22, M8, R2). This observation is consistent with observations that high levels of IgM rheumatoid factor, self-associating IgG rheumatoid factors forming intermediate-sized complexes, and perhaps low-molecular-weight (8 S) IgM rheumatoid factors are highly associated with more severe disease and vasculitic complications (A9, C3, H12, J2, M8, S32, P11, P12, T10, W22). Of the complement-dependent tests, the Raji assay is probably the best in terms of detecting intermediate-sized complex-

es which have fixed complement (L2, M10, T15). Although this test tends to have a lower sensitivity for rheumatoid arthritis in most (C5, H3, M8, T15) but not all (G22, L2) studies, it nevertheless correlates quite well with extra-articular manifestations of the disease (G22, H3, M8, T15).

Collectively, these studies support the notion that immune complexes are of major pathogenic importance in rheumatoid arthritis, and population studies do show correlations with disease activity and associations with extra-articular manifestations. Does this mean that the tests are of value in the management of individual patients? Some studies have shown that individual patients who improve generally have a fall in immune complex levels (N7, U2, W2, Z4) and elevated immune complex levels may be one of the earliest manifestations of rheumatoid arthritis (J9). Our own studies have shown that while immune complex assays are as good as any other test in reflecting disease activity, they really are no better than simpler tests such as the erythrocyte sedimentation rate (G22, L2, M8). With respect to diagnosis of rheumatoid arthritis or discrimination of extra-articular subsets of this disease, their value is at best limited to the use of a negative test result in ruling out a diagnosis. This is because the differential diagnosis is usually not between rheumatoid arthritis (or a subset of this disease), which may have a high incidence of positivity, and normals, which by definition have a low incidence of positivity, but between rheumatoid arthritis (or an extra-articular subset) and other diseases (or non-extra-articular subsets) that may themselves be characterized by a high incidence of positivity.

7.1.2. *Systemic Lupus Erythematosus*

In spite of the strong evidence for the role of DNA/anti-DNA complexes in causing the kidney lesions of systemic lupus (A6, K13, K15, W19), it is not certain that the material assayed in serum as immune complexes is actually composed of DNA linked to specific antibody. Several groups have demonstrated DNA (D2) or anti-DNA (H10) in immune complex material from sera, but careful studies by others have failed to repeat these findings (A7, H27, I8). DNA, by itself, does not persist in the circulation (G10, I7). Because of the multiplicity of autoantibodies found in lupus, any of a number of antigen–antibody systems may be involved (T4). Based on direct examination and the reactivity patterns with various immune complex assays, the immune complex material found in systemic lupus tends to be macromolecular (>19 S) and complement fixing (A5, A7, C5, D2, E1, F12, G1, L2, M10, N6, T15).

Immune complex measurements have been widely used in patients with systemic lupus because of the strong evidence that this disease is mediated by immune complex injury. In general, a good correlation has been found between levels of immune complexes, anti-DNA titers, depressed comple-

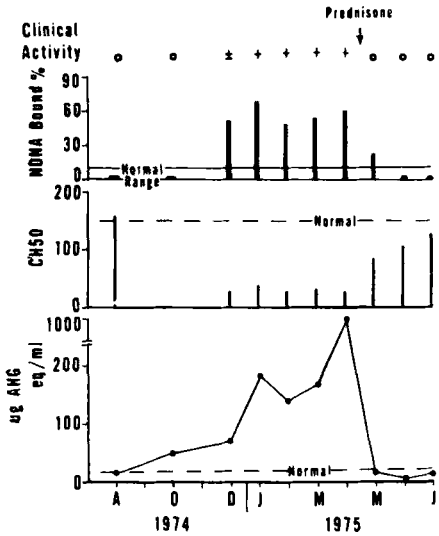

FIG. 5. Serial study of a woman with systemic lupus erythematosus showing correlation among levels of immune complexes measured by the Raji assay, complement levels, antibodies to native DNA, and clinical activity. (Reproduced with permission, A. N. Theofilopoulos, C. B. Wilson, and F. J. Dixon, *J. Clin. Invest.* 57, 169–182, 1976, by copyright permission of the American Society for Clinical Investigation.)

ment levels, and the degree of clinical activity (Fig. 5), although not all studies agree (A1, C5, D2, D5, E1, F12, G1, H14, I2, K3, L2, L11, L18, M10, N6, P19, T11, T15). These studies are population studies and support the notion that immune complexes are of pathogenic importance in lupus, but the range of values obtained with different degrees of activity may be such that a test result in an individual patient may have little discriminatory power. Our own experience has been that serial measurements are not very good predictors, particularly in estimating the extent of renal damage that is occurring (F34, K9, P2). The use of these tests in the clinical management of patients with systemic lupus is the subject of an excellent review by Inman (I1).

7.1.3. *Necrotizing Vasculitis*

Polyarteritis nodosa is a severe systemic necrotizing vasculitis of medium- and large-sized arteries. Gocke *et al.* first reported an association between circulating hepatitis B antigen (HBsAg) and polyarteritis (G13). Since then, numberous studies have demonstrated HBsAg–antibody complexes in the circulation and in vascular lesions of patients with polyarteritis (C18, D12, 13, G15, G20, I4, M16, T15, T19, T20G). As many as half of the cases of

polyarteritis may be associated with hepatitis B antigenemia (F3, S19, T18, T20). Clinically, these cases cannot be distinguished from those without HBsAg(C11, G15, P18, S19, T18). In one longitudinal study, only 3 of 266 chronic HBsAg carriers developed vasculitis (D8), and host factors that are important in the development of vasculitis are far from clear. Not all patients with HBsAg–antibody complexes develop vasculitis (N6, P18), and vascular lesions associated with HBsAg–antibody complexes are not confined to large- and medium-sized arteries (C18, G15, G20, K6, L13). No particular serologic profile or antigenic response to hepatitis B characterizes these patients (C11, G20, I4, T18, T20) and some have suggested that cellular immune responses (P18) or the host response to extrahepatic viral replication (S22) are important contributing factors.

In the WHO study (L2) and in other studies (C20, F3, F13, K5, M11, T15, T18), about half the sera from "miscellaneous" vasculitis patients had detectable immune complex material, and there was little or no correlation or concordance among the tests. Fye *et al.* and Kaufman *et al.* found that immune complex levels correlated with disease activity (F13, K5). Gupta and Kohler reported that while serum HBsAg levels remain constant, the amount of HBsAg involved in complex formation did correlate with clinical course (G20), and Pernice *et al.* describe a hepatitis antigen-specific immune complex assay that correlates with disease activity (P6). Others have not found any clinically useful relationships between immune complex levels and clinical course or disease manifestation (C20, F3, G15, I4, S19, T18). Perhaps this is because of the episodic or evanescent nature of immune complex formation and deposition, the possible irrelevance of circulating material to that which is deposited in tissue, or the heterogeneous nature of the disease in terms of presentation, course, and etiology, and the small numbers of patients in any one center available for clinical studies.

7.1.4. *Chronic Hypocomplementemic Urticarial Vasculitis*

The syndrome of hypocomplementemia, i.e., chronic urticaria with cutaneous vasculitis (venulitis) in association with a low-molecular-weight (7 S) C1q precipitins, was first described in the early 1970s (A5, M12). Since then, it has been established as a clinical entity with a relatively benign clinical course and is distinct from chronic urticaria, systemic lupus, or hereditary angioedema. Diagnostic criteria (and exclusions) have been proposed (S14). The syndrome is an unusual cause of cutaneous vasculitis (M1) but is recognized with increasing frequency. A characteristic feature is the presence of low-molecular-weight C1q precipitins with depletion of serum C1q and early complement components (A5, M12, S14). The C1q binding material has been shown to be an unusual 7 S IgG (M6). The demonstration of this material using immune complex assays based on C1q binding in conjunction

with serum fractionation is an important adjunct (criterion) for the diagnosis of this disease.

7.1.5. *Giant Cell Arteritis*

Temporal or giant cell arteritis is a granulomatous form of vascular inflammation that primarily affects branches of the internal and external carotid artery and is often associated with polymyalgia rheumatica. Immunoglobulin deposits have been detected in vessel walls (L16) and circulating immune complexes have been detected by the Raji assay (P1). The mechanisms by which immune complexes elicit a granulomatous response have been proposed by Fauci *et al.* (F3). An initial report of an association with hepatitis B infection (B1) has not been confirmed (B15, E3). Although circulating immune complex levels correlate with disease activity (P1), it is unlikely that any test will replace the erythrocyte sedimentation rate for monitoring disease activity and therapy.

7.1.6. *Essential Mixed Cryoglobulinemia*

Arthralgia, palpable purpura, and weakness often associated with renal disease, sicca complex, and hepatosplenomegaly in the absence of any known systemic disease distinguishes the syndrome of essential mixed cryoglobulinemia from other diseases associated with cryoglobulins. Meltzer and Franklin first established this syndrome as a distinct clinical entity (M15). Cryoglobulins are Type III (B16) mixed cryoglobulins containing IgM, IgG, occasionally IgA, complement components, and often rheumatoid factors (B2, B16, M15). Levo *et al.* found HBsAg, anti-HBsAg, or both in 14 of 19 cryoprecipitates examined (L13). This finding has been confirmed but the frequency of association of HBsAg–antibody complexes with this syndrome has been debated (B11, G2, G3, G15, P14, S22). In one case, HBsAg, immunoglobulin, and complement were demonstrated in vascular lesions (G15). Similar findings have been observed in glomerular lesions (C18, K14), and hepatitis B infection has been proposed as a major cause of membranous or membranoproliferative glomerular disease especially in children from certain geographic areas (K11, S25, T1). Taken together, at least some cases of essential mixed cryoglobulinemia are a result of hepatitis B infection, usually in association with mild inflammatory liver disease.

7.2. Complications of Infectious Diseases

7.2.1. *Endocarditis*

The extravalvular manifestations of infective endocarditis have many clinical features of an immune complex disease and immunologic studies of

tissue lesions are compatible with immune complex deposition (G18, L14). Bayer *et al.* found positive Raji cell tests in 28 of 29 patients studied and found that levels of complexes correlated with extravalvular manifestations and normalized with resolution of infection (B5). That these immune complexes actually do contain bacterial antigen has been demonstrated by Inman *et al.* (I3). Rheumatoid factors are also frequently found in endocarditis (C7, W9). Although immune complexes and rheumatoid factors appear together, the absolute levels do not correlate and rheumatoid factor titers tend to peak later than immune complex levels (C7). This lead Carson *et al.* to suggest that rheumatoid factor production in endocarditis requires a stimulus by other immune complexes that do not contain rheumatoid factor (C7). The differential diagnosis of septicemia is a frequent therapeutic problem, and immune complex assay results may be of value. Bayer *et al.* found that immune complexes were elevated in 98% of endocarditis patients but in only 44% of noncardiac septicemias. Thus, a negative test result is helpful in ruling out endocarditis as the cause of a positive blood culture (B5).

7.2.2. *Neisseria gonorrhoeae Infection*

This disease, more than any other, exemplifies the interpretive difficulties that can be encountered when trying to distinguish the role of the host response from that of the organism in the pathogenesis of a disease. The role of the host immune response in gonococcal infections has been an enigma. Many normal sera contain antigonococcal antibodies, and patients respond to gonococcal infection with a prompt antibody response; yet infection or reinfection commonly occurs despite the presence of such antibodies (K7). In disseminated gonococcal infection (DGI), a syndrome with many features of an acute immune complex disease, the immune response may actually be responsible for the lesions found in this syndrome. Even this is far from clear. Although the skin lesions in DGI resemble an acute allergic vasculitis histologically (S20) and have been shown to contain gonococcal antigen (T21), to our knowledge, only one group has examined these lesions for immune deposits. Scherer and Braun-Falco found deposits of C3 but not immunoglobulin in a case of DGI and proposed that activation of the alternative complement pathway by gonococcal endotoxin is responsible for the tissue lesions (S6). Circulating immune complexes have been detected with the Raji and C1q binding assays, but generally have not been detected with the monoclonal rheumatoid factor assay or cryoglobulin assay (L20, M3, W1). We detected immune complexes containing gonococcal antigen, IgG, IgM, and, with particularly high frequency, IgA in the majority of sera from patients with DGI (Fig. 6). However, we also found a 5–40% rate of positivity (depending on the test) in patients with gonococcal infection localized in the genitourinary tract, compared to a 15–20% incidence in other studies

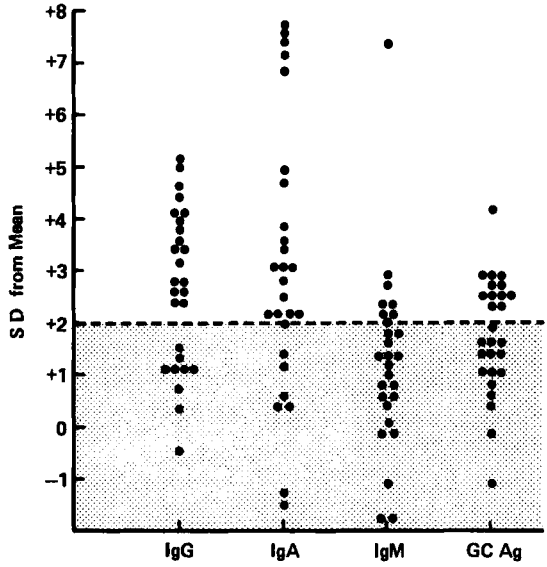

FIG. 6. Immune complexes in sera from patients with disseminated gonococcal (GC) infection. Immune complexes were run in the staphylococci binding assay. Isotype-specific reagents and an anti-GC reagent were used to detect immune complex material. Standard deviations were determined on a panel of 27 normal sera.

(L20, M3, W1). We have also found, as did Manicourt and Orloff (M3), that immune complexes persisted for up to 6 days after penicillin therapy, at a time when the patients were clinically asymptomatic. Thus, while it is quite clear that immune complexes are found in DGI sera, that they contain gonococcal antigens, and that tissue lesions resemble acute allergic vasculitis and contain gonococcal antigenic material, it is still not clear whether these lesions are caused by viable organisms or gonococcal products and, in either case, if antibody binding and/or complement activation is required to elicit the lesions.

In the face of so much evidence that the immune response in gonococcal infection is irrelevant, nonprotective, or even deleterious are observations that indicate that the host response does participate in the resolution of infection. *In vitro*, antibody- and complement-mediated lysis is an effective bactericidal mechanism (R4, S9). DGI, unlike local infections, does appear to protect against dissemination on subsequent exposure (R14), and patients with deficiencies of terminal complement components are particularly prone to disseminated infection (P8). By and large, characteristics of the bacteria, rather than host factors, appear to be more important in allowing gonococci to elude immune destruction and cause local, locally invasive, or dissemi-

nated infections. Thus, the production of an IgA protease by pathogenic strains of *Neisseria* may explain the failure of local immunity (where IgA predominates) and why dissemination to the blood (where other Ig classes predominate) is rare (M22). Strains of gonococci that cause DGI tend to be resistant to the bactericidal effect of serum (S9), a feature which correlates with certain principal outer memberane phenotypes (POMP) (J8). Manicourt and Orloff suggested that immune complexes may facilitate entrance of viable gonococci into the joints (M3). We found that the majority of patients with DGI are infected with strains of gonococci that are resistant to the bactericidal effect of normal sera. A minority (3/29) were infected with relatively sensitive strains. These patients had IgA-containing complexes and IgA from these patients blocked the bactericidal activity of normal serum. Thus, adaptations of the bacteria which allow it to elude immune destruction and cause disseminated infection are, for the most part, associated with an innate absolute or relative resistance to serum lysis. However, in some cases, the immune response in the host of appropriate type may facilitate dissemination by otherwise serum-sensitive strains.

7.2.3. *Acute Hepatitis B*

Acute hepatitis is occasionally accompanied or preceded by a syndrome of arthralgia, arthritis, rash, urticaria, and occasionally proteinuria (M18). This syndrome often dominates the early presentation of hepatitis and can present as a diagnostic problem before clinical or chemical evidence of acute hepatitis is manifest. Wands *et al.* demonstrated that cryoprecipitates from acute hepatitis patients contain IgM, IgG, and HBsAg (W7). Cryoprecipitates from patients with arthritis were higher in concentration than those from patients without arthritis. They were further distinguished by the presence of IgA and complement components and by their ability to fix complement and generate the anaphylatoxin, C3a (W7). Dienstag *et al.* extended these findings and demonstrated deposition of IgM, C3, and HBsAg in skin lesions (D4).

7.2.4. *Lyme Arthritis*

Lyme disease, first reported in 1975, typically begins with a unique skin lesion, erythema chronicum migrans, accompanied by systemic symptoms. Late sequelae include neurologic or cardiac complications and arthritis. The disease is caused by a spirochete and is transmitted by ticks (S34). The majority of patients have C1q-reactive material in their sera early in the course of disease. In most patients immune complex levels subside. However, in those likely to develop neurologic or cardiac sequelae, immune complex levels persist, and, thus, the test is of prognostic value. Those patients

destined to develop arthritis do not usually have detectable serum levels of immune complexes, but abnormal C1q binding is uniformly present in synovial fluids (H11).

7.2.5. *Viral Diseases*

Dengue virus has become a prototype example of the importance of host response in the manifestation of a viral infection. Dengue virus has four serotypes. Infection with one serotype usually results in a self-limited febrile disease, a protective antibody response to the infecting serotype, and a heterologous (presumably low-affinity and cross-reactive) antibody response to the other serotypes (H5). A second infection with a different serotype may result in a severe syndrome, Dengue hemorrhagic fever, with thrombocytopenia and disseminated intravascular coagulation. Immune complexes are found in sera and complement consumption occurs by both the classic and alternative pathways (B10, R10, T15). Although the precise mechanisms leading to coagulopathy are unknown, it is likely that immune complex-mediated platelet activation and/or complement activation leads to kinin and coagulation pathway activation. Dengue virus replicates in macrophages and heterologous, nonneutralizing antibodies enhance infectivity (H6, H7, T9). Thus, these circumstances are consistent with features known to predispose to immune complex disease: enhanced antigen load and low-affinity, heterologous antibody.

Immune complex technology has similarly been helpful in understanding the pathogenesis of a number of viral diseases, especially the herpes group (C8, S33, W3).

The acquired immune deficiency syndrome (AIDS), a newly recognized syndrome, is characterized by progressive lymphopenia, defects in T cell-mediated immunity (especially T-helper cell function), and susceptibility to life-threatening opportunisitic infection. The disease is caused by a human retrovirus (HTLV-III) and is transmitted by intimate sexual contact and exposure to blood, which explains its occurrence in particular risk groups: homosexual men, intravenous drug abusers, hemophiliacs, and other recipients of blood products (F4, P17, S1). Although the virus is tropic for T cells, B cell abnormalities (which are presumably secondary) occur as well (L4). Immune complexes are detected in the majority of these patients (Table 4), but immune complex disease is an unusual complication (F4, G21, M9, M21, R1, W4). Indeed, immune complexes are detected as frequently as T cell abnormalities and both tests are equivalent in discriminating AIDS patients from risk group controls. Both tests are highly associated with evidence (antibody) of exposure to HTLV-III, but neither test performs as well as HTLV-III serology (S1, CDC unpublished comparison).

TABLE 4

IMMUNOLOGIC TESTS IN HOMOSEXUAL MEN WHO HAVE AIDS, CHRONIC UNEXPLAINED LYMPHADENOPATHY, OR WHO ARE ASYMPTOMATIC

Test	Percent abnormal or positive			
	Homosexual men			Random blood donors
	AIDS	Lymphadenopathy	Asymptomatic	
Low T helper/suppressor cell ratio	85(91)[a]	65(123)	27(122)	3(60)
Low T helper cells	80(75)	28(122)	8(117)	3(36)
Positive antihepatitis B core	88(101)	78(105)	80(152)	6(189)
High β_2-microglobulin	58(24)	63(89)	21(62)	3(81)
High α_1-thymosin	63(51)	57(35)	88(41)	4(70)
Positive immune complexes (SBA)	82(67)	93(118)	54(85)	5(196)
Positive immune complexes (C1qBA)	66(65)	75(118)	51(85)	7(164)
HTLV-III/LAV antibody	96(24)	94(50)	44(120)	0(120)

[a] Numbers in parentheses are number tested.

7.3. Renal Disease (Glomerulonephritis)

In general, the glomerulonephritides that are associated with systemic disease such as systemic lupus, those that are complications of systemic disease such as hepatitis B infection or endocarditis, and acute poststreptococcal nephritis are frequently positive for circulating immune complexes, whereas patients with chronic forms of glomerular disease are infrequently or episodically positive (C16, L2, T11, T23, V1, W26). There are curious and perhaps informative exceptions. Patients with steroid-responsive nephrotic syndrome ("minimal lesion" glomerulonephritis), a disease of children for which there is little or no histologic evidence of immune damage, often have circulating complexes at levels that correlate with therapeutic response (L12). Berger's disease (IgA nephropathy) and Henoch–Schonlein purpura are associated with circulating IgA-containing complexes, a feature which would be undetected by most immune complex assays unless manipulations designed to detect IgA are used (L10, W25). Despite the fact that glomerular tissue has been enormously useful for the detection and analysis of immune complex materials and the fact that over 90% of glomerular disease is immune mediated (W17), it may seem paradoxical that testing of serum for immune complexes is not of greater clinical value. Immune complex testing does not outperform other measures for the diagnosis of systemic disease (with renal complications) or renal biopsy for definitive and prognostic information. Nor would immune complex testing supplant urinalysis and chemical tests for monitoring renal disease activity.

7.4. Neoplasia

Tumors may express new antigens which are recognized as foreign, and the host may mount an immune response to them. It is conceivable that antibodies to tumor-associated antigens, whether cell bound or shed into the circulation, may result in immune complex disease or that antibodies or complexes might enhance or inhibit cell-mediated destruction of tumor cells. If so, circulating immune complex levels might be expected to reflect tumor load, complications, or prognosis and there is some evidence in multiple systems that this is so.

The continuous presence or production of tumor antigens along with an antibody response represents a situation, not unlike chronic autoimmune disease, in which the immune complexes may be continually forming. Overt immune complex-mediated disease such as nephritis does occasionally occur, and the presence of tumor-associated antigens and antibodies in immune deposits has been well documented in several instances (C17, D1,

L15, O1, W32). Remission of immune complex disease and circulating immune complex levels after successful therapy of the tumor provides further evidence of a cause and effect relationship (H23).

In spite of this well-documented association, the low frequency of such complications in malignancies is surprising in view of the high prevalence of immune complex-like materials in the sera of individuals with tumors. It is not known whether the rarity of this complication reflects special properties of the complexes formed or other factors. However, subclinical immune complex deposition with vascular inflammation may be a much more frequent occurrence than generally realized and may contribute to the general systemic symptoms associated with malignancies such as malaise, weight loss, fever, and weakness (S38, T7).

In several large studies, the detection of immune complexes has not been shown to be of sufficient discriminatory power to distinguish benign from malignant disease or local from metastatic disease (H17, R7, S2, T7). In patients with established malignancy, however, the presence of immune complexes in the circulation has on the whole proven to be a poor prognostic sign, and levels have correlated with successful or unsuccessful therapeutic responses (Fig. 7). Well-documented examples include acute and chronic myelogenous leukemia (C1, C2, H26), neuroblastoma (B13), lymphoma (A12), breast cancer (H22), melanoma (T7, T15), and ovarian cancer (P16).

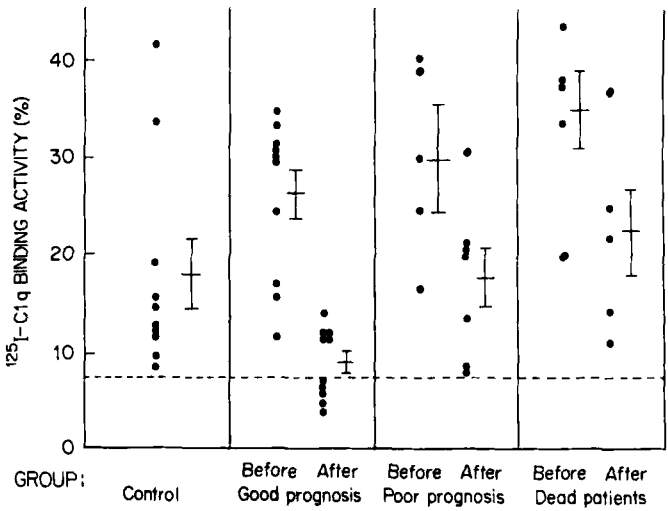

FIG. 7. C1q binding activities in women with breast cancer according to clinicopathologic staging. Horizontal line indicates binding activity in normal sera. (Reproduced with permission, K. Hoffken, I. D. Meredith, R. A. Robbins *et al. Br. Med. J.* 2, 218–220, 1977.)

7.5. Other Diseases

There are a number of other diseases not elaborated upon here in which immune complex technology supports the contention that these diseases (or complications of these diseases) are immune complex mediated. Testing in these diseases has also been shown to correlate with disease activity and/or to predict a therapeutic response. Generally, however, the tests are not used in clinical management because there are better or easier modes of making these assessments. Examples include mixed connective tissue disease (H48); certain manifestations of progressive systemic sclerosis (P9, S18, S23); subsets of juvenile rheumatoid arthritis (R6); poststreptococcal sequelae (V1); distinction of lepromatous and tuberculoid leprosy (L2); complications of syphilis or antitreponeme therapy (G4, W8); therapeutic response prediction in idiopathic interstitial lung disease (D10); renal transplant rejection (O3); Bechet's disease (G24, L8, L11); extrahepatic manifestations of chronic hepatitis (T16, W5, W6); pemphigus (T5); assessment of gluten sensitivity in dermatitis herpetiformis with celiac disease (Z1); and systemic complications of Crohn's disease and ulcerative colitis (H21, N1).

8. Conclusions

The value of immune complex technology can be viewed on three levels, each of equal importance but perhaps in descending order with respect to the applied relevance of current technology. First, the technology which was fostered by observations in experimental animals has, in turn, been very successfully applied to explain the pathophysiologic mechanisms in many human diseases. Second, in clinical studies of groups, the technology supports the notion that manifestations of disease are reflected in the levels or types of immune complexes found and has been of some value in clinical trials. Third, the value of an individual measurement in an individual patient has been the least fruitful. The reasons for this are that immune complexes reflect a pathophysiologic process and not a specific clinical diagnosis and that equivalent or easier methods are often available to make the same clinical assessment of disease activity. Nevertheless, the judicious use of immune complex testing does have current application in selected clinical settings. Continued refinements in technique and larger, more carefully designed studies with prolonged follow-up hopefully will establish the limits and specific applicability of the technology. Finally, continued pursuit of methods for defining the components (antigen, antibody specificity, polyspecificity, and idiotypy) of immune complexes may unravel some of the etiologic mysteries of many diseases currently considered idiopathic.

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ADVANCES IN NONISOTOPIC IMMUNOASSAY

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1. Introduction

The essential criteria for a useful analytical technique are specificity, sensitivity, accuracy, precision, simplicity, rapidity, economy, wide applicability, and freedom from hazard. It is well known that radioimmunoassay (RIA) was developed in 1959 by Yalow and Berson (Y1). Since then the radioimmunoassay method has been widely used in the field of clinical chemistry. Radioimmunoassay has inherent in it the advantages listed above. However, this method always requires special facilities for use and disposal of radioisotopes and consideration must be given to the fact that the labeled substances have short half-lives. Immunoassay methods are explosively increasing in use and development as an analytic technique in basic science as well as in clinical laboratory medicine (L1–L3, V1). With these points as background, efforts have been made to develop nonisotopic immunoassay methods or alternative immunoassay methods that are based on antigen–antibody reactions but do not involve use of a radioisotope.

Forerunners of nonisotopic immunoassay had already appeared before radioimmunoassay was developed. For example, nephelometry is based on precipitation, which is known as the classical immune reaction, and the ideas of particle immunoassay and viroimmunoassay seem to have developed from the hemagglutination test. The principles of enzyme and fluorescence immunoassay had already been used as enzyme and fluorescence antibody techniques in histochemical analysis. In 1971, two groups reported use of an enzyme immunoassay (E5, V2). Leute *et al.* reported spin immunoassay, which has spurred recent development of nonisotopic immunoassays (L5).

Up to the present time, various techniques of nonisotopic immunoassay have been developed. Substances to be measured by these techniques include hormones (peptide and nonpeptide), biologically active trace substances, carrier proteins, immunoglobulins, viruses, tumor markers, antibodies against microorganisms (viruses, bacteria, and parasites), autoantibodies, etc. Innumerable papers concerning nonisotopic immunoassay have been published, and enzyme (I5, K1, N5, N6, P1, S2, V4, W9) and fluores-

TABLE 1
CLASSIFICATION OF NONISOTOPIC IMMUNOASSAY

- Test sample
  - Antigen
    - Protein and peptide
    - Hapten
      - Heterologous
      - Homologous
  - Antibody
    - Antibody to exogenous antigen
    - Autoantibody
- Marker
  - Labeled method
    - Competitive method (immunoassay in a narrow sense)
    - Noncompetitive method (immunometric assay)
  - Nonlabeled method
- Bound/free separation
  - Separation method (heterogeneous)
  - Nonseparation method (homogeneous)
- Reaction
  - Liquid phase
  - Solid phase

cence immunoassays (K1, M1, M10, U2) have been extensively and expertly reviewed. Therefore, in this article the subject of nonisotopic immunoassay will focus on reviews of various types of assay systems, introduction of the monoclonal antibody, and application to preventive medicine.

2. GENERAL CLASSIFICATION OF NONISOTOPIC IMMUNOASSAY

Immunoassay is a type of binding or ligand assay that depends on the antigen–antibody reaction. The various nonisotopic immunoassays that have been used can be divided into several groups as shown in Table 1.

2.1. TEST SAMPLE

The immunoassays are divided into two groups according to whether the substances to be tested are antigens or antibodies. The hapten enzyme immunoassay is divided into heterologous and homologous methods (V3). In the homologous enzyme immunoassay of heptens, the methods for coupling of haptens with enzymes are the same as those used for the coupling of haptens with carrier proteins that are used for immunizing. In this case, the antibodies bind the enzyme-labeled hapten much more strongly than they bind the unlabeled hapten and thus less enzyme-labeled hapten is displaced

TABLE 2

COMPARISON OF ED_{50} VALUES OF THYROXINE (T_4) IN VARIOUS TYPES OF HETEROLOGOUS AND HOMOLOGOUS ENZYME IMMUNOASSAYS OF T_4[a,b]

Conjugate for immunization[c]	T_4–enzyme conjugate[c]						
	T_4–GOD[E][d]	T_4–GOD[G]	T_4–s–GOD[E]	T_4–g–GOD[E]	T_4–ALP[C]	T_4–ALP[G]	T_4–GAL[M]
T_4–BSA[E][d]	>[e]	1300	>	>	—	—	—
T_4–BSA[G]	705	113	>	>	—	—	—
T_4–s–BSA[E]	205	95	220	300	—	—	—
T_4–g–BSA[E]	33	14	207	120	—	—	—
T_4–BSA[C]	—	—	—	—	>[f]	1200[f]	0.054[f]

[a] The ED_{50} value is the T_4 concentration (pg per assay tube) corresponding to 50% binding at zero concentration of T_4.

[b] From Tsuji *et al.* (T1).

[c] Abbreviations: BSA, bovine serum albumin; GOD, glucose oxidase; ALP, alkaline phosphatase; GAL, β-D-galactosidase; S, succinate; g, glutarate; E, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; C, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; G, glutaraldehyde; M, 4-(maleimidomethyl)cyclohexane-1-carboxylic acid succinamide ester.

[d] [ ] indicates preparation of conjugate.

[e] > indicates over 5000 pg.

[f] From Miyai *et al.* (H5, M6).

by unlabeled hapten. In the heterologous enzyme immunoassay, a significant displacement can be achieved by decreasing the affinity between the enzyme-labeled hapten and antibodies using different methods for coupling of hapten–carrier protein and hapten–enzyme. Van Weemen and Schuurs described three types of heterology: hapten, bridge, and site heterology used for enzyme immunoassay (V3). Table 2 shows sensitivities of various types of homologous and heterologous enzyme immunoassays of thyroxine (H5, M6, T1). The sensitive assays with the smaller values of ED_{50} were obtained by the heterologous system.

2.2. Assay Systems

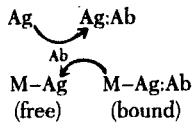
The assay systems are divided into two groups: those using labeled and those using nonlabeled methodology. In the former, the antigen–antibody reaction is detected using marker-labeled substance. In the latter, the antigen–antibody complex is detected directly without markers.

TABLE 3
Various Markers for Nonisotopic Immunoassays

Name of assay	Marker	Detection
Labeled method		
Enzyme immunoassay	Enzymes, coenzymes, enzyme modulator, etc.	Enzyme activity
Fluorescence immunoassay	Fluorescence	Fluorescence intensity, polarization, etc.
Phosphorescence immunoassay	Phosphorescence	Phosphorescence intensity
Luminescence immunoassay	Chemi- or bioluminescence	Luminescence intensity
Spin immunoassay	Free radicals	Electron spin resonance
Viroimmunoassay	Bacteriophages	Cytolysis
Metal immunoassay	Metals	Atomic absorption spectrometry
Particle immunoassay	Colloidal (metal) particles, latex particles	Atomic absorption spectrometry, Turbidimetry, counting
Nonlabeled method		
Immunodiffusion method	Immunoprecipitin	Turbidity
Nephelometric immunoassay	Immunoprecipitin	Nephelometry (light scattering)
Immunosenser	Electric response derived from antigen–antibody reaction	Chemically modified electrodes

TABLE 4
PRINCIPLE CLASSIFICATION OF HETEROGENEOUS IMMUNOASSAYS[a]

Competitive



Noncompetitive

Two-site immunometric (sandwich)

$$\text{Ag} + \text{S–Ab} + \text{M–Ab} \rightarrow \underset{\text{(bound)}}{\text{S–Ab:Ag:M–Ab}} + \underset{\text{(free)}}{\text{M–Ab}}$$

Immunometric (narrow sense)

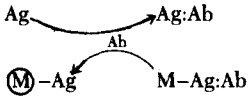
$$\text{Ag} + \text{M–Ab} + \text{S–Ag} \rightarrow \underset{\text{(free)}}{\text{M–Ab:Ag}} + \underset{\text{(bound)}}{\text{M–Ab:S–Ag}}$$

[a] Abbreviations: Ag, unlabeled antigen; Ab, unlabeled antibody; M, marker; S, solid phase.

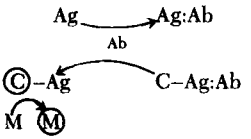
In the labeled method, the method for detecting marker requires sensitivity and simplicity. Furthermore, the affinity of the antigen–antibody reaction must be stable when a reagent is labeled with marker. Table 3 shows various markers for nonisotopic immunoassays. The labeled methods are also divided into competitive and noncompetitive methods. As an example of a competitive method, in a conventional heterogeneous immunoassay the limited amount of antibody used is insufficient to bind all the antigen (Table 4). The marker-labeled antigen is employed to enable determination of the proportion of the total antigen (both labeled and unlabeled antigen) present in the bound and/or free form. If the amounts of labeled antigen and antibody are kept constant, the proportion of the labeled antigen in the bound form is inversely related to the initial amount of unlabeled antigen in the standard or test sample. As an example of a noncompetitive method, in the two-site sandwich immunometric assay, antigen in the standard or test sample is allowed to react with the solid-phase-coupled antibody. Then an excess of labeled antibodies which bind to another site of the antigen is added. The portion of the labeled antibodies bound is related to the initial amount of antigen in the standard or test sample. A separation of bound and free forms is necessary in labeled heterogeneous immunoassay methods. Separation steps are not necessary in homogeneous immunoassays where antigen–antibody reactions result in changes either in the nature or the

TABLE 5
PRINCIPLE CLASSIFICATION OF HOMOGENEOUS IMMUNOASSAYS[a]

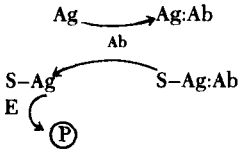
A. Homogeneous immunoassay based on marker-labeled antigen



B. Modulator or prosthetic group-labeled antigen

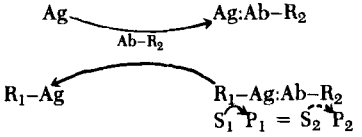


C. Reactant-labeled antigen



D. Linkage of plural reactants

a)



b) $Ag + R_1Ab + R_2Ab \rightarrow R_1Ab:Ag:R_2Ab$
$S_1 \rightarrow P_1 = S_2 \rightarrow P_2$

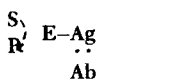
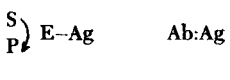
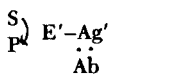
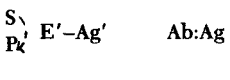
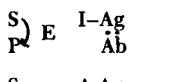
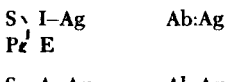
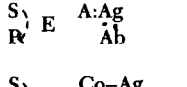
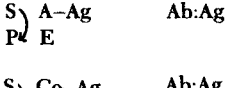
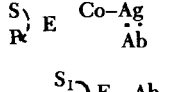
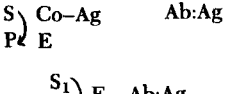
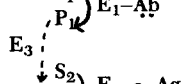
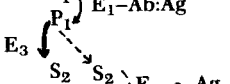
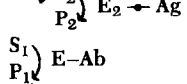
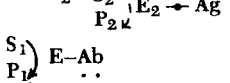
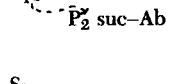
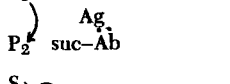
[a] Labeled methods for determination of antigen are shown. Abbreviations: Ag, unlabeled antigen; Ab, unlabeled antibody; M, marker (usually inactive); Ⓜ, marker with changed activity (usually active); C, modulator or prosthetic group (usually inactive); Ⓒ, modulator or prosthetic group with changed activity (usually active); R, reactant; S, stimulant or substrate; Ⓟ, product; E, enzyme.

intensity of the signal of the marker. Various types of homogeneous immunoassays have been developed, as shown in Tables 5 and 6. The methods for detecting antigen–antibody reactions have been improved in nonlabeled methods such as laser nephelometry or immunosensor techniques. These will be described later.

Immunoassay systems can also be divided into two types in which reactions occur in the liquid phase or between solid and liquid phases.

TABLE 6

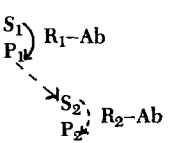
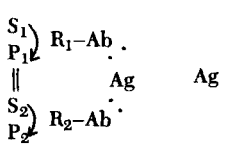
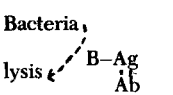
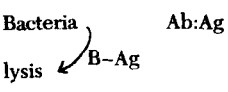
VARIOUS HOMOGENEOUS NONISOTOPIC IMMUNOASSAYS[a]

System	Type[b]	Antigen to be determined[c] (−)	Antigen to be determined[c] (+)	Result
Enzyme immunoassay				
Homogeneous enzme immunoassay developed by Rubenstein	A			[P ↑]
E′: malate dehydrogenase Ag′: thyroxine				[P ↓]
Enzyme modulator-mediated immunoassay	B			[P ↓]
				[P ↑]
Enzyme immunoassay with enzyme prosthetic group	B			[P ↑]
Enzyme channeling immunoassay	D			[P_2 ↓]
				[P_2 ↑]
Enzyme enhancement immunoassay	D			
Enzyme membrane immunoassay	A			[P ↓]

Fluorescence immunoassay					
Substrate-labeled fluorescent immunoassay	C	F–S–Ag Ab E	F–S–Ag (F) E	Ab:Ag	[(F) ↑]
Enzymatic hydrolysis of antigen–fluorescent dye conjugate immunoassay	C	F–Ag Ab E	F–Ag (F) E	Ab:Ag	[(F) ↑]
Antibody-enhanced hydrolysis of steroid–fluorescent dye conjugate immunoassay	C	F–Ag F Ab	F–Ag	Ab:Ag	[(F) ↓]
Fluorescence polarization immunoassay	A	(F)–Ag Ab	F–Ag	Ab:Ag	[(F) ↓]
Fluorescence quenching immunoassay	A	F–Ag Ab	(F)–Ag	Ab:Ag	[(F) ↑]
Fluorescence enhancement immunoassay	A	(F)–Ag Ab	F–Ag	Ab:Ag	[(F) ↓]
Fluorescence protection immunoassay	A	(F)–Ag anti-F Ab	Anti-F:F–Ag	Ab:Ag	[(F) ↓]
Fluorescence immunoassay based on excitation by internal reflection spectroscopy or cytometric detection	A	(F)–Ab:Ag ⊷ F–Ab Ab ⊷ (F)–Ag:Ab ⊷	F–Ab:Ag Ag ⊷ (F)–Ab:Ag:Ab ⊷ F–Ag Ag:Ab ⊷		[(F) ↓] [(F) ↑] [(F) ↓]
Fluorescence excitation transfer immunoassay	D	F–Ag Q–Ab	(F)–Ag	Ag Q–Ab	[(F) ↑]
		(F)–Ab Q–Ab	F–Ab: Q–Ab: Ag		[(F) ↓]

(*continued*)

TABLE 6 (*Continued*)

System	Type[b]	Antigen to be determined[c] (−)	Antigen to be determined[c] (+)		Result
Others					
Proximal linkage immunoassay	*D*				[P_2 ↑]
Spin immunoassay	A	Fr–Ag Ab	Ⓕr–Ag	Ab:Ag	[*Fr* ↑]
Viroimmunoassay	A			Ab:Ag	[Lysis ↑]
Particle immunoassay	A	Pa–Ab Pa–Ab	Pa–Ab Ag Pa–Ab		[Agglutination ↑]
		Pa–Ag: Pa–Ag: Ab	Pa–Ag Pa–Ag	Ab:Ag	[Agglutination ↓]
		Pa–Ab: Pa–Ab: Ag	Pa–Ab:Ag Pa–Ab:Ag		[Agglutination ↓]
		Pa–Ag:Ab_1: Pa–Ag:Ab_1: Ab_2	Pa–Ag Pa–Ag	Ag:Ab_1:Ab_2 Ag:Ab_1:Ab_2	[Agglutination ↓]

[a] Schemes indicate determination of antigen.

[b] Type: See Table 5.

[c] Abbreviations: Ag, antigen; Ab, antibody; Ag:Ab, antigen–antibody complex; –, coupling, ⊸, solid phase; E, enzyme; S, substrate; P, product; I, inhibitor; A, enhancer; Co, coenzyme; Suc–Ab, succinylated antibody, F, fluorescer; Q, quencher; Fr, free radical; B, bacteriophage; Ⓕ, detectable fluorescence or fluorescence polarization; Ⓕr, detectable free radical; Ⓑ, active bacteriophage; Pa, particle; R_1, R_2, reaction.

2.3. Homogeneous Immunoassays

The term homogeneous immunoassay may be defined as an immunoassay in which the extent of the antigen–antibody reaction can be determined without physical separation of the free and bound forms. This term is usually used for immunoassays such as enzyme and fluorescence immunoassays in which labeled substances (markers) are used. It does not include immunoassay systems such as nephelometry in which no labeled substances are used. Homogeneous immunoassays are widely used as routine tests because the procedures involved are simple. The principle of homogeneous immunoassay is based on changes in signals of the indicators by the antigen–antibody reaction (N5, U2).

A sophisticated homogeneous enzyme immunoassay of morphine was first developed by Rubenstein *et al.* in 1972 (R2). Since then various systems have been reported, as listed in Table 6. In this table, nonisotopic immunoassays are classified according to the markers used, such as enzymes, fluorescence, bacteriophages, or free radicals. Ngo and Lenhoff, (N5), Shuurs and Van Weemen (S2), and Ullman *et al.* (U2) have reviewed and classified homogeneous enzyme immunoassays and fluorescence immunoassays. In the following sections, homogeneous nonisotopic immunoassays are classified into several groups according to the mechanisms used for detection of the antigen–antibody reaction, as shown in Table 5.

2.3.1. *Homogeneous Immunoassay Based on Marker-Labeled Antigen*

In this system, a change in activity of a marker coupled with antigen, caused by binding to an antibody, is measured. When unlabeled antigen is added, it competes with marker-labeled antigen for binding to the antibody. Usually the free form of marker-labeled antigen has increased activity. Any change in the activity of the markers can be stoichiometrically related to the concentration of the unlabeled antigen in the sample. The procedure in this method is simple, but the method is usually not applicable to antigens that are macromolecules. Immunoassays included in this category are as follows: homogeneous enzyme immunoassay developed by Rubenstein (R2), enzyme membrane immunoassay (F3), fluorescence polarization immunoassay (D2), fluorescence quenching immunoassay (S4), fluorescence enhancement immunoassay (S6), fluorescence protection immunoassay (N2), fluorescence immunoassay based on excitation by internal reflection spectroscopy (K13), spin immunoassay (L5), viroimmunoassay (H2), and particle immunoassay (C4). Alternatively, marker-labeled antibody can be used to measure antibody.

2.3.2. *Homogeneous Immunoassay Based on Modulator or Prosthetic Group-Labeled Antigen*

In this type of assay, an antigen is coupled with a modulator or prosthetic group which enhances or inhibits the activity of the marker. The activity of the modulator or prosthetic group coupled with the antigen is inhibited by antibody binding and the inhibition or enhancement is reversed by adding unlabeled antigen. Thus the amount of added unlabeled antigen can be determined by measuring the change in activity of the marker. Enzyme modulator-mediated immunoassay (modulator may be an inhibitor or an activator) (N4) and enzyme immunoassay with enzyme prosthetic group (coenzyme) (N4) are included in this category.

2.3.3. *Homogeneous Immunoassay Based on Reactant-Labeled Antigen*

In this type of assay, antigen is coupled with the reactant. Usually an enzyme substrate (reactant) and enzyme reaction are used. The enzyme can bind to the free form of the substrate–antigen conjugate and the substrate is transformed to the product. However, when antibody is bound to substrate–antigen conjugate (bound form) the enzyme reaction does not take place. When the amount of unlabeled antigen is increased, the free form of the substrate–antigen conjugate increases and the product of the enzyme reaction is increased. Thus, the amount of product formed is proportional to the amount of added unlabeled antigen. Substrate-labeled fluorescent immunoassay (B7), enzymatic hydrolysis of antigen–fluorescent dye conjugate immunoassay, and antibody-enhanced hydrolysis of steroid–fluorescent dye conjugate immunoassay (K9) are included in this category.

2.3.4. *Homogeneous Immunoassay Based on Linkage of Plural Reactants*

Several homogeneous immunoassays have been developed in which all of the reactions of markers occur when the reactants are closely linked by the antigen–antibody reaction.

In the proximal linkage immunoassay system, two kinds of monoclonal antibodies that can bind to two different antigenic determinants on one antigen are selected. The selected pairs of monoclonal antibodies are conjugated with the proper pair of reactant molecules (R_1, R_2) as shown in Table 7 (S3). When unlabeled antigen is added, each reactant-labeled monoclonal antibody binds to a proximal antigenic site of the antigen and the whole process proceeds.

$$S_1 \xrightarrow{R_1} P_1 = S_2 \xrightarrow{R_2} P_2$$

Thus the amount of the final product (P_2) is increased.

TABLE 7

REACTANTS FOR PROXIMAL LINKAGE IMMUNOASSAYS[a]

System	S_1	R_1	$P_1 = S_2$	R_2	P_2
Fluorescence quenching	340 nm	Fluorescein	460 nm	Rhodamine	520 nm
Proximal enzyme linking	ATP + glucose	Hexokinase	Glucose-6-P + NAD^+	Glucose-6-P dehyrogenase	Gluconolactone-6-P + NADH
Proximal enzyme linking	Glucose	Glucose oxidase	Peroxide + DAB	Peroxidase	Oxidized DAB
Bioluminescence	NADH + FMN	NAD oxidoreductase	$FMNH_2$	Luciferase	$h\nu$
Allosteric activation	Fructose-6-P	Phosphofructokinase	Phosphoenolpyruvate + NADH (MDH)	Phosphoenolpyruvate carboxylase	NAD^+
Allosteric inhibition	Oxalacetate + glutamate	Aspartate aminotransferase	Phosphoenolpyruvate + NADH	Phosphoenolpyruvate carboxylase	Oxalacetate + NAD^+
Enzyme/substrate	Peroxide	Peroxidase	(Luminol)	Luminol	$h\nu$

[a] This table is modified from Fig. 7, Sevier *et al.* (S3). R_1 and R_2 are reactants, such as fluorophores or enzymes; S_1 is the initial stimulant, such as excitation light or substrate for R_1; and P_1 is a product of reaction of R_1. P_1 can become the next stimulant (S_2) for R_2, and P_2 is a product of R_2.

$$S_1 \xrightarrow{R_1} P_1 = S_2 \xrightarrow{R_2} P_2$$

The original methods of fluorescence exciting transfer immunoassay (U1) and enzyme channeling immunoassay (L9) are similar to the proximal linkage immunoassay. In the former, two reactants are coupled to solid antigen and antibody and, in the latter, they are coupled to two kinds of monoclonal antibodies. The enzyme enhancement immunoassay (G4) also falls in this category.

3. Enzyme Immunoassay

Enzyme immunoassays using enzymes as markers have a number of advantages. Detection limits for enzymes are very low, because enzyme reactions can be amplified as catalytic reactions. The enzyme-labeled antigen and antibody are considerably stable. No expensive equipment is required to determine the enzyme activity.

Since the first report on enzyme immunoassay appeared in 1971 (E5, V2) relevant techniques have been developed and applications are increasing. There are many reports on new coupling agents for preparing conjugates of

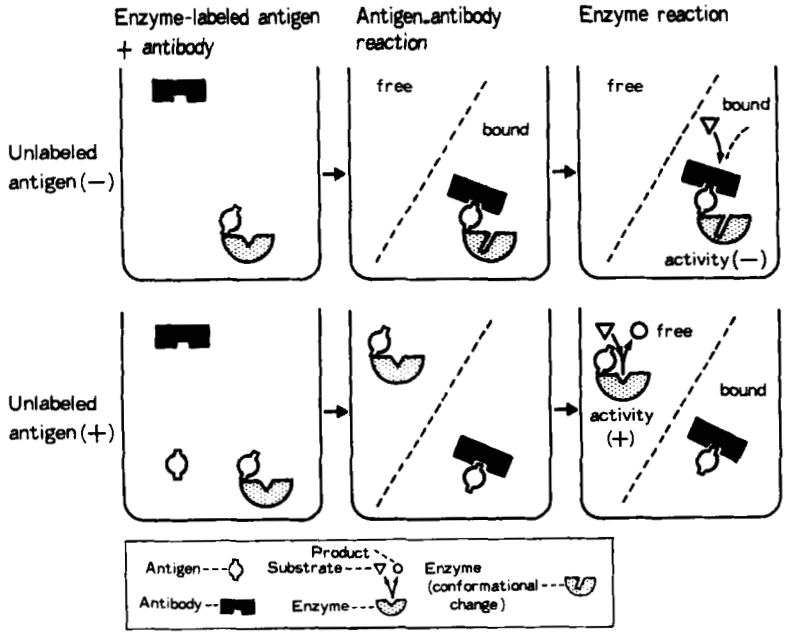


FIG. 1. Schematic representation of the principle of homogeneous enzyme immunoassay developed by Rubenstein (EMIT). [Cited and modified from Fig. IV-4, Miyai, K., in Ishikawa *et al.*, eds. (I5).]

enzymes and antigen or antibody (D6, I4, I5). The sensitivity of determination of enzyme activity can be improved by using a fluorescent or luminescent substrate (T2). To simplify the assay procedure, extensive studies have been directed toward the development of nonseparation, or homogeneous, methods. In the following section, various types of homogeneous enzyme immunoassay are described. The schemes are shown in Table 6, and the abbreviations in this table are cited in parentheses in the text. Some schematic representations are also illustrated in Figs. 1–3. These schemes are shown in a simplified manner and not quantitatively.

3.1. Homogeneous Enzyme Immunoassay Developed by Rubenstein

This system was the first homogeneous immunoassay reported (R2). As shown in Fig. 1 and Table 6, in this system a morphine derivative (antigen) is coupled with lysozyme (enzyme) which has enzyme activity [E–Ag]. When an antibody against morphine [Ab] is bound to the morphine–lysozyme conjugate [E–Ag:Ab], the large substrate, *Micrococcus luteus* [S], is unable to gain access to the catalytic site of lysozyme and thus the enzyme activity is inhibited. When unlabeled morphine [Ag] is added, it competes with the morphine–lysozyme conjugate for binding to the antibody [Ab:Ag]. Then the amount of the bound form with little or no enzyme activity [E–Ag:Ab] decreases and the amount of the free form with enzyme activity [E–Ag] increases. Thus, an increase of added morphine is detectable as an increase of lysozyme activity. Subsequently, other enzymes with high turnover rates, such as glucose-6-phosphate dehydrogenase, malate dehydrogenase, and β-D-galactosidase have been used in this system. In these cases, inhibition of enzyme activity on binding to antibody seems to be caused by a conformational change of the enzymes.

As an exception, malate dehydrogenase coupled with thyroxine [E–Ag] has no enzyme activity, and this inhibition is reversed when antibody to thyroxine is bound to the enzyme-labeled thyroxine [E–Ag:Ab] (U3). When the amount of unlabeled thyroxine [Ag] is increased, the enzyme activity is decreased. An EMIT kit (Syva) and autoanalyzers based on this principle are available for routine use. This method was previously used only for measuring substances with small molecular weights but recently it has been applied to high-molecular-weight substances. For example, Gibbons *et al.* constructed a homogeneous enzyme immunoassay for IgG using anti-IgG, IgG/β-D-galactosidase conjugate, and a synthetic macromolecular substrate (*o*-nitrophenyl-β-galactoside/dextran conjugate) (G3).

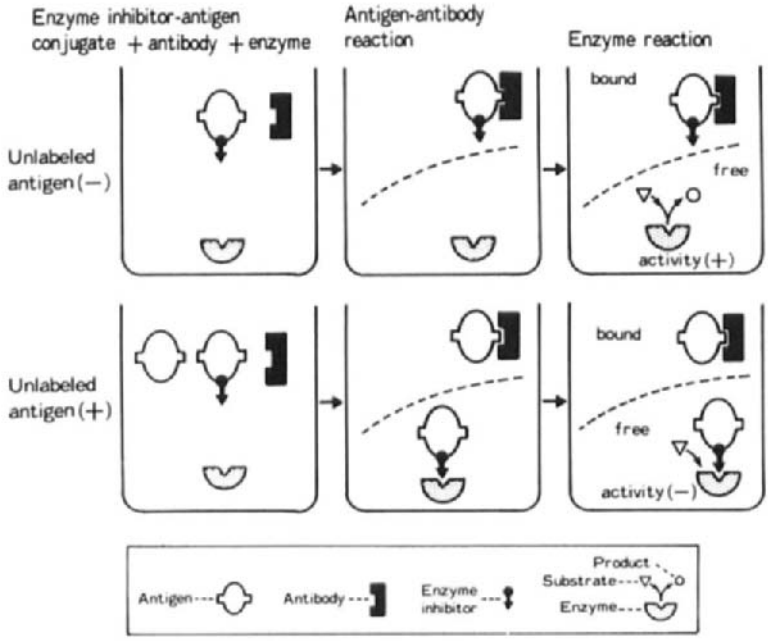


FIG. 2. Schematic representation of the principle of enzyme inhibitor immunoassay (TETRAZYME). [Cited and modified from Fig. IV-5, Miyai, K., in Ishikawa *et al.*, eds. (I5).]

3.2. ENZYME MODULATOR-MEDIATED IMMUNOASSAY (EMMIA)

The EMMIA system was developed by Ngo and Lenhoff (N3, N4). In this assay, enzyme activity is modulated by an enzyme modulator which is coupled to antigen (free form) but not by the complex of enzyme modulator–antigen and antibody (bound form). As shown in Fig. 2 and Table 6, in an enzyme inhibitor immunoassay, an enzyme inhibitor is used as a negative modulator. For example, the reaction mixture for measuring thyroxine consists of acetylcholine inhibitor–thyroxine conjugate [I–Ag], acetylcholinesterase [E], unlabeled thyroxine [Ag], and antithyroxine antibody [Ab]. When the amount of unlabeled thyroxine, which binds to antibody [Ab:Ag], is increased, the free form of acetylcholine inhibitor–thyroxine conjugate [I–Ag] increases, and the enzyme activity decreases. Therefore, the enzyme activity is inversely proportional to the concentration of unlabeled thyroxine. A TETRAZYME kit (Abbott) is now available for measuring thyroxine.

Ngo and Lenhoff pointed out that for EMMIA an enzyme modulator with high affinity for the enzyme must be used, and they proposed the following

substances for this purpose: avidin, which can inhibit biotin-dependent enzymes; coformycin, which inhibits adenosine deaminase; and an antibody to wild-type *Escherichia coli*, β-galactosidase, which can activate the activity of a mutant enzyme known to be inactive by itself (N4). The prosthetic group described in Section 3.3 may be considered as one of the positive modulators.

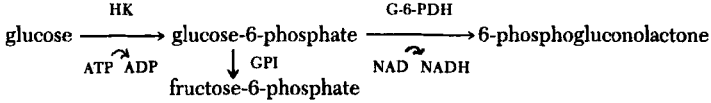
3.3. Homogeneous Enzyme Immunoassay with Enzyme Prosthetic Group Labeling

This type of enzyme immunoassay was also developed by Ngo and Lenhoff (N4). In this system an antigen is coupled with an enzyme prosthetic group (coenzyme). The free form of coenzyme–antigen conjugate [Co–Ag] can bind to the apoenzyme with high affinity to form an enzymatically active holoenzyme. Antibody binds to the coenzyme–antigen conjugate forming an antigen–antibody complex [Co–Ag:Ab] that cannot combine with apoenzyme [E], and thus the enzyme activity is decreased. When the amount of unlabeled antigen [Ag] is increased, the free form of coenzyme–antigen conjugate is increased and the enzyme activity is increased. For example, flavin adenine dinucleotide (FAD) and apoglucose oxidase can be used as coenzyme and apoenzyme, respectively. Kohen *et al.* reported steroid immunoassays utilizing a steroid-NAD derivative conjugate and alcohol dehydrogenase or malic dehydrogenase (K9).

3.4. Enzyme Channeling Immunoassay

Litman *et al.* developed a homogeneous enzyme immunoassay based on the rate enhancement resulting from immobilization of two enzymes in close proximity to each other (L9). This method is included in the category of proximal linkage immunoassay described in Section 2.3.4. The two enzymes [E_1] and [E_2] catalyze two consecutive reactions—transformation of the substrate [S_1] to the product [$P_1 = S_2$] and subsequently of [$P_1 = S_2$] to [P_2]. E_1 is coupled with antibody [E_1–Ab] and E_2 is coimmobilized with antigen [Ag] on a solid material such as fine beads [$E_2 \leftarrow$ Ag]. Alternatively, E_1 can be coupled to antigen and E_2 can be coimmobilized with antibody. When E_1-labeled antibody is bound to immobilized antigen, E_1 and E_2 are located close together, the overall reaction ($S_1 \rightarrow P_1 = S_2 \rightarrow P_2$) is enhanced, and the rate of P_2 formation is increased. When unlabeled antigen is added, it competes with immobilized antigen for binding to E_1-labeled antibody and results in an increase in [E_1–Ab:Ag], which can catalyze $S_1 \rightarrow P_1 = S_2$, but not $P_1 = S_2 \rightarrow P_2$. Thus, the formation of the final product P_2 is decreased. The background due to the reactions of the free forms of E_1 and E_2 can be

reduced by removing $P_1 = S_2$. Thus a third enzyme E_3 is added to act as a scavenger to remove $P_1 = S_2$. Litman *et al.* reported a procedure for this type of immunoassay for human IgG (L9). The enzymes used were hexokinase (HK), glucose-6-phosphate dehydrogenase (G-6-PDH) and glucose phosphate isomerase (GPI). The assay mixture consisted of glucose [S_1], ATP, HK–anti-IgG (antibody) conjugate [E_1–Ab], unlabeled IgG [Ag], agarose beads on which G-6-PDH and IgG are coimmobilized [E_2 ⇠ Ag], NAD, and GPI [E_3]. When HK–anti-IgG is bound to IgG on agarose beads [E_1–Ab:Ag ⇠ E_2], the following reaction is enhanced and NADH formation is increased.



When the amount of unlabeled IgG is increased, binding of HK–anti-IgG to IgG on the beads is decreased, and NADH formation decreases.

3.5. Enzyme Enhancement Immunoassay

This method was reported by Gibbons *et al.* of the Ullman group (G4). The technique involves unlabeled antigen [Ag], β-galactosidase-labeled antibody [E–Ab], succinylated antibody [Suc–Ab] and a macromolecular substrate, *o*-nitrophenyl-β-galactoside [S_1]. The β-galactosidase-labeled antibody and the succinylated antibody form an immune complex in the presence of unlabeled antigen [E–Ab:Ag:Suc–Ab]. The β-galactosidase within this negatively charged microenvironment produced a product [P_1] that forms a second light-scattering phase [P_2], whereas the product [P_1] produced by the free form of β-galactosidase–antibody [E–Ab] remains soluble. Thus, the amount of added antigen can be determined by measuring the increase in light scattering. This method was applied to assays of IgG and C-reactive protein as well as of specific antibodies.

3.6. Enzyme Membrane Immunoassay (EMIA)

This method was developed by Collaborative Research Inc. (F3). The technique, based on the use of immunoreactive liposomes, may be classified as another type of homogeneous immunoassay. The liposomes are microscopic vesicles (200–1000 nm in diameter) consisting of a relatively impermeable lipid bilayer that delineates and separates an internal aqueous compartment from the external aqueous medium. The principle is as follows (Fig. 3 and Table 6). An enzyme, alkaline phosphatase, is encapsulated in the liposomes [E] and sequestered from the substrate, *p*-nitrophenyl phos-

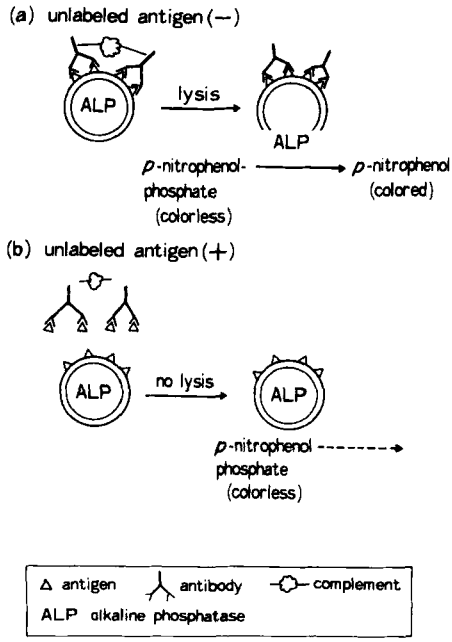


FIG. 3. Schematic representation of the principle of enzyme membrane immunoassay (EMIA).

phate [S], which is in solution outside the liposomes. Antigen molecules are attached on the surface of the liposomes (IMMUNO SOMES) [Ⓔ-Ag]. When antibody and complement are added, the antibody binds to the antigen and causes lysis of the liposomes [EAg:Ab]. Thus, the enzyme is liberated from the liposomes and interacts with its substrate. When unlabeled antigen is added, it competes with the antigen on the liposomes for the binding to antibody, resulting in reduced complement-mediated immunoreactive liposome lysis, and decreased enzyme activity. The EMIA system has been used to determine thyroxine, human chorionic gonadotropin, IgG, chymosin, etc. Freytag and Litchfield (F2) reported liposome-mediated immunoassays for small haptens using a hapten–melittin conjugate which causes lysis of the lipid vesicles independent of complement.

4. Fluorescence and Luminescence Immunoassay

Fluorescent or luminescent substances are known to be excellent markers for antigen–antibody reactions. The fluorescent and luminescent substances with low molecular weight can be coupled easily to antigen or antibody

without loss of affinity of the antigen –antibody reaction. Recent developments in instrumentation make it possible to detect very low concentrations of fluorescent substances (S7). There are three types of immunoassays utilizing fluorescent or luminescent substances, as follows: (1) The substances are directly coupled to antigen or antibody. (2) The substances are coupled to substrate in an enzyme reaction that is modulated by an antigen–antibody reaction. (3) The substances are coupled to substrate for detecting enzyme activity in an enzyme immunoassay. For type (1), various heterogeneous fluorescence immunoassays are available (M1) and many papers on luminescence immunoassays have been published (cf. I1, W2, W4–W6). Type (3) is already described in Section 3 on enzyme immunoassays (T2), and type (2) will be described in Sections 4.1–4.2. The development of homogeneous immunoassay systems using fluorescence and luminescence is included in this discussion.

4.1. Substrate-Labeled Fluorescent Immunoassay

This method was developed by Burd *et al.* (B7) and reviewed by Boguslaski *et al.* (B1). Table 6 and Fig. 4 illustrate the principle. For example, for measuring gentamicin (B6), the mixture contained umbelliferyl-β-galactoside/gentamicin conjugate (fluorogenic enzyme–substrate-labeled antigen) [F–S–Ag], β-galactosidase [E], unlabeled gentamicin [Ag], and antibody to gentamicin [Ab]. When antibody is bound to fluorogenic enzyme–substrate-labeled gentamicin [F–S–Ag:Ab], the enzyme reaction does not take place. When unlabeled gentamicin [Ag] is increased, it competes with fluorogenic enzyme–substrate-labeled gentamicin for binding to antibody, and the free form of fluorogenic enzyme–substrate-labeled gentamicin [F–S–Ag] increases; then, β-galactosidase [E] can catalyze the reaction of fluorogenic enzyme–substrate [F–S] to fluorogenic product [F]. Thus the amount of gentamicin can be measured as an increase in fluorescence intensity. Kits for the Therapeutic Drug Assay (TDA, Ames) are available for measuring drugs such as antiepileptics (phenytoin, phenobarbital, primidone, etc.) and antibiotics (gentamicin, amikacin, tobramycin, etc.).

4.2. Enzymatic Hydrolysis of Antigen–Fluorescent Dye Conjugate Immunoassay

In this assay, marker-labeled antigen itself acts as substrate. Kohen *et al.* (K9) developed a homogeneous immunoassay for steroid using a steroid–fluorescent dye conjugate that yields fluorescent products upon hydrolysis with enzyme. The steroid–fluorescent dye conjugate is inactive as a substrate when bound to the antibody to steroid [F–Ag:Ab]. But when unlabeled steroid [Ag] is added, it binds competitively to the antibody [Ab:Ag], and the free form of steroid–fluorescent dye conjugate [F–Ag], which is

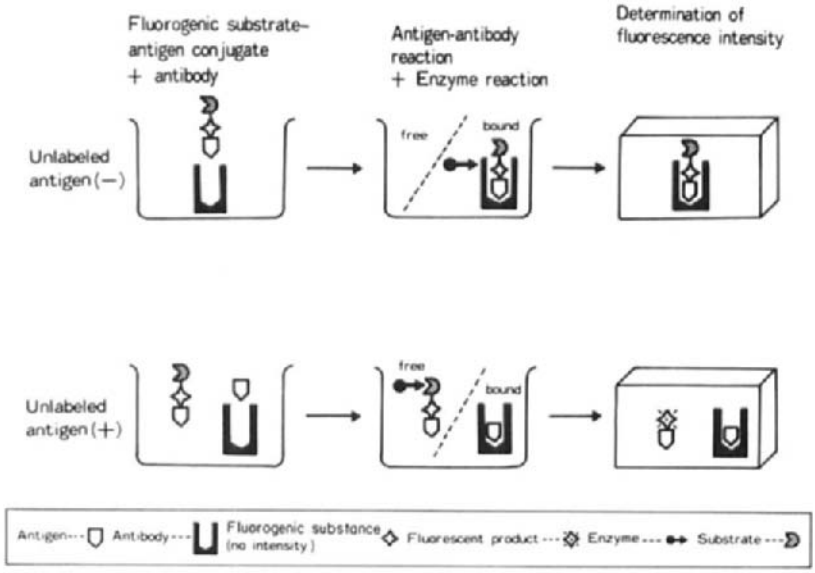


FIG. 4. Schematic representation of the principle of substrate-labeled fluorescent immunoassay (TDA, Ames). [Cited and modified from Fig. B-5, Miyai, K., in Miyai *et al.*, eds. (M10).]

active as substrate, increases. Thus, the enzyme [E] reaction takes place and the total fluorescence intensity increases. For example, cortisol was determined using cortisol 21-hemisuccinate/umbelliferone conjugate and hog liver esterase. The determinations of testosterone (K11), 17-hydroxyprogesterone (K10), and estriol (G2) by this method have also been reported.

4.3. Antibody-Enhanced Hydrolysis of Steroid–Fluorescent Dye Conjugate Immunoassay

This sytem, which was also developed by Kohen *et al.*, is similar to that described in Section 4.2 except that nonenzymatic hydrolysis is enhanced by antibody binding (K9–K11). For example, testosterone-1-carboxymethylthioether was conjugated through an ester bond with a fluorescent compound (testosterone–umbelliferone conjugate). The testosterone–umbelliferone conjugate [F–Ag] was devoid of fluorescence but yielded a fluorescent product [F] upon incubation with the antibody to testosterone [Ab]. When unlabeled testosterone [Ag] was added, the amount of the free form of testosterone–umbelliferone conjugate was increased, and thus that of the fluorescent product was decreased (K11). A similar assay system was developed for determining 17α-hydroxyprogesterone by using 17α-hydroxyprogesterone-7α-carboxyethylthioether/umbelliferone (K10).

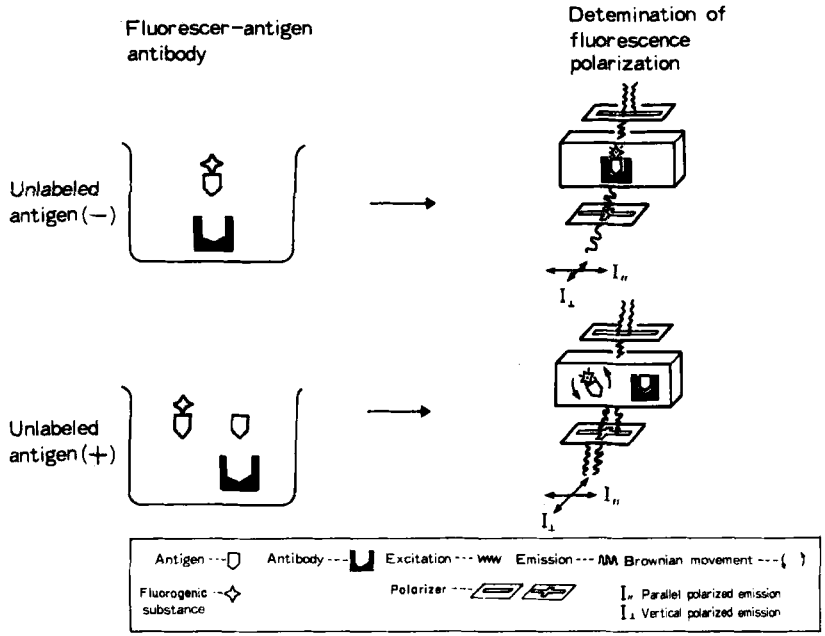


FIG. 5. Schematic representation of the principle of fluorescence polarization immunoassay (TDX, Abbott). [Cited and modified from Fig. B-1, Miyai, K., in Miyai *et al.*, eds. (M10).]

4.4. FLUORESCENCE POLARIZATION IMMUNOASSAY

The principle of this method was reported and reviewed by Dandliker *et al.* (D1–D3). As illustrated in Fig. 5, when the exciting light beam is polarized and directed to a fluorescent substance that is assumed to be rigidly fixed, the parallel polarized emission ($I_{\parallel}$) is increased while the vertical polarized emission ($I_{\perp}$) is decreased. Thus, the polarization $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$ is increased. On the contrary, when the rotational Brownian movement of the substance is increased, $I_{\parallel}$ is decreased, $I_{\perp}$ is increased, and P is decreased. In the fluorescence polarization immunoassay, the rotational movement of the complex of the fluorescer-labeled antigen and antibody (bound form) [F–Ag:Ab] is slow and thus the polarization is increased. When unlabeled antigen [Ag] is added, the free form of the labeled antigen with rapid movement [F–Ag] is increased and the polarization is decreased. Therefore, the degree of polarization is inversely correlated with the amount of added unlabeled antigen.

The method is simple and rapid, but it has limitations for practical use. One is that Brownian movement is influenced by factors such as temperature and viscosity, and thus conditions must be strictly controlled. Another is that

TABLE 8
COMPARISON OF THREE TYPES OF FLUORESCENCE IMMUNOASSAY (FIA)[a]

	Polarization FIA	Quenching FIA	Solid-phase FIA
Sample size (μl)	10	100	10
Procedure time (hours) 100–150 assays	5	5	5
Sensitivity (ng/tube)	0.1	3	0.2
Measurable range (μg/dl)	1–100	3–100	2–100
Intra-assay CV (%)	7.2–11.6	7.7–10.5	11.6
Inter-assay CV (%)	4.6–10.6	10.7–13.3	12.1
B/F separation	Homogeneous	Homogeneous	Heterogeneous
Correlation FIA (y)/RIA (x)	$y = 1.08x - 0.13$ $r = 0.95$	$y = 0.89x + 0.88$ $r = 0.97$	$y = 0.98x + 0.04$ $r = 0.98$

[a] From Miyai *et al.* (M12).

this method is suitable mainly for the assay of small molecular substances, because the changes in rotational movement of antigen with large molecules upon binding to antibody is much less than that wich labeled small molecules. This method has been used to measure gentamicin (W3), phenytoin (M4), thyroxine (N1), cortisol (K5, K6), insulin (S9, Y2), and others. TDX kits (Abbott) have become available. Table 8 shows our data obtained for comparison of two types of homogeneous fluorescence immunoassay (M12), i.e., fluorescence quenching [Q] (K7), and polarization [P] (K5, K6) immunoassays with a heterogeneous solid-phase fluorescence immunoassay [S] (K8) for cortisol. Direct assay could be done by [P] and [S], but a methanol extraction step was necessary for [Q]. The sensitivity of [Q] was lower than the sensitivities of [P] and [S].

4.5. FLUORESCENCE QUENCHING IMMUNOASSAY

In certain cases, antibody binding to hapten (antigen)–fluorophor (marker) conjugate affects the fluorescence quantum yield (E2). For example, binding of anticortisol antibody [Ab] to the cortisol–fluorescein isothiocyanate (FITC) conjugate [F–Ag] causes a decrease in fluorescence intensity of FITC by quenching [F–Ag:Ab]. When unlabeled cortisol [Ag] is added, the free form of cortisol–FITC [F–Ag] increases and the fluorescence intensity increases (K7). The procedure in the fluorescence quenching immunoassay is simple and rapid. However, the degree of quenching depends on the nature of the hapten; thus, application of the method is limited to certain substances such as gentamicin (S4).

4.6. Fluorescence Enhancement Immunoassay

As a special case, the fluorescence intensity of the fluorophor–thyroxine conjugate [F–Ag] is increased when antibody to thyroxine is bound to it [F–Ag:Ab]. When unlabeled thyroxine [Ag] is added, the fluorescence enhancement is decreased (S6).

4.7. Fluorescence Protection Immunoassay (Indirect Fluorescence Quenching Immunoassay)

In this method, the effect of antifluorescein antibody [anti-F] is to decrease the signal of the free form of fluorescein-labeled antigen [anti-F:F–Ag]. In the presence of antibody [Ab], which binds to fluorescein-labeled antigen [F–Ag:Ab], the fluorescence is "protected" from the quenching effect with antifluorescein. When the amount of unlabeled antigen [Ag] that binds to antibody is increased, the free form of fluorescein-labeled antigen [F–Ag] increases and the fluorescence intensity decreases due to the effect of antifluorescein. Thyroxine (H3) and large molecular substances such as human serum albumin (N2) and IgG (Z1) can be determined by fluorescence protection immunoassay.

4.8. Fluorescence Immunoassay Based on Excitation by Internal Reflection, Spectroscopy, or Cytometric Detection

This technique was reported by Kronick and Little (K13). In this method, fluorescein-labeled antibody [F–Ab] is bound to antigen adsorbed on a quartz plate [Ag –•]. By internal reflection spectroscopy, an area of only a few angstroms perpendicular to the surface of the quartz plate is illuminated by exciting radiation. Thus, only the fluorescence of fluorescein-labeled antibody bound to antigen on the quartz plate [F–Ab:Ag –•] is measured. When unlabeled antigen [Ag] is added, the antigen competitively binds to the fluorescein-labeled antibody [F–Ab:Ag], which is far from the quartz plate [Ag –•] and cannot be excited. Therefore, measurement of a decrease in the fluorescent signal gives a measure of the concentration of unlabeled antigen. Phenylarsonic acid and morphine were measured by this technique.

This method was modified by Lisi *et al.* (L8) using antibody-coated microspheres [Ab–•]. Added unlabeled antigen [Ag] and fluorescein-labeled antibody [F–Ab] bind to the antibody-coated microspheres as in the conventional sandwich immunoassay [F–Ab:Ag:Ab –•]. After the reaction is complete the suspension is introduced into a flow cytometer with a laser light source. By gating fluorescence light accumulation on scattered light pulses,

microsphere-associated fluorescence may be selectively measured. Therefore, if the amount of unlabeled antigen is increased, the amount of fluorescein-labeled antibody bound to the microspheres is increased and the fluorescence is increased. Human IgG was measured in a model experiment. Alternatively, unlabeled antigen [Ag] can react competitively with fluorescein-labeled antigen [F–Ag] and antibody-coated microspheres [Ab –●].

4.9. Fluorescence Excitation Transfer Immunoassay

This elegant method was developed by Ullman *et al.* (U1). In this system the fluorophor is coupled to antigen [F–Ag] and the energy acceptor or quencher is coupled to antibody [Q–Ab]. When the fluorescence-labeled antigen is allowed to bind to quencher-labeled antibody [F–Ag:Ab–Q], the fluorescence intensity is reduced. When unlabeled antigen [Ag] is added, some of the quencher-labeled antibody will be used up [Q–Ab:Ag] and be unavailable for binding to fluorescer-labeled antigen. Thus, the fluorescence intensity of fluorescer-labeled antigen [F–Ag] increases with an increase in the concentration of unlabeled antigen. For example, the fluorescence intensity of fluorescein [F]-labeled morphine is reduced when it binds to rhodamine [Q]-labeled antibody. When unlabeled morphine is added it binds to rhodamine-labeled antibody that does not bind to fluorescein-labeled morphine and thus the fluorescence intensity is increased. Similar assays were developed for human IgG, the light chain of IgG, albumin, and complement C3. Lim *et al.* (L7) state that the assay is capable of detecting nanomolar concentrations of albumin.

Alternatively, it was found that excitation transfer could be employed in assays in which the antigen is labeled indirectly through an antibody. For example, a mixture of morphine–albumin conjugate (unlabeled antigen [Ag]), fluorescein-labeled antimorphine–albumin conjugate [F–Ab], and rhodamine-labeled antimorphine–albumin conjugate [Q–Ab] form an immune complex [F–Ab:Ag:Ab–Q] and the fluorescence intensity is quenched. Thus, the concentration of added morphine–albumin conjugate [Ag] can be determined by a decreased fluorescence intensity (U1). This type of fluorescence excitation transfer immunoassay can be included in the category of proximal linkage immunoassay described in Section 2.3.4.

5. Other Immunoassays

5.1. Spin Immunoassay

Spin immunoassay is also a type of homogeneous immunoassay. A change in the electron spin resonance (ESR) spectrum of a spin (marker)-labeled

hapten (antigen) by antibody binding [Fr–Ag:Ab] is an indicator of the antigen–antibody reaction. When unlabeled antigen [Ag] is added, it competitively binds to antibody [Ag:Ab]. The free form of the spin-labeled antigen [Fr–Ag] increases and the ESR changes. Leute *et al.* (L5) first reported the spin immunoassay of a morphine analogue and since then the method has been used for drug monitoring under the name of FRAT (Free Radical Assay Technique).

5.2. VIROIMMUNOASSAY

Viroimmunoassay, first reported by Haimovich *et al.* (H2), is also included in the category of homogeneous immunoassay. In this type of assay, the bacteriolytic activity (plaque forming) of bacteriophage (marker), which is coupled with antigen [B–Ag], is inactivated by antibody binding [B–Ag:Ab]. Addition of unlabeled antigen [Ag], which competitively binds to antibody [Ag:Ab], inhibits the inactivation, and thus increases plaque formation. Viroimmunoassay has been applied to measure rabbit IgG, insulin, lysozyme (M3), prostaglandins (D4), estradiol (A5), antioxazolone antibodies (J1), etc., but it is not widely used because the procedure is complex.

5.3. PARTICLE IMMUNOASSAY

For many years, antigen–antibody reactions have been studied by agglutination techniques using red cells or "latex" particles which were coated with antigen or antibody. In this case, particles are regarded as a labeled marker. Descriptions of several methods that have been devised for detecting agglutination follow.

5.3.1. *Particle Counting Immunoassay (PACIA)*

In 1977, Cambiaso *et al.* (C4) showed that agglutination could be quantitatively measured by counting latex particles with a device designed for counting blood cells. The principle is based on the reduction of the total number of particles when they are agglutinated.

Macromolecular substances with multiple antigenic determinant [Ag] can be determined by agglutination of particles which are coated with specific antibodies [Pa–Ab:Ag:Ab–Pa]. This method can detect immunoglobulins, human placental lactogens, α-fetoprotein, etc. The sensitivity is approximately 10 μg/liter. Alternatively, antigen-coated particles are used; these particles agglutinate with antibodies. Thus, antibodies can be determined by this system. Cambiaso *et al.* reported automated determination of immune complexes by their inhibitory effect on the agglutination of IgG-coated particles by rheumatoid factor or C1q (C3).

Hapten can also be determined by the particle immunoassay as reported by Cambiaso *et al.* (C4). In the first system the concentration of the added hapten [Ag] can be measured by its inhibitory activity on the agglutination of hapten-coated latex [Pa–Ag] with the specific antibody. They showed a model experiment of this system using dinitrophenol. In the second system the particles are coated with antibody [Pa–Ab] and agglutinated by hapten conjugate [Pa–Ab:Ag–conj:Ab–Pa]; the free hapten can be determined by its inhibitory effect on this agglutination. Thyroxine in a concentration of 10 μg/liter can be measured using thyroxine-coupled dextran and antithyroxine-coated particles. In the third system, hapten-coated particle [Pa–Ag] is agglutinated by a mixture of IgG fraction of antihapten antibodies [Ab_1] and IgM fraction of anti-IgG antibodies (rheumatoid factor) [Ab_2]. Hapten can be determined by its inhibitory activity in the agglutination of hapten-coated particles.

The automated system of the particle counting immunoassay is now commercially available as the product IMPACT (Immunoassay by Particle Counting), which can measure C-reactive protein, ferritin, human placental lactogen, thyroxine, α-fetoprotein (C7), IgE (M2), digoxin (C6), somatotropin (C5), and others.

5.3.2. *Photometric Immunoassay of Particle Agglutination*

Latex agglutination reactions can be determined by near-infrared turbidimetry (S10). The turbidity caused by agglutination of the antigen-coated latex (0.1 to 0.8 μm in diameter) with antibody increases in the near-infrared region, but not in the visible or ultraviolet region; this phenomenon is supported theoretically by the Mie scattering theory. Hapten can also be measured as follows. When latex coated with antibodies to hapten, polyhapten, and hapten to be tested are mixed, agglutination is inhibited by the hapten to be tested. The system is now automated and commercially available (Latex Photometric Immunoassay System, LPIA, Mitsubishi Chemical Industries, and Latex Agglutination Photometric Assay System, LA-SYSTEM, Analytical Ir.struments). This system can be used for measuring α-fetoprotein, β_2-microglobulin, IgE, C-reactive protein, etc.

Alternatively, Briggs *et al.* (B4) reported a fluorescent immunoassay based on the correlation of fluctuations in particle number measures of the amount of tagged species bound to micrometer-sized beads. A homogeneous competitive assay based on this principle can detect 1 ng of gentamicin per ml from a total sample volume of 10 μl.

5.3.3. *Sol Particle Immunoassay (SPIA)*

Sol particle immunoassay was developed by Leuvering *et al.* of the Organon group (L6). They used inorganic (metal) colloidal particles as a label

for immunoassay, and several techniques such as colorimetry and carbon rod atomic absorption spectrophotometry to measure the amounts of labeled substances. As a model experiment, a sandwich method for human placental lactogen (hPL) and human chorionic gonadotropin (hCG) was described. Samples containing hPL were added into a well of a plate coated with anti-hPL, and incubated. Gold particle–anti-hPL conjugate was then added and the wells were washed. Finally, visible absorbance at 540 nm or absorbance in a carbon rod atomic absorption spectrophotometer at 242.8 nm was measured. The sensitivity was about 1.4 pmol/liter, which was equal to that of competitive radioimmunoassay.

5.4. Metal Immunoassay

Cais *et al.* reported on the feasibility of a new nonisotopic immunoassay, metal immunoassay, that uses metal atoms as labeling agents for antigens (C1). The basic requirements for a metal immunoassay are (1) preparation of reagents (antibodies and metallohaptens) and (2) development of a method for quantifying the metal content. The metallohapten (labeled antigen) and unlabeled antigen to be tested are competitively reacted with antibody. The bound and free forms are separated and the amount of metal present in the bound or free forms is determined. When the amount of unlabeled antigen increases, the metal concentration in the bound form decreases. The metal content can be determined by suitable analytical methods such as emission, absorption, and fluorescence spectrometry, electrochemical methods, and neutron activation. As an example, estrogen steroids were labeled with iron or manganese complexes and atomic absorption spectrometry was used for quantitation of the metal concentration. Sol particle immunoassay (described in Section 5.3.3) is also included in the category of metal immunoassay.

5.5. Nephelometric Immunoassay

In 1967, Ritchie reported a direct technique for measurement of albumin and immunoglobulins by the development of antigen–antibody precipitates in liquid media. In this method turbidity produced by the antigen–antibody complex was determined by measuring absorbance at 420 nm with a spectrophotometer (R1).

In 1970, Eckman *et al.* devised the automation of a quantitative immunochemical analysis of transferrin (E1). In this automated flow system, diluted samples were allowed to react with antitransferrin antiserum serially and the degree of light scattering of the resulting turbidity was measured in the fluorometer, used as a nephelometer. The optimal conditions for nephelometry were extensively studied. Subsequently, Buffone reported

the modification of a commercially available analyzer system to allow the performance of light scattering measurements by incorporation of a laser and applied this to the kinetic measurement of the IgG–anti-IgG reaction (B5).

Hapten can also be measured by the nephelometric inhibition (competitive nephelometric) immunoassay. Cambiaso *et al.* described the assay of hapten by use of nephelometry to measure the light scattering produced when immunoprecipitation of specific antibody and hapten–carrier protein conjugate is competitively inhibited by hapten (C2). Nishikawa *et al.* also reported a competitive nephelometric immunoassay of theophylline in plasma (N7). Finley *et al.* measure phenytoin and phenobarbital by a rate nephelometric inhibition immunoassay in which reaction times range from 30 to 50 seconds (F1). The lowest concentration of progesterone measured was 10 ng/liter. Thus, the sensitivity of the nephelometric inhibition immunoassay for hapten was shown to be greater than that of conventional nephelometry for protein (approximately 1 mg/liter). A laser–nephelometer immunoassay system is now commercially available for measuring albumin, immunoglobulins, complements, transferrin, haptoglobin, α_1-antitrypsin, C-reactive protein, fibrinogen, β-lipoprotein, drugs, and others. (Hyland, and Chugai). The sensitivity of this method has been considerably improved, but its application is still limited to quantitating substances that are present in relatively high concentration in body fluids.

5.6. Electrochemical Immunoassay

Ambitious attempts to develop an electrochemical immunosensor have been made. Homogeneous systems can be easily analyzed by this method.

5.6.1. *Direct Immunosensor*

Yamamoto *et al.* developed a direct immunosensor (Y3). The immunoelectrode was made of titanium wire, on which antigen or antibody was chemically fixed. The electric potential changes when an antigen–antibody reaction occurs. For example, the potential of the electrodes sensitized with anti-hCG antibody shifted to positive polarity when hCG was added. Aizawa *et al.* also reported a direct immunosensor (A1). The sensitivity is insufficient for clinical use but it is possible that the methods will be improved in the near future.

5.6.2. *Enzyme Immunoassay Using Electrodes*

Aizawa *et al.* reported the development of a competitive enzyme immunoassay using electrodes (A2). In this method, antibody immobilized on the membrane is attached to the electrodes for oxygen. When catalase–antigen conjugate binds to the immobilized antibody, production of oxygen can be

easily detected by the oxygen sensor. When antigen is added, it competes with the catalase–antigen conjugate for binding sites on the immobilized antibodies. Thus, when antigen is increased the oxygen production is decreased. Ion-selective electrodes for iodide (B2) or fluoride (A4) can be used for determination of enzyme activity. Boitieux *et al.* devised a computerized enzyme immunosensor (sandwich method) and measured hepatitis B surface antigen (B3).

5.6.3. *Liposome Immunosensor*

Haga *et al.* developed another type of immunosensor by combining an enzyme membrane immunoassay and an enzyme sensor using oxygen electrodes (H1). In this assay antigen molecules (theophylline) are attached on the surface of the liposomes and an enzyme (horseradish peroxidase) is encapsulated in the sensitized liposome. When antibody (antitheophylline antibody) and complement are added, the enzyme is released by the liposome lysis. The enzyme activity with the NADH–NAD reaction can be determined by the oxygen electrode. When antigen is added, it competitively binds to antibodies, then liposome lysis and enzyme activity are decreased. The sensitivity of this method for theophylline determination was reported as 0.7 ng/ml.

5.6.4. *Immunoassay Utilizing Electroactively Labeled Antigen and Polarography*

Wehmeyer *et al.* developed a new immunoassay in which the binding of electroactively labeled antigen with antibody and its displacement by unlabeled antigen were monitored by differential pulse polarography (W8). They used estriol labeled in the 2 and 4 positions with nitro groups, which were electroactive. The addition of antiestrogen antibody reduced the peak current proportionately and subsequent addition of unlabeled estrogen produced incremental increases in peak reduction current. This method, in principle, can be used as one of the homogeneous immunoassays.

5.6.5. *Potentiometric Ionophore-Modulation Immunoassay (PIMIA)*

This method was developed by Keating and Rechnitz (K4) and its principle is as follows. An antigen is coupled to an ionophore (antigen–carrier conjugate). This conjugate is incorporated into a plastic support membrane that is mounted in the sensing tip of a conventional potentiometric membrane electrode. The electrode is exposed to a constant activity of a marker ion chosen for its compatibility with the ionophore portion of the conjugate. When an antibody capable of binding the antigen portion of the conjugate is added, a potential change proportional to the antibody concentration is pro-

duced. This method also can be used for detection of antigens in a manner comparable to that of the competitive immunoassay. Keating and Rechnitz used a digoxin/benzo-15-crown-5/PVC electrode, which can determine K^+, and determined antidigoxin antibody and digoxin.

6. Nonisotopic Immunoassay with Monoclonal or Clonotype Antibody

Since Kohler and Milstein developed the hybridoma technique (K12), monoclonal antibodies to various substances have become available. Monoclonal antibodies have been introduced into many radioimmunoassays as well as into nonisotopic immunoassays (S3). Immunoassays using monoclonal antibodies have the following attributes. Desirable monoclonal antibodies have been available for a long time. Since monoclonal antibodies usually react with a single determinant of the antigen, by immunoassay it is possible to distinguish one antigen from another structurally and from immunochemically similar antigens. However, the affinity constants of monoclonal antibodies have usually been found to be less than those of original polyclonal antibodies (S5, S8). Thus, competitive immunoassays utilizing monoclonal antibodies are specific, but not as sensitive as those utilizing polyclonal antibodies.

The use of monoclonal antibodies is very desirable in assays requiring labeled antibody or solid-phase antibody. Labeled polyclonal antibodies used in such assays usually require affinity purification to avoid nonspecific binding of the markers with other antibodies. In some cases of immunoassay with solid-phase antibody, polyclonal antibodies must be separated from total immunoglobulin to increase the titers of specific antibody. On the contrary, monoclonal antibodies need not be purified further. Two-site enzyme immunometric assay, particularly the simultaneous procedure, in which antigen and enzyme-labeled antibody are simultaneously placed in an antibody-coated tube, is better when two different monoclonal antibodies are used for labeling and coating, respectively, because this avoids competition between binding sites. As mentioned previously, use of a monoclonal antibody is essential in proximal linkage immunoassay (S3).

6.1. Enzyme Immunoassay with Monoclonal Antibody

As an example of enzyme immunoassay with monoclonal antibody, Wada *et al.* reported a sandwich enzyme immunoassay of glycoprotein tropic hormones (W1). Human glycoprotein tropic hormones such as human chorionic gonadotropin, human luteinizing hormone (hLH), human follicle-stimulating hormone (hFSH), and human thyroid-stimulating hormone (hTSH) are

known to consist of α and β subunits. The structures of the α subunit of these hormones were studied by DNA coding and found to be identical. On the other hand, the structures of the β subunits of these hormones are known to be different and to be specific. In this assay, the anti-α subunit monoclonal antibody was coated on the surface of the tubes, and the monoclonal antibody against the β subunit of hCG, hLH, or hTSH was coupled to horseradish peroxidase (HRP). The HRP-labeled anti-β subunit and the serum samples were then placed in anti-α subunit-coated tubes. After incubation, excess HRP-labeled anti-β subunit was removed, and the enzyme activity in the tubes was measured. Cross-reactions between the closely related hCG and hLH were apparently greater by this method than by competitive radioimmunoassay with the same monoclonal antibodies. The affinity of the antibodies did not appear to be as critical to sensitivity in this method as in radioimmunoassay. The advantage of this method is that three different hormones could be measured using only one type of anti-α subunit-coated tube.

6.2. Fluorescence and Enzyme Immunoassays with Clonotype Antibody

Recently, immunoassays using clonotype antibody have been developed in our laboratory. The clonotype antibody was prepared from polyclonal antibody by isoelectric focusing (E3) or chromatofocusing techniques (E4). As a model experiment, IgG fractions prepared from rabbit antithyroxine antisera were fractionated by polybuffer exchange chromatography using the Pharmacia Fast Protein Liquid Chromatography (FPLC) system as shown in Fig. 6. On Scatchard plot analysis of double-antibody radioimmunoassay with [^{125}I]thyroxine, typical curves were obtained with these fractions, but the curvatures of the lines were smaller than was obtained with the initial polyclonal antibody. The highest affinity constant of the initial antithyroxine IgG was $2.5 \times 10^9\ M^{-1}$ while the constants of the fractions ranged from 2.4×10^9 to $6.0 \times 10^9\ M^{-1}$. As shown in Fig. 6, ED_{50} (the thyroxine concentration corresponding to 50% binding of radioactivity at zero concentration of thyroxine) in the assay with the initial antibody was $1.7 \times 10^{-9}\ M$. The values of ED_{50} were in the range $(0.83–2.5) \times 10^{-9}\ M$ in assays of fractions with clonotype antibodies. The clonotype antibodies were applied to nonisotopic immunoassays (M5). Fluorescence polarization immunoassay of thyroxine was carried out using fluorescer–thyroxine conjugate (Abbott) and these antithyroxine–IgG fractions. The ED_{50} value was $7.6 \times 10^{-9}\ M$ for the initial antithyroxine and the values for the fractions ranged from $3.3 \times 10^{-9}\ M$ to infinitesimal. Similarly, double-antibody enzyme immunoassay of thyroxine with β-D-galactosidase/thyroxine conjugate was carried out. The

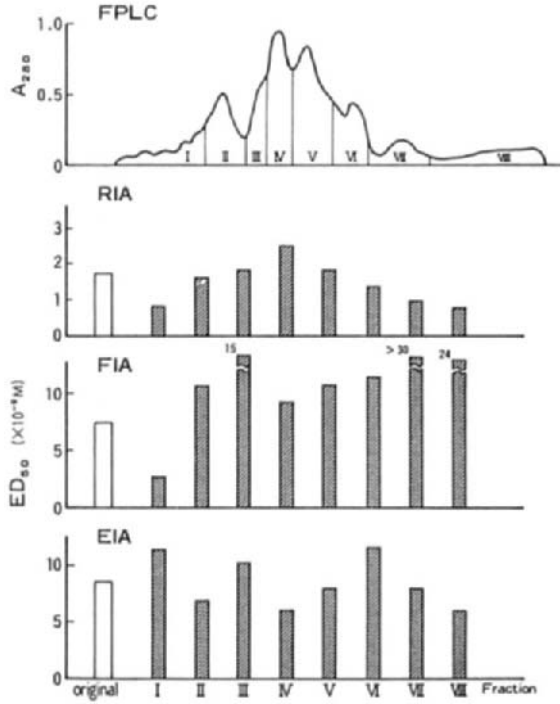


FIG. 6. Comparison of immunoassays using clonotype antibodies. The ED_{50} values (thyroxine concentration corresponding to 50% of bound form at zero concentration of thyroxine) in thyroxine immunoassays using clonotype antithyroxine antibody prepared by fast protein liquid chromatography (FPLC). RIA, double-antibody radioimmunoassay; FIA, fluorescence polarization immunoassay; EIA, enzyme immunoassay. [Cited from Miyai *et al.* (M5).]

ED_{50} for the initial antithyroxine was 9.2×10^{-9} *M* and the values for the fractions were in the range $(6.3–13.0) \times 10^{-9}$ *M*. However, the clonotype antibodies with which the most sensitive assay could be achieved differed for different types of immunoassays; i.e., fractions I and VIII for radioimmunoassay, fraction I for fluorescence immunoassay, and fractions II, IV, and VIII for enzyme immunoassay in these experiments. The sensitivities of immunoassays may be improved by using appropriate clonotype antibodies.

7. Application of Nonisotopic Immunoassay to Neonatal Screening

During the past 20 years, neonatal screening programs using the Guthrie test for inborn errors of metabolism such as phenylketonuria have become more widely used. The characteristics of diseases in which mass screening is

TABLE 9
RECOMMENDED IMMUNOASSAYS FOR MASS SCREENING

Disease	Test[a]	Method[b]
Hypothyroidism	TSH	RIA, EIA
	T_4 + TBG	RIA, EIA (FIA)
	Free T_4	RIA, EIA
Congenital adrenal hyperplasia	17-OH-progesterone	RIA, EIA
Cystic fibrosis	Trypsin	RIA
Atopic disease	IgE	RIA (EIA)
Neural tube defect	α-Fetoprotein	RIA (EIA)

[a] TSH, Thyrotropin; T_4, thyroxine; TBG, thyroxine-binding globulin.

[b] RIA, Radioimmunoassay; EIA, enzyme immunoassay; FIA, fluoroimmunoassay. Procedures in parentheses have shown promising results.

useful are as follows: The diseases are fairly common, impairments are irreversible when not treated, treatment is effective on early diagnosis, early diagnosis is difficult by clinical features alone, and the diseases are detectable at an early stage by a suitable screening method. Since in these screening programs a large number of small samples must be tested, suitable screening methods must be sensitive, simple, labor saving, economical, well popularized and nonhazardous. The most important recent development in this field has been the introduction of radioimmunoassay, but it needs special facilities. Moreover, the numbers of samples taken for mass screening are so large that a nonisotopic assay would be preferable.

Table 9 lists various congenital disorders and the immunoassay methods recommended for their mass screening. Most of the substances listed in Table 9 have been measured by radioimmunoassay, but a nonisotopic immunoassay such as enzyme immunoassay is now recommended. Sections 7.1 and 7.2 describe examples of mass screening by enzyme immunoassay.

7.1. MASS SCREENING FOR NEONATAL HYPOTHYROIDISM

Because the irreversible mental retardation caused by neonatal hypothyroidism can be prevented by early treatment with thyroid hormone, the importance of early diagnosis of this disease has been emphasized. The difficulty of its early diagnosis by clinical features alone and its relatively high incidence have prompted the organization of various mass screening programs that include measuring thyroxine (T_4) (D5) or TSH (I3, M11) by radioimmunoassay.

Measurement of total thyroxine is useful for detecting all types of the disease except rare cases of peripheral resitance to thyroid hormone, but it gives false-positive results for patients with a deficiency of thyroxine-binding globulin (TBG) who do not need to be treated. Therefore, TBG should be measured when the total T_4 concentration is found to be low. Alternatively, measurement of free T_4 is effective for detecting these types of diseases. However, measurement of either total or free T_4 alone does not detect mild cases of primary hypothyroidism and increases the percentage of false-negative results. Thus, the International Conference on Neonatal Screening recommended measurement of TSH which is increased in patients with primary hypothyroidism, although this method gives false-negative results for rare cases of secondary or tertiary hypothyroidism.

Serum samples obtained from cord blood can be used, but small amounts of blood can be taken easily on filter paper by heel puncture and the dried blood sample on filter paper (blood disks) can be sent to special laboratories by mail. Therefore, the latter method is now widely used in neonatal screening programs.

Table 10 gives a list of enzyme immunoassays of substances related to neonatal hypothyroid screening. The methods for measuring substances in dried blood can be used for mass screening and those for tests on serum can be used for babies who are recalled.

7.1.1. *Enzyme Immunoassay of Thyrotropin*

In 1976, we developed a double-antibody enzyme immunoassay of TSH using alkaline phosphatase, but this method was not very sensitive (M8). As shown in Table 10, several other methods were later reported (A3, I2, K2, K3, M7) and two methods have been used to measure TSH in dried blood samples in screening for neonatal hypothyroidism in Japan.

One is a competitive double-antibody solid-phase method using peroxidase/TSH conjugates, which was reported by Kato *et al.* (K3). Their pilot study on mass screening for neonatal hypothyroidism has been successful.

Another method is a semiautomated noncompetitive one-step sandwich method using β-D-galactosidase/anti-TSH conjugate. The procedure for this method, which we developed, is briefly as follows (M7, M13). Polystyrene tubes are coated with the IgG fraction of anti-TSH by the method of Yang and Kennedy (Y5). A conjugate of β-D-galactosidase and anti-TSH–IgG, which is purified by affinity chromatography, is prepared by a modification of the method of Ishikawa and Kato using *N,N'-o*-phenylenedimaleimide (I4). When kept at 4°C, the conjugate can be used for at least 6 months. On the first day, two 3-mm dried blood disks (equivalent to about 5.4 μl of blood) and normal rabbit serum IgG solution are placed in anti-TSH-coated tubes, shaken, and then allowed to stand for 40 minutes. Then, anti-TSH–

TABLE 10

ENZYME IMMUNOASSAY FOR SUBSTANCES RELATED TO NEONATAL HYPOTHYROID SCREENING

Substances	Specimen[a]	Enzyme	Method for coupling	System	Author (year) (reference)	Name of kit (company)
Thyrotropin	Serum	Alkaline phosphatase	Glutaraldehyde	Double antibody	Miyai *et al.* (1976)(M8)	
	Serum	Glucose oxidase	Periodate–Schiff base	Double antibody	Albert *et al.* (1978)(A3)	
	Serum	Peroxidase	Periodate–Schiff base	Double-antibody solid phase	Kato *et al.* (1979)(K2)	
	Blood disk	Peroxidase	Periodate–Schiff base	Double-antibody solid phase	Kato *et al.* (1980)(K3)	(Fuji Rebio)
	Blood disk	β-D-Galactosidase	Dimaleimide	Solid phase	Miyai *et al.* (1981)(M7)	(Eiken ICL)
	Serum	β-D-Galactosidase	Dimaleimide	Solid phase	Imagawa *et al.* (1982)(I2)	
Thyroxine	Serum	Malate dehydrogenase		(NaOH)–homogeneous	Ullman *et al.* (1975)(U3)	EMIT (Syva)
	Serum	Peroxidase	Hydroxysuccinamide	(ANS)–solid phase	Albert *et al.* (1978)(A3)	ENZYMUN–T_4 TEST (Boehringer)
	Serum	β-D-Galactosidase	Maleimide	(ANS)–double antibody	Monji *et al.* (1978)(M14)	

	Serum	Peroxidase	Periodate–Schiff base	(ANS)–solid phase	Schall *et al.* (1978)(S1)	(Organon)
	Serum	Acetylcholinesterase		(ANS)–enzyme inhibitor immunoassay		TETRAZYME (Abbott)
	Blood disk	Alkaline phosphatase	Glutaraldehyde	(ethanol)–double antibody	Miyai *et al.* (1980)(M6)	
	Serum	β-D-Galactosidase	Maleimide	(NaOH)–double-antibody solid phase	Yamamoto *et al.* (1981)(Y4)	
	Blood disk	Glucose oxidase	Glutaraldehyde	(ANS)–double-antibody solid phase	Tsuji *et al.* (1984)(T1)	
Free thyroxine	Serum	Peroxidase		(ANS)–solid phase	Weetall *et al.* (1982)(W7)	
	Serum	β-D-Galactosidase	Maleimide	Double antibody	Ito *et al.* (1984)(I6)	
	Blood disk	β-D-Galactosidase	Maleimide	Double antibody	Hata *et al.* (1985)(H5)	
Thyroxine-binding globulin	Serum	β-D-Galactosidase	Maleimide	Double antibody	Miyai *et al.* (1982)(M9)	
	Blood disk	β-D-Galactosidase	Maleimide	Double antibody	Hata *et al.* (1983)(H4)	

[a] Blood disk: dried blood samples on filter paper.

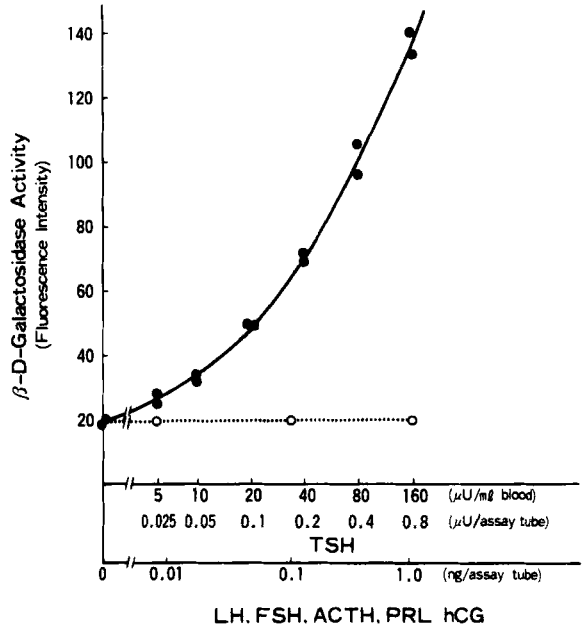


FIG. 7. A typical standard curve for enzyme immunoassay of thyroid stimulating hormone (TSH) (●——●) in dried blood spots on filter paper. LH, FSH, ACTH, PRL, and hCG (○···○) show no cross-reactivity in this assay. One unit of fluorescence intensity corresponds to the intensity of 5×10^{-9} of 4-methylumbelliferone per liter. [Cited from Miyai *et al.* (M13).]

IgG/β-D-galactosidase conjugate is added and the mixtures are incubated for 20–24 hours at 25°C. On the second day, washing solution is poured into each tube and the contents are discarded by inverting the racks of tubes with vigorous shaking, after covering the racks with a net to prevent the tubes from falling out. This washing step is repeated three times. The enzyme activity in the tubes is then measured in an analyzer specifically manufactured for this purpose. The substrate, 4-methylumbelliferyl-β-D-galactopyranoside, is introduced into each tube, and after incubation for 40 minutes at 37°C, glycine buffer, pH 10.3, is added to stop the enzyme reaction. Then the solution is sucked out and its fluorescence measured at 450 nm with excitation at 370 nm. The calibration curve, measured values, and the distribution of the values are computed automatically.

Concentrations of $(10–160) \times 10^{-3}$IU/liter of TSH in blood can be measured. There is no cross-reactivity with hLH, hFSH, adrenocorticotropic hormone, prolactin, or hCG (Fig. 7). TSH in dried blood samples is stable at room temperature for at least 1 month. The mean coefficients of variation were 12.1% (within assay) and 14.0% (between assays). A good correlation

was found between values for TSH determined by this method and by radioimmunoassay ($r = 0.94$). In pilot tests on 17,160 newborn infants in the general population, five cases of primary hypothyroidism were detected by both enzyme immunoassay and radioimmunoassay. It is emphasized that this method is sensitive and simple and is very promising for use in neonatal hypothyroid screening.

7.1.2. *Enzyme Immunoassay of Thyroxine*

7.1.2.1. *Total Thyroxine.* Since the homogeneous enzyme immunoassay for T_4 was developed by Ullman *et al.* (U3), excellent methods have been reported, as shown in Table 10 (A3, G1, M6, M14, S1, T1, T2, Y4). For neonatal hypothyroid screening, some kits are available for determining T_4 in serum but not in dried-blood samples. In 1980 we developed a double-antibody enzyme immunoassay for T_4 (M6). In this method, a site-heterologous system was used, namely, T_4–bovine serum albumin (BSA) conjugate for immunization was prepared with carbodiimide, and T_4–alkaline phosphatase conjugate was prepared with glutaraldehyde. This method could be used for measuring T_4 in dried blood samples, but was not useful for mass screening because the procedure was complicated. Subsequently, Tsuji and colleagues developed a sensitive double-antibody solid-phase chemiluminescence enzyme immunoassay for T_4 (T1). In this method a bridge-heterologous system is used, namely, T_4–glucose hemiglutamate/BSA conjugate for immunization was prepared with carbodiimide and T_4–glucose oxidase conjugate prepared with glutaraldehyde. The sensitivity is shown in Table 2. A 3-mm blood disk (2.7 μl of blood) is mixed with rabbit anti-T_4–hemiglutarate/BSA antiserum, T_4–glucose oxidase conjugate and polyacetal beads coated with goat anti-rabbit γ-globulin antiserum. The mixture is incubated for 3 hours at 37°C, and then glucose is added. The mixture is further incubated overnight at 4°C, and chemiluminescence is determined using bis(2,4,6-trichorophenyl)oxalate with H_2O_2 in the presence of fluorescent dye. The minimum detectable concentration of T_4 in a blood disk is 2.5 μg/liter, which is equivalent to 5 pg per tube. This method has been used in a pilot study of neonatal hypothyroid screening and a good correlation has been found between this method and radioimmunoassay for measuring T_4 in 492 dried blood specimens.

7.1.2.2. *Free Thyroxine.* Measurement of free T_4 radioimmunoassay in dried blood samples has been found to be useful to avoid false-positive results for low TBG in neonatal hypothyroid screening. Enzyme immunoassay of free T_4 in serum was developed by Weetall *et al.* (W7) and subsequently by us (I6).

Recently, we developed a sensitive double-antibody enzyme immu-

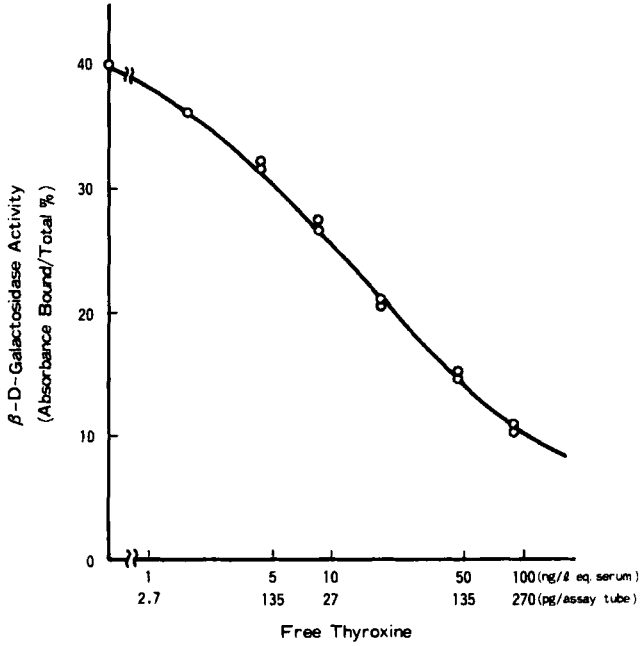


FIG. 8. A typical standard curve for enzyme immunoassay of free thyroxine in dried blood samples on filter paper. [Cited and modified from Hata *et al.* (H5).]

noassay for determination of free T_4 in dried blood samples (H5). The method is briefly as follows. T_4/β-D-galactosidase conjugate was prepared using 4-(maleimidomethyl)cyclohexane-1-carboxylic acid succinimide ester. Anti-T_4 serum was obtained from rabbits immunized with T_4–BSA conjugate which was prepared using 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate. Two 3–mm-diameter blood disks are soaked in anti-T_4 solution for 30 minutes and T_4/β-D-galactosidase conjugate is added, then the mixtures are incubated for 5 hours at room temperature. After adding the second antibody, the mixtures are incubated for 20 hours at room temperature and centrifuged. The resulting precipitates are washed and suspended in *o*-nitrophenyl-β-D-galactopyranoside solution. After the suspension is incubated for 2 hours at 37°C, sodium carbonate solution is added to stop the enzyme reaction and absorbance at 405 nm is measured. Figure 8 shows a typical standard curve.

The minimum detectable dose of T_4 was calculated as 5.1 fg per assay tube. Free T_4 at a concentration of 1.9 to 90 ng/liter in dried blood samples could be measured. The mean coefficients of variation were 5.7% (within assay) and 5.5% (between assays), respectively. The free T_4 concentrations in dried blood samples determined by the enzyme immunoassay correlated

well with those in serum samples determined by radioimmunoassay (r = 0.98). Since this method can detect hyper- and hypothyroidism even in subjects with alteration of albumin or TBG concentrations, it may be useful for screening or in the follow-up of babies with thyroid abnormalities.

7.1.3. *Enzyme Immunoassay of Thyroxine-Binding Globulin*

We developed a double-antibody competitive enzyme immunoassay for determination of TBG in serum (M9) and in dried blood samples on filter paper (H4). This method for dried blood samples is briefly as follows. TBG/β-D-galactosidase conjugate is prepared using *m*-maleimidobenzoyl-*N*-hydroxy-succinimide ester. Two 3-mm-diameter blood disks are incubated in diluted anti-TBG serum for 30 minutes at room temperature. Then, TBG/β-D-galactosidase conjugate is added and the mixtures are incubated for 20 hours at room temperature. After adding the second antibody, the mixtures are incubated for 30 minutes and centrifuged. The substrate, *o*-nitrophenyl-β-D-galactopyranoside is added to the washed precipitate and the suspension is incubated for 2 hours at 37°C. Sodium carbonate solution is added to stop the enzyme reaction, the mixtures are centrifuged, and the absorbance of the supernatant at 405 nm is measured.

The measurable concentration range of TBG is 3.3 to 52 mg/liter (Fig. 9).

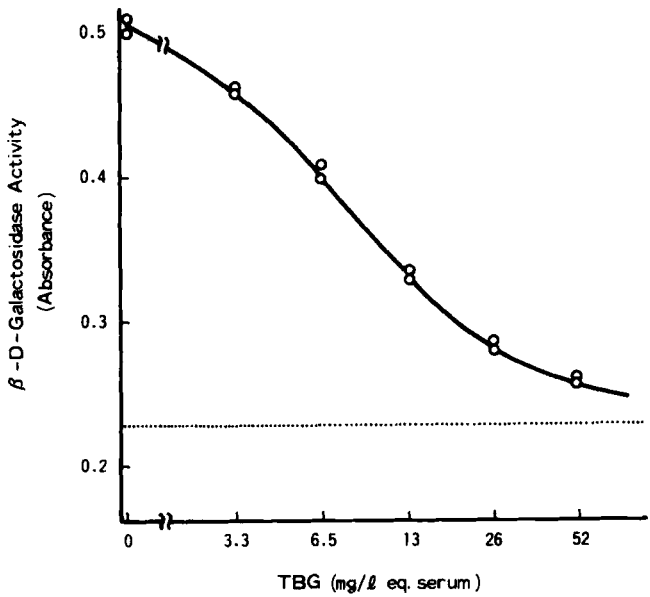


FIG. 9. A typical standard curve for enzyme immunoassay of thyroxine-binding globulin (TBG) in dried blood samples on filter paper. [Cited from Hata *et al.* (H4).]

TBG in dried blood samples on filter paper is stable for at least 1 month when kept dry at room temperature. The mean coefficients of variation are 6.6% (within assay) and 5.9% (between assays). The concentrations of TBG in dried blood samples determined by this method correlated well with those in serum determined by radioimmunoassay ($r = 0.95$) and by enzyme immunoassay ($r = 0.96$). This method is applicable for detecting patients with congenital TBG deficiency who do not need to be treated and avoids the false-positive results obtained on neonatal screening with T_4.

7.2. Mass Screening for Other Congenital Disorders

Pang *et al.* reported a successful mass screening for congenital adrenal hyperplasia by measuring 17α-hydroxyprogesterone in dried blood samples on filter paper (P2). They used radioimmunoassay but recently Arakawa *et al.* developed a chemiluminescence enzyme immunoassay of 17α-hydroxyprogesterone for this purpose (A6).

Cystic fibrosis is a lethal autosomal recessive disorder in Caucasians, with an incidence of 1:2000 in the general population. Newborn babies with this disease have increased serum trypsin concentrations. Thus, Crossley *et al.* developed a radioimmunoassay for human trypsin in dried blood samples on filter paper and used it for neonatal screening for this disease (C9). A nonisotopic immunoassay, such as enzyme immunoassay of trypsin, will be developed for this purpose.

Atopic diseases including atopic dermatitis and bronchial asthma develop during the infantile period and are found to be predictable at birth by detecting a high immunoglobulin E (IgE) concentration in cord blood. Croner *et al.* reported a radioimmunoassay of IgE for screening for these disorders (C8). A method of enzyme immunoassay of IgE in serum has been developed (H6) and may be used for this purpose.

Neural tube defect is also a tragic disorder. Until now, iatrogenic abortion has been the only treatment but the possibility of preventing this disease by periconceptional vitamin supplementation has been suggested. Leighton *et al.* reported that the maternal plasma α-fetoprotein concentration determined by radioimmunoassay is elevated between 16 and 26 weeks gestation in pregnancies associated with neural tube defect (L4). They suggested that α-fetoprotein in maternal blood should be measured routinely in all pregnancies. Enzyme immunoassay of α-fetoprotein (P3) could be used for this purpose.

8. Conclusion

Nonisotopic immunoassay has the following advantages when compared with radioimmunoassay. The procedure can be performed in a routine labo-

ratory, the substance labeled with marker can be used for a longer period, and further separation of bound and free forms is not necessary in homogeneous systems. However, in nonisotopic immunoassay, labeling and detection are rather complicated and the method is sometimes insensitive, although this can be improved. No method satisfies all essential criteria for a useful analytical technique and so the relative importance of the criteria must be considered according to the desired purpose of the method. Since the nonisotopic immunoassay has many advantages, its application should result in remarkable progress in laboratory medicine.

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ISOTOPE-DILUTION MASS SPECTROMETRY IN CLINICAL CHEMISTRY

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1. Introduction

1.1. Gas Chromatography/Mass Spectrometry in Clinical Chemistry—A General Overview

Mass spectrometry (MS) is being used increasingly as an analytical tool to solve complex problems in biochemistry and medicine. As a matter of fact, there are five ways that mass spectrometry can be applied to problems in these areas (Roboz, 1975): (1) confirmation of identity, (2) identification of unknowns, (3) elucidation of the structure of complex molecules, (4) quantitation of selected constituents present in ultratrace quantities, and (5) dynamic analysis of major constituents *in vivo*.

Initially, the technique was used mainly for qualitative purposes (Morris, 1980; Roboz, 1975; Waller, 1972; Waller and Dermer, 1980). In this context, integrated gas chromatography/mass spectrometry (GC/MS) has proved to be one of the most versatile and powerful tools in analytical chemistry (Halpern, 1981; Mellon, 1981). Because of its unique capability to separate and simultaneously identify the components of even very complex mixtures, GC/MS is especially well suited for the identification and structure elucidation of unknown constituents, as well as for the confirmation of peak identity in, e.g., a metabolic profile. This has proved of great value in the diagnosis of metabolic disorders and has led to the discovery of a number of new metabolites and diseases (Halpern, 1981; Roboz, 1975). The introduction of high-resolution capillary gas chromatography and of alternative ionization methods, new sampling and desorption techniques, and especially powerful data systems have marked further progress in this area (Burlingame *et al.*, 1982; Hill and Whelan, 1984).

In addition to these qualitative applications, it was soon realized that a mass spectrometer, coupled to a gas chromatograph, could also be used as a specific quantitation device. A major development in this field was the introduction of the technique of "selected ion monitoring" (SIM) or "mass fragmentography" (MF) (Sweeley *et al.*, 1966; Hammar *et al.*, 1968). The extensive possibilities of this new quantitation method rely on the selective monitoring of only one or a restricted number of relevant m/z values. Molecules which do not generate ions at these specific values are not detected. Because of this selectivity and the time averaging of the signal achievable in the fixed focus mode, the noise level is greatly reduced and the sensitivity is increased by a factor of up to 1000 compared to that obtained for the same ions during a mass scan (Halpern, 1981).

Due to this unsurpassed combination of specificity and sensitivity, quantitative SIM has revolutionized the measurement of low-molecular-weight organics down to the picogram level. Some typical examples of applications

include amino acids (Bengtsson *et al.*, 1981; Lehmann *et al.*, 1981), analysis of drugs and metabolites (Garland and Powell, 1981), bioavailability and metabolism studies using stable, isotope-labeled drugs (Eichelbaum *et al.*, 1981; Schmid *et al.*, 1980), biocides (Kuehl *et al.*, 1980), biological markers (Abbott *et al.*, 1980; Muskiet, 1982), carbohydrates (Ashraf *et al.*, 1980; Petit *et al.*, 1980), fatty acids (Stan and Scheutwinkel-Reich, 1980; Vine, 1980), inorganic compounds (Schulten and Lehmann, 1978; Schulten *et al.*, 1983), lipids (Vergey *et al.*, 1981), neurotransmitters (Matthews *et al.*, 1978; Suzuki *et al.*, 1980), nucleic acid components (Jardine *et al.*, 1980; Johnson *et al.*, 1980), peptides (Desiderio *et al.*, 1980), pollutants (Kimble and Gross, 1980; Zakett *et al.*, 1981), prostaglandins (Chiabrando *et al.*, 1980; Rosello *et al.*, 1981), steroids (Adlercreutz, 1977; Gaskell and Pike, 1978; Siekmann, 1978), urinary organic acids (Hunt *et al.*, 1982; Sweeley *et al.*, 1981), and vitamins (Chiang, 1980; Holmen *et al.*, 1981).

Today, the development of quantitative procedures has become at least as important in biomedical mass spectrometry as identification work. Since excellent reviews on the current status of quantitative MS are available (Burlingame *et al.*, 1982; De Leenheer and Cruyl, 1980; Garland and Powell, 1981; Halpern, 1981; Millard, 1978a; Schulten and Lehmann, 1978; Sweeley *et al.*, 1977), this contribution will focus on one of the new developments in this field: the use of isotope-dilution mass spectrometry (IDMS) in the development of reference methodology.

1.2. Isotope-Dilution Mass Spectrometry—Principle and Scope

In quantitative mass spectrometry, the signal intensity depends not only on the amount of sample, but also on a number of other variables such as the ionization yield, focusing of the ion beam, and the amplification factor of the detector. As it is very difficult to keep these parameters constant over the whole period of analysis, nearly all quantitative applications of MS are based on a comparison of the ion current obtained from the component of interest, with the ion current obtained from a standard. In quantitative SIM this can be accomplished either by the continuous admission of a reference sample at a constant rate, concurrently with the sample under investigation, or by the use of an internal standard (IS) which is added to the sample prior to MS analysis (Halpern, 1981). The choice of this IS is of primary importance in the design of a new assay and was subject to some controversy in the late 1970s (Claeys *et al.*, 1977; Lee and Millard, 1975; Millard, 1978b; Self, 1979). Ideally, an IS should compensate for all possible losses during sample isolation, purification, derivatization, and separation steps and at the same time minimize variances due to the measurement process. In practice, the

selection of an IS should be based on a consideration of its effect upon limiting errors at each stage of the analysis, with the greatest weight being attached to the reduction of the largest errors (Millard, 1978b). There are three possible choices:

1. A stable isotope-labeled analog of the analyte, with (nearly) the same retention time on GC but a different *m/z* value.
2. A close homolog of the compound of interest, which can be chosen to give the same *m/z* value and which has a different retention time of GC.
3. A compound of the same chemical class that may either have the same *m/z* value, in which case the retention time must be different, or a different *m/z* value, in which case the retention time may be different or the same.

Millard and Lee came to the conclusion that for the mass spectrometric part of the assay, a type 2 internal standard is superior to the other two, as the mass spectrometer can then be operated in the single-ion mode, which increases both the sensitivity and precision of the instrument (Lee and Millard, 1975; Lee and Millard, 1978; Millard, 1978b). For biological work, the most important sources of error however do not lie in the mass spectrometric measurement, but in the extraction, evaporation, and derivatization stages (Millard, 1978b). Although a type 2 internal standard may compensate for these losses as well, we agree with Claeys *et al.* (1977) that, where one aims at the highest obtainable precision and accuracy (as in the development of reference methodology), isotope-dilution mass spectrometry, with a stable or radioactive isotope-labeled analog of the analyte as IS, is to be preferred. As the physicochemical properties of such an IS are virtually identical to those of the analyte, no separation takes place during the isolation, derivatization, cleanup, and chromatographic steps. Only during the final, mass spectrometric step of the analysis does a differentiation take place based upon the difference in molecular weight between the labeled and unlabeled molecules. As the number of ions produced is proportional to the number of molecules in the sample, the mole ratio of compound and internal standard can be calculated from the isotopic ratio measured. Quantitative determinations are made by comparing the isotopic distribution in an unknown sample with the isotopic ratio of standard mixtures with known concentrations of analyte and internal standard.

The most important advantage of such an IDMS procedure is that it compensates in an ideal way both for losses during sample workup and for variations in mass spectrometric response. As demonstrated by Claeys *et al.* (1977), the use of a stable isotope-labeled internal standard, as opposed to the use of homologs, produces the lowest variance factors due to instrumental stability and sample manipulating errors. Moreover, IDMS can be used both with and without prior chromatographic separation, as for instance in

the direct-inlet analysis of inorganic ions (Schulten and Lehmann, 1978; Schulten *et al.*, 1983). Finally, the addition of an excess (10:1 to 1000:1) of a stable isotope-labeled analog could act as a carrier to minimize adsorptive and other losses of analyte during the sample workup and chromatographic separation (cf. Section 2.1).

2. General Approaches

2.1. Analytical Setup

IDMS involves the precise addition of an isotopically labeled form of the analyte to an accurately measured sample of the specimen, e.g., serum. After an appropriate equilibration time, the analyte and its labeled internal standard are isolated from the sample with a suitable extraction and purification step, and an aliquot is introduced, either directly or after (gas) chromatographic separation from remaining interferences, into the mass spectrometer. The latter accurately measures the ratio of analyte to internal standard using the intensities of an equivalent ion in the spectrum of each. From this ratio, the concentration of analyte is calculated by comparison with the ratios of the same ions in standard calibration mixtures. Critical points in this procedure are as follows:

1. The accurate sampling (by volume or weight) of the biological material.
2. The selection of a suitable internal standard.
3. The precise addition of this internal standard to both sample and calibration standards.
4. The equilibration of the sample with the IS.
5. The avoidance of discrimination between analyte and IS during the various steps of the analytical procedure.
6. The accurate and precise measurement of the isotopic ratio and its conversion into a mass ratio.
7. The purity of the calibration standards.

Accurate sampling supposes a proper homogenization of the specimen. It can be performed either volumetrically with a calibrated pipet or by weighing. It should be realized that, together with the precise addition of the IS and the accurate measurement of calibration solutions, this is the only step where eventual losses are not compensated for by the presence of a labeled analog. Thus, accuracy and precision may be ruined regardless of how good and precise the subsequent IDMS analysis may be. This is a matter of the utmost importance in the development of definitive methods and will be further discussed in Section 4.4.2.

The selection and/or synthesis of a labeled IS are treated in Sections 2.2 and 2.3. An important assumption is that the isotopically labeled form of the analyte will act during the various stages of the assay in an identical manner to the analyte itself. This is not always obvious.

First of all, it is almost impossible to prove that during the extraction of the sample, the recovery of internal standard and analyte will be the same. Especially in the case of an endogenous compound, specific protein binding may play an important role. A complete equilibration of the labeled analog with the matrix is a prerequisite but does not guarantee a true reproduction of the natural binding process (Vessman, 1980). Moreover, standard addition experiments do not validate equilibration, but at best demonstrate accuracy and precision of the extraction and measurement of the externally added compound (Lawson *et al.*, 1980). In any case, the IS should be added to the sample in a minimal amount of (organic) solvent (e.g., 20 μl/ml) in order to minimize its influence on the biological matrix.

Subsequent stages of the assay rely both on the equivalence of analyte and IS and on their reproducible behavior with respect to each other (Lawson *et al.*, 1980). Although it is generally accepted that compounds that are as closely related as an analyte and its labeled analog will display the same chemical and physical properties, a significant difference in retention time may be observed during high-resolution gas chromatographic or liquid chromatographic separation steps (De Ridder and Koppens, 1978; Oates *et al.*, 1978). This is especially true when analogs with more than four deuterium atoms are used as IS (Dehennin *et al.*, 1980). A theoretical consideration of these effects on ratio calculations has been reported by Matthews and Hayes (1976).

Also during the sample workup, a certain discrimination may occur. One should avoid situations in which the label is situated in a position that affects solvation properties, pK_a values, or derivatization kinetics (Millard, 1978b). Differences in the peak ratio of thyroxine and its $^2H_2$-labeled IS, before and after ion exchange chromatography, however, were ascribed to interfering material originating from the column or solvents (Moller *et al.*, 1983).

An important point of discussion is the so-called "carrier effect" of deuterated analogs. When very small (subnanogram) amounts of analyte are measured, a departure from linearity is often observed (Fig. 1a), due to adsorption of the compound on glassware, chromatography columns, molecular separators, etc. (Millard, 1978a,b). It was shown on several occasions (Samuelsson *et al.*, 1970; Gaffney *et al.*, 1971; Haskins *et al.*, 1978) that the addition of an excess of deuterium-labeled IS (from 10:1 to 1000:1) could enhance the sensitivity of the assay and restore linearity in the (sub)nanogram range (Fig. 1b) by minimizing these adsorption phenomena. Adsorption-reducing compounds or carriers have been used from Tswett's days

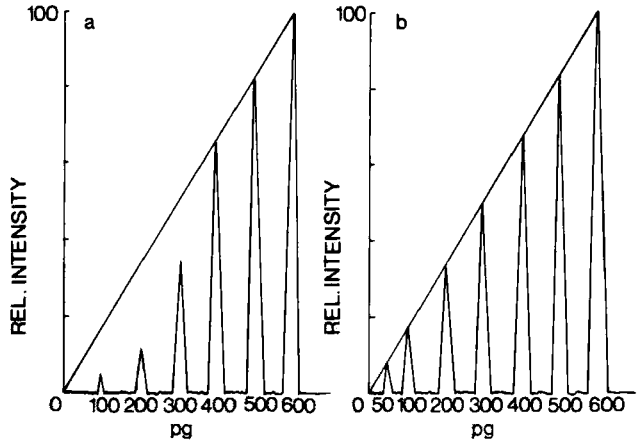

FIG. 1. Effect of the addition of an excess of labeled analog on the linearity of an IDMS assay: (a) without and (b) with carrier added as described by Millard (1978a).

onward (Self, 1979), but labeled analogs would have a distinct advantage in that their chromatographic retention and (column) adsorption properties are nearly the same as those of the analyte (Gaffney *et al.*, 1971). Millard *et al.* (1977), however, could not demonstrate any carrier effect due to the addition of the deuterated analog of octopamine at either the solvent extraction, or GC/MS stage. Conversely, Haskins and co-workers, in a study designed to prove the existence of a carrier effect in the diphenoxylate assay, showed a sevenfold increase in sensitivity when the tetradeuterated analog was used as carrier and internal standard (Haskins *et al.*, 1978). According to Self (1979), these conflicting results might be explained by the existence of two types of adsorption phenoma: irreversible adsorption at component specific sites and nonspecific reversible adsorption at active sites on the GC column, glassware, etc. The addition of an excess of an isotopically nonpure IS would introduce a sufficient amount of unlabeled analog to saturate the specific adsorption sites. The deuterium-labeled analog, on the other hand, would compete in a reversible way for the nonspecific adsorption sites and thus cause a frontal displacement of the nonlabeled compound. As these adsorption phenomena are highly dependent on the type of column used (Haskins *et al.*, 1978), one can imagine that with either a well-deactivated system (column, glassware, separator, and ion source) or an IS of high isotopic purity, the deuterated analog would lose its carrier effect (Self, 1979). It is important to note that in this case, the use of a large excess of labeled IS would decrease the sensitivity and precision of the analysis (Millard, 1978b). Thus, an excess of IS should *not* be used unless a carrier effect has clearly been demonstrated.

Finally, the presence of heavy isotopes may significantly alter the fragmentation pattern and ionization efficiency of a labeled IS. Thus, the rate of cleavage of C[^{2}H] bonds can be up to 14 times slower than similar cleavage of C[^{1}H] bonds (Haskins, 1982). These *primary isotope effects* give rise to different intensities for the same fragment ion in the labeled and nonlabeled analog. *Secondary isotope effects* occur when the presence of an isotope influences the cleavage of bonds between unlabeled atoms (Millard and Shaw, 1966). Although certain fragment ions sometimes are not used for quantitative SIM because of these isotopic effects, the differences are constant in time and do not prevent quantitative analysis.

The accurate and precise measurement of isotope ratios forms the cornerstone of each IDMS analysis. First, it is important to introduce the compounds of interest into the ion source in a pure and concentrated form. Depending on the nature of the sample, the concentration of the analyte and the specificity of the ions formed, a direct introduction of the purified sample may be possible, or a preliminary chromatographic separation may be needed. The first technique is most often used in the IDMS assay of inorganic ions (Schulten *et al.*, 1983). For organic compounds however, a chromatographic separation prior to MS analysis is usually indicated. GC is well suited for on-line coupling with MS (GC/MS), and good results have also been reported on the use of liquid chromatography/MS combinations (LC/MS) (Arpino and Guiochon, 1979; Mellon, 1981; Smith *et al.*, 1981). A preliminary chromatographic separation enhances the specificity and sensitivity of the assay and allows the simultaneous determination of several sample components. Nevertheless, it should be realized that a goal such as "absolute specificity" is almost unattainable, even if one applies techniques of highest resolution such as capillary GC and/or high-resolution MS (De Leenheer and Cruyl, 1980; Gaskell and Pike, 1978; Hummel and Shadoff, 1980).

The selection of the ions to be monitored is also of the utmost importance, as it will influence both the specificity and sensitivity of the assay. As a general rule, monitoring of the molecular ion, if sufficiently intense, will be preferred to the monitoring of fragment ions, because of its greater specificity for the compound to be determined. As for fragment ions, recording of ions at higher m/z values generally keeps interferences from instrumental background, column bleed, and coeluting sample components at a minimum (De Leenheer and Cruyl, 1980; Gaskell, 1982). One should also avoid the monitoring of m/z values typical for column bleed or sample workup steps. Eventually, an increase in molecular weight by the formation of suitable derivatives may be considered (Halpern, 1981). A very interesting example of selective derivatization was given by Colby and McCaman (1978), who reported a great improvement in selectivity by the formation of dinitro-

phenylethyl derivatives of γ-aminobutyric acid instead of trimethylsilyl ethers. This paper also illustrates very well the point that even a highly sophisticated technique such as GC/SIM does not offer absolute specificity and that other steps such as derivatization and cleanup should be given thoughtful consideration in view of their impact on sensitivity and specificity. Another possibility is the simultaneous monitoring of several characteristic fragment ions. It will allow the detection of unexpected interferences by a change in the peak ratio of different channels. Multiple ion detection (MID), however, has an adverse effect on sensitivity and instrumental stability (Halpern, 1981) and should not be used when highest precision and accuracy are required. An alternative approach is to monitor metastable peaks corresponding to fragmentations occurring in the first field-free region of a double-focusing MS (Halpern, 1981). This highly selective technique allows detection limits in the low picogram range (Harvey *et al.*, 1980). All this of course requires a good knowledge of the mass spectrometric properties of the compound of interest. To a certain extent, optimal conditions can be predicted on the basis of theoretical considerations (Colby *et al.*, 1979), but always should be verified experimentally to obtain the most accurate and precise results for a given compound in a given matrix.

After proper adjustment of the instrumental parameters for an optimal detection of the chosen ions (Eldjarn *et al.*, 1978; Matthews and Hayes, 1976; Matthews *et al.*, 1978; Sweeley *et al.*, 1977), analyte can be measured and a calibration can be performed. The latter involves the correlation of the measured isotope ratios of analyte and IS with their mass ratios, in at least six calibration standards. These standards should cover the useful concentration range which, in its turn, depends on the spiking level and m/z values selected (Colby *et al.*, 1981) and on the calibration model adopted (cf. Section 3). Alternatively, a "bracketing" procedure may be adopted with standards closely surrounding the anticipated concentration of the analyte (Cohen *et al.*, 1980; Yap *et al.*, 1983) (cf. Section 3.5). Calibration standards should be prepared from concentrated stock solutions in order to avoid adsorptive losses (Millard, 1978b). To account for matrix effects on isolation and derivatization, calibration should be performed with spiked samples, processed in exactly the same way as the unknown. If the analyte is an exogenous compound (e.g., a drug), a pooled drug-free sample provides an excellent biological matrix for spiking. For endogenous components, the intercept of the calibration graph yields the basal level of the compound in the pooled sample, to be subtracted each time from the value obtained on the unknown (De Leenheer and Cruyl, 1980). Another possibility is to prepare blanks by removing the endogenous compound from the matrix as described by Eldjarn *et al.* (1978). In the analysis of glucose in serum, for example, the serum can be treated with glucose oxidase to remove glucose completely and in a

highly specific manner. The glucose-free serum can then be used as a blank. In a similar way one can remove urea completely by treatment with urease, uric acid by treatment with uricase, and so on. As for the standards, it is logical that no accurate measurement can be performed without knowledge of the exact purity of the calibration material. This is again a matter of the utmost importance in the development of definitive methods, where well-characterized primary reference material of highest and certified purity must be used (cf. Section 4.2).

Finally, the calibration curve seldom is linear, due to mutual interference of cluster ions of the analyte and unlabeled molecules in the IS. Although this problem may be circumvented to some extent by calibration over a very narrow concentration range (bracketing) and/or a proper choice of m/z ratios and spiking level (Colby *et al.*, 1981; Yap *et al.*, 1983), a mathematical data reduction as described in Section 3 generally is the best approach.

2.2. Selection of Internal Standard

On theoretical grounds, the molecular weight of the IS has to be increased, preferably by at least 3 mass units, to avoid interference of natural isotopes of the analyte on the m/z value of the labeled compound. If not, a proper mathematical treatment of calibration data may still allow an accurate calibration (cf. Section 3), but highly nonlinear calibration curves will cause a distortion of the normal result distribution (Picart *et al.*, 1978) (Fig. 2). A difference of more than 4–5 mass units, on the other hand, will cause a net chromatographic separation between IS and analyte and thus negatively influence the precision of the assay (Matthews and Hayes, 1976) and abolish any carrier effect during the GC run and transfer to the ion source (Dehennin *et al.* 1980; Picart *et al.*, 1978). Moreover, the label has to be present at a stable position in the molecule to avoid an isotopic exchange during the IDMS assay or in stock solutions (De Leenheer and Cruyl, 1980; Haskins, 1982). This is especially true for molecules labeled with deuterium (^2H) and tritium (^3H). Finally, extraction and derivatization must not show any isotope effect (Millard, 1978b).

In practice, two possibilities exist: the use of a stable isotope-labeled molecule or a radiolabeled IS. The most commonly used stable isotope is deuterium, which can be incorporated relatively easily into molecules. ^{15}N, ^{13}C, and ^{18}O are used too, but to a lesser extent. Considerable efforts have been made to increase the availability and range of stable isotope-labeled compounds. In the United States, the National Stable Isotope Resource Center has been established at the Los Alamos Scientific Laboratories to help meet the need for stable isotope-labeled compounds. Funds have been made available for the synthesis of many labeled compounds in sufficient

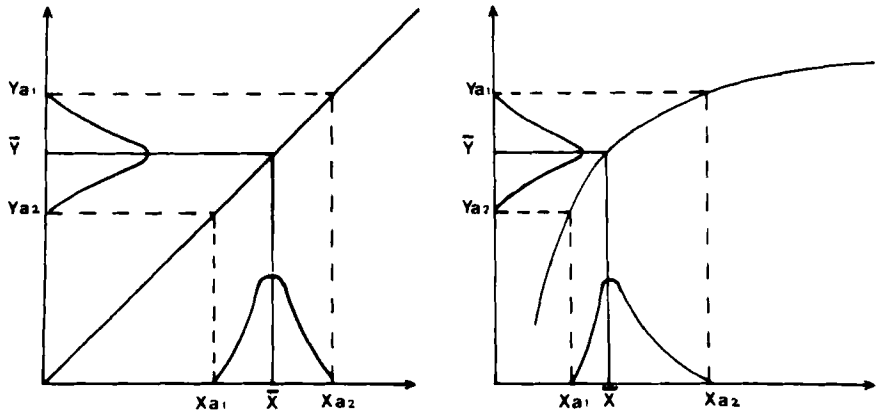

FIG. 2. Distortion of normal results distribution using linear and nonlinear calibration curves as described by Picart *et al.* (1978). Copyright Elsevier Science Publishers B.V., Amsterdam.

purity for IDMS analysis (Halpern, 1981). A number of commercial firms nowadays have such compounds in their product line. For a general review on the use of stable isotopes in biomedicine we refer to Haskins (1982) and Krahmer and McCloskey (1978).

As an alternative to a stable isotope-labeled IS, ^{14}C- or ^{3}H-radiolabeled analogs can be used. They are generally less expensive and more readily available than stable isotope-labeled molecules, and save one from the often considerable effort (and expense!) of synthesizing a labeled analog. From the MS point of view, these radiolabeled analogs can be considered identical in their applicability to stable isotope-labeled analogs (De Leenheer and Cruyl, 1980). However, the use of radioisotopes could lead to a contamination of the instrument when large numbers of analyses are performed (Halpern, 1981). Moreover, isotopic enrichments of ^{14}C-labeled molecules are often rather low, while ^{3}H-labeled analogs present a potential health hazard due to the presence of significant tritium activities in the vapor phase (Dehennin *et al.*, 1980). Thus, if a radiolabeled internal standard is used, all necessary precautions should be taken to protect the analyst from radiation, while the isotopic purity of the compound should be controlled at regular intervals to check for radiolysis.

2.3. Synthesis of Labeled Compounds

If no suitable IS is available, one must synthesize it. The simplest approach is an exchange of mobile hydrogen atoms in the unlabeled molecule with ^{2}H atoms of a deuterated solvent. Good results have been obtained

with an acid- and a base-catalyzed proton exchange in 17- or 20-oxosteroids and phenolic steroids, respectively (Dehennin *et al.*, 1980). In general, however, one can expect these labels to be lost fairly easily during the various steps of the IDMS assay, e.g., by proton exchange with solvent molecules or water vapor. A better approach, but more laborious and costly, is to incorporate ^{13}C or ^{2}H in a stable position of the molecule starting from a labeled precursor molecule, or by synthesis with a deuterium-labeled reagent such as $C^{2}H_3I$ (Jonckheere *et al.*, 1980). When a precursor containing a double bond or reducible keto group is available, deuterium atoms can also be incorporated by catalytic reduction in the presence of deuterium gas or by reduction with sodium borodeuteride (Dehennin *et al.*, 1980).

Stable isotope-labeled derivatives (De Leenheer and Cruyl, 1980) are much easier to prepare (incorporation of the isotope via a labeled derivatizing agent), but do not fulfill all the purposes of the stable isotope-labeled analog, as they only account for losses *after* the derivatization process, such as variations in ionization efficiency and mass spectrometric response.

2.4. Characterization of the Isotope Enrichment of the IS

Even when highly enriched compounds are used in the synthesis of a labeled molecule, the labeling reaction never will be 100% complete. This results in the presence of a number of unlabeled and partially labeled molecules in the IS, which will give a response at the same m/z value as the unlabeled analyte. An exact knowledge of the incorporation efficiency is required as it influences both the detection limit and precision of an assay (Dehennin *et al.*, 1980). Furthermore, the relative isotopic abundances of labeled and unlabeled molecule should be known to allow accurate calibration (cf. Section 3).

There are several ways to calculate the isotopic enrichment of an IS (Beynon and Williams, 1963; Biemann, 1962; Millard, 1978a). The fastest and most accurate approach (Jonckheere, 1982) is to compare similar ion clusters of the labeled and unlabeled molecule and to use computer facilities to calculate the contribution of each variant to the ion cluster of the labeled molecule. In this, it is preferable to use the ion cluster of the intact molecule (i.e., molecular ion) to avoid isotope effects in the fragmentation process and the possibility of different fragment ions with the same m/z value (low-resolution MS).

First of all, the isotope composition of the labeled compound is measured by recording a selected ion current profile corresponding to the molecular ions of zero-, mono-, di-, tri-, and n-labeled analog. Preferably the integration time on each channel should be adjusted in order to produce ion statis-

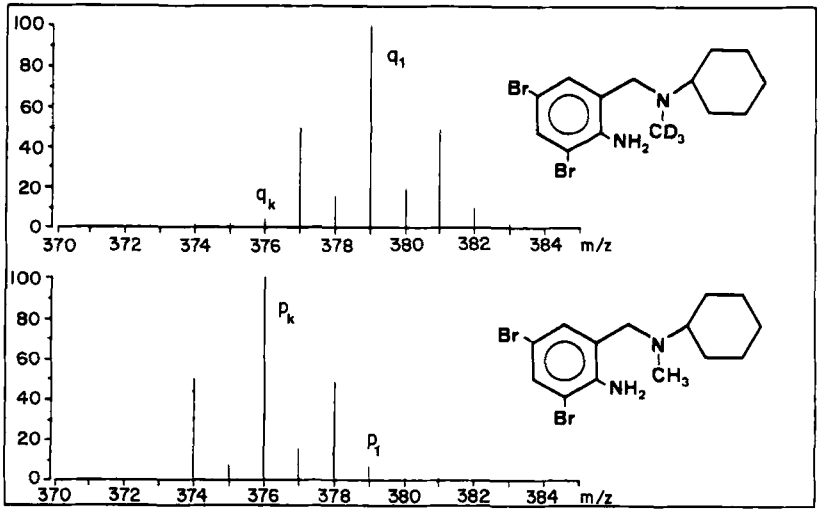

FIG. 3. Ion clusters of the molecular ions of [$^2H_3$]bromhexine and unlabeled bromhexine. Reprinted from Jonckheere (1982) with permission of the author.

tical errors which are similar (Dehennin *et al.* 1980). These experimental values are then compared with the ion cluster of the pure and unlabeled compound. This is shown in Fig. 3 with bromhexine and [$^2H_3$]bromhexine as an example. The intensity at m/z 374 (0.4) in the spectrum of [$^2H_3$]bromhexine can originate only from unlabeled bromhexine, present as an impurity in [$^2H_3$]bromhexine. On the basis of the relative intensities of the M + 1, M + 2, and M + 3 peaks in the ion cluster of the unlabeled molecule, one can then calculate the contribution of unlabeled bromhexine to the intensities at m/z 375, 376, and 377 in the ion cluster of [$^2H_3$]bromhexine. Thus, the intensity of the ion at m/z 375 (1.90) becomes, after correction, $1.90 - (0.4 \times 0.2172) = 1.81$. This intensity only originates from [$^2H_1$]bromhexine and can be used to calculate in an analogous way the contribution of monosubstituted bromhexine to the ion cluster of the labeled compound, and so on. This gives rise to the following linear equations:

$$
\begin{aligned}
y_1 &= a_1x_1 \\
y_2 &= a_2x_1 + a_1x_2 \\
y_3 &= a_3x_1 + a_2x_2 + a_1x_3 \\
y_n &= a_nx_1 + a_{n-1}x_2 + a_{n-2}x_3 + \cdots + a_1x_n
\end{aligned}
$$

in which y_i represents the intensity of the ions in the cluster of the labeled compound, a_i the intensities in the cluster of the unlabeled compound, and

x_i the concentration of the respective isotopic variants. In matrix notation this can be written as

$$[Y] = [A] \cdot [X]$$

from which $[X]$ is solved as

$$[X] = [Y] \cdot [A]^{-1}$$

A computer program builds up the $[A]$-matrix on the basis of the theoretical cluster (a_i) of the unlabeled molecule and calculates its inverse, $[A]^{-1}$. The $[Y]$-vector is built up from the experimentally measured intensities (y_i) of the labeled component. After calculation, the $[X]$-vector then gives the concentration of each variant (x_i).

A more complex situation arises when a label is used with a high natural abundance of stable isotopes, e.g., ^{13}C that occurs in nature as 1.07% (compared to 0.016% for ^{2}H). In that case the cluster will have to be adapted for each variant. Again, a computer program can be used to calculate the theoretical clusters for each variant and thus build up a corrected matrix $[A]$ (Jonckheere, 1982).

2.5. Instrumental Requirements

In GC/MS, analytical packed columns are still often used, mainly because of their well-established characteristics, simple injection technique, high sample capacity, and the fact that most instrumentation has been designed for operation with such columns. However, the separation efficiency and transfer to the MS are in most cases inferior to open tubular (capillary) columns.

Today, the introduction of fused silica capillary columns (Dandenau and Zerenner, 1979) has greatly expanded the possibilities of installation in mass spectrometric systems, as compared to the less rugged glass open tubular columns. These columns offer a high separation efficiency, shorter analysis times, and a better sensitivity due to the narrower band width of the eluting analyte and the potential for direct coupling to the MS ion source, allowing almost quantitative sample transfer. In our opinion the use of capillary columns is often mandatory in order to obtain adequate results in trace analysis of substances of biochemical interest. Moreover, a single column can be used to cover a wide range of applications which previously required the use of several liquid phases.

Sample introduction remains one of the most critical aspects in capillary GC. It can be achieved by direct injection (Chauhan and Darbre, 1981; Grob and Grob, 1972; Sonchik and Walker, 1979), stream splitting (Schomburg *et al.*, 1977, 1981), splitless injection (Grob and Grob, 1969a,b; Grob and

Romann, 1981), desorption from a cold trap (Kalman *et al.*, 1980; Rijks *et al.*, 1979), solid injection using an all-glass moving needle (De Jong, 1981; Van den Berg and Cox, 1972), and nonvaporizing cold on-column injection (Galli *et al.*, 1979; Knauss *et al.*, 1981; Schomburg *et al.*, 1981). As the amount of biological sample available is often limited, methods involving stream splitting cannot be used for trace analysis. Moreover, a distinct discrimination of higher boiling compounds will occur (Schomburg *et al.*, 1977, 1981). Direct injection, on the other hand, is restricted to the use of less efficient medium-bore open tubular columns and results in a large, broad solvent peak and rapid contamination of the column with biological material. These problems can be circumvented by a splitless injection that combines good chromatographic performance with high sensitivity and ease of operation and automation. The technique is quite popular, e.g., in steroid profiling, but has several drawbacks and pitfalls. Like direct and split injection, it is a vaporizing injection technique which is not suitable for thermolabile compounds and will tend to discriminate higher boiling compounds (Schomburg *et al.*, 1981). The extent of this discrimination strongly depends on the type of injector used and experimental conditions chosen. Thus, 95% of the sample should be transferred to the column in order to obtain a satisfactory quantitation (Grob and Romann, 1981) and special care should be taken in syringe handling to avoid sample discrimination (Galli *et al.*, 1979; Grob and Rennhard, 1980; Schomburg *et al.*, 1977, 1981).

In our opinion, solid (or falling needle) and cold on-column injection at present offer the best precision, accuracy, and sensitivity for quantitation purposes (De Leenheer *et al.*, 1982; Verzele *et al.*, 1980). Each design has its own advantages. With the falling needle injector, higher molecular weight matrix components tend to polymerize on the needle during injection and thus do not enter the column, while all of the solvent is evaporated before injection. Both features significantly increase column lifetime and thus make this injection device especially well suited for routine analysis of biological samples. Moreover, injection can be performed at an elevated oven temperature, which obviates the need for long temperature programs (De Jong, 1981). Finally, the falling needle injector allows the introduction of larger sample amounts (up to 3 μl) into the chromatographic system, thus contributing to greater overall sensitivity (De Leenheer and Gelijkens, 1978). The fact that it cannot be used quantitatively for compounds with a boiling point lower than 300°C is no major drawback for most clinical applications. The cold on-column approach, on the other hand, allows the introduction of thermolabile compounds under very mild conditions and is the only injection technique which is completely free of discrimination effects for both low- and high-boiling substances (Schomburg *et al.*, 1981). It does however require extensive temperature programming and considerable

experience is needed to avoid problems of peak broadening and peak splitting (Galli *et al.*, 1979; Grob and Neukom, 1980; Grob, 1981, 1982). Moreover, the whole of the sample, including high-boiling impurities and excess of derivatization reagents, is introduced into the column, which is a serious threat to column life and performance. As an alternative, a novel splitless injection technique may be considered that features cold sample introduction into an insert or small packed precolumn that can be heated separately (Poy *et al.*, 1981; Schomburg *et al.*, 1983). This new technique features improved precision and accuracy as compared to the classical splitless injection, and has the advantage over cold on-column injection in that it can be automated. High-boiling contaminants remain in the insert, which can be cleaned separately.

As far as the column is concerned, a good coating efficiency, inertness, and long-term stability are of particular importance for quantitative purposes. Good quality columns covering the whole range of nonpolar, medium polar, and polar stationary phases are commercially available now. Compared to borosilicate or soft glass, the fused silica material not only offers a higher mechanical strength and inherent straightness, but also a more inert surface (with slightly acidic character) and a low metal oxide content (<1 ppm) (Lipsky *et al.*, 1980). Still, proper deactivation is necessary: persilylation techniques (Godefroot *et al.*, 1980; Grob *et al.*, 1979) and polysiloxane deactivation (P.D.) (Houtermans and Boodt, 1979) appear to be the most effective at present, while a pyrocarbon treatment also has been shown to yield good results (Pretorius and Desty, 1981). In comparison with the older carbowax type deactivation, these columns show much lower bleeding characteristics and a highly improved temperature stability, while their quasi-perfect inertness has been demonstrated by direct chromatography of underivatized barbiturates and steroids (Kovarich and Munari, 1982). Film stability has been greatly improved both by the evolution in coating techniques and the introduction of chemically bonded (Blomberg *et al.*, 1981) or high temperature cross-linked (Sandra *et al.*, 1981) phases. The latter have a quasi-zero solubility in commonly used injection solvents, which is of particular importance in splitless and on-column injection; nevertheless, the use of a so-called "retention gap" still may be required in cases where interaction of the solvent with the stationary phase causes peak splitting (Grob, 1982). Another feature of these chemically bonded phases is that they can be cleaned periodically with an organic solvent to remove nonvolatile deposits.

A poorly designed GC/MS interface can easily compromise the performance of both the capillary GC system and the mass spectrometer. The interface should provide an inert transfer surface, yield full sample transmission, and maintain the chromatographic performance of the column.

Due to the lower carrier gas flow rate, instruments with a differentially

pumped ion-source/mass-analyzer system can generally accommodate the full effluent of a narrow-bore open tubular column. As a result, the capillary column can be coupled directly to the ion source, which guarantees a complete sample transfer through a highly inert interface. In this configuration the column outlet is operated at reduced pressure, which is hardly detrimental to column performance. On the contrary, the optimal carrier gas velocity shifts to higher values, thus permitting even faster analyses (Cramers *et al.*, 1981). As an alternative to this direct coupling, the availability of fused silica restrictors and transfer lines has revived interest in the open split coupling method (Henneberg *et al.*, 1975). Though sample transfer is not always quantitative, this technique allows the capillary column to be operated at atmospheric outlet pressure, independently of the ion-source vacuum system. Furthermore, unwanted effluent components (e.g., solvent peak) can be vented by changing the helium purge flow through the interface.

When, on the other hand, a nondifferentially pumped system is used, direct coupling generally will be impossible. In that case either an open split device or a carrier gas separator must be installed. When using a jet or membrane separator, one should keep in mind that these devices have been designed for packed columns. Thus, make-up gas must be added to the column effluent to restore optimal working pressure and purge unswept volumes.

Finally, to increase analysis speed, hydrogen can be used as carrier, but instruments with a nondifferentially pumped ion source may present some difficulty in handling this gas.

As for the mass spectrometric system itself, the application of capillary columns requires the use of fast scanning devices (quadrupole or laminated magnet) and improved data systems capable of collecting enough data points across the narrow chromatographic peaks in order to maintain the required precision for quantitation (De Leenheer *et al.*, 1982). Built-in integration algorithms often lack the precision and accuracy required for reference methodology. Therefore off-line data processing and/or manual peak integration may be required to produce highly reproducible peak ratio measurements (Cohen *et al.*, 1980; Jonckheere and De Leenheer, 1983).

3. Mathematical Techniques

3.1. Essentials

Although an extensive mathematical treatment of all calibration models is certainly beyond the scope of this article, it should be realized that correct

data reduction is the only way to obtain accurate results. Accurate use of a standard curve requires an exact knowledge of its form, whereas the precision of the analysis is more a function of chemical and instrumental parameters. Thus, sufficient sample should be injected to allow good ion statistics, and the ratio of analyte and IS ideally approaches 1 (Chiabrando *et al.*, 1980; Colby *et al.*, 1981; Matthews and Hayes, 1976). Calibration curves in IDMS are essentially nonlinear, due to the presence of unlabeled material in the IS and the natural abundance of higher isotopes in the analyte (cf. Section 2.4). According to Pickup and McPherson (1976), the relationship between the measured isotope ratio R_m and the actual amount of labeled and unlabeled material can be written as

$$R_m = [(x/M_1)p_i + (y/M_2)q_i]/[(x/M_1)p_j + (y/M_2)q_j] \quad (1)$$

where R_m is the ratio of the abundance of the isotopic form representing the natural material to the abundance of the isotopic form representing the labeled internal standard; x and y are the masses of analyte and labeled material present in the sample; M_1 and M_2 their respective molecular masses; p_i and q_i represent the abundance of the isotopic form (i) corresponding to nonlabeled material, in unlabeled and labeled material, respectively; and p_j and q_j represent the abundance of the isotope (j) corresponding to labeled material, in unlabeled and labeled material, respectively. Similarly, the relationship between the mass ratio x/y and isotope ratio R_m can be written as

$$x/y = (M_1/M_2)[(q_i/q_j - R_m)/(R_m - p_i/p_j)]\ (q_j/p_j) \quad (2)$$

As M_1, M_2, p_i, p_j, q_i, and q_j are constant for a given analytical situation, a calibration can be performed by measuring R_m for a number of standards with a known x/y ratio. It is clear that the actual values of p_i, p_j, q_i, and q_j will determine the form of this relationship. There are four possibilities:

1. *Both* p_j *and* q_i *are negligible.* In other words, there is no mutual interference of labeled and unlabeled product. Equation (2) can then be reduced to

$$x/y = (M_1/M_2)\ R_m\ (q_j/p_i) \quad (3)$$

and the relationship between the mass ratio x/y and isotopic ratio R_m will be linear. This situation, however, is rather hypothetical in IDMS, as there will always be some unlabeled material present in the IS, even in the case of a high isotopic enrichment.

2. *Only* p_j *is negligible* (labeled IS with a sufficient mass increment to avoid interference of naturally abundant isotopes on its measuring channel). Under these circumstances, a linear relationship will be obtained also:

$$x/y = (M_1/M_2)\, R_m\, (q_j/p_i) - (M_1/M_2)\, (q_i/p_i) \quad (4)$$

3. q_i *is negligible but* p_j *is not.* In this case the following nonlinear relationship is obtained.

$$x/y = (M_1/M_2)\, [R_m\, q_j/(p_i - R_m p_j)] \quad (5)$$

4. *Both* p_j *and* q_i *are not negligible.* In this case Eq. (2) is valid and the relationship is again nonlinear.

In practice, this means that

1. If there is no mutual interference of labeled compound and analyte, the calibration curve is a straight line which passes through the origin.
2. If the IS contributes to the signal of the analyte, but the reverse is not true, a linear calibration curve with a positive intercept is obtained. Provided that the variance on the isotope ratios measured is uniform throughout the whole calibration range, linear regression analysis may be applied. Otherwise, weighting factors should be introduced, e.g., the reciprocals of the variances at different concentration levels (Claeys *et al.*, 1977; Schoeller, 1976).
3. If natural isotopic forms of the unlabeled analyte interfere with the m/z value of the IS, a curved calibration line will be obtained which passes through the origin.
4. If, on the other hand, there is a mutual interference of both unlabeled material in the IS and natural isotopic forms of the analyte, the calibration line will also be curved, but will no longer pass through the origin.

Each of the preceding situations was illustrated by Picart and co-workers (Picart *et al.*, 1978) with a practical example.

From the foregoing theory, it will be clear that in IDMS, the relationship between isotope ratios and mole ratios can only be calculated by linear regression analysis if situation 1 or 2 is true. In all other cases, the assumption of a linear relationship will introduce a systematic error which results in significant inaccuracy. Although several authors still neglect this fact, a number of solutions have recently been proposed to deal with this problem of nonlinearity. Two different approaches can be discerned (Jonckheere, 1982): (a) "theoretical methods" based on the basic equation of IDMS Eq. (1), similar equations, or an approximation of this formula, and (b) "empirical methods" that use a mathematical transformation of empirical data to mask the effect of nonlinearity. Our group, on the other hand, has worked out an alternative approach based upon polynomial regression analysis (Jonckheere *et al.*, 1982).

Bracketing calibration represents a different situation, as only a very nar-

row concentration range is covered. This will be treated separately in Section 3.5.

3.2. Theoretical Methods

3.2.1. *Colby–McCaman Model*

Colby and McCaman (1979) and Colby *et al.* (1981) described an approach based upon an IDMS equation derived from the original work of Inghram (1954) and Hintenberger (1956):

$$X/Y = (R_y - R_m)\,(R_x + 1)/(R_m - R_x)\,(R_y + 1) \tag{6}$$

in which X/Y represents the mole ratio of unlabeled and labeled product; R_x the isotope ratio of the pure unlabeled component; R_y the isotope ratio of the labeled component; and R_m the isotope ratio of a mixture of unlabeled and labeled components.

As a matter of fact, Eq. (6) can be derived from Eq. (2) if one assumes the presence of only two isotopic forms of labeled compound and analyte; in other words, if $p_i + p_j = q_i + q_j = 1$. As $R_x = p_i/p_j$ and $R_y = q_i/q_j$, Eq. (2) can be rewritten as

$$x/y = (M_1/M_2)\,[(R_y - R_m)/(R_m - R_x)]\,(q_j/p_j)$$

or, as $q_j = 1/(R_y + 1)$ and $p_j = 1/(R_x + 1)$,

$$X/Y = (R_y - R_m)\,(R_x + 1)/(R_m - R_x)\,(R_y + 1)$$

Based on this IDMS approximation, corrected isotope ratios can be calculated to construct a linear calibration curve. First R_x and R_y are measured on the pure analyte and IS. Then, the R_m values of a number of calibration standards with a known X/Y ratio are determined. By substituting these R_x, R_y, and R_m values into Eq. (6), the corrected mole ratio $(X/Y)_{calc}$ is obtained, which then can be used to construct a linear calibration curve. Unknown mole ratios are read from this calibration line after recalculation of the isotopic ratio according to Eq. (6).

This Colby and McCaman approach is universal, i.e., applicable to each type of ion interference (1,2,3, or 4, Section 3.1), but its validity entirely depends on the assumption that there are only two isotopic forms of labeled compound and analyte ($p_i + p_j = q_i + q_j = 1$). This certainly is not true for organic compounds, as demonstrated by Jonckheere for the previously cited example of bromhexine and [$^2H_3$]bromhexine ($p_i = 0.4282$; $p_j = 0.0340$; $q_i = 0.0121$; $q_j = 0.4045$) (Jonckheere, 1982). As a consequence, the approximate character of the model may result in an inaccurate calibration, especially if it is used over a wider concentration range (Jonckheere, 1982). Moreover, the experimental determination of R_x and R_y has a considerable

impact on accuracy. As these values are determined by measuring the isotope ratio of pure analyte and "pure" labeled product, and p_j and q_i are very small in comparison with p_i and q_j, the respective ratios p_i/p_j and q_i/q_j will be subject to relatively large errors (Jonckheere *et al.*, 1982). Colby himself stated limits to the concentration range on which acceptable quantitation can be expected (Colby *et al.*, 1981). According to him, low propagated errors and acceptable quantitation can be expected when

$$2R_y < R_m < 0.5R_x$$

while m/z values should be selected to yield the largest log R_x/R_y value (on condition that there are no extenuating circumstances such as interferences). Finally, the optimum quantity of labeled analog to spike into a sample was defined as that which yields an intensity ratio R_m equal to the square root of the product of R_x times R_y (Colby *et al.*, 1981):

$$R_m = (R_x R_y)^{1/2}$$

3.2.2. *Linear Extrapolation of Calibration Results*

A very simple method to avoid nonlinearity is based upon a linear extrapolation of Eq. (2) (Gambert *et al.*, 1979). Only one standard mixture is used. First, the isotope ratio R_{ms} corresponding to a known concentration of unlabeled and labeled compound (X_s/Y_s) is measured. After separate determination of p_i/p_j and q_i/q_j on the "pure" products, the unknown concentration X_x is calculated by linear extrapolation of the mole ratio (X_x/Y_x) corresponding to the isotopic ratio in the unknown (R_{mx}):

$$\frac{X_x/Y_x}{X_s/Y_s} = \frac{[(q_i/q_j - R_{mx})/(R_{mx} - p_i/p_j)]\,(q_j/p_j)}{[(q_i/q_j - R_{ms})/(R_{ms} - p_i/p_j)]\,(q_j/p_i)}$$

or, as $Y_x = Y_s$,

$$X_x = \frac{(q_i/q_j - R_{mx})\,(R_{ms} - p_i/p_j)}{(R_{mx} - p_i/p_j)\,(q_i/q_j - R_{ms})}\,X_s \tag{7}$$

It is clear that this method should only be used over a very small concentration interval and that again, the experimental determination of q_i/q_j and p_i/p_j is a limiting factor to the accuracy of the method (Jonckheere *et al.*, 1982).

3.2.3. *Siekmann Model*

An analogous approach was used by Siekmann in the development of definitive methods for steroid hormones (Siekmann, 1978, 1979). Based upon the theoretical model,

$$x = [(I - f_1 I^*/(I^* - f_2 I)]\,y \tag{8}$$

The amount of analyte x in a sample is calculated from the isotopic intensities I and I^* and the amount of labeled component y, determined in a standard mixture with a known concentration of analyte. The factors f_1 and f_2, which represent the signal ratio I/I^* (q_i/q_j) in the "pure" labeled product and the inverse ratio I^*/I (p_j/p_i) of pure analyte, were determined originally by measuring the isotopic interferences in the labeled product and analyte. Later, a correction of Eq. (8) was proposed (Siekmann, 1982) in which a "proportional factor" f_0 was introduced:

$$x/y = [(I/I^* - f_1)/(1 - f_2 I/I^*)]f_0 \tag{9}$$

A more accurate estimate of f_1, f_2, and f_0 was obtained by using three standard solutions with isotopic ratios of 0.75/1, 1/1, and 1.25/1. Owing to the principle of linear extrapolation, this calibration method is restricted to a narrow concentration range (Jonckheere *et al.*, 1982).

3.2.4. *Garland Model*

The approach described by Garland and collaborators (Garland and Powell, 1981; Min *et al.*, 1978) is generally applicable and is different from the previous ones in that they use nonlinear calibration curves. By dividing both numerator and denominator by p_i, Eq. (1) was rewritten as

$$R_m = \frac{x/M_1 + (y/M_2)(q_i/p_i)}{(x/M_1)(p_j/p_i) + (y/M_2)(q_j/p_i)}$$

With the exception of x, all parameters are constant for a given analytical situation and the relationship has the following form:

$$R_m = \frac{x/M_1 + C_1}{(x/M_1)C_2 + C_3} \tag{10}$$

Calibration is performed by measuring R_m for a number of standard mixtures with a known concentration of analyte x. Depending on the type of ion interference, linear regression analysis is applied if both C_1 and C_2 (type 1 ion interference, Section 3.1) or only C_2 (type 2) are negligible. In all other cases (types 3 and 4), a "NONLIN" computer program is used to calculate the nonlinear calibration curve after experimental determination of C_1, C_2, and C_3 on the pure components. Apart from the fact that the exact form of the "NONLIN" data regression was not specified, the experimental determination of ion interferences and the assumption that $q_j = p_i$ are limiting factors to accuracy (Jonckheere, 1982).

3.2.5. *Bush–Trager Model*

Recently, Bush and Trager published an approach which allows the "linearization" of the calibration curve in all types of ion interference (1,2,3, and

4, Section 3.1) (Bush and Trager, 1981). This was achieved by working with the complete ion cluster of the molecule and inverting the mole ratio. Starting from the formula

$$\frac{X}{Y} = \frac{m_x + m_x^{+1} + m_x^{+2} + \cdots + m_x^{+n} + \cdots + m_x^{+(n+q)}}{m_y + m_y^{+1} + m_y^{+2} + \cdots + m_y^{+n} + \cdots + m_y^{+(n+q)}} \tag{11}$$

the mole ratio (X/Y) was defined as the ratio of the sum of all ion abundances (m) in the clusters (with $n + q$ ions) of the analyte (X) and its labeled analog (Y) with mass increment n.

The ion abundance at the masses monitored for X and Y are denoted by m and m^{+n}, respectively. The subscript x or y denotes the specific contribution of compound X or Y to these ion abundances, while in the case of a mixed contribution, subscript u is utilized. As all the ions in a given cluster are related to one another, the mole ratio (X/Y) can be expressed as a function of the ratio of the most prominent ions in each cluster:

$$X/Y = f(m_x/m_y^{+n}) \tag{12}$$

In case of an ion overlap, the abundances actually measured in the mass spectrometer (m_u and m_u^{+n}) are no longer equal to m_x and m_y^{+n} and the function to be derived is

$$X/Y = f(m_u/m_u^{+n}) \tag{13}$$

In order to facilitate the derivation of this function, four general constants were defined: h_x, h_y, k_x, and i_y. The equation

$$h_x = \frac{m_x^{+1} + m_x^{+2} + \cdots + m_x^{+(n+q)}}{m_x}$$

represents the fraction of the ion cluster of X that does not give a response at the m/z value monitored for X. In an analogous way, h_y represents the fraction of the ion cluster of Y that is not detected at the m/z value monitored for Y:

$$h_y = \frac{m_y + m_y^{+1} + m_y^{+2} + \cdots + m_y^{+(n-1)} + m_y^{+(n+1)} + \cdots + m_y^{+(n+q)}}{m_y^{+n}}$$

The values k_x and i_y are constant factors representing the interference of the ion cluster of X at the m/z value of Y and vice versa:

$$k_x = m_y^{+n}/m_x \qquad \text{and} \qquad i_y = m_y/(m_y^{+n} - k_x m_y)$$

Since unlabeled Y is identical to X, the $k_x m_y$ term corrects m_y^{+n} for potential ion intensity falling at m_y^{+n} but actually due to the natural isotopic abundance of unlabeled Y.

In case 1(Section 3.1), where there are no mutual ion interferences, m_x^{+n} and $m_y = 0$ and the actual form of the function (Eq. 13) is

$$X/Y = [(1 + h_x)/(1 + h_y)]\ (m_x/m_y^{+n}) \tag{14}$$

In case 2, $m_y \neq 0$ but $m_x{}^{+n} = 0$ and Eq. (13) takes the following form:

$$\frac{X}{Y} = \frac{1 + h_x}{1 + h_y} \cdot \frac{m_u}{m_y^{+n}} - \frac{i_y(1 + h_x)}{1 + h_y} \tag{15}$$

Since by definition $m_x^{+n} = 0$, k_x must equal zero and i_y reduces to m_y/m_y^{+n}. Both relationships are linear and a direct proportionality exists between molar ratios and measured ion intensities.

In case 3 ($m_y = 0$ but $m_x^{+n} \neq 0$), a linear relationship was obtained by inverting the mole ratios:

$$\frac{Y}{X} = \frac{1 + h_y}{1 + h_x} \cdot \frac{m_u^{+n}}{m_x} - \frac{k_x(1 + h_y)}{1 + h_x} \tag{16}$$

as then the ion intensity appearing in the denominator is due only to a single compound.

Finally, in case 4, where both components interfere, a linear solution was obtained by correcting X for the fraction of Y which is not labeled and thus identical with X. This fraction is expressed as

$$l = m_y(1 + h_x)/m_y^{+n}(1 + h_y)$$

and the corrected mole ratio becomes

$$\frac{Y}{X + lY} = \frac{(1 + k_x i_y)(1 + h_y)}{1 + h_x} \cdot \frac{m_u^{+n}}{m_u} - \frac{(k_x + k_x^2 i_y)(1 + h_y)}{1 + h_x} \tag{17}$$

According to the authors, l is determined experimentally from the mass spectrum of "pure" labeled component.

The practical performance of a calibration thus is identical in all four cases of ion interference. One determines the isotope ratio m_u/m_u^{+n} for a number of standards with a known mole ratio X/Y and performs a linear regression on these data. If there is interference by the unlabeled compound, the inverse mole ratio Y/X is used as a variable and if both unlabeled and labeled compound interfere, $Y/(X + lY)$ is used as variable. In the latter case, the introduction of the experimental factor l, however, will be an important source of error propagation, especially if $q_j \neq p_i$ (Jonckheere, 1982; Jonckheere *et al.*, 1982).

3.3. Empirical Methods

In a review article on the use of linear regression analysis for the reduction of isotope-dilution data, Schoeller (1976) described a number of strategies for

the treatment of nonlinear calibration data. First, a critical review was given of the criteria to which the data must conform for a regression line to be an accurate estimate: (1) the variance on x values (mole ratios) must be negligible with respect to that on y values (measured isotope ratios); (2) for each x value, the population of y values (mean $\pm$ random error) must be independent from all other y populations; (3) the standard deviations on each population of y values must be equal; and (4) the relationship between x and the mean of each population of y values must be linear. The first point can easily be tested by an analysis of variance (Schoeller, 1976), while the second criterion is seldom violated in IDMS except in case of "memory effects" of the mass spectrometer. The third assumption (equal standard deviation throughout the calibration range) is often not valid. Therefore, Schoeller recommended a weighted regression analysis to avoid inaccuracy near the origin of the calibration line. Finally, it was recognized that each set of calibration data has to be tested for linearity. Schoeller therefore proposed a graphical test, either by visual inspection of the calibration line, or by plotting the residuals (difference between the observed isotope ratio and the isotope ratio calculated from the regression line) versus the mole ratio. In case of a significant departure from linearity, several options for handling the data were suggested.

The simplest approach was to omit the nonlinear portion of the data. If this produced a calibration that encompassed too small a range of mole ratios, a plot of the inverse ratios was suggested. According to the author, this reversal of the roles of major and minor components in the linear region, under certain circumstances will extend the linear range (Schoeller, 1976). Although based upon empirical observations, it can be deduced from the theory given previously (Eq. 5), that, in the case of an ion interference of type 3 (Section 3.1), this approach effectively will yield a linear solution (Jonckheere, 1982). This does not hold, however, for the more frequent type 4 interference. Therefore, Schoeller suggested that if neither of these simple techniques was successful, the data should be transformed to a linear model by calculation of the observed atom% excesses according to Campbell (1974):

$$\text{atom\% excess} = [R'/(R' + 1)] \times 100$$

where R' is the isotopic ratio corrected for natural abundance contributions (Schoeller, 1976). From these, the dilution of each mixture is calculated and a plot of the observed dilution versus theoretical dilution can then be made. According to Schoeller, this technique will yield a linear calibration but requires a considerable amount of data reduction, while accompanying propagation of errors may sensitize the calibration to errors in the measurement of the isotope ratios of the pure undiluted analyte and its IS. Moreover, the calculation of the atom% excess truncates the original data and thus masks the nonlinearity rather than compensating for it (Jonckheere, 1982). The

same applies (Jonckheere *et al.*, 1982) to the use of a log–log regression (Van Langenhove *et al.*, 1982). According to Jonckheere, these empirical approaches are only of limited value when a highly accurate calibration is required.

3.4. Polynomial Regression Analysis

Instead of artificially transforming the data to a linear model, our group developed an approach in which the relation between isotope ratios and mole ratios is described by means of a polynomial regression (Jonckheere *et al.*, 1982). In this, the basic IDMS equation [Eq. (1)] is seen as a rational function:

$$R_m = a_0 + a_1 (x/y) + a_2 (x/y)^2 + \cdots + a_n(x/y)^n \qquad (18)$$

whose degree (n) depends on the actual values of the ion interferences p_j and q_i.

For a given set of data, different polynomial functions are calculated, starting from a first degree (linear equation) up to a fourth degree. The use of higher order polynomials is not advisable, in order to avoid oscillating of the curve through the measuring points. The residuals around the different models, i.e., the absolute differences between given mole ratios and the values calculated by the polynomial, are then used for a statistical evaluation for goodness of fit. This is done by testing the difference between the residuals of two consecutive models against the residuals of the highest degree model by means of an F-test for significance ($p = .95$). The flow scheme for this model testing is given in Fig. 4. Each model is tested against the next higher order model and the best one is then tested again with the following higher order model. This test indicates that, if the lower order model is true, there is only a 5% chance of choosing the wrong model. The use of weighted regression has also been included to compensate for the nonconstant variance of the analytical data points. As is evident from statistical literature (Schwartz, 1979), the accuracy of the calibration curve is almost invariably increased when weighting factors are incorporated, taking into account the experimentally determined variances at each measurement point.

The calculation procedure, as described, was tested by applying it to a set of synthetic data, generated with Eq. (1) for the analytical situation in the previously cited example of bromhexine and its trideuterated analog (Jonckheere *et al.*, 1982). By means of a computer program, "CLUSTR," based on the probability theory of Pickup and McPherson, the different parameters of Eq. (1) were calculated. These values were entered in Eq. (1) to obtain the isotope ratios covering the range 1.67–53.6 ng of bromhexine per 53.6 ng of labeled analog. The data points obtained were then used in the regression

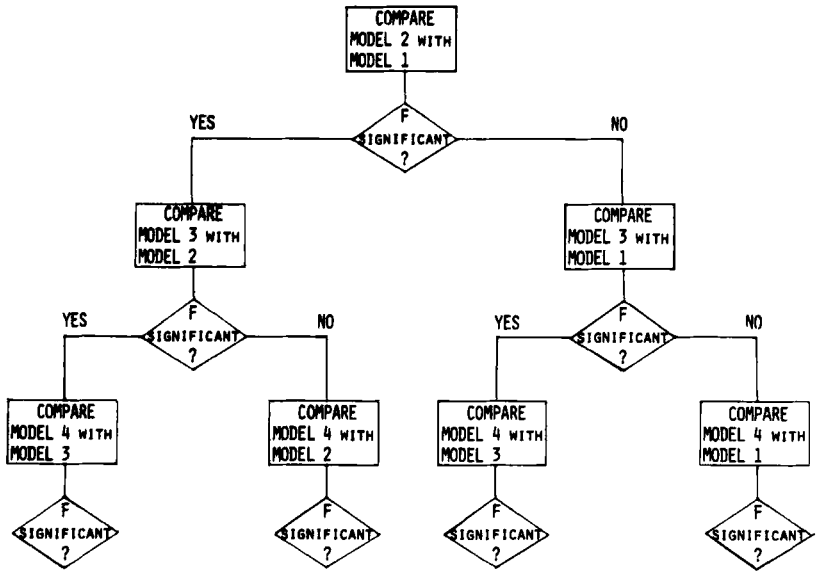

FIG. 4. Flow scheme of model testing in polynomial regression analysis. Reprinted with permission from *Anal. Chem.* **55**, 153–155 (1982). Copyright ACS.

analysis program, to give the four polynomials and the subsequent model test. The function thus produced was used further to calculate the mole ratios, which were then listed against the theoretical data points. From this, it could be concluded that the model appropriately described the curvature of the calibration curve over the whole concentration range.

Given the evidence of this theoretical example, the polynomial regression model was also applied to experimental data from published IDMS studies. The given isotope ratios were reentered in the chosen model and, for the purpose of comparison, in a linear equation (Jonckheere *et al.*, 1982). The difference between the calculated mole ratios and the given mole ratios is graphically represented in Fig. 5. Clearly, the higher order equation was more appropriate to describe the IDMS calibration curve than the linear model. It is important to note that the use of the correlation coefficient as a means of evaluating goodness of fit of linear regression should clearly be discouraged (Van Arendonk *et al.*, 1981). Its statistical significance indicates only that there exists "a" relationship between x and y values, without evaluating linearity. This is clearly seen in Fig. 5 where, despite a high linear correlation coefficient, the higher order model is more appropriate as proved by the F-test for significance (Jonckheere *et al.*, 1982). Polynomial regression also compared favorably with the "theoretical" models described

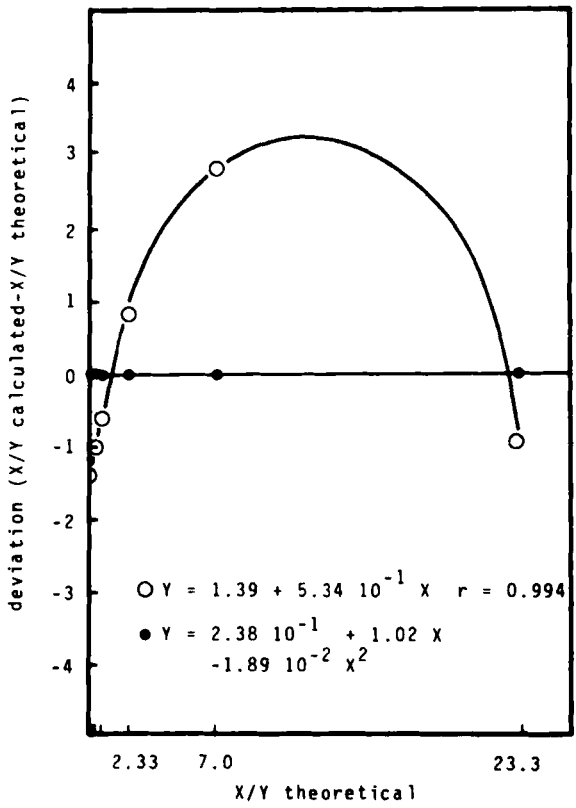

FIG. 5. Difference between calculated and given mole ratios for a linear and polynomial regression line, constructed from the data in Ref. C11, describing an IDMS assay for γ-aminobutyric acid (GABA) with [2,2-$^2H_2$]GABA as internal standard. Reprinted with permission from *Anal. Chem.* **55**, 153–155 (1982). Copyright ACS.

above, yielding the lowest residuals and thus the most accurate calibration (Jonckheere, 1982).

From this it can be concluded that the curvature of IDMS calibration curves can be described very accurately by means of higher order polynomials. The ability to check different models allows one to adapt the same calculation procedure regardless of the actual analytical situation. This is especially important in cases where, due to low efficiency of the synthesis, a large amount of unlabeled or partially labeled material is present in the IS. The setup of analyses with an IS of low mass increment is also facilitated (Jonckheere *et al.*, 1982). In contrast to other calibration methods, no initial estimates of the amount of unlabeled product and/or influence of naturally

occurring isotopes are necessary. The proposed method is even more valuable in cases where some separation of analyte and IS occurs (multiply labeled IS and/or high resolution columns). This effect destroys the validity of the basic IDMS equation [Eq. (1)] and subsequent calibration procedures based on this formula. Indeed the ion overlap is only partial and no accurate estimation of the interferences can be obtained by separate measurement of the pure product and IS (Jonckheere *et al.*, 1982).

Finally, it is obvious that the presented polynomial regression analysis with model testing requires a reasonable computational facility. A computer program, RAMP, is available in FORTRAN IV or HPL-BASIC (Jonckheere *et al.*, 1982).

3.5. Bracketing

Apart from the problem of nonlinearity, the calibration curve approach has another pitfall: measured ion abundance ratios can change with time, leading to the possibility of significant errors since the calibration and sample measurements cannot be simultaneous (Schoeller, 1980). In order to minimize the effect of instrumental drift and to optimize precision, the National Bureau of Standards (NBS) proposed a "bracketing" protocol for the development of definitive (i.e., essentially bias-free and precise) IDMS methods (Cohen *et al.*, 1980; White *et al.*, 1982; Yap *et al.*, 1983). It involves the measurement of each sample between measurements of calibration standards whose ion abundances most closely surround the ion abundance ratio of the sample. Measurements are made according to a strict protocol, used with samples prepared under restrictive conditions:

1. Initially each sample is measured to determine its approximate concentration, then calibration standards are made whose concentrations closely surround the anticipated levels of the samples. All dilutions are made in tared flasks or by weighing aliquots in a calibrated syringe.
2. Samples and standards are prepared in such a way that the ratio of analyte and labeled IS is within a narrow range near 1:1. This ensures the attainment of linearity and provides optimum measurement conditions.
3. Relatively large and uniform quantities are injected into the GC/MS in order to minimize background effects at the masses of interest and to circumvent possible nonlinear response of the mass spectrometer ion source to sample size.
4. Each sample is measured between the nearest pair of calibration solutions, with a concentration just below and above that of the sample. All measurements are performed in duplicate and are validated only if the iso-

tope ratios agree within 1%. If not, a third measurement is performed, which then should agree, otherwise the measurements are rejected. Apart from improving the precision, this rule helps to ensure that the instrument is in proper working condition. On a second day, the whole series of measurements is repeated in reverse order in order to minimize systematic errors (memory effects).

5. In their original paper, Cohen *et al.* (1980) also recommended that neither the aliquots taken from a particular serum pool nor the amount of IS added should be equal. Thus the intensity ratios observed usually differ, as do the pairs of calibration mixtures needed for bracketing them. This minimizes the effect of any error in a single calibration mixture, but complicates manipulations and calculations. When larger series of the same serum pool have to be analyzed, as for our BCR work (cf. Section 4.4), we prefer to prepare the standards from several independent stock solutions, and to keep the amount of internal standard constant for both samples and calibration standards.

The molar ratio of analyte and IS in the sample (X/Y) is calculated by linear interpolation between the bracketing mixtures, using Eq. (19):

$$\frac{X}{Y} = \frac{R_{MS} - R_{ML}}{R_{MH} - R_{ML}} (R_{WH} - R_{WL}) + R_{WL} \tag{19}$$

where R_{MS}, R_{MH}, and R_{ML} are the measured ion abundance ratios in the sample and surrounding "high" and "low" standards, respectively, and R_{WH} and R_{WL} represent the molar ratio of analyte to IS in the "high" and "low" calibration mixtures (Yap *et al.*, 1983).

Yap *et al.* (1983) also investigated the difference between using linear and nonlinear models in bracketing procedures and came to the conclusion that, as long as the isotopic abundance of the labeled form in the analyte (p_j) was smaller than 10%, the deviation due to the mode of interpolation is negligible. Only when the unlabeled sample contains large amounts of the labeled isotope, and the two bracketing standards differ widely in composition, does the deviation due to different modes of interpolation become significant.

Apart from the fact that a linear calibration can be performed, "bracketing" offers excellent precision and accuracy. With the determination of serum cholesterol as an example, Cohen *et al.* (1980) showed that the replication error on five different serum pools was characterized by a CV of 0.17% with a set-to-set variability of 0.32%. For each serum average, a standard error (considering all causes of variability combined) of 0.16% CV was obtained. The undetected systematic error (bias) in this study was estimated to be smaller than 0.5%, while White *et al.* (1982), using two different IDMS methods, found serum glucose concentrations to agree within 1%.

4. Reference Methodology

4.1. Accuracy Concept

The biomedical and medical sciences have increasingly demonstrated that human health and disease are strictly related to molecular processes (Engel, 1977). Each change at the molecular or cellular level is reflected in an alteration of a certain compound in a biological matrix such as tissue, serum, or urine (Spiro, 1975). Consequently, the exact quantitative determination of these components is of the highest diagnostic value (Boutwell, 1975).

A wide variety of techniques and methods has been introduced into clinical chemistry and judged especially on the criteria of usefulness, availability, and stability.

At present the choice of a new method is greatly influenced by the instrumentation which is available in the clinical laboratory. It is clear that the use of so many different techniques and methods has greatly (and negatively) influenced the correlation among the results obtained by laboratories.

Although clinical diagnosis is possible in the laboratory using precise but inaccurate results, the reliability and diagnostic value of the test can be greatly enhanced when the results are more highly accurate. In this way, reference intervals can be compared with other laboratories or with those obtained by international scientific studies. Epidemiological surveys are only meaningful if results obtained on different occasions (in time and/or space) are comparable. Thus, national and international efforts have been directed toward obtaining compatible results from the different laboratories. These activities must be clearly distinguished from standardization where the overall objective is to establish an initial basis for comparison of measurement results. For this purpose standard or reference methods are usually proposed. However, although a reference method may have been fully evaluated, e.g., with regard to its possible bias when compared with other techniques, it must be kept in mind that a user should still verify its performance when applying that method in his own laboratory. On the other hand, the promotion of the use of reference methods could discourage people from looking for improvement of techniques since, by definition, these methods would already provide satisfactory results. The effect of standardization can be substantially improved by the use of certified reference materials (Eisenhart, 1968). These materials can serve to check the reliability of the reference methods when applied by a particular laboratory. They can be employed by manufacturers or highly specialized laboratories, who would like to develop new techniques and make sure that they provide accurate results, i.e., with a close agreement between the "true" value and the measured value (Cali, 1973).

4.2. Hierarchical Structure of Methods and Materials

In the field of clinical chemistry a complete structure of methods and materials was proposed in the late 1970s for better accuracy and compatibility of laboratory results (Boutwell, 1977; Alvares *et al.*, 1982). The system includes primary and secondary reference materials and control materials, as well as definitive, reference, and laboratory methods. Each of the materials or methods would guarantee the transfer of accuracy from the highest level to the final routine level of the laboratories. For this transfer the three types of materials would in principle be used in conjunction with the three levels of methods. A schematic overview of this system would be as follows:

Primary reference material (pure, or in matrix)	Definitive method
Secondary reference material (pure, or in matrix)	Reference method
Control material (commercial)	Laboratory method

In this scheme, the primary reference material is defined as a chemical substance of the highest (and known) purity, or a well-characterized substance in a matrix. This classification of materials is, however, fairly arbitrary. It is ideal when used in connection with standards characterized in terms of biological activity. Primary standards are thus the International Reference Preparations (IRP) produced by the World Health Organization (WHO). In this case the primary standard for a particular antibiotic is the WHO reference preparation which constitutes the unit of that antibiotic. When people wish to use it they have to prepare a large batch of samples calibrated to the primary. This is then called a secondary standard. However, for well-defined chemical parameters, the term certified reference material is preferred.

Several definitions have been proposed in recent years for the different methods. From the various definitions given in Sections 4.2.1–4.2.3 it is clear that no consensus has been reached. One important organization, i.e., the International Standards Organization (ISO), does not endorse these definitions, in particular those for definitive methods.

4.2.1. *Definitive Method*

1. "A definitive method is one which, after exhaustive investigation, is found to have no known source of inaccuracy or ambiguity" [International Federation of Clinical Chemistry (IFCC), 1976]. This is really a very straightforward definition which accepts no source of error, no inaccuracy,

and no matrix effect. The result obtained by such a method must be the "true value." However, the method must still be subjected to rigorous investigation for possible sources of error. This definition is really too idealistic. More realistic definitions have been given by Cali *et al.* (1977) and by the AACC (Boutwell, 1978).

2. "A definitive method is one that, after exhaustive investigation, provides analytical results that are accurate, i.e., are free of systematic errors, to the extent required for the intended end-use(s)" (Cali *et al.*, 1977). The resulting accuracy can be specified and proved on theoretical and experimental grounds, usually from first principles. The result obtained is termed the definitive value and is the best approximation to the "true value."

3. "A definitive method is an analytical method that is capable of providing highest accuracy among all methods for determining that analyte, and of sufficient accuracy for the stated end-purposes of the definitive method" (Boutwell, 1978).

Definitions 2 and 3 allow an evolution in the different techniques and methods as definitive methods for the same analyte (Leijnse, 1982). Indeed, even though systematic errors were investigated during the initial research work, later technical advances may uncover errors that were undetected during the original measurements. The end use and end purposes of the definitive method include the evaluation of the accuracy of reference methods and its application to the quantitation of analytes in certified reference materials present in a biological matrix.

Isotope-dilution mass spectrometry combines the separating power of GC together with the specific detection of the mass spectrometer; it also allows the use of the "ideal" internal standard, i.e., a labeled internal standard. For these reasons, IDMS has been envisaged to provide definitive methods for organic analytes of a molecular weight below 500 (Bjorkhem *et al.*, 1976; Siekmann, 1979). However, it is not from the application per se of a method, but rather from the accuracy and precision obtained by this method, that one can conclude whether a method is definitive or not. In this way other techniques, e.g., GC coupled to other specific detectors and high-performance liquid chromatography (HPLC), can be verified for accuracy and precision in obtaining definitive values. IDMS is really a powerful tool for quantitative analyses of a certain compound of interest. However, high technology and expensive instrumentation together with high operational costs and wide experience are essential in this work.

4.2.2. *Reference Method*

Several definitions have also been given by the IFCC and other research groups:

1. "A reference method is one which after exhaustive investigation has been shown to have negligible inaccuracy in comparison with its imprecision" [International Federation of Clinical Chemistry (IFCC), 1979]. With its comparison of inaccuracy and imprecision this definition clearly refers to the principles of quality control in clinical chemistry. Indeed, statistical models such as Youden plots are used to find out whether the error in a pair of results happens by chance (imprecision of the method) or is systematic (inaccuracy) (Youden, 1967). If the results are close to the true values, inaccuracy is negligible in comparison with imprecision. As demonstrated earlier, each analytical procedure has a certain degree of imprecision; consequently, the total absence of systematic error can never be proved. Only as the influence of a systematic error is evident in comparison with the influence of chance or random error can the systematic error be demonstrated.

2. "A reference method is a technique that can be accomplished with generally available equipment and that, after exhaustive investigation, has been shown to have acceptable known inaccuracy in comparison with a definitive method" (Brown *et al.*, 1976). This definition compares definitive and reference methods with regard to their inaccuracy. However, as there is no "absolute" method and the "true value" remains unknown, a classification based on this criterion remains questionable. The other criterion is precision, but methods cannot be classified as definitive or reference based on this criterion alone. Also, the third definition clearly describes the hierarchy in methods and materials.

3. "A reference method is an analytical method with thoroughly documented accuracy, precision and low susceptibility to interferences. The accuracy and precision shall be demonstrated by direct comparison with the definitive method and primary reference material or, where not available, with other well-characterized and documented analytical approach(es)" (Boutwell, 1977). As long as accuracy and imprecision are within the limits, each technique or method is acceptable as a reference method. However, for reference methods one always looks for a method easily applicable in the laboratory. Therefore, the expensive instrumentation and the relatively low sample capacity make IDMS suitable as a definitive method rather than as a reference method. For some applications, however, IDMS is the method of choice, allowing a more specific detection than the existing methods in the laboratory.

4.2.3. *Laboratory Method*

All methods commonly used in the clinical laboratory with a sufficient precision, accuracy, and specificity can be called "laboratory methods." Several attempts have been made to select some of these methods as "recommended" or "selected" methods. Although this system of selecting laboratory methods could diminish the wide discrepancy between laboratory

methods and reference methods, heterogeneity in criteria again hampers this classification.

4.3. International Efforts

The hierarchy established among the different methods and materials suggests an easy organization (Tietz, 1979). However, serious practical problems have hampered fast progress. In the United States and in Europe, the National Committee for Clinical Laboratory Standards (NCCLS) and the European Committee for Clinical Laboratory Standards (ECCLS) have been founded. These standardization organizations promote the development and the ultimate use of reference materials and methods. The National Bureau of Standards (NBS) and the Centers for Disease Control (CDC) in the United States (Bayse, 1982), and the European Community Bureau of Reference (BCR) in Europe, undertake projects oriented more toward the preparation of certified reference materials (BCR, 1982).

In spite of the work done by these organizations, only a few products are readily available to clinical chemists as certified reference materials, i.e., in a highly purified and certified form. All these products are purified and distributed by the NBS (NBS, 1980). The most important reference materials in the field of clinical chemistry are listed in Table 1.

In routine determinations especially, matrix effects adversely influence the accuracy, but even a highly accurate and specific method such as IDMS can also suffer seriously from these matrix effects. However, at present only a few certified matrix reference materials are available. A transfer of accuracy among the various clinical laboratories is possible only by means of these materials.

TABLE 1
Certified Reference Materials Issued by the NBS

Material	Purity (%)	NBS SRM number[a]
Cholesterol	99.8	911a
Urea	99.7	912
Uric acid	99.7	913
Creatinine	99.8	914
Bilirubin	99.0	916
D-Glucose	99.8	917
D-Mannitol	99.8	920
Cortisol	98.9	921

[a] SRM = Standard Reference Material.

NBS and BCR coordinate research groups to validate and certify these matrix reference materials. Four important points have to be considered before a matrix reference material is certified. First, the production of the material has to deliver the different samples homogeneously within the overall uncertainty limits provided, i.e., the difference between the samples must be smaller than the uncertainty of the measurements. As these measurements become more precise the homogeneity requirements also increase. Only if inhomogeneity is negligible in comparison with the precision of the measurement methods is it possible to certify the material. Second, the stability of the material has to be studied thoroughly, especially for materials of biological origin that can degrade even in the lyophilized form. A statement must be given on the certificate reporting the expected shelflife of the sample after which the "true values" of the certificate are no longer valid. Third, special precautions that should be taken, e.g., storage, opening of the vials, and reconstitution, also have to be mentioned on the certificate. Finally, the "true value" has to be placed on the certificate. These "true values" should include a realistic statement of uncertainty accounting for unresolved systematic errors. Therefore, definitive methods (although they are too expensive for routine analyses) should be used for the measurements as they significantly reduce the possibility for errors. A listing of some of the certified materials is given in Table 2.

Different approaches can be used to assign values to reference materials, i.e., by one laboratory using a definitive method; by statistical consensus, or by coincidence of results obtained by several accurate procedures. If only one laboratory has developed an expertise far superior to what can be found anywhere else, there is no alternative to certification by that one single

TABLE 2
CERTIFIED MATRIX REFERENCE MATERIALS WITH THE METHOD OF CERTIFICATION

Material in "human serum"	Method	Organization
Calcium	IDMS	NBS
Cholesterol	ID–GC/MS	NBS
D-Glucose	ID–GC/MS	NBS
Uric acid	ID–GC/MS	NBS
Lithium	IDMS	NBS
Potassium	IDMS	NBS
Magnesium	IDMS	NBS
Cortisol[a]	ID–GC/MS	BCR

[a] The production of certified matrix reference materials of cortisol by the BCR currently is in a final stage.

laboratory. However, certification by one laboratory using a so-called definitive method does not give a total guarantee against some bias or accidental errors. Therefore, an independent confirmation by at least one other laboratory is necessary. A second approach consists of certification on a statistical consensus of several laboratories. In this approach the results of a great number of laboratories are subjected to statistical analysis and the outliers are rejected. The mean value is then claimed not to differ greatly from the "true" value. However, a priori this is not correct, as an accurate but minority method could be eliminated because its results would appear as outliers. Therefore, this method is not recommended for certification. The last procedure of certification is based on several methods and several laboratories. To prevent a bias by one single method, different methods used by several participating laboratories should be included. The results obtained by each method are then subjected to statistical analysis for consistency and if there are differences between the methods the causes of discrepancies are identified. This can lead to the elimination of one or more particular procedures if their inaccuracy can be demonstrated; even one single method used in a few laboratories can be applied for certification (Gilbert, 1976, 1978). The so-called definitive methods, especially isotope-dilution mass spectrometry, should be included whenever possible in the certification process (Bjorkhem *et al.*, 1981; Cohen *et al.*, 1981; Freudenthal, 1981). However, the question may arise whether a laboratory using the more current procedures can obtain an acceptable result by use of a reference material certified by one sophisticated method. In this situation it is recommended to examine the possibility of improving the classical methods and to investigate the possible causes of errors. It is clear that the use of a correction factor is never allowed.

4.4. Selected Example

In 1975 a BCR Clinical Chemistry Working Group was founded for the preparation and the distribution of reference materials in the clinical chemistry field. Due to the major problems encountered in the labs during steroid determinations, a project for the preparation of a matrix reference material was accepted. The task of the BCR Expert Group on Steroid Hormone Analysis consisted of the following points:

1. Production of a human serum "reference material" with well-known concentrations of cortisol. The "low level" ampoules should contain approximately 100 ng/ml cortisol, whereas the "high level" ampoules should contain 300 ng/ml cortisol, respectively.
2. The determination of the cortisol level in the reference materials by reliable analytical procedures, specifically ID–GC/MS. Alternatively,

HPLC is used in this project in order to obtain a certified value not biased by a particular procedure. The first step is an intercomparison after which all the results are discussed in detail until the causes of discrepancies are identified and a clear indication can be obtained to establish the most accurate method and the most accurate value. In this way measurements are improved and accuracy on a wide scale can also be improved significantly. It was shown that the HPLC–UV method had not yet been adequately developed for the purposes of certification measurement and for this reason HPLC was only employed to obtain comparative data for future assessment.

3. Control of the stability of the steroid hormone in the reference material.

In the following sections we will briefly describe the different problems encountered during the elaboration of this project.

4.4.1. *Frozen Serum versus Lyophilized Serum*

Customarily, frozen serum stored at −70°C or below is used for internal quality control of serum steroid analysis. However, for international reference preparations it becomes very difficult to transport the material in the frozen state, due to the postal and customs delays.

In the past few years great technical progress has been made in lyophilization. Lyophilization and ampouling under nitrogen also overcomes the problems of destructive postal delays. Therefore, the BCR has decided to prepare the reference material as lyophilized human serum. Some people believe that lyophilization alters the properties of the serum to such an extent that problems can arise in matrix-dependent measuring techniques such as radioimmunoassay (RIA) and competitive protein binding (CPB) (Menson and Adams, 1977; Fraser *et al.*, 1978; DiSilvio, 1979). Indeed, before the lyophilized material can be used it should be checked to determine whether the material can be satisfactorily reconstituted and whether it can be assayed by the different techniques currently in use in clinical laboratories.

4.4.2. *Opening and Reconstitution of the Ampoules*

It is assumed that all ampoules contain the same amount of dry powder. Since sealed glass ampoules are used, dispersion of the powder takes place during transport and mailing. In order to prevent additional variability and losses of material it is recommended to centrifuge the ampoules and open them as close as possible to the top rounding, taking care that no glass particles fall into the ampoule. Following this procedure precision coefficients (CV) on estimated powder content can reach 0.2%; probably the powder content precision is even better, but higher precision weighing suffers greatly from the presence of moisture. It is clear that this variability is

directly linked to the accuracy and precision of the final expression of the results. The water for reconstitution (1.250 ml) is added by means of a Hamilton Diluter-Dispenser. A total reconstitution time of 45 minutes is allowed. The serum samples are taken by means of a calibrated glass pipette of 1.00 ml. Each volumetric step (addition of water or sampling) is controlled by a weighing procedure. Indeed, imprecision of pipets directly influences the precision of the final weight/volume expressions. By taking into account the weight of the samples, this imprecision is compensated for; the final results should then be expressed in weight/weight units.

4.4.3. *Calibration*

To ensure the accuracy of the calibration, Standard Reference Material (SRM) No. 921, certified by the National Bureau of Standards to contain 98.9 ± 0.2% cortisol, is used as a primary reference material. Taking into account the stated impurity, each day appropriate amounts of cortisol are weighed and dissolved in a weighed amount of methanol to obtain two independent stock solutions. Also, for the subsequent dilution of working and calibration solutions, weighing is used. Each day, calibration standards are made by weighing varying amounts of the working solutions so that the anticipated concentration is enclosed within ±15%. This procedure of "bracketing standardization" allows one to evaluate the consistency of the two separate working solutions and also excludes nonlinearity of the calibration graph due to large differences in concentrations. This technique was introduced in 1978 by the NBS working group (Cohen *et al.*, 1980), and is already described more extensively in Section 3.5. Calibration standards are also taken through the sample workup procedure. For calibration standards the use of spiked serum is not recommended since endogenous cortisol will offset the calibration line and will result in higher signals than obtained for the serum samples themselves—far from the ideal 1/1 ratio between the peak of cortisol and the internal standard peak. Therefore, the use of water or an albumin solution is preferable. Internal standardization is carried out by volumetric addition of the same amount of [1,2-$^2H_2$]cortisol to both the serum and calibration mixtures. All necessary steps should be taken to prevent any change of concentration of these standard solutions. The imprecision of the addition of the internal standard should be kept as low as possible; as the final calculations are based on ratios, the accuracy of this addition is of minor importance. After addition of the standard and the internal standard all samples are left to equilibrate overnight at 4°C.

4.4.4. *Measurement Protocol*

In order to compensate for short-term or long-term drift effects, a protocol is followed which alternatively measures serum and calibration standards.

The following protocol was proposed and consisted of 4 measuring days. Each day, four (or six at day 1) standards and four samples are analyzed. The calibration curves are constructed by least squares regression analysis and statistically tested for nonlinearity by means of an *F*-test on the residuals. The amount of cortisol in the serum samples is obtained by linear interpolation on the daily calibration curve. Preliminary experiments were also set up to determine the influence of the use of peak height or peak area ratios. For the cortisol measurement, some separation takes place between syn and anti isomers, therefore the use of peak heights is less favorable.

An extensive description of the sample manipulation together with the derivatization conditions and the gas chromatographic/mass spectrometric conditions used in our laboratory has already been published (Jonckheere and De Leenheer, 1983).

5. Other Applications

Because of the cost of the equipment and the expertise required for its maintenance and use, the main application of quantitative MS is for research purposes for which sensitivity and specificity are of paramount importance. One of the first examples of selected ion monitoring using a stable isotope-labeled IS was an assay for prostaglandin E_1 (Samuelsson *et al.*, 1970). Other typical applications include the analysis of biogenic amines and their metabolites in cerebrospinal fluid and brain tissue; and analysis of steroids, bile acids, polyamines, and prostaglandins in serum and urine; and the determination of ^{13}C-labeled compounds in urine by GC, followed by pyrolysis to CO_2 and $^{13}C/^{12}C$ ratio detection (Halpern, 1981; Hill and Whelan, 1984). GC/MS has also been used extensively for quantitative drug analysis. Apart from its specificity, which makes the technique ideally suited for the validation of routine assays, GC/SIM generally offers a much higher sensitivity than other purely chromatographic procedures, while it has the advantage over immunoassays of a great versatility and consequent speed of method development. Thus, if only a limited number of samples have to be analyzed, an operational method employing GC/MS may often be developed in a matter of weeks, whereas the development of an immunoassay, involving antigen synthesis, antibody production, and testing, may take months of work (Gaskell, 1982). Horning *et al.* (1977) have reviewed the use of GC/MS in drug quantitation, discussing the problems of extraction methods, choice of derivatives, and internal standards; and a book has been published on the GC/MS analysis of drugs and their metabolites (Gudzinowicz and Gudzinowicz, 1979). Many of these methods involve the use of a radiolabeled IS (Hill and Whelan, 1984). Just as for reference methodology, here, too, the

increased precision and accuracy of quantitative GC/MS and ID/SIM in particular have led to a growing recognition that variability in the collection and handling of biological specimens such as blood may lead to changes in test values and calls for a careful examination of these steps (Halpern, 1981). Finally, MS techniques also have been used for trace elemental analysis, an area of rising interest and concern in clinical chemistry. Although other techniques such as atomic absorption spectroscopy, neutron activation analysis, and polarography also have been used with considerable success, MS has the advantage that it can be used to determine parts per million levels of several metals simultaneously with high precision and accuracy (Halpern, 1981). Two approaches have been followed: spark-source and field-desorption MS with direct sample introduction and GC/MS after complexing with chelating agents (Schulten and Lehmann, 1978; Halpern, 1981).

6. New Developments and Future Trends

Quantitative mass spectrometry is still a fast-growing technique and much remains to be done. In the field of reference methodology, the hierarchical structure described above has been realized only for a few analytes. The lack of high purity reference materials as well as methodological problems limits the number of assays available. In order to accelerate the establishment of accuracy control programs, the assignment of values to external quality control matrices should be encouraged. Since external quality control programs are well established in most countries, such materials could be used as secondary matrix reference materials. Under these circumstances, the validation with reference methodologies would provide a constant pressure to improve the accuracy of field methods (De Leenheer *et al.*, 1982).

Technological advances in the areas of ionization methods, ion sources, and high-mass detection are now opening new perspectives in the study of low-volatility high-mass substances of biological importance. Fast-atom bombardment (FAB), ^{252}Cf plasma and laser desorption, and molecular secondary-ion MS (SIMS) especially show promise in this field (Day *et al.*, 1980; Barber *et al.*, 1981; Cotter and Tabet, 1984). On-line LC/MS techniques (Arpino and Guiochon, 1979; McFadden, 1979; Mellon, 1981; Smith *et al.*, 1981) have now been available for a number of years in selected laboratories, and studies involving structural and quantitative work on drugs and biogenic compounds have demonstrated the potential of this approach in the biomedical field. Widespread use is limited, however, by the inherent technological complexity of this technique.

An interesting development from a viewpoint of selectivity was the introduction, some years ago, of "soft" ionization techniques such as chemical

ionization. Not only does this produce characteristic ions incorporating the intact molecule, but a judicious choice of the reagent gas also provides a means of selective ionization of target compounds in complex mixtures. Thus, instead of achieving an indiscriminate ionization of all volatile mixture components as in the conventional electron impact (EI) mode, conditions may be adjusted to effect ionization only of chosen compound types (Gaskell, 1982). Another rapidly developing technique is that of negative chemical ionization (NCI) mass spectrometry. Major ion formation processes include resonance or dissociative capture of low-energy electrons and ion–molecule interactions (Hunt and Crow, 1978; Dougherty, 1981). Detection of analytes with high electron affinites may be uniquely sensitive and selective (Markey *et al.*, 1978; Halpern, 1981). It is particularly noteworthy here that the high sensitivity of detection is attributable in part to the suitable choice of derivatives, which no doubt have a major part to play in the more widespread application of this technique (Gaskell, 1982).

Further improvement in selectivity may also be expected from the use of multisector instruments developed for MS/MS (McLafferty and Bockhoff, 1978; Glish *et al.*, 1980; Hunt *et al.*, 1980). Designs involving electric and magnetic sectors and/or several quadrupole mass filters have been described and used for structure elucidation and quantification. The GC/MS/MS setup may well be the most selective combination available today. Although operation at high MS resolution necessarily reduces signal intensity, *useful* sensitivity in the analysis of complex mixtures may be increased by a much greater reduction in the signal attributable to instrument background and matrix constituents (Harvey *et al.*, 1980; Gaskell, 1982). The complexity and cost of these techniques, however, prevent a more generalized use in the near future. Meanwhile, there is no doubt that isotope dilution in combination with "classical" MS techniques has a great future in clinical chemistry.

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CLINICAL CHEMISTRY OF VITAMIN B_{12}

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1. Introduction

It was recognized by Minot and Murphy (M1) as early as 1926 that an active principle present in liver restored normal erythropoiesis in patients with pernicious anemia. The treatment required the patient to consume large quantities of raw liver. Prior to this form of therapy pernicious anemia had a uniformly fatal outcome. Extracts of liver containing the active principle, which could be given by intramuscular injection, later became available, and this was followed in 1948 by the isolation of a pure crystalline material from liver almost simultaneously by Rickes and his colleagues (R1) in the United States and Smith and Parker (S1) in England. Rickes called this material vitamin B_{12}. The isolation of vitamin B_{12} not only had substantial benefits for patients with pernicious anemia and other vitamin B_{12} deficiency states but also resulted in a great surge of research into all aspects of vitamin B_{12} metabolism. Some 35 years and several thousand publications later there still remain many unanswered questions.

Vitamin B_{12} is essential for normal health and development in man and animals. The vitamin is unusual insofar as only very small (microgram) quantities are required to satisfy the daily requirement, yet relatively large quantities are stored in the liver, sufficient to supply normal requirements for a period of at least 3 years.

1.1. Nomenclature

Vitamin B_{12} is the name which has been used since its first introduction by Rickes and his colleagues in 1948 (R1). However, vitamin B_{12} is a generic term which includes cyanocobalamin, hydroxocobalamin, adenosylcobalamin, and methylcobalamin. This group of compounds has been called cobalamins or corrinoids, the generic term used for all compounds containing a

corrin nucleus. The name corrin refers to the core of the vitamin B_{12} molecule and is not derived from the fact that the vitamin contains cobalt. Recommendations on nomenclature have been published by the *Journal of Nutrition*, "Nomenclature Policy: Generic Descriptors and Trivial Names for Vitamins and Related Compounds." These recommendations state that "The term vitamin B_{12} should be used as the generic descriptor for all corrinoids exhibiting qualitatively the biological activity of cyanocobalamin. The term corrinoid should be used as the generic descriptor for all compounds containing the corrin nucleus and thus chemically related to cyanocobalamin. The term corrinoid is not synonymous with the term vitamin B_{12}" (A1). More detailed recommendations on the nomenclature of the corrinoids have been made by the IUPAC–IUB Commission on Biochemical Nomenclature (I1).

2. Chemistry and Biochemistry

2.1. Chemistry

The vitamin B_{12} molecule is a complex structure made up of a corrin ring with a centrally located cobalt atom. Held almost at right angles to the corrin ring is a nucleotide 5,6-dimethylbenzimidazole, one nitrogen of which is attached to the ribose while the other is bonded to the cobalt atom of the corrin structure (Fig. 1). Identification of the structure of vitamin B_{12} was finalized by the outstanding work of Hodgkin *et al.* (H1) using X-ray crystallography.

The various forms of the vitamin have either a cyanide, hydroxo, deoxyadenosyl, or a methyl group attached to the cobalt atom. When exposed to light, cyanocobalamin, deoxyadenosylcobalamin, and methylcobalamin are converted to hydroxocobalamin or aquocobalamin by photolysis. Hydroxocobalamin has an OH^- group and aquocobalamin an H_2O group bound to the cobalt atom. Prolonged exposure to sunlight results in the conversion of 10% of cyanocobalamin to hydroxocobalamin for each 30 minutes of exposure (D1). This change can be reversed in the dark (V1) and there is no loss of vitamin activity. Because of this light sensitivity the biologically active form of the vitamin, that is, the coenzyme form deoxyadenosylcobalamin, was not recognized until 1958 (B1). Deoxyadenosylcobalamin is the principal cobalamin present in human liver, which is the major storage organ.

The vitamin is stable in aqueous solution at room temperature. It may be heated to 120°C with little loss of activity provided the pH is kept between 4 and 6. However, it breaks down rapidly when exposed to a pH below 2 or above 9. Crystals of cyanocobalamin are dark red, needle-like, and contain about 12% water.

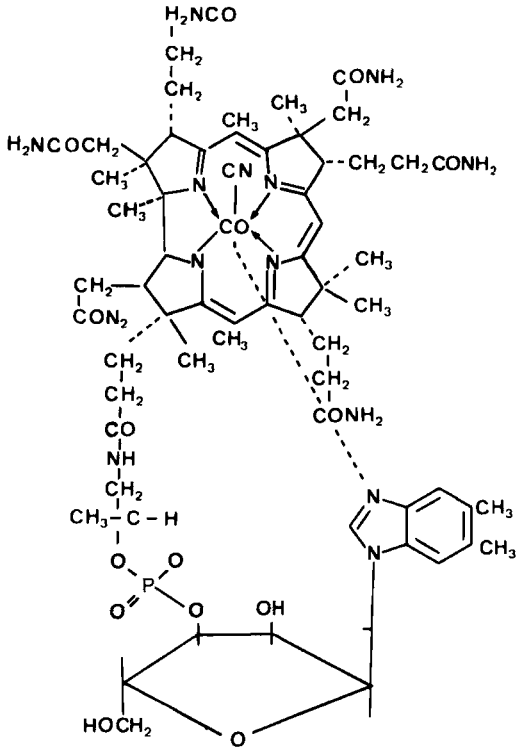

FIG. 1. Vitamin B_{12} (cyanocobalamin).

2.2. BIOCHEMISTRY

2.2.1. *Vitamin B_{12}-Mediated Reactions*

Although numerous enzymatic reactions requiring vitamin B_{12} have been described, and 10 reactions for adenosylcobalamin alone have been identified, only three pathways in man have so far been recognized, one of which has only recently been identified (P1). Two of these require the vitamin in the adenosyl form and the other in the methyl form. These cobalamin coenzymes are formed by a complex reaction sequence which results in the formation of a covalent carbon–cobalt bond between the cobalt nucleus of the vitamin and the methyl or 5′-deoxy-5′-adenosyl ligand, with resulting coenzyme specificity. Adenosylcobalamin is required in the conversion of methylmalonate to succinate (Fig. 2), while methylcobalamin is required by a B_{12}-dependent methionine synthetase that enables the methyl group to be transferred from 5-methyltetrahydrofolate to homocysteine to form methionine (Fig. 3).

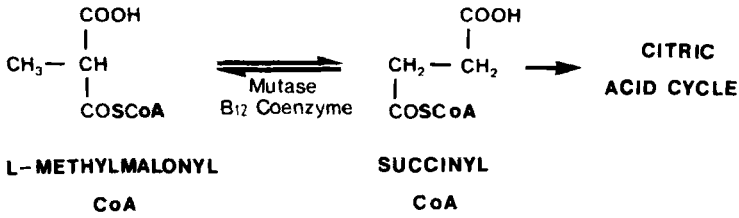

FIG. 2. Conversion of methylmalonate to succinate.

There are two clinical defects associated with a deficiency of vitamin B_{12}: a megaloblastic hemopoiesis and associated anemia and a neurological deficit usually presenting as subacute degeneration of the spinal cord. While this type of neurological change is perhaps the most common, other changes have also been described. Megaloblastic erythropoiesis was recognized many years ago as resulting from a reduction in DNA synthesis due to a lack of folate, the availability of which is mediated by vitamin B_{12}. However, there was no clear understanding of how the neurological changes came about. It did not appear to be related to folate metabolism because folate corrects the anemia caused by a vitamin B_{12} deficiency, but it has no beneficial effect on the neuropathy. In fact, it is likely to cause a deterioration in the neurological state. It had been suggested that the neuropathy was due to inactivation of the B_{12}-dependent enzyme methylmalonyl-CoA mutase (F1). This in turn would then result in abnormal fatty acid synthesis and it was proposed that these abnormal fatty acids accumulated in nervous tissue. This hypothesis left a number of questions unanswered and it was not until 1981, when Scott and his associates (S2) published the results of their studies on monkeys, that a satisfactory solution was presented. They maintained two groups of monkeys in an atmosphere of nitrous oxide. This had been shown previously to change the cobalt atom in vitamin B_{12} from the reduced to the oxidized form, and in this form the vitamin cannot be used by methionine synthetase. After 10 weeks exposure to the gas the monkeys developed

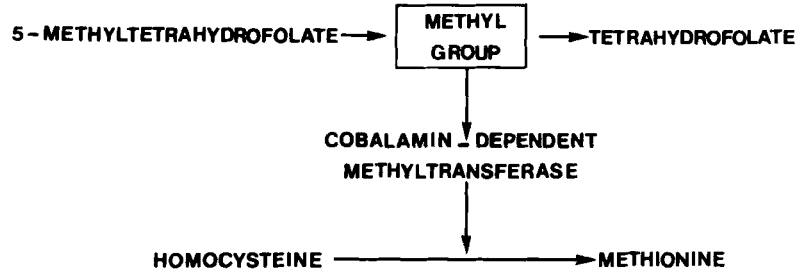

FIG. 3. Conversion of homocysteine to methionine.

subacute combined degeneration of the spinal cord. The diet of one group of monkeys was supplemented with methionine and in this group there was no clinically apparent neurological change.

These important findings indicate that the neurological changes resulting from a deficiency of vitamin B_{12} are due to the inability of methyltetrahydrofolate to donote its methyl group to homocysteine to give methionine. This is a vitamin B_{12}-mediated step. A lack of methionine decreases the availability of S-adenosylmethionine (SAM), which suppresses important methylation reactions, particularly those required to maintain myelin. Scott and Weir (S3) suggested that a decrease in SAM causes more folate to be directed along the 5-methyltetrahydrofolate pathway and away from the cycles that produce purines and pyrimidines required for DNA synthesis. While this reduction in DNA synthesis may then result in the development of a megaloblastic erythropoiesis, the 5-methyltetrahydrofolate cannot donate its methyl group to homocysteine due to the lack of vitamin B_{12}. This may then result in an abnormal accumulation of 5-methyltetrahydrofolate in the plasma. This has been noted particularly in patients with marked neuropathy but with only a modest degree of anemia (W1). Because the folate cycle is blocked, the amount of folate available for DNA synthesis is further reduced.

In 1980 Poston (P1) proposed that vitamin B_{12} was required for the conversion of the branched-chain amino acid β-leucine to leucine. He found circulating β-leucine levels elevated in patients with vitamin B_{12} deficiency. The concentration of leucine on the other hand was found to be much lower. He suggested that 2,3-aminomutase, which catalyzes the interconversion of β-leucine and leucine, is a vitamin B_{12}-dependent enzyme which is consequently reduced in patients with pernicious anemia. The enzyme has been found in the liver of several animals and in human leucocytes, and *in vitro* experiments have shown it to be adenosylcobalamin dependent (P2).

2.2.2. *Biosynthesis*

Vitamin B_{12} is unique in that it is found exclusively as a product of microorganisms. However, not all microorganisms have this facility and some require an exogenous source for growth while others appear not to require the vitamin. *Streptomyces griseus* was first used commercially to produce streptomycin but was later found also to produce large quantities of vitamin B_{12} [up to 24 mg per liter of fermentation liquor (S4)]. The addition of an isotope of cobalt such as ^{57}Co or ^{58}Co to the medium used to grow the vitamin B_{12}-producing organisms results in incorporation of the radiolabel into the vitamin. Sewerage sludge is recognized as a good source of B_{12} because it represents an excellent medium for bacterial growth and consequent production of the vitamin.

Cobalt is essential for the synthesis of vitamin B_{12} by microorganisms.

Ruminant animals obtain their B_{12} from bacterial synthesis that takes place in the rumen. As a result, these animals may suffer from vitamin B_{12} deficiency when they are grazed on cobalt-deficient pastures because the rumen bacteria will be unable to produce the vitamin.

2.2.3. *Synthesis*

Vitamin B_{12} has been successfully synthesized (W2), but this was a major undertaking involving 100 people over 11 years. The synthesis was completed in two stages; first, the corrinoid cobyric acid, which is a natural substance, was converted to vitamin B_{12}, then work was continued on the synthesis of the cobyric acid. The procedure is complex and still not of commercial significance. However, the synthesis of such a complex molecule was a great achievement at the time.

2.2.4. *Vitamin B_{12} Absorption and Transport*

Vitamin B_{12} can be absorbed when present in physiological amounts only if it is first bound to a specific protein—the so-called intrinsic factor—that tightly binds to the vitamin. The complex then passes through the jejunum to the ileum, which contains receptor sites for the vitamin B_{12}/intrinsic factor complex. Calcium ions are required for the reaction between ileal receptors and the intrinsic factor/vitamin B_{12} complex. The reaction is inhibited by EDTA and reduced by a pH below 5.4. The vitamin appears to be separated from intrinsic factor at the ileal receptor sites and is then bound to another protein carrier, transcobalamin II, which transports the vitamin and permits its uptake by a number of tissues. The subject has been well reviewed by Jacob and her colleagues (J1). Removal of 60 cm of ileum may impair vitamin B_{12} absorption and with the loss of 180 cm absorption is almost always affected.

2.2.5. *Intrinsic Factor*

Following a series of studies, Castle and his colleagues (C1–C3) concluded that there was a lack of a particular property in the gastric contents of patients with pernicious anemia. This was followed by the observation that gastric juice, if given orally with liver, enhanced the effect of the liver some 30 times (F2). We now know that in man this intrinsic factor is a glycoprotein secreted by the parietal cells in the fundic mucosa of the stomach. It has a molecular weight of approximately 44,000 and contains 15% carbohydrate (A2), including 6.9% hexose, 4.1% hexosamine residues, and 1.7% sialic acid (V2). One molecule of intrinsic factor will bind one molecule of vitamin B_{12} (A3,C4). The reaction is rapid and the resulting complex appears to be stable. When bound to vitamin B_{12}, the intrinsic factor is reconformed to produce a more compact molecule that is resistant to proteolytic action and

this enables the complex to remain intact on its route to the ileum. Intrinsic factor is heat labile and is destroyed by heating for 5 minutes at 100°C (C5). When degraded by peptic digestion it may still bind the vitamin. However, this type of complex does not provide the right conditions to permit absorption of the vitamin. R-Proteins (see Section 2.2.7) are also found in gastric juice and although they are able to bind the vitamin, the complex is usually released again in the duodenum where the R-proteins are broken down by pancreatic enzymes (S5).

2.2.6. *Transcobalamins and Other Vitamin B_{12} Binding Proteins*

The term transcobalamin is used to describe the vitamin B_{12} binding proteins present in plasma. Three such binding proteins have been described: transcobalamins I (S6), II (A4), and III (S6) (TC I, TC II, and TC III). TC I and TC III are classified as R-proteins, a group of proteins that are able to bind the vitamin B_{12} but do not appear to have any useful biological function. The term "R-protein" was originally used by Grasbeck to describe a vitamin B_{12} binding protein in human gastric juice which was devoid of intrinsic factor activity (G1). It was designated "R" because of its rapid mobility on electrophoresis. Progress in the study of the transcobalamins has been slow because of the very small quantities present in plasma. For example, the concentration of TC II is of the order of 25 μg/liter (H2). The only physiologically important plasma vitamin B_{12} transport protein is TC II, and it is this protein that appears to deliver the vitamin to the liver and all other tissues (A4). The half-life of TC II has been variously estimated as 5 minutes (H3), 90 minutes (H4), and 28 hours (E1). At present, convincing evidence of the half-life of TC II is lacking. The protein has a molecular weight of approximately 35,000 (H5) and the turnover rate has been calculated as 10.03 nmol per 24 hours, although this figure would depend on the calculated half-life (E2).

The site of TC II synthesis has been the subject of much study and it has been shown that *de novo* synthesis occurs in the liver (E3,S7,T1), blood monocytes (R2,R3), and ileal enterocytes (C6,R4). Like intrinsic factor, TC II contains a single vitamin B_{12} binding site per molecule. However, unlike intrinsic factor, TC II will bind analogs of the vitamin (H6). A number of genetically determined variants of TC II have been described and were recognized by their altered electrophoretic mobility (D2,F3) and these are discussed in Section 8.1.

2.2.7. *R-Proteins*

These have been found in saliva, amniotic fluid, plasma, granulocytes, platelets, milk, and gastric juice, and it was suggested by Stenman (S8) that the R-protein in these different cells and fluids is a single microhetero-

geneous protein with a variable carbohydrate composition. Further characterization of the R-proteins associated with chronic myeloid leukemia showed that the increase in vitamin B_{12} binding capacity was due to an increase in the more acid isoproteins compared with those found in healthy subjects. In polycythemia vera, and in most examples of leukocytosis, the increased vitamin B_{12} binding capacity was found to be due to an increase in the least acid isoproteins (S9). Estimates of the molecular weight of R-proteins range from 72,000 to 150,000 depending on the separation technique used. The high values obtained are probably due to the 33–40% carbohydrate content of these proteins (A4). The sialic acid content of individual R-proteins has been found to correlate with their electrophoretic mobility (B2).

Approximately 90% of the unsaturated vitamin B_{12} binding capacity of sonicates of peripheral blood granulocytes appears to be TC III, while the remainder is TC I (S6). Granulocytes have not been found to contain TC II. Studies on the delivery of vitamin B_{12} to human lymphocytes by TC I, TC II, and TC III have shown that in lymphocyte cultures the uptake of $[^{57}Co]B_{12}$ from its complex with TC II was substantially higher than from complexes with other binders. It was concluded from these experiments that TC III, like TC I, did not promote uptake of vitamin B_{12} by human hemopoietic cells and that these two transcobalamins appear to be storage proteins (M2).

3. Daily Requirements and Occurrence in Foods

3.1. Daily Requirements

Baker and Mathan (B3) studied the hematological responses to varying amounts of vitamin B_{12} in five Indian subjects with a vitamin B_{12}-deficiency anemia. They concluded that daily amounts ranging from 0.07 to 0.25 μg of dietary vitamin B_{12} were inadequate but that amounts ranging from 0.3 to 0.65 μg were adequate or possibly more than adequate. They suggested that the daily intake should be in the region of 0.5 μg/day and that an intake of 1.0 μg/day would adequately cover the needs of the majority of the population. This is less than the 2 μg/day recommended by the World Health Organization (T2), but is similar to that suggested by Sullivan and Herbert (S10) and Darby and his associates (D3).

3.2. Food Sources of Vitamin B_{12}

Vitamin B_{12} is found only in foodstuff of animal origin such as meat, offal, eggs, milk, and cheese. The vitamin is not present in any fruits, nuts, or green leaf vegetables. Claims to the contrary are usually the result of con-

tamination of the products in question with insects, insect droppings, or vitamin B_{12}-producing bacteria.

Cow's milk contains between 1.6 and 6.2 μg of vitamin B_{12} per 500 ml and this is sufficient to provide the daily requirement of man. However, if milk is boiled much of the vitamin B_{12} content may be lost and that remaining may not be sufficient to prevent overt signs of vitamin B_{12} deficiency. Pasteurization of milk results in the loss of up to 10% of vitamin B_{12} (C7).

In certain health food literature, *Spirulina*, a blue-green algae, has been claimed to be a source of vitamin B_{12}. It appears that this was based on the results obtained from the United States Pharmacopeia microbiological assay for vitamin B_{12}. This assay uses *Lactobacillus leichmannii* as the test organism and it is known that this organism responds to some vitamin B_{12} analogs. Herbert and Drivas (H7) found that analogs of the vitamin accounted for more than 80% of what appeared to be "vitamin B_{12}."

It has also been suggested that seaweed is a good source of vitamin B_{12} and that 100 g will provide the normal daily requirement. The nodules on the roots of nitrogen-fixing plants are also thought to be a source of the vitamin (L1). As there is enough vitamin B_{12} in sea water, at least in some parts of the oceans, to provide the needs of certain vitamin B_{12}-dependent organisms, it may be that seaweed has the ability to concentrate the vitamin. However, it is more likely that any vitamin that may be present in both seaweed and the root nodules of nitrogen-fixing plants is the result of bacterial synthesis.

4. Methods for the Measurement of Vitamin B_{12} Status

Although measurement of the concentration of vitamin B_{12} in serum is the most widely used test for the investigation of a possible deficiency, there are a number of other tests used to measure absorption, the integrity of the methylmalonyl metabolic pathway, and the presence of antibodies to intrinsic factor. These additional tests are also described in this section.

Vitamin B_{12} may be assayed using biological or radiolabeled vitamin dilution methods. However, because the serum concentration of the vitamin is in the order of nanograms per liter, chemical methods have not proved to be sufficiently sensitive. Most of the vitamin present in serum is bound to a protein and it must first be released before commencing an assay. None of the currently available methods can directly measure protein-bound vitamin. Denaturing the serum protein will release the vitamin and this may be done using heat or chemical means. While the microbiological methods are considered by many to be more accurate and, with the possible excep-

tion of oxidized cobalamin, closely reflect the concentration of biologically active vitamin, they may take several days to complete.

Measurements using radioisotope dilution techniques are rapid and the more recently designed kits appear to be reliable in skilled hands.

4.1. Biological Assays

Several methods using bacteria, protozoa, euglenoid flagellates, and diatoms as test organisms have been described for the measurement of the vitamin. They vary in their sensitivity and specificity.

One of the first clinically useful assay techniques was described by Hutner and his associates in 1949 shortly after the isolation of the vitamin (H8) and used *Euglena gracilis* var. *bacillaris* as the test organism. Ross (R5) used *Euglena* to measure vitamin B_{12} in blood and other body fluids and for many years this method was widely used in clinical practice. The organism is sensitive to as little as 1.0 ng/liter. The bacillaris strain was replaced by the more vigorously growing Z strain of the organism in 1955 (H9). Although *Euglena* responds to some B_{12} pseudovitamins, these have not been found in mammalian blood or tissue, so for practical purposes the assay reflects true vitamin B_{12}. The organism grows well in a simple sugar medium and requires an acid pH which serves to inhibit growth of many potential contaminants. A major disadvantage is that growth of the organism is slow and it is usually 4 to 5 days before results can be read. The organism appears not to be affected by the presence of antibiotics or drugs that may be present in the samples to be assayed, although Herbert and his colleagues claimed that its growth could be inhibited by the presence of chlorpromazine (H10). However, a careful study of the effect of chlorpromazine failed to confirm that the drug had any inhibitory effect on *Euglena* (D4).

Assays using bacteria as the test organisms have the advantage of requiring only a relatively short growth period, and results can usually be read in less than 24 hours. Assays using *L. leichmannii* (R6) and *Escherichia coli* (G2, S11) as the test organisms have been described. The technique using *L. leichmannii* was later modified for use as an automated method (D5). This method used a chloramphenicol-resistant mutant of the organism, which permitted the test to be run without the need for sterilization or aseptic addition by the simple expedient of adding chloramphenicol to the assay medium. This was effective in inhibiting most contaminants likely to be encountered during the short growth period required for the test. Heat was used to disassociate the vitamin from its protein carrier and precipitation of the protein was avoided by first reducing the pH to 3.6. The pH of the assay medium was adjusted so that the final pH in the test preparation was 5.9. The use of *L. leichmannii* as a test organism has the disadvantage that growth

may be suppressed in the presence of antibiotics and folate antagonists such as trimethoprim. Results also tend to be somewhat higher than those obtained using *Euglena* because the organism responds to purine and pyrimidine deoxyriboses as well as to vitamin B_{12} (F4).

Ford (F5) designed an assay technique using the protozoan *Ochromonas malhamensis*. The organism is rather fastidious and difficult to handle but has the advantage of responding only to cyanocobalamin, although the presence of methionine does have a vitamin B_{12}-sparing effect. The method has not been used in clinical medicine but is valuable for determining the vitamin B_{12} concentration in feces, etc., which may also contain significant concentrations of vitamin B_{12} analogs.

Davis and Nicol (D6) designed an assay using the small centric diatom *Cyclotella nana* Hustedt. The diatom is resistant to a wide range of salt concentrations, making it one of the few organisms suitable for measuring the vitamin B_{12} content of estuarine and sea waters, a matter of some importance in the study of marine productivity. The organism appears equally sensitive to both cyanocobalamin and hydroxocabalamin and shows little response to vitamin B_{12} analogs. Growth of the organisms used in these biological assays is usually measured in a colorimeter. However, *C. nana* does not produce sufficient turbidity to enable measurements to be made in this way. As the diatom uses silicon from the medium in a linear fashion, the amount of free silicon remaining in the medium can be measured chemically and related to growth.

4.2. Radioisotope Dilution Assays

Radioisotope dilution (radiodilution) techniques for measuring vitamin B_{12} were first introduced in 1961 (B4,R7). A simplified method suitable for clinical use was described by Lau *et al.* in 1965 (L2) and this was quickly taken up by many laboratories throughout the world. These early assays were designed to measure the vitamin B_{12} concentration in serum. The sample is first acidified and heated to free the vitamin from its protein binder, and a volume of radiolabeled vitamin is added (usually $[^{57}Co]B_{12}$) followed by the addition of a known amount of intrinsic factor sufficient to bind approximately 60 to 80% of the added $[^{57}Co]B_{12}$. Albumin-coated charcoal is then added to the preparation, the charcoal behaving as a sponge and the albumin coat as a molecular sieve, resulting in the absorption of the free vitamin onto the charcoal. After removal of the coated charcoal by centrifugation the activity of the bound vitamin in the supernatant is measured in a gamma counter. Results are obtained by comparing with a series of standards and compare well with those obtained using the *Euglena* assay.

A number of variations of the radiodilution technique have since been

described and these have used a variety of vitamin B_{12} binding agents such as chicken serum (G3), human saliva (C8), oyster toadfish serum (B5), and human serum (T3). Many of these methods gave higher results than methods using intrinsic factor as the binder. Linnell (L3) considered that the discrepancy was due to problems with technique but there is persuasive evidence that the problem resulted from the use of vitamin B_{12} binding agents that, unlike intrinsic factor, bound not only the biologically active vitamin but also biologically inactive vitamin B_{12} analogs. In 1978 it was reported that some patients with pernicious anemia appeared to have a normal serum level of vitamin B_{12} when measured by the radiodilution assay (C9,K1) and this resulted in manufacturers modifying their assay kits in such a way that they were no longer sensitive to vitamin B_{12} analogs. This was generally effected by using only purified intrinsic factor as the vitamin B_{12} binder. For some years it had been appreciated that there were problems with the radiodilution assays. Raven *et al.* (R8) found that some postgastrectomy patients had a normal serum vitamin B_{12} concentration when assayed by radiodilution and abnormally low results when assayed microbiologically. We have found that some patients with diabetes mellitus have high serum vitamin B_{12} concentrations when measured isotopically but show borderline results when measured using *E. gracilis*. Zacharakis (Z1) compared results obtained using saturation analysis with intrinsic factor and R-protein as binding agents. Of 210 patients with pernicious anemia, 3 had results which overlapped the normal range when R-protein binder was used, and 1 of 117 samples from patients with pernicious anemia was reported as "just normal" using gastric juice binder.

Another problem associated with saturation analysis is that abnormally low results may be obtained unless cyanide is present when the vitamin is freed from its binder. It appears that forms other than cyanocobalamin are difficult to separate completely from the binding protein. Early studies that failed to recognize this not infrequently found that results from patients with pernicious anemia gave negative values (R9). A recent study by Brown *et al.* (B6) examined the effect of varying the concentration of cyanide used in the test. They found that an excess of cyanide resulted in a significant increase in apparent vitamin B_{12} levels in sera from patients who were deficient in the vitamin, but it had little effect on sera from normal patients. They found the mean of 12 vitamin B_{12}-deficient sera to be 49 ng/liter when 3 mg/liter of cyanide was used in the extraction mixture, 104 ng/liter in the presence of 30 mg/liter of cyanide, and 196 ng/liter when 300 mg/liter of cyanide was used. The authors emphasized that cyanide was necessary to convert all of the several forms of vitamin B_{12} present in serum to cyanocobalamin but warned that the concentration should not be greater than 5 mg/liter.

The marketing of kits for the measurement of vitamin B_{12} is a highly

competitive business and this results in regular changes and innovations, of which only a few offer a real improvement in technique. "No-boil" kits have appeared that use alkali rather than heat to denature the protein binder, and this has been followed by a solid-phase test in which the vitamin B_{12} binder is immobilized on cellulose particles. Results obtained using these methods are similar to those obtained with kits using the more conventional approach.

4.3. Measurement of Vitamin B_{12} Absorption

The most common cause of vitamin B_{12} malabsorption is due to a lack of intrinsic factor associated with pernicious anemia. However, there are other causes such as ileal malfunction and gastrectomy. Schilling (S12,13) originally introduced a method of assessing absorption by measuring the radioactivity appearing in the urine following an oral dose of radiolabeled vitamin B_{12}. Of unlabeled vitamin B_{12}, 1 mg was usually given parenterally following the oral dose and this served to flush the absorbed isotope-labeled vitamin from the bloodstream into the urine. Normal subjects were found to excrete between 16 and 45% of the labeled vitamin in their urine while patients with pernicious anemia and those who had had a total gastrectomy usually excreted less than 5%. Adding intrinsic factor to the labeled vitamin B_{12} resulted in a marked increase in absorption of the vitamin in these patients. However, when malabsorption was not due to a lack of intrinsic factor the addition of this material did not improve absorption.

Four isotopes of cobalt have been used to produce radiolabeled vitamin B_{12}: ^{56}Co, ^{57}Co, ^{58}Co, and ^{60}Co. Although ^{56}Co has a short half-life of 77 days it results in the greatest radiation exposure; ^{60}Co has a half-life of 5.27 years, which also results in undesirable exposure. For this reason these two isotopes are seldom used. The isotope most commonly used is ^{58}Co, though ^{57}Co is equally suitable. In 1963 a dual-isotope test was described which used both ^{57}Co- and ^{58}Co-labeled vitamin B_{12} (K2) that, it was claimed, permitted a rapid differential diagnosis of vitamin B_{12} malabsorption to be made. An oral dose of free cyanocobalamin labeled with ^{58}Co was given, and if absorption was impaired this would be reflected by a reduction in the amount of the vitamin appearing in the urine, as would be the case in a patient having a deficiency of intrinsic factor. At the same time an oral dose of cyanocobalamin labeled with ^{57}Co and bound to intrinsic factor was given. Absorption of this and the resulting level of ^{57}Co activity in the urine is independent of the patient's ability to secrete intrinsic factor but is a measure of his ability to absorb the vitamin in the ileum. The activity of the individual isotopes can be counted without difficulty because of the substantial difference in the energy of their gamma emissions. Pulse height ana-

lyzers and dual scalers can make counting very simple but any scintillation counter can be used by varying the discriminator bias. The test has the advantage that complete 24-hour samples of urine, although desirable, are not essential because results can be expressed as the ratio of the two isotopes excreted, i.e., a ratio of 1 indicates that both isotopes have been absorbed equally, while a ratio of 2 indicates that the patient is not producing intrinsic factor. This test has been made available as a commercial kit.

Absorption tests of this kind are not without their problems. Radiation produced by the label tends to degrade the parent molecule and this may result in a loss of up to 25% of the vitamin activity by the end of 4 weeks. This can be partly overcome by storing the vitamin in a diluted form. The ^{57}Co or ^{58}Co freed by degradation of the molecule may be readily absorbed. Degrading of the vitamin may be monitored by measuring its concentration using a microbiological assay. Renal failure may result in poor excretion of the vitamin. The administration of other isotopes may produce a misleadingly high urinary activity and the taking of a meal shortly before the test dose can result in dilution of the label with a consequent reduction in absorption. The validity of the dual-isotope single-stage absorption test has recently been questioned. Fairbanks *et al.* (F6) found that in some patients $[^{58}Co]B_{12}$ appeared to replace some of the $[^{57}Co]B_{12}$ on the intrinsic factor molecule. This resulted in the apparent absorption of $[^{58}Co]B_{12}$ without added intrinsic factor and suggested that absorption of the vitamin was normal, or if it was abnormal that the abnormality was not due to a lack of intrinsic factor. It was thought that the exchange of the isotope-labeled vitamins on the intrinsic factor molecule took place when there was a prolongation of the transit time from the stomach to the ileum or when there was an excessive gastric acidity. This last point would be unlikely to affect the results in patients with pernicious anemia who have a histamine fast achlorhydria. This was an important observation because a number of patients who had pernicious anemia were missed because of the apparent absorption of free $[^{58}Co]B_{12}$. The one-stage absorption test has also been found to give an excessive number of intermediate or ambiguous results compared with the two-stage Schilling test (F7).

Vitamin B_{12} deficiency may result in the abnormal development of many body cells including the mucosal cells lining the intestine, and this in itself can result in malabsorption of the vitamin. This may be reversed following treatment and it is important therefore not to undertake tests of absorption until the patient has been adequately treated with vitamin B_{12} and a suitable period for recovery has elapsed.

It is usual to give a test dose of between 0.5 and 1.0 μg of the labeled vitamin for the standard Schilling test, and in the case of the dual-isotope technique 0.25 μg each of ^{57}Co- and ^{58}Co-labeled vitamin. It has been found

that if test doses of the vitamin exceed 2.0 μg the percentage of the dose excreted is reduced (E4).

Absorption studies are usually performed on fasting patients, as the presence in the stomach of food containing vitamin B_{12} may dilute the dose of radioactive vitamin and result in apparent poor absorption. On the other hand, it is possible that testing absorption by giving an oral dose of pure vitamin B_{12} in aqueous solution provides an ideal situation which is seldom likely to occur in practice. To approximate more closely the physiological process, some workers have given the test dose of the vitamin bound to protein such as egg (D7) or chicken serum (D8). Malabsorption of protein-bound vitamin which is not detected by administering the vitamin in the free form may reflect a clinically important vitamin B_{12} deficiency. An interesting observation made in India by Desai *et al.* (D9) was that black coffee enhanced the absorption of the vitamin. Taking 170 ml of black coffee with the labeled vitamin was found to reduce the number of equivocal results.

4.4. The Deoxyuridine Suppression Test

Thymidine is required by replicating cells and may be obtained via a synthetic pathway or a salvage pathway which recycles preformed thymidine. The synthetic pathway depends on the methylation of deoxyuridine, the methyl donor being 5,10-methylenetetrahydrofolate. This reaction is vitamin B_{12} dependent and a deficiency will result in an accumulation of 5,10-methylenetetrahydrofolate due to the inability to transfer the methyl group. In the presence of deoxyuridine and adequate folate, thymidine will be synthesized. However, when the available folate is reduced, thymidine will be obtained via the salvage pathway. Killmann (K3) designed a test based on this reaction that made it possible to determine the role played by vitamin B_{12} or folate in the pathogenesis of disordered hemopoiesis. The method underwent a number of modifications but the technique described by Wickramasinghe (W3) appeared to be reliable and is relatively simple to perform.

Bone marrow is placed into a balanced salt solution containing preservative-free heparin and a single cell suspension prepared by passing the marrow through a fine wire mesh. Alternatively, a small Potter–Elvehjem tissue homogenizer (H11) may be used. The nucleated cell count is adjusted to $5–10 \times 10^6$ ml of balanced salt solution and deoxyuridine added. Following incubation, 5 μCi of tritiated thymidine is added and the mixture incubated for an additional hour. The cells are then washed and the nucleated cell count determined. A 0.1 ml aliquot is then placed on a filter paper disk and dried. The activity of the dried disks is then measured in a scintillation

fluid using a beta counter. The amount of labeled thymidine incorporated into the DNA of 10^6 nucleated cells is calculated and the results are expressed as the percentage uptake when compared with the activity of a sample which had not been preincubated with deoxyuridine. This is called the deoxyuridine-depressed value. Wickramasinghe (W3) found the normal range to be 1.4–1.8%.

A depressed uptake of deoxyuridine brought about by a deficiency of vitamin B_{12} can be reversed as reflected by a reduction in labeled thymidine uptake by the addition of as little as 1 μg of the vitamin. Folic acid added to a concentration of 50 μg/ml of the culture will correct abnormal results due to folate deficiency and may partially correct abnormal results due to vitamin B_{12} deficiency. Abnormal results may occur in bone marrow from patients with iron deficiency and from patients being treated with 5-fluorouracil (B7).

4.5. Measurement of Methylmalonic Acid

Methylmalonyl-CoA mutase is a cobalamin-linked enzyme of mitochondria that catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA. A reduction of this enzyme due to vitamin B_{12} deficiency will result in a metabolic block with the urinary excretion of methylmalonic acid, and the measurement of this metabolite has been used to confirm a deficiency of vitamin B_{12}. The test has also been useful in investigating rare abnormalities of this enzyme that result in the excretion of methylmalonic acid in the presence of adequate vitamin B_{12}. Given an oral loading dose of valine or isoleucine will increase the urinary excretion of methylmalonic acid in patients with a vitamin B_{12} deficiency (G4). However, Chanarin and his colleagues (C10) found that one-quarter of their patients with pernicious anemia excreted a normal concentration of methylmalonic acid even after a loading dose of valine. Normal subjects excrete up to 15 mg of methylmalonic acid in their urine over a 24-hour period (C11).

Methylmalonic acid is technically difficult to measure and this has precluded its use as a routine clinical test for vitamin B_{12} deficiency, although it remains a valuable tool for investigating congenital defects of the methylmalonase-CoA pathway. It may be assayed colorimetrically following separation by ion-exchange chromatography as described by Oace and Chen (O1), or by using gas chromatography following thin layer chromatography and extraction with diethyl ether as described by Gompertz (G5). A rapid and sensitive spectrophotometric method has also been devised by Frenkel and Kitchens (F8). This is based on the conversion of methylmalonic acid to propionate by heat decarboxylation and measurement of the propionate increase over the endogenous amount in the noncarboxylated specimen.

4.6. Assay of Antibodies to Intrinsic Factor

Schwartz (S14) observed that 18 of 52 patients with pernicious anemia had antibodies to intrinsic factor in their serum, and in the past this information has been helpful in establishing a diagnosis of pernicious anemia. A rapid, sensitive, and reproducible method for measuring intrinsic factor antibodies was described by Gottlieb and his colleagues (G6). The method took advantage of the fact that following contact with its antibody, intrinsic factor will no longer bind vitamin B_{12}. Unbound vitamin was absorbed onto precoated charcoal and the amount of bound vitamin remaining was governed by the concentration of intrinsic factor used and the titer of the antibody being assayed. Radiolabeled vitamin B_{12} was used in this assay; the radioactivity of the supernatant following absorption was compared with a duplicate assay without the added antibody and this enabled the titer of the antibody to be calculated.

5. Effect of Drugs on Vitamin B_{12} Metabolism and Absorption

A number of drugs are known to affect absorption of vitamin B_{12} in man. These include neomycin, potassium chloride, *p*-aminosalicylic acid, and colchicine. This section is not a comprehensive review but rather examines a few of the more commonly used drugs which may affect absorption and utilization of the vitamin in man.

5.1. Nitrous Oxide Inhibition of Vitamin B_{12}

In 1956 Lassen *et al.* reported that 6 of 13 patients with tetanus developed hematological changes with granulocytopenia and thrombocytopenia following prolonged treatment with nitrous oxide anesthesia (L4). Green and Eastwood (G7) subjected rats to nitrous oxide inhalation for periods of up to 6 days and found that they developed neutropenia and bone marrow depression. However, they found that the megakaryocytes appeared to be unaffected. Until that time nitrous oxide had been considered a safe, inert gas, although this may in some measure have been due to a lack of study of its properties. Kripe *et al.* (K4) also studied the effect of nitrous oxide exposure on rats and found evidence of bone marrow injury by the third day of treatment using a mixture of 40% nitrous oxide, 20% oxygen, and 40% nitrogen. The bone marrow was characterized by a reduction in myeloid and lymphoid activity with intracytoplasmic vacuolization of myeloid precursors. The megakaryocytes appeared unaffected.

Amess *et al.* (A5) noted a high incidence of megaloblastic change in pa-

tients receiving mixtures of 50% nitrous oxide and 50% oxygen for short periods. Further work by Cullen *et al.* using DNA flow microfluorimetry showed that patients receiving nitrous oxide had an increased proportion of early S-phase cells with a decrease in late S, G_2, and mitotic cells in their bone marrow (C12). The changes were similar to those seen following the use of cytotoxic drugs with S-phase specificity. These authors concluded that the bone marrow changes were probably the result of an acute vitamin B_{12} deficiency. Nitrous oxide appears to inhibit the cytosol enzyme methionine synthetase and, in the rat, exposure to 50% nitrous oxide was found to result in a 70% inhibition of this enzyme. Interestingly, inhibition of the mitochondrial enzyme methylmalonyl-CoA mutase was observed only following prolonged exposure to the gas and this was only seen in rats. It appeared that nitrous oxide was able to modify the vitamin in such a manner that the resulting methionine synthetase was without activity (K5).

Nitrous oxide reacts rapidly with a number of transition-metal complexes having a metallic ion linked directly to an organic compound, and vitamin B_{12} with its central cobalt atom fits into this group (B8). These complexes are able to activate nitrous oxide, releasing free nitrogen and oxygen. In the course of this reaction, vitamin B_{12} is changed from the reduced cob(I)alamin form to the oxidized form, cob(III)alamin. The vitamin is required in the reduced form in the methionine synthetase pathway and once the cobalt atom is oxidized the vitamin is no longer able to function as a coenzyme in this system (C13). Methylmalonyl-CoA appears unaffected by the change from the reduced to the oxidized form of the vitamin, at least in the short term. Linnell and his colleagues (L5) studied the synthesis of methylcobalamin and adenosylcobalamin in normal human lymphocytes exposed to nitrous oxide. Results showed that methylcobalamin synthesis was markedly depressed, whereas adenosylcobalamin synthesis was not.

Myeloneuropathy may occur in patients exposed to the gas for prolonged periods. Layzer (L6) described neurological changes in 15 patients; 14 were dentists and 12 of these had abused nitrous oxide. The fifteenth patient was a hospital technician who had inhaled the gas by blowing up a surgical glove with nitrous oxide from a wall outlet and then inhaling the gas from the glove. Neurological symptoms included numbness or paresthesia of hands and legs, inability to walk, impotence, and impairment of equilibrium or gait. The autonomic nervous system was not involved except in those with altered sexual function and sphincter control. In general, the myelopathy was similar to that found in subacute combined degeneration of the spinal cord and it appeared to be the result of inactivation of vitamin B_{12} by the gas.

Because vitamin B_{12} in the oxidized form inactivates methionine synthetase, it could be expected to interfere with folate metabolism because 5-methyltetrahydrofolate would be unable to donate its methyl group. This

would prevent methylation of homocysteine to form methionine with consequent release of tetrahydrofolate. The impaired utilization of methyltetrahydrofolate probably accounts for the rise in serum folate levels found 8 hours after starting nitrous oxide inhalation (L7). However, this rise was transient and body stores of folate then appeared to fall. The intracellular formation of pentaglutamate that occurs once folate enters a cell is inhibited by nitrous oxide, and this metabolic block may lead to tissue depletion. Following withdrawal of nitrous oxide and the return of normal vitamin B_{12} function, a dietary source of folate is required to restore the vitamin pool to its preexposure level (L8).

Although exposure to nitrous oxide is associated with an acute vitamin B_{12} deficiency due to oxidation of the vitamin, the serum vitamin B_{12} concentration remains normal even when measured using a biological system with *E. gracilis* as the test organism (A6). The organism is apparently able to use both reduced and oxidized forms of the vitamin.

5.2. Ascorbic Acid

It was observed by Herbert and Jacob (H12) that the daily ingestion of 0.5 g or more of ascorbic acid destroyed substantial amounts of vitamin B_{12} when taken within 1 hour of a meal. When taken in this way the ascorbate was found to have a direct effect on the vitamin. Patients taking 2 g of oral ascorbic acid daily have been found to have a low serum vitamin B_{12} level. However, bone marrow studies in two patients showed normoblastic erythropoesis and a normal deoxyuridine suppression test although one showed some hypersegmentation of the granulocyte nuclei (H13). However, other workers have failed to demonstrate that ascorbic acid has any affect on food vitamin B_{12} (M3). In another study, serum vitamin B_{12} concentrations were measured in 20 children with myelomeningocele who were taking at least 1.5 g of ascorbic acid daily. Half of these children had been taking ascorbic acid for more than 3 years. There was no significant difference in serum vitamin B_{12} concentrations in children taking ascorbic acid as compared to control children not taking ascorbate (E5). Hogenkamp (H14) concluded that among the naturally occurring forms of vitamin B_{12}, only aquocobalamin was readily reduced and subsequently destroyed by ascorbic acid. The major cobalamins in food are methylcobalamin and adenosylcobalamin, and it was questioned whether these cobalamins were destroyed by ascorbate. In a later series of experiments Herbert (H15) found that incubation for 30 minutes at 37°C at pH 7.0 in 0.5% ascorbic acid resulted in a substantial loss of activity of hydroxocobalamin and cyanocabalamin. Herbert also raised the interesting question as to whether the use of ascorbic acid in the salting

of fresh meats to prevent oxidative browning of the red color results in the formation of vitamin B_{12} analogs.

5.3. Biguanides

The biguanide hypoglycemic agents metformin and phenformin have been found to cause malabsorption of vitamin B_{12}. In a group of 25 diabetic patients on long-term treatment with phenformin, the Schilling test was found to be abnormal in 10. The test was repeated with the addition of intrinsic factor in 6 patients and 5 failed to show any improvement. None of the patients had any evidence of anemia (J2). The effect was reversible and absorption returned to normal 2–8 weeks following cessation of the drug. Only one case of megaloblastic anemia asociated with malabsorption of vitamin B_{12} due to long-term treatment with metformin has been reported (C14). The patient had been treated with metformin for 8 years and was clinically anemic with lassitude and general debility. Her hemoglobin was 89 g/liter, her serum vitamin B_{12} was 60 ng/liter, and a bone marrow biopsy showed megaloblastic hyperplasia. There was no evidence of neurological disease. A modified Schilling test showed malabsorption of vitamin B_{12} that was not improved by the addition of intrinsic factor. The patient continued to take metformin and in addition was given a monthly injection of vitamin B_{12}.

5.4. Chlorpromazine

Herbert and his associates (H10) found that growth of *E. gracilis* was suppressed by chlorpromazine and that serum from patients treated with this drug appeared to have a reduced level of vitamin B_{12}. Similar results were obtained by Jacob and Zondag (J3) using an assay with *L. leichmannii* as the test organism. However, the same sera assayed by a radiodilution method were found to give much higher results. In a carefully designed study we were unable to show that chlorpromazine had any effect on the growth of *E. gracilis* (D4). Voogd and Burg (V3) also found normal serum vitamin B_{12} levels in 20 patients receiving 50–600 mg of chlorpromazine daily using a microbiological assay with *L. leichmannii* as the test organism.

5.5. Oral Contraceptive Agents

A significantly reduced serum concentration of vitamin B_{12} has been found in women taking oral contraceptive steroids when compared with a large group of women not taking these agents (D10). However, all of the women

taking oral contraceptives had a vitamin B_{12} concentration which was within the reference "normal" range. It appears that the vitamin B_{12} level in these women reestablishes itself at a different and somewhat lower level. Similar findings have been made by a number of workers including Costanzi *et al.* (C15), who, in addition to finding a reduced serum concentration of vitamin B_{12}, observed that there was no correlation between this finding and the concentration of vitamin B_{12} binding protein, which remained unchanged.

5.6. Vitamin B_{12} Deficiency in Alcoholics

Vitamin B_{12} depletion could be expected in chronic alcoholics since their diet is often low in animal protein, and, although many alcoholic beverages are the result of bacterial fermentation, they have nevertheless been found to be essentially free of vitamin B_{12} (L9). Reduced levels of serum vitamin B_{12} have been reported in alcoholics by some workers (H16,L9) while others have found the concentration to be normal or elevated (D11). Because of the liver damage often associated with alcoholism, serum levels of the vitamin may be normal or elevated even though liver stores of the vitamin are reduced (R10,S15). An elevated level of serum vitamin B_{12} binding protein may also serve to increase the vitamin B_{12} level. The interpretation of serum vitamin B_{12} levels in alcoholics is of very limited importance since a clinically significant deficiency of the vitamin very rarely occurs.

The gastritis and chronic pancreatitis associated with chronic alcoholism may result in a reduction of the amount of vitamin B_{12} absorbed but this has not been found to result in a clinical deficiency (M4). Alcohol can also cause damage to the ileum. Lindenbaum and Lieber gave alcohol to human volunteers for periods of 13–37 days and found that absorption of the vitamin was impaired in six of eight volunteers and this was not corrected by the addition of intrinsic factor or pancreatin (L10,L11). Biopsy of the ileum showed ultrastructural evidence of mitochondrial damage (R11). It has been shown previously that folate deficiency may result in a reduction in the serum vitamin B_{12} level (H16) and the low serum vitamin B_{12} levels found in some alcoholics is probably secondary to folate depletion, which is common in this condition (L12). How folate is able to influence the serum vitamin B_{12} level is not clear.

6. Vitamin B_{12} and Pregnancy

Except for the rare patient with an undiagnosed pernicious anemia, vitamin B_{12} deficiency is unusual in pregnant women consuming a "normal"

varied diet. In India, chronic tropical sprue has been associated with vitamin B_{12} malabsorption and a low serum level of the vitamin (B9).

Lactating women consuming diets low in animal protein and dairy products may have difficulty in providing enough vitamin B_{12} for both their own and their babies' needs. These women should be offered supplementary oral vitamin B_{12} when they are not prepared to change their dietary habits, for religious or other reasons. The vitamin in tablet form may be obtained from some health food stores and is bacterial in origin.

Levels of transcobalamins appear to change during pregnancy. In a study of 193 women at various stages of pregnancy it was found that the levels of transcobalamins I and III rose steadily through the pregnancy and fell during the puerperium. On the other hand, transcobalamin II decreased in the second trimester, rose sharply in the third, and fell in the puerperium. It has been suggested that this fluctuation reflects the needs of the fetus at that particular time (F9).

7. Clinical Conditions Associated with Vitamin B_{12} Deficiency

7.1. Pernicious Anemia

This term was used in the past to describe an anemia of insidious onset with a usually fatal outcome. The anemia is now known to respond to treatment with parenterally administered vitamin B_{12}, and so it can no longer be termed pernicious. However, the term pernicious anemia is now so firmly established in describing the anemia that it seems unlikely to be changed. The disease, although well known, is uncommon and affects some 127 per 100,000 people in the United Kingdom (S16). European countries and North America have an incidence of the disease not greatly different from the United Kingdom. Pernicious anemia appears to become more common with advancing age and it is unusual to find it in people below the age of 40 years. Females are more often affected than males, and Chanarin quotes a ratio of 10:7 (C16). It appears to affect most races.

Patients may present with lassitude, unsteadiness of gait, and paresthesia. They frequently have blue eyes and prematurely white or graying hair and their skin often has a pale lemon tint. The tongue may be beefy red and smooth and chelosis may be present. They may have a severe anemia with a hemoglobin which may be as low as 40 g/liter. The anemia is macrocytic and is usually accompanied by a granulocytopenia and thrombocytopenia. In the stained blood film the granulocytes may appear abnormally large with a hypersegmented nucleus, and it is generally accepted that this is one of the

earliest features of both vitamin B_{12} and folate deficiency. The erythrocytes, too, are frequently very large and the mean corpuscular volume (MCV) may reach 140 fl. These cells are, however, very fragile and as the hemoglobin decreases, many of the larger cells fail to survive and the MCV sometimes appears to decrease. Because of the more rapid breakdown of erythrocytes, the serum or plasma may appear slightly icteric.

Bone marrow biopsy shows a varying degree of megaloblastosis and when the anemia is mild there may be only an occasional megaloblast, although it is usually possible to find some giant metamyelocytes. These cells may be up to 25 μm in diameter and contain a large horseshoe-shaped nucleus. As the hemoglobin falls the bone marrow may show an increasing number of large primitive megaloblasts. The nucleus of these cells has a fine chromatin network, in contrast to the normoblast in which the chromatin is coarsely clumped. The nucleus and cytoplasm of the megaloblast appear to lack synchrony in their rate of maturation and the cytoplasm may become hemoglobinized while the nucleus remains relatively immature. Megakaryocytes are also affected and this is likely to be associated with a considerable degree of ineffective thrombopoiesis.

Examination of the bone marrow, although important, will only confirm that the hemopoiesis is megaloblastic. A deficiency of folic acid will also cause a megaloblastic anemia and it is not possible to identify the cause on the basis of morphology. A serum assay of both vitamins will usually indicate which is responsible. If the patient is vitamin B_{12} deficient, the next step is to carry out a vitamin B_{12} absorption test to confirm that the deficiency is due to a lack of intrinsic factor. Preferably this should not be done until the patient's vitamin B_{12} and hemoglobin levels have returned to normal, since the gastric and intestinal cells are also affected by a lack of vitamin B_{12}; aborption may be less than optimal if it is attempted too early. Patients with pernicious anemia also have a histamine-fast achlorhydria and gastric atrophy. The disease appears to have an autoimmune basis and antibodies to intrinsic factor can be demonstrated in the serum of more than half of affected patients.

In females it has been shown that untreated pernicious anemia is associated with infertility. Female patients who have previously failed to conceive became pregnant within a few months of starting treatment with vitamin B_{12} (H17,J4). On the other hand, in a study of vitamin B_{12} levels in pregnancy, Armstrong and his associates (A7) found three women with a previously undiagnosed pernicious anemia. Infantile vitamin B_{12} deficiency may occur in the rare instance when a baby is breast fed by a mother who has an undiagnosed pernicious anemia (H19). Hoey and his colleagues (H19) reported a 14-month-old breast-fed girl presenting with severe neurological changes. She had been breast fed for 14 months without supplements. Her

development was normal, but 6 weeks before admission to the hospital she had a respiratory infection, glossitis, and a skin rash. She became lethargic, her motor skills regressed, and she could no longer stand or sit upright. On investigation, her hemoglobin was 94 g/liter and her serum vitamin B_{12} was 40 ng/liter. Her mother was also found to have a low serum concentration of vitamin B_{12} (129 ng/liter), but this was not as low as might be expected in pernicious anemia. However, the mother's pernicious anemia was confirmed by a Schilling test.

7.1.1. *Pernicious Anemia and Neuropathy*

Neuropathy may develop in the presence of a vitamin B_{12} deficiency regardless of the cause, although it is most commonly associated with pernicious anemia. The presenting signs are usually unsteadiness when walking (some patients state that they feel as if they are walking on cotton wool), numbness, and a sensation of pins and needles. Clinical examination reveals a pronounced loss of vibration sense and exaggerated knee jerks. The peripheral nerves and posterior columns of the spinal cord are affected first and this may be followed by changes in the lateral columns. Butler *et al.* (B10) described two patients with electric shocklike dysesthesia produced by neck flexion (Lhermitte's sign). This symptom is associated with damage to the posterior columns of the spinal cord, and both patients had pernicious anemia. Severe neuropathy may occur without anemia, and Perold (P3) described a 31-year-old white women presenting with a 1-month history of progressive unsteadiness of gait and a tendency to fall toward the left. She also complained of a numb feeling in both legs. Her general physical condition was good. She had a hemoglobin concentration of 132 g/liter and an MCV of 96 fl. After a further 2 weeks she was even more ataxic and the deep reflexes in both legs were clearly exaggerated. The combination of posterior and lateral column signs suggested vitamin B_{12} deficiency and after blood had been taken for the measurement of this vitamin she was given 1 mg intramuscularly. Seven days later she felt much improved and with further treatment she became symptom free. This patient had a serum vitamin B_{12} concentration of 100 ng/liter (reference range 300–1000 ng/liter); she was achlorhydric, and a Schilling test showed malabsorption of the vitamin, which was corrected by the addition of intrinsic factor.

In a study of peripheral nerve involvement in 20 patients with untreated penicious anemia, Cox-Klazinga and Endtz (C17) found 13 to have signs of peripheral nerve dysfunction with reduced conduction velocities. Interestingly, 16 of these patients were also found to have reduced blood thiamin levels. Clinical signs of peripheral nerve involvement were reported by Pallis and Lewis (P4) to be common in patients with untreated pernicious

anemia. Peripheral neuropathy has also been reported in patients with vitamin B_{12} deficiency following gastrectomy (K6).

Cerebral involvement may occur in 25–60% of patients with untreated pernicious anemia. However, the changes are usually relatively mild (G8,S17). Occasionally, patients present with serious psychiatric changes and this has been described as megaloblastic madness. Patients may be greatly agitated (H20) or may be depressed. Some have hallucinations while others suffer from an acute confusional state and, rarely, they may be violent. Although many patients with neurological signs also have anemia, this is not invariably so and occasionally the hemoglobin may be within normal limits (P3). It is also of interest that a raised level of serum folate usually accompanies moderate or severe neurological involvement but appears unrelated to the degree of anemia (W1). Treatment with folic acid may result in rapid and severe neurological deterioration. Kosik *et al.* (K7) described a 23-year-old woman who had taken folic acid intermittently for a "mild anemia." She was admitted to the hospital because she was confused and at this time had diminished knee and ankle jerks. The confusion increased and was associated with drowsiness and weakness. Two weeks before being transferred to another hospital, she developed a paranoid psychosis and 7 days later became paralyzed in all four limbs. At this time her hemoglobin was 92 g/liter and her MCV was 100 fl. A peripheral blood smear showed some hypersegmented neutrophils, a bone marrow biopsy showed a megaloblastic erythropoiesis, and her serum vitamin B_{12} was 86 ng/liter. A Schilling test showed her to absorb 4.1% of the dose of free vitamin, which improved to 16.6% with the addition of intrinsic factor. Treatment with vitamin B_{12} resulted in a return to normal mental function but she remained paraplegic with a T10 sensory level.

In a study of vitamin B_{12} levels in 835 consecutive psychiatric patients, Elsborg *et al.* (E6) found a reduced serum concentration in 10%. Of these 1 patient had pernicious anemia, 7 were postgastrectomy, and 1 had previously had a small bowel resection; the remaining 72 patients were considered to have a nutritional deficiency.

7.1.2. *Congenital Deficiency of Intrinsic Factor*

This condition has often been referred to in the past as juvenile pernicious anemia but it appears to be a quite separate entity. Confusion probably arose because there is a deficiency of intrinsic factor resulting in vitamin B_{12} malabsorption in both conditions. However, it differs from the disease in adults in that free acid is present in the gastric secretion (A8,L3,M5), the gastric mucosa is usually normal, and antibodies to intrinsic factor are not a feature. Megaloblastic anemia usually develops during the first 2 years of life but this depends on the amount of residual intrinsic factor available, and

occasionally the diagnosis is made in much older children (L14). The disorder is extremely rare and may be difficult to treat because following a course of vitamin B_{12} the child will appear to be perfectly well, and it is not easy for parents to appreciate that health will be maintained only if the child has regular injections of the vitamin. Waters and Murphy (W4) described three brothers with the disease, one of whom was seen on four occasions over a period of 8 years with anemia and a megaloblastic bone marrow. On two occasions the anemia developed 15 months after vitamin B_{12} injections had been stopped.

7.1.3. *Treatment*

Treatment of both pernicious anemia and a congenital absence of intrinsic factor is with regular injections of vitamin B_{12}. It is usual to give several injections of 1 mg at weekly intervals, and this is then followed by a monthly injection. If reasonable body stores of the vitamin have been built up, clearly patients should remain fit for many months without additional injections of the vitamin, but it is usually better to get the patient accustomed to a regular routine. Attempts have been made to give patients vitamin B_{12} orally together with hog intrinsic factor. Although this works, many patients develop antibodies to hog intrinsic factor and suffer a relapse of their anemia and such treatment is not to be recommended. A small percentage of an oral dose of the vitamin will be absorbed by diffusion and it is possible to treat the disease successfully with massive oral doses of the vitamin. Crosby (C18) described a 101-year-old woman with pernicious anemia and weighing only 32 kg. Because she found the injections of vitamin B_{12} painful she refused them and was given instead 1 mg of the vitamin orally twice weekly. Three years later her hemoglobin was 110 g/liter and she remained well.

Two forms of vitamin B_{12} are available for treatment, cyanocobalamin and hydroxocobalamin, both showing similar activity in man. Hydroxocobalamin is retained in the body more efficiently than cyanocobalamin. It disperses more slowly from the injection site (K8) and therefore a larger percentage of the dose is retained.

7.2. Nutritional Deficiency of Vitamin B_{12}

A nutritional deficiency of vitamin B_{12} is uncommon and is found mainly among those who for religious reasons or habit do not eat meat or dairy products. The concentration of vitamin B_{12} in foods not of animal origin is negligible although it must be remembered that insects and their droppings may contribute a not insigificant amount of the vitamin in unwashed fruit and vegetables. Studies in South Africa showed that the fruit bat (*Rousettus aegypticus*) developed a neurological deficit due to vitamin B_{12} deficiency

when fed "clean" fruit in the laboratory rather than natural "bush" fruits that were probably contaminated to a significant degree with insects and their droppings (T4).

Hindus eat very little food of animal origin and their diet consists largely of rice, chapatis made from wheat flour, spiced vegetables, and lentils, with some fresh fruits. Milk is consumed by some and this may be their only known source of vitamin B_{12}. However, milk is often boiled for prolonged periods and boiling for as little as 5 minutes will result in a 30% loss of the vitamin (F10). Megaloblastic anemia is three times as common in Indian immigrants living in Britain as in the white community. In a study of 25 Indian patients with megaloblastic anemia, 6 were found to be folate deficient, 4 had a nutritional deficiency of vitamin B_{12}, 7 had a mixed deficiency of both folate and vitamin B_{12}, 3 were shown to have pernicious anemia, 3 had evidence of malabsorption, and in 2 the reason for malabsorption of vitamin B_{12} remained unexplained (B11).

Jathar and Inamdar-Deshmukh (J5) examined the serum and erythrocyte vitamin B_{12} levels in lactovegetarian pregnant Indian women. They found that the mean serum level of the vitamin in 48 third trimester women was 68 ng/liter compared with 178 ng/liter in nonpregnant controls. However, the mean erythrocyte levels of the vitamin were not significantly different between the two groups, being 157 ng/liter in the nonpregnant group compared with 126 ng/liter in the pregnant women. This suggests that although serum levels were low, tissue concentrations were maintained. The major form of cobalamin in plasma is methylcobalamin while in erythrocytes it is 5-deoxyadenosylcobalamin (L15) and it may be that the 5-deoxyadenosyl form is elaborated on a priority basis at the expense of methylcobalamin. A fall in the plasma level of the vitamin has also been reported in pregnant Caucasian women (M6).

Armstrong *et al.* (A9) studied the vitamin B_{12} and folate levels in 562 Seventh-Day Adventist Church members. Seventh-Day Adventists do not drink alcohol or smoke tobacco, and they are advised not to eat flesh foods, although this is not a condition of church membership. In this study 431 were vegetarians. It is also recommended that they exercise moderation in their use of dairy products such as butter, cheese, eggs, and cream. There were 122 volunteers with serum vitamin B_{12} levels of less than 160 ng/liter, which was the lower limit of the reference range. Of these, 21 (10 men and 11 women) had a serum B_{12} level below 100 ng/liter, and of these, 4 had a serum concentration below 80 ng/liter. There were 431 volunteers who ate meat less than once per month and their mean serum vitamin B_{12} concentration was 223 ng/liter compared with 84 volunteers eating meat at least once per week who had a mean serum vitamin B_{12} concentration of 341 ng/liter. A similar difference was seen with respect to the eating of eggs: 72 volunteers

consuming less than one egg per month had a mean serum vitamin B_{12} level of 199 ng/liter compared with 275 volunteers who ate at least one egg per week who had a mean serum vitamin B_{12} level of 232 ng/liter.

After 1 year, a follow-up study of 21 volunteers with an initial serum vitamin B_{12} level of 100 ng/liter or less showed that the serum vitamin B_{12} was still 100 ng/liter or below in 6 volunteers and 160 ng/liter or less in 8. None had developed a macrocytic anemia and none had any clinical evidence of vitamin B_{12} deficency, although one 68-year-old male with a persistently low serum vitamin B_{12} concentration was found to have malabsorption of the vitamin, which was corrected by intrinsic factor. No unequivocal neurological evidence of vitamin B_{12} deficency was found in any of the volunteers. Interestingly, subjects with a serum vitamin B_{12} level below 160 ng/liter had a significantly higher mean corpuscular volume and mean corpuscular hemoglobin and lower total erythrocyte and leukocyte counts than the rest of the group. Those taking part in this study had been Senventh-Day Adventists for from 4 months to 80 years; only 29 had been members for less than 2 years. It would appear from this work that a serum vitamin B_{12} concentration of 100 ng/liter is unlikely to be of any clinical significance and is not important when it is known to be due to abstinence from meat and/or dairy products. Such people may, however, benefit by taking vitamin B_{12} supplements in situations of special stress such as blood loss or pregnancy.

Hirwe *et al.* (H21) examined the fertility of 134 Indian male lactovegetarians attending an infertility clinic: 60 were oligospermic and 24 were azoospermic. None had any evidence of anemia. Although there was an association between serum and seminal plasma vitamin B_{12} levels, treatment of five oligospermic patients with weekly intramuscular injections of 500 μg of hydroxocobalamin for 8 weeks had no effect on the sperm concentration, morphology, or motility. There was, however, a marked rise in seminal plasma vitamin B_{12}. It appears from this work that vitamin B_{12} deficiency is unlikely to affect male fertility in those patients who have a sufficient intake of the vitamin to maintain a normal hemoglobin. Although claims have been made for an improvement in the sperm count in oligospermic patients following treatment with vitamin B_{12} (P5,S18), these patients were also treated with testosterone.

7.3. Parasitic Infestation of the Intestine and Vitamin B_{12} Absorption

7.3.1. *Diphyllobothrium latum*

Although the fish tapeworm *Diphyllobothrium latum* has the reputation for causing a megaloblastic anemia in man by competing for dietary vitamin

B_{12}, only a small percentage of those infected develop a clinically significant deficiency of the vitamin. The tapeworm is found in the vicinity of many of the great lakes of the world and has been reported in Europe, the Baltic States, Russia, Japan, China, and North America. Although the worm has a wide distribution, it has only been associated with the occurrence of a megaloblastic anemia in Finland, the Baltic States, and Russia. It is not clear whether this is due to an unrecognized species difference or to the particular eating habits of the people in the area. In man, the tapeworm usually grows to a length of 3 to 4 m, but may reach a length of 20 m (B12). It may have 3000 or more segments and can produce as many as 1 million eggs daily. The eggs can mature only in the presence of water and the ciliated embryo takes 11–15 days to develop. The ciliated embryo must be ingested by the first host, a small crustacean, within 12 hours, otherwise it dies. In the crustacean the ciliated embryophore is lost and the larva migrates into the body cavity of the host where it undergoes further development. If the crustacean is now swallowed by one of the small plankton-feeding fish the larva bores its way through the intestinal wall and is found principally in the fat of the fish's mesentery. These small fish provide food for a variety of larger fish and these, if eaten by man either raw or inadequately cooked, will result in infestation with the tapeworm. In addition to infecting man, adult forms of the worm have been found in cats, dogs, foxes, bears, and seals (B13). It has been estimated that the carrier rate for *D. latum* in Finland is about 20% of the population and that worldwide there are about 9 million carriers (B12).

The tapeworm appears to have a high requirement for vitamin B_{12}; Nyberg (N1) found that in five patients with megaloblastic anemia, between 83 and 100% of an oral dose of radiolabeled vitamin B_{12} could be recovered from the worm following its expulsion. With larger doses of the vitamin, for example 3–10 μg, the uptake by the worm was less complete and some remained available for absorption by the host. The serum level of vitamin B_{12} was found to be reduced in patients with a megaloblastic anemia due to infestation with *D. latum* (N2,P5). In nonanemic carriers the uptake of vitamin B_{12} by the worm was significantly less than that found in carriers with megaloblastic anemia, nevertheless nonanemic carriers were found to have a lower serum level of the vitamin than a group of noninfected controls (P6). The amount of dietary vitamin B_{12} removed by the worm depends at least to some extent on the worm's location within the small bowel. If it is located in the jejunum it can compete more effectively with the host for the vitamin, whereas if it is attached to the terminal ileum the host may be able to absorb a greater proportion of the vitamin (B14). Neurological involvement may be present and this is similar to that found in other forms of vitamin B_{12} deficiency. The neuropathy may occur without significant anemia (B15,B16).

Expulsion of the worm results in recovery from the anemia and the speed with which this occurs depends on the level of dietary vitamin B_{12} (B17).

7.3.2. *Fasciolopsis buski*

Fasciolopsiasis is endemic in Thailand (M7,S19) but is also found in China, Taiwan, Malaysia, Kalimantan, and the Bengal Assam regions of India. This parasite lives in the small intestine, particularly the duodenum of man and pigs. It attaches itself to the gut wall and this results in foci of inflammation that sometimes lead to hemorrhage and abscess formation. Each worm may lay up to 25,000 eggs per day and in water the eggs hatch in about 3 to 7 weeks. The miracidium enters snails of the genus *Segmentina*, in which a sporocyst forms, and after several days cercariae are released from the snail and make their way to water plants that when eaten by man result in infestation with the worm. Areekul and his colleagues (A10) studied 100 patients from central Thailand, all of whom had eggs of *F. buski* in their feces. Blood samples were taken for the measurement of vitamin B_{12} and the mean value was found to be significantly lower than that found in normal (uninfected) subjects. Of 14 patients having a serum concentration of less than 100 ng/liter, 3 of 9 patients tested were found to have impaired absorption of vitamin B_{12} as determined by a modified Schilling test. Although 34 of the patients were found to have a hematocrit of less than 36%, there was no report of any megaloblastic anemia. It appears that infestation with *F. buski* may result in impaired absorption of vitamin B_{12}, but how this occurs is not clear and requires further study.

7.3.3. *Giardia lamblia*

There have been a number of reports of infestation with *G. lamblia* interfering with vitamin B_{12} and xylose absorption (A11,A12,W5). Gheorghescu *et al.* (G9) studied a group of 40 adult patients: 31 were infested with *Giardia* alone, 6 with *Giardia* and *Strongyloides*, and 3 with *Giardia* and *Taenia*. Of the 31 patients, 5 (16%) infected with *Giardia* alone were found to malabsorb vitamin B_{12} on the basis of the Schilling test. Those with additional *Strongyloides* or *Taenia* infestation appeared to have normal absorption of vitamin B_{12} (W5). Patients were treated with appropriate antiparasitic agents and were reexamined at intervals of 6 and 12 months. At 6 months the vitamin absorption test in one patient remained abnormal but at 12 months, absorption had returned to normal in all treated patients. The cause of the vitamin B_{12} malabsorption is not known but may be due to the excessive uptake of the vitamin by the parasite. Serum vitamin B_{12} levels have been found to be reduced in children with giardiasis compared with healthy controls (R12), but no actual deficiency of the vitamin has been reported.

7.4. Gastrectomy

Patients who have had a total gastrectomy can no longer secrete intrinsic factor and must eventually develop a vitamin B_{12} deficiency. In a series of 27 patients who had undergone a total gastrectomy, macrocytes appeared in the peripheral blood of some at 6 months, and after 2 years they were present in all of the 19 survivors. Megaloblastic change in the bone marrow was first seen after 2 years and was present in all survivors after 7 years (P7). MacLean and Sundberg (M8) examined the bone marrow of 14 gastrectomized patients and found 13 to be megaloblastic. Two of these patients also had a vitamin B_{12} neuropathy. It is unusual for a megaloblastic anemia to appear in less than 2 years from the time of the operation. However, in some patients vitamin B_{12} absorption may be compromised before the operation, resulting in an earlier than expected reduction in vitamin B_{12} stores. Such is the case with some patients who have had surgery for gastric carcinoma. Treatment is the same as that required by all patients who have an inability to absorb the vitamin through a lack of intrinsic factor, i.e., regular injections of vitamin B_{12} on a life-long basis. Oral preparations of the vitamin together with intrinsic factor can be used but they have generally not found favor.

7.5. Partial Gastrectomy

Where only part of the stomach is removed the absorption of vitamin B_{12} will depend on the extent of the operation. Lous and Schwartz (L16) studied 119 patients and 37 were found to have impaired vitamin B_{12} absorption. In a subsequent series of studies, malabsorption of the vitamin was found to affect 32–43% of patients (R13,R14). However, only about 5% of patients eventually show clinical evidence of vitamin B_{12} deficiency (D12). The results of the Schilling test were found to improve from a mean excretion of 3.9 and 5.2% to 10.3 and 11.7% when the test dose was given with a meal, and this probably had something to do with the stimulation of gastric secretions (T5). Partial gastrectomy for gastric ulcer results in a more serious and more frequent malabsorption of vitamin B_{12} than when the operation is done for a duodenal ulcer (J6), although this is not invariably so. Ardeman and Chanarin (A13) found that patients who had more than 10 units of intrinsic factor per milliliter of gastric juice following histamine stimulation absorbed vitamin B_{12} normally. There appears to be a rough correlation between vitamin B_{12} malabsorption and other tests of intestinal function such as xylose absorption and the measurement of fecal fat (R14). Following partial gastrectomy the ability to release vitamin B_{12} from food may be impaired, and this

together with a reduced quantity of intrinsic factor can result in a substantial reduction in absorbable vitamin.

Many studies have confirmed a low serum vitamin B_{12} concentration in patients following a partial gastrectomy. The level tends to fall slowly over several years, and by 8 years following the operation approximately 14% of patients were found to be deficient (B18,H22,M9). The measurement of serum vitamin B_{12} levels in these patients has presented some problems. Levels found to be low by microbiological methods using *Euglena* as the test organism have been normal when assayed by radiolabeled saturation analysis (R8). There is little doubt now that results given by the microbiological assay were correct and that similar results would have been obtained by laboratories using saturation analysis if purified intrinsic factor had been used as the binder in the test. Unfortunately, at the time these observations were made a considerable number of variations of the saturation analysis technique were available, many of them using binders that were not specific for biologically active vitamin B_{12}. This raises a question about the nature of the B_{12} analogs in the serum of these patients that were recognized as vitamin B_{12} by the binders used in a particular saturation analysis. Work in our own laboratory suggested that there were indeed B_{12} analogs present in the serum of some patients who have had a partial gastrectomy, and also in the serum of some diabetics. The reason for this is not clear and requires further study.

Iron deficiency may be present in postgastrectomy patients and occasionally this may mask an underlying megaloblastic anemia. Treatment of the iron deficiency will unmask the megaloblastic process and macrocytes will appear in the peripheral blood. Iron therapy appears able to influence the serum vitamin B_{12} level and Williams (W6) noted a substantial increase in the serum vitamin level following treatment with oral iron for 3 months. Of his patients, 36 had a low serum vitamin level before iron therapy, and in 26 the level rose to within the normal range.

7.6. Gastritis

Patients with severe atrophic gastritis may have impaired absorption of vitamin B_{12} and a reduced serum level of the vitamin, but this is not accompanied by either megaloblastic anemia or neuropathy. Parietal-cell antibodies have been found in 33% of patients with gastritis, none of these patients having pernicious anemia (I2,V4). Intrinsic factor antibodies were not found, and this was not surprising since it is rare to find antibodies to intrinsic factor in the absence of pernicious anemia. Patients with superficial gastritis usually have normal vitamin B_{12} absorption and normal serum levels of the vitamin.

8. Deranged Vitamin B_{12} Metabolism due to Abnormal Transport Proteins

There are three classes of vitamin B_{12} binding proteins: intrinsic factor, which facilitates absorption of the vitamin from the ileum; R-proteins, which include TC I and TC III (the function of these proteins is not clear, but they probably act as mobile storage forms); and TC II, which is the transport protein responsible for carrying the vitamin to the tissues. Abnormalities of intrinsic factor or TC II could be expected to have serious consequences for the patient, and in recent years a number of such abnormalities have been reported.

8.1. Hereditary Absence of Transcobalamin II

Four patients have been described so far with a hereditary absence of TC II (B19,H23,H24). The patients, all infants, presented with a megaloblastic anemia and a normal serum vitamin B_{12} concentration. This was not unexpected as the most abundant of the serum protein binders are R-proteins. The deficiency appears to be inherited as an autosomal recessive trait. Hematological abnormalities are usually apparent during the first few weeks of life. Patients respond to treatment with large doses of vitamin B_{12} given parenterally, usually 1 mg two or three times weekly, and presumably the vitamin enters the cells by passive diffusion. Treatment with folic acid is ineffective and may precipitate serious neurological complications. Thomas and his associates (T6) reinvestigated a patient initially thought to have a dihydrofolate reductase deficiency and who was treated for 2 years with folinic acid. From the age of 12 months his mental and motor development had regressed and he suffered frequent chest infections. His neurological state continued to deteriorate and further laboratory studies showed him to have a normal dihydrofolate reductase level but a hereditary TC II deficiency. The folinic acid therapy was stopped and treatment started with hydroxocobalamin. His mental and neurological state slowly improved but at the age of 7 years his intellectual development was severely retarded. Because of the lack of carrier protein, if treatment is stopped tissues are rapidly depleted of the vitamin and a megaloblastic anemia soon develops. For example, vitamin supplementation was stopped in one child who had up to that time been receiving 2 mg each week. The bone marrow prior to cessation showed normal morphological development of all cell lines. The study was terminated after 6 weeks because of rapidly progressive anemia with thrombocytopenia and neutropenia. The hemoglobin had fallen from 130 to 70 g/liter, and the platelets from 300 to 50 $\times$ 10^9/liter. The patient's serum vitamin B_{12} level at this time was 1200 ng/liter. Treatment with 1 mg of vitamin B_{12} given intramuscularly every 2 weeks resulted in only a partial

hematological response and 1 mg given at weekly intervals was necessary to effect a complete hematological remission (S20).

Seligman *et al.* (S21) reported an interesting patient whose parents both had an abnormality of TC II. The father appeared to be a heterozygote for transcobalamin deficency while the mother was a heterozygote for an abnormal, inactive form of TC II. The daughter had both abnormalities and presented with a hemoglobin of 54 g/liter, reduced leukocyte and platelet counts, and a megaloblastic bone marrow. Her serum vitamin B_{12} concentration was normal and within 3 weeks of starting treatment with 1 mg of vitamin B_{12} each week she had a full hematological remission.

8.2. Abnormal Transcobalamin II

An abnormal form of TC II has been described by Haurani *et al.* (H25). It was found in a 34-year-old women who had presented first as a child with a severe megaloblastic anemia that had responded to treatment with very large doses of vitamin B_{12} given parenterally. The patient was unusual in that she also responded to treatment with folic acid and was treated for 10 years with this vitamin alone without developing any signs of posterior or lateral column degeneration of the spinal cord. During periods of relapse the patient was found to have an elevated serum concentration of vitamin B_{12} with a peak concentration of 4384 ng/liter, of which 3824 ng/liter was bound to TC II. The patient had a TC II concentration of 6400 ng/liter compared with 917 $\pm$ 91 ng/liter found in 10 normal controls. This transcobalamin reacted with antibody raised against normal human TC II in a normal fashion but did not facilitate the uptake of the vitamin by human cells.

8.3. Antibodies to Transcobalamin II

Occasionally, patients have been seen who have developed antibodies to TC II. This does not appear to interfere with the uptake of vitamin B_{12} but it does seem to affect the ability to transfer the vitamin to cells. Such an antibody was found in a 38-year-old man admitted to the hospital with a pulmonary abcess. He was found to have a very high serum vitamin B_{12} level, which on one occasion reached 21,980 ng/liter. The unsaturated B_{12} binding capacity was also greatly increased with a peak of 49,530 ng/liter (reference range 600–1600 ng/liter). Most of this unsaturated vitamin B_{12} binding capacity was due to the elaboration of a circulating antibody to TC II, the resulting complex representing an abnormal binder. The patient did not have any clinical signs related to this abnormality, therefore active transfer of the vitamin to cells must have been possible (C19).

8.4. Abnormal Intrinsic Factor

The hereditary absence of intrinsic factor has already been discussed. However, an abnormal form of intrinsic factor has been described in a 13-year-old boy (K9) who presented with glossitis and a megaloblastic anemia. He did not appear to have classical pernicious anemia on the basis of normal gastric mucosa, normal gastric acidity, and the absence of antibodies to intrinsic factor. Generalized malabsorption was also ruled out by the results of radiographic and laboratory studies. The patient's gastric juice contained a normal amount of vitamin B_{12} binding protein, which behaved in a manner similar to intrinsic factor with respect to its immunological properties, apparent affinity for vitamin B_{12}, and its chromatographic mobility. However, normal gastric juice corrected the patient's vitamin B_{12} malabsorption. The patient's gastric juice was given as part of a Schilling test to a patient who had had a total gastrectomy; this resulted in a 48-hour urinary excretion of only 2.8% of the labeled vitamin compared with 24.1% when normal gastric juice was substituted. The patient's mother and father, who were first cousins, both had a similar intrinsic factor abnormality except that they appeared to be heterozygotes. Their gastric juice, given to the same gastrectomized patient as part of a Schilling test, resulted in the excretion of 17.9 and 17.3% of the dose, respectively.

8.5. Vitamin B_{12} Binding Proteins and Malignancy

Malignant disease may be associated with an increase of vitamin B_{12} binding protein or the appearance of abnormal forms of the binding proteins. Kane and his colleagues (K10) found 3 of 44 patients with hepatocellular carcinoma to have grossly raised levels of tumor-related vitamin B_{12} binding protein. The unsaturated vitamin B_{12} binding capacity fell during partial remission following treatment with adriamycin in one patient and following resection in a second patient. The level increased with the advance of the disease. None of these patients had cirrhosis, which in itself may increase the unsaturated vitamin B_{12} binding capacity. Increased levels of vitamin B_{12} binding protein have been found in hepatoma tumor tissue compared with normal liver tissue (B20), and this provides some support for the hypothesis that the hepatoma cells are the site for the synthesis of this unusual vitamin B_{12} binding protein. Alternatively, the hepatoma cells may modify only TC III (A14).

An unusual vitamin B_{12} binding protein has been described by Jacob *et al.* (J7). The patient was a 59-year-old black male with widely metastasized carcinoma of the lung. The tumor produced a protein that complexed with R-protein, forming a macromolecular complex which was distinct from TC I, II, or III, and 90% of the endogenous vitamin B_{12} was held in this complex.

8.6. Deficiency or Absence of R-Type Vitamin B_{12} Binding Proteins

An absence of R-type binding protein has been reported in two adult siblings by Carmel and Herbert (C20). R-Type protein was virtually absent from their leukocytes and saliva, and as was expected they had very low levels of serum vitamin B_{12}. The absence of the protein did not appear to have any adverse effects. Other members of this family have also been found to have an absence of, or very low levels of, R-protein (H26). There was no general deficiency of plasma glycoproteins in these patients and the amounts of thyroid binding globulin, thyroxine, ceruloplasmin, and transferrin were all normal.

8.7. Vitamin B_{12} Absorption and Exocrine Pancreatic Dysfunction

Patients with exocrine pancreatic dysfunction may malabsorb vitamin B_{12} because a considerable part of the dietary vitamin may bind to R-proteins in the stomach. The R-proteins are normally broken down by pancreatic enzymes. The vitamin is released and binding to intrinsic factor then takes place. If the R-proteins are not degraded, then the vitamin B_{12} will remain bound to these proteins and will not be absorbed (M10). Harms and his colleagues (H27) measured vitamin B_{12} absorption in 19 children with exocrine pancreatic insufficiency and found the average absorption to be 8.0% compared to 59.2% in a control group. Adding pancreatin to the test dose of radiolabeled vitamin B_{12} increased absorption of the vitamin to an average of 61%.

Brugge *et al.* (B21) described a simple dual-isotope test for pancreatic exocrine function. They gave their patients radiolabeled [^{57}Co]B_{12} bound to R-protein and [^{58}Co]B_{12} bound to intrinsic factor; if significantly less of the R-protein-bound vitamin was absorbed compared to the intrinsic-factor-bound vitamin this provided good evidence for pancreatic exocrine dysfunction.

8.8. Congenital Inability to Absorb Vitamin B_{12}

8.8.1. *Immerslund–Najman–Gräsbeck Syndrome*

The Immerslund–Najman–Gräsbeck syndrome is a term used to describe a congenital malabsorption of vitamin B_{12}. It is perhaps the most common of the familial vitamin B_{12} absorption disorders and more than 100 cases have been described (C21). Although the majority of patients present during the first 2 years of life, a few have been seen much later. The disorder is charac-

terized by vitamin B_{12} malabsorption and proteinuria, and patients (particularly the older ones) present initially with a megaloblastic anemia. The serum vitamin B_{12} level is very low but the serum folate concentration is usually normal. There is impaired absorption of vitamin B_{12} that is not improved by the addition of intrinsic factor. Other tests of absorption are normal and it appears that the solitary defect is malabsorption of vitamin B_{12} associated with proteinuria. The nature of the defect is uncertain. Ultrastructure examination of the renal glomeruli has shown mild alterations of the glomerular basement membranes and it has been suggested that an increase in permeability may lead to proteinuria without ultrastructural alterations (R15). The kidneys behave normally in their concentrating power and their clearance of urea and creatinine. Patients respond dramatically to treatment with vitamin B_{12} but this has no effect on the proteinuria. Untreated patients may develop the neurological changes associated with vitamin B_{12} deficiency and this can result in serious and irreversible damage, particularly in very young infants.

The disorder is inherited in an autosomal recessive manner and the sexes are equally affected. As would be expected it has an increased frequency in communities that tend to be inbred (B22).

9. Inborn Errors of Metabolism in Cobalamin-Dependent Systems

9.1. Methylmalonic Aciduria

S-Methylmalonyl-CoA mutase (EC 5.4.99.2) is a deoxyadenoxyladenosylcobalamin-dependent enzyme of mitochondria required to catalyze the conversion of methylmalonyl-CoA to succinyl-CoA. A decrease in the activity of methylmalonyl-CoA mutase leads to the urinary excretion of large amounts of methylmalonic acid (C22). The biochemical lesion may be at the mutase level due to an abnormality of apoenzyme protein or an inability to elaborate the required coenzyme form of vitamin B_{12}, i.e., adenosylcobalamin. In rare cases the abnormality may be due to an inability to convert the D form of methylmalonyl-CoA mutase to the L form as a result of a defective racemase (EC 5.1.99.1) (K11). In patients, the nature of the abnormality can be determined by tissue culture studies (D13) and by clinical trial, since patients with a defect in adenosylcobalamin production will show clinical improvement when treated with very large doses of vitamin B_{12} (M11).

The varient of the disorder which does not respond to treatment with vitamin B_{12} may be controlled by restricting the intake of certain branched-

chain amino acids associated with methylmalonic acid production such as valine, threonine, methionine, and isoleucine (N3).

The disorder is inherited as an autosomal recessive characteristic and it is rare, with fewer than 30 cases so far described. Severely affected infants show symptoms within a few days of birth. A mild or benign form has been reported in adults (G10). Infants with the disorder fail to thrive or grow, they may be mentally retarded, and they have intermittent hypoglycemia or hyperglycemia. Protein intolerance is also a feature, with vomiting and ketosis.

Whlean *et al.* (W7) described a follow-up, extending over several years, of two infants with methylmalonic aciduria unresponsive to treatment with vitamin B_{12}. The first patient, a boy, was the child of two first cousins; delivery followed an uneventful pregnancy. The child had convulsions 4 days after birth and was found to have a profound metabolic acidosis, and was excreting a large amount of methylmalonic acid in his urine. His serum vitamin B_{12} concentration was normal. Further studies confirmed a diagnosis of methylmalonic aciduria.

He was provided with a diet restricted in protein but was found to limit his own protein intake to 1.0 to 1.2 g/kg. Treatment with large doses of vitamin B_{12} for a period of 4 months made no difference to the concentration of methylmalonic acid excreted in his urine. He required frequent hospitalization for anorexia, vomiting, and dehydration. At 18 months of age he developed renal failure, and at 3 years he became oliguric. His physical development appeared normal for the first 12 months and then it deteriorated. He started to walk at $3\frac{1}{2}$ years, but at this time he also had hepatomegaly and persistent vomiting. On the basis of a developmental screening test he was found to be 12 to 18 months behind in all areas tested.

The second child was a female delivered at 33 weeks gestation by cesarean section because of premature rupture of the membranes. Her parents were not related and a previous child had died at the age of 5 weeks with severe metabolic ketoacidosis of undetermined cause. One hour after birth she was found to be flaccid and unresponsive, and had a convulsion. At this time she was hyopoglycemic, with a blood glucose of 0.8 mmol/liter. This was corrected and she began breast feeding. At 8 days of age she became lethargic and started to vomit. Her condition rapidly deteriorated and she became comatose. She had severe metabolic acidosis and was found to be excreting a large amount of methylmalonic acid. The acidosis was corrected and she was given 2 mg of vitamin B_{12} daily and her dietary protein intake was restricted. The vitamin B_{12} therapy appeared to be without effect. At 6 months of age she had a life-threatening coma. Examination at 12 months showed her physical and mental development to be normal in all areas tested.

Metabolic studies failed to detect any methylmalonyl-CoA mutase in skin fibroblasts from the first patient, while in the second patient the level was found to be 10% of normal. Two forms of vitamin B_{12} were tried on each of the patients, cyanocobalamin and hydroxocobalamin, both without apparent effect.

Vitamin B_{12}-responsive methylmalonic aciduria is easier to treat than the nonresponsive variant, although a restriction on dietary protein may still be needed. Morrow and Burkel (M12) described the successful treatment and long-term follow-up of a boy aged 8½ years. He had done well until 6 months of age when he developed a severe ketoacidosis. This was repeated at the age of 1 year, and he went into a coma. At this stage a diagnosis of methylmalonic aciduria was made. Before treatment his urine methylmalonate excretion was 367–443 mg/day. He was given 1 mg of hydroxocobalamin daily by intramuscular injection and dietary protein was restricted to 1 g/kg daily. The urinary methylmalonate decreased to 68 mg/day over a 7-day period. His physical development appeared to be normal and intelligence tests were reported as well above average. He continued to excrete up to 266 mg/day of methylmalonate in his urine.

Prenatal diagnosis of the disorder may be made (A15). Mothers carrying an affected fetus excrete large quantities of methylmalonic acid. Free methylmalonic acid is also present in the amniotic fluid and cultures of the amniotic cells enable the nature of the defect to be determined. In the vitamin B_{12}-responsive disorder, treatment of the mother with large doses of vitamin B_{12} was found to reduce the maternal excretion of methylmalonic acid.

9.2. Homocystinuria

Homocystinuria may result from one or several abnormalities in the mechanism whereby homocysteine is methylated to form methionine. About half of the patients respond to treatment with pyridoxine and it is thought that the vitamin overcomes a block at the homocysteine/cystathionine level by mass action (C23). However, Schuh *et al.* (S22) have recently described a patient who responded to vitamin B_{12}. The infant presented with severe developmental delay, homocystinuria, and a megaloblastic anemia. Treatment with cyanocobalamin was without effect but treatment with hydroxocobalamin resulted in a rapid clinical improvement, and the homocystinuria disappeared. Methionine synthetase activity in cell extracts was normal, while cultured fibroblasts showed an absolute growth requirement for methionine. The defect appeared to be limited to methylcobalamin accumulation and an inability to transfer the methyl group from 5-methyltetrahydrofolate to homocysteine.

9.3. Methylmalonylaciduria with Homocysteinuria

This is another rare inherited disorder of vitamin B_{12} metabolism in which both coenzyme forms, adenosylcobalamin and methylcobalamin, are affected. Methylcobalamin is required for the transfer of the methyl group of 5-methyltetrahydrofolate to homocysteine to give methionine. Lack of methylcobalamin results in deficient activity of N^5-methyltetrahydrofolate-homocysteine methyltransferase, resulting in a reduced ability to methylate homocysteine. A failure of methionine synthetase would produce a similar result.

Only five patients have so far been described with the disorder and two of these were brothers, offspring of a consanguineous marriage. All patients excreted large quantities of methylmalonate and homocysteine. The clinical severity of the disorder depends on the level of residual coenzyme; one boy had developed normally while two other children died, one at $7\frac{1}{2}$ weeks and the other at 7 years. Only two of the patients were found to be anemic; one was normocytic and normoblastic, while the other was megaloblastic. However, there seemed to be some uncertainty as to whether the magaloblastosis may have been caused by a deficiency of folate resulting from an inadequate diet. Although the patient had a normal serum level of folate this may not have been a reliable index of folate status in view of the methylcobalamin deficiency, and neither erythrocyte nor liver folate was measured (D13). One patient with a severe form of the disorder has been successfully treated (A16). The patient, a female born in 1969, was the fifth child of unrelated parents. At the age of 18 months it was noted that the child was not developing normally; she would not respond to verbal commands and appeared not to comprehend spoken words. At the age of 3 years she had an attack of influenza that was followed by episodic lethargy. She had poor control over her legs while walking and her mother thought that there had been some regression in the volume of her speech and vocabulary, as well as a deterioration in her mental state. Urinary excretion of methylmalonic acid was found to be greatly increased, at 934 mg per 24 hours, and urinary homocysteine was slightly increased. The child was treated with 1 mg/day hydroxocobalamin (intramuscularly), 50 mg/day pyridoxine, 15 mg/day folic acid, and 5 mg/day choline. Dietary protein was restricted to 30 g/day. The hydroxocobalamin was given on the basis of earlier observations that large doses of the vitamin reduced homocysteinuria and methylmalonic aciduria. Folate was given on the assumption that reduced methyltetrahydrofolate-homocysteine methyltransferase activity could be improved by increasing the amount of tetrahydrofolate. Pyridoxine has been shown to lower serum methionine and homocysteine, and it should lower the toxic effects of homo-

cysteine on various body tissues. Choline accelerates the methylation of homocysteine by acting as a methyl donor. On this treatment the patient showed a marked physical and mental improvement during the ensuing 12 months.

10. Reactions to Treatment with Vitamin B_{12}

Vitamin B_{12} is frequently given in very large doses, and injections of 1 mg are used as initial treatment in many deficiency states. Very few adverse effects resulting from exposure to large doses of the vitamin have been reported. Malten (M13) described a 55-year-old woman with psoriasis typus inversus. Her palms and soles were erythematous and often showed crops of tiny vesicles. She was prescribed a course of vitamin B_{12}, and on the evening of the day on which she had her third injection of 250 μg she had a sudden bullous flare on her hands, and to a lesser extent on her feet. This cleared in 2 days without any specific treatment. The patient was later patch tested with a concentrated solution of the vitamin but this proved to be negative. Hovding (H28) reported an anaphylactic reaction in a patient following an injection of vitamin B_{12} and there have been several others (S23,Y1), with one fatal reaction (R16).

Positive intradermal skin tests using pure vitamin B_{12} were seen in 2 out of 1000 individuals tested, although they did not react to an intramuscular injection (B23).

In addition to its use in relieving a deficiency state and certain metabolic abnormalities, vitamin B_{12} has also been used as a "tonic" and untoward reactions have been exceedingly rare.

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PLASMA LIPOPROTEINS, APOLIPOPROTEINS, AND PROTEINS CONCERNED WITH LIPID METABOLISM

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1. Introduction. A Note on Clinical Trials of Lowering Plasma Cholesterol

In this article we shall review recent advances in plasma lipoproteins, apolipoproteins, and those proteins concerned with lipoprotein lipid metabolism. This is a rapidly growing field, far too large to enable all aspects to be covered in a single paper. In order to keep to a manageable size, we shall focus on areas in which there have been recent major advances. Our treatment of other areas will be brief, as adequate reviews are in most cases available. Clinical details of primary disorders of plasma lipid metabolism are very well covered in a series of reviews in "The Metabolic Basis of Inherited Disease" (5th Ed., J. B. Stanbury, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein, and M. S. Brown, eds.) (B52, G13, G20, H24, N8, S3, S56). Disordered lipid metabolism, and especially secondary disorders of lipid metabolism, are equally well covered in "Metabolic Control and Disease" (8th Ed., P. K. Bondy and L. E. Rosenberg, eds.) (H16).

The major interest in plasma lipoprotein metabolism for the clinical chemist arises because of the relationship with human disease, especially coronary heart disease. In this respect it is worth noting some of the major recent advances in clinical studies and epidemiology before returning to discuss plasma lipoprotein metabolism.

1.1. The Relationship between Plasma Cholesterol and the Risk of Developing Coronary Heart Disease

It has been known for some years that elevated plasma cholesterol concentrations and particular lipoprotein abnormalities are causal factors in the development of coronary heart disease, and that it is possible to lower plasma cholesterol concentrations in individuals or populations.

Over the past few years noninvasive techniques for visualizing plaques *in vivo* have been refined, so that it has become possible to demonstrate a positive effect of cholesterol-lowering, in a secondary prevention trial with cholestyramine and a cholesterol-lowering diet, on coronary artery disease as assessed by angiography (L13)

The noninvasive demonstration that lowering plasma cholesterol sufficiently results in either regression or lack of progression of atherosclerosis in man (and the very large amount of evidence to support this from animal studies) leads one to expect that direct evidence can be obtained that choles-

terol-lowering in susceptible individuals will lower the risk of myocardial infarction or death from coronary heart disease. Recent studies have confirmed this expectation.

Although previous intervention studies reported have been congruent, suggesting that lowering plasma cholesterol decreases the risk of myocardial infarction or death from coronary heart disease, nearly all of these have been flawed in some way, so that their findings could not be taken as conclusive evidence. For instance, although the New York Anti-Coronary Club Study (R13), the Los Angeles Veterans Administration Study (D3), and the Finnish Mental Hospital Study (T12) reported encouraging results, these studies were not blinded, and in some cases they had other imperfections in design. Other studies [e.g., the Newcastle (G33) and Edinburgh (R11) clofibrate studies, and the Coronary Drug Project Studies (C18–C21)] were secondary prevention studies in which medication to lower lipid levels was used, and in which for various reasons the results were not conclusive. It might be argued that real benefits from lowering plasma cholesterol may not easily be demonstrated in a study population of individuals who had already suffered a myocardial infarction, whereas they might be expected in a primary prevention trial.

Other studies examined the effect of reducing more than one risk factor. For instance, the Multiple Risk Factor Intervention Trial (M45) failed to achieve a sufficient difference in cholesterol levels between its study and control groups to draw any valid conclusions on the effect of cholesterol-lowering (although both study and control groups had a substantial decrease in risk factor levels including plasma cholesterol, and fewer coronary heart disease episodes than expected if there were no risk factor changes). The Oslo study on the effects of a cholesterol-lowering diet and smoking cessation resulted in a 47% lower incidence of coronary heart disease in the hypercholesterolemic study group (H25). Most of the reduction in coronary heart disease was attributed by the investigators to the fall in plasma cholesterol. Another Oslo trial, the Oslo Diet Heart Study, was a secondary prevention study using diet to lower plasma cholesterol (L10). It achieved a 35% reduction in coronary heart disease incidence after a 5-year follow-up.

The Lipid Research Clinics Coronary Primary Prevention Trial (L16, L17) is a landmark double-blind study in which cholestyramine, a bile acid sequestrant that is not absorbed from the gut, was used to lower plasma cholesterol. The investigators recruited 3806 men, with a Type II hyperlipoproteinemia phenotype and in good health, into the study. All were prescribed a cholesterol-lowering diet. Subjects were randomly assigned to a treatment group (who were prescribed 24 g cholestyramine daily) and a group with similar baseline characteristics who received an inactive placebo. A 19% lower incidence of coronary heart disease over a mean of 7.4 years in

the cholestyramine-treated men was accompanied by falls of 8% and 12% in plasma total and low-density-lipoprotein (LDL) cholesterol levels (relative to placebo-treated men). Other analyses of the data were all consistent with this finding, and with the assumption of a causal relationship between a fall in total or LDL cholesterol and a fall in coronary heart disease risk. This trial has clearly shown that a fall in plasma cholesterol induced by cholestyramine in hypercholesterolemic men is associated with a reduction of coronary heart disease risk. As a rough approximation, over the study period every 1% fall in plasma cholesterol was associated with a 2% fall in coronary heart disease risk. Other published studies are also consistent with this approximation. Because coronary heart disease risk from hypercholesterolemia is a continuous variable in both sexes and at all ages, and because cholestyramine may be thought of as having a physiological effect resulting in enhanced removal of LDL from the blood by hepatic receptors, it is reasonable to extrapolate these findings to other patient groups and to reductions in plasma cholesterol by diet.

Taken together, these studies indicate that lowering plasma cholesterol lowers coronary heart disease risk. The importance of research on normal and abnormal plasma lipoprotein metabolism is emphasized, and is, in part, the justification for this review.

There is evidence that links apoB and apoA-I levels to the incidence of coronary heart disease, and that suggests that plasma apolipoproteins may serve as a better marker of risk for atherosclerosis than plasma lipid levels. Details are discussed in Sections 4.1.5 and 4.4.10; a useful review has been published (B59).

2. Nomenclature and General Structure of Lipoproteins

2.1. General Structure

Hydrophobic lipids (triacylglycerols and cholesteryl esters) are virtually completely insoluble in water; they are solubilized for transport in plasma by incorporation into lipoproteins. Lipoproteins are spherical complexes containing triacylglycerol (triglyceride) and cholesteryl ester surrounded by a layer containing phospholipids, unesterified cholesterol, and specific apolipoproteins.

This general model is now universally accepted, though there are minor reservations [e.g., both cholesteryl ester (S39) and triglyceride (H4) have limited solubility in phospholipid, and hence a fraction of these hydrophobic "core" lipids is probably found in the outer coat of lipoprotein lipid].

2.2. Lipoprotein Classification

Lipoproteins are most conveniently classified according to their hydrated densities, i.e., in practical terms, the density limits defining their isolation in the preparative ultracentrifuge.[1] Although each density class defines a heterogeneous group of particles, there are enough similarities in each density class for this to be a useful classification. Other classifications are possible; that based originally on paper electrophoresis (β-lipoproteins = LDL; pre-β-lipoproteins = VLDL; α-lipoproteins = HDL) is now less often used.

It should also be remembered that plasma lipoproteins are constantly in a state of metabolic flux. Some large particles are converted to smaller particles, and vice versa. Lipids and most apolipoproteins exchange or are transferred between particles and particles are released into and removed from the circulation.

Lipoproteins interact with enzymes (e.g., lecithin:cholesterol acyltransferase, or lipases), with lipid transfer proteins, or with receptors on cell surfaces. The composition of a lipoprotein class depends upon the results of these kinds of interactions. Ultracentrifugation itself may result in minor changes in lipoprotein composition (see Sections 6 and 6.1).

2.3. Lipoprotein Structure

The core structure of lipoproteins has been examined by differential scanning calorimetry (D6, D7, T1). A reversible phase transition occurs in human LDL (in which the core lipid is mostly cholesteryl ester) as the temperature is increased from below about 20°C to 40°C. The transition has been shown to be due to an order–disorder transition of cholesteryl esters. It is thought that, below the transition temperature in the smectic state, there is a two-dimensional ordering of core cholesteryl ester molecules into 3.6–nm-thick layers, with the long axes of the cholesteryl ester molecules aligned. Above the transition temperature a more disordered state applies. The more saturated cholesteryl esters have a higher transition temperature than unsaturated esters, and studies on LDL from normocholesterolemic and hypercholesterolemic animals suggest that, at body temperature, the degree of cholesteryl ester saturation is an important determinant of the degree of order of the cholesteryl esters in the lipoprotein core. Another

[1]Chylomicrons $d < 0.95$ g/ml; very low-density lipoproteins (VLDL) $d = 0.95–1.006$ g/ml; intermediate-density lipoproteins (IDL) $d = 1.006–1.019$ g/ml; low-density lipoproteins (LDL) $d = 1.019–1.063$ g/ml; high-density lipoprotein subfraction 2 (HDL_2) $d = 1.063–1.125$ g/ml; high-density lipoprotein subfraction 3 (HDL_3) $d = 1.125–1.21$ g/ml; very high-density lipoproteins (VHDL) $d > 1.21$ g/ml.

determinant is the triglyceride content; VLDL, in which the cholesteryl esters appear to be dissolved in triglyceride, shows no thermal transition, and decreasing triglyceride content of LDL is associated with a higher transition temperature (D6, D7, T1). However, when triglyceride-rich lipoproteins are cooled below 23–26°C, as is usual during ultracentrifugation, crystallization of core triglyceride begins and the density increases (C16).

Subfractions of human high-density lipoprotein, HDL_2 and HDL_3 particles, display no thermal transitions. No doubt the cooperative molecular interactions normally present in bulk lipid of the same composition are prevented by the small size of the core (e.g., HDL_3 contains about 32 cholesterol ester, 10 triglyceride, and 5 cholesterol molecules in the core) (L20). However, larger HDL particles from hypercholesterolemic animals do display thermal transitions. It has been suggested that the smallest particle that could accommodate a layered arrangement of extended cholesteryl ester molecules would have a diameter of about 14 nm, which is larger than human HDL_2 but characteristic of various particles that have been labeled HDL_1 or HDL_c found particularly in plasma from hypercholesterolemic animals (A30, T1, T3).

Other evidence supporting this concept of lipoprotein structure includes X-ray small angle scattering studies. For example, LDL and an HDL fraction (LpC, i.e., apoC-rich HDL) have been shown to have low electron density cores (suggesting lipid cores) and high electron density shells (L1). Below the phase transition temperature the X-ray scattering pattern of LDL shows a fringe, corresponding to a Bragg spacing of 36 Å (and thus consistent with the smectic state), which disappears above the transition temperature (A29, D6, D7).

In summary, therefore, one can say that the core of lipoprotein particles is disordered (or liquid) at body temperature, with the partial exception of some LDL and HDL_1 containing a high proportion of saturated cholesteryl esters. The biological implications of lipoprotein core structure are unknown.

There is abundant evidence to support the concept that the outer layer of plasma lipoproteins is a monolayer of polar lipids (phospholipids, mainly phosphatidylcholine, and cholesterol) and apolipoproteins with the hydrophilic aspect of the apolipoproteins and the polar head groups of phospholipids on the surface. The evidence has been reviewed by others [e.g., (S24)] and will not further be examined here. Nuclear magnetic resonance studies on HDL have shown that about 40% of unesterified cholesterol molecules are in the lipoprotein core, and 60% are associated with phospholipid molecules in the surface. Neither surface nor core is saturated with cholesterol (L20). Presumably, unesterified cholesterol is also found in the core of other lipoproteins.

3. General Properties of Apolipoproteins

Although other systems of nomenclature have been used in the past—e.g., the C-terminal amino acid nomenclature (apo-Gln-I, etc.) (F21), and Fractions III, IV, V, etc. (S4)—the A, B, C system developed initially by Gustafson *et al.* (G34, G35) then Alaupovic *et al.* (A6) has become universally accepted.

3.1. Apolipoprotein Functions

The apolipoproteins serve at least three important kinds of functions in lipoprotein metabolism.

3.1.1. *Structural*

All apolipoproteins bind lipid in an aqueous environment. The primary amino acid sequences of several apolipoproteins are known [apoA-I (B1, B43), apoA-II (B45), apoC-I (J8, S33), apoC-II (J3), apoC-III (B46) and apoE (R4)]. There are no long sequences of hydrophobic amino acids in these primary structures, as there are in some membrane proteins. However, as a number of groups have shown, the sequences permit regions of amphipathic α-helices to be modeled, so that hydrophilic amino acid residues lie on one side of the helix, and hydrophobic residues on the opposite side (A26, B42, J5, O7, S22). The hydrophilic regions of amphipathic helices would thus be oriented toward the surface of the lipoprotein, and the hydrophobic regions toward the interior of the apolipoprotein or of the lipoprotein itself. Pownall and colleagues have studied the energetics of apolipoprotein–lipid binding, and shown that the magnitude of the free energy change on binding is less than predicted, even when corrections are made for the presence of hydrophilic residues on the outside of the amphipathic helix. This finding has led to the proposal that there is a gradation of polarity across the lipoprotein particle, so that the outer part of the particle has less hydrophobicity than the inner part (P24, P25). The group at the Baylor College of Medicine, Houston, have identified probable amphipathic helical lipid-binding regions in the apolipoproteins for which the primary structure is known, have synthesized segments including these regions, and have carried out extensive studies on the properties of lipid-binding regions and the nature of apolipoprotein–lipid interactions [see (S42, S52)]. Detailed reviews have been published (B42, O7).

With one exception, all apolipoproteins appear to exchange between plasma lipoproteins. The exception is apoB, an apolipoprotein that is present in all chylomicrons and VLDL, LDL, and Lp(a) particles, and which seems to be structurally essential to the integrity of these particles. It appears likely

that, in order to exchange between lipoproteins, apolipoproteins must be water soluble, and apoB, alone of the apolipoproteins, is insoluble in an aqueous medium. Tracer experiments indicate that an apoB molecule remains with its parent lipoprotein particle throughout its existence.

The properties of the apolipoproteins in solution have been extensively studied. Two points are relevant to the present discussion.

1. Apolipoproteins, when lipid-free in an aqueous environment, appear to be loosely folded, with some exposure of hydrophobic amino acid residues to the solvent. Evidence to support this conclusion comes from denaturation studies by Tall (T1). This group studied heat- and urea-induced denaturation of apoA-I, and showed that the free energy difference between the folded and unfolded states of the apolipoprotein is quite small (2.4 kcal/mol at 37°C) compared with that of other small globular proteins like myoglobin or ribonuclease (about 10 kcal/mol). In this loosely folded state hydrophobic sites on the free apolipoproteins are probably readily accessible to lipid, so that recombination readily occurs. Many studies have shown that the recombination of apolipoproteins with lipid result in major changes consistent with the formation of a highly ordered molecule with an increased helical content as judged by circular dichroism [e.g., (J6, L22, L26)] and a movement of tryptophan residues to a more hydrophobic environment (J7).

2. Apolipoproteins self-associate in solution. (It should be remembered that association differs from aggregation. Aggregation is the irreversible, association the reversible, formation of higher molecular weight species.) Thus apoA-I is considered to exist, in aqueous solution, in a monomer–dimer–tetramer–octomer system, apoA-II in a monomer–dimer system, and apoC-I in a monomer–dimer–tetramer system (O7). These observations are clearly in accord with the notion that apolipoproteins in solution, lacking primary hydrophobic sequences, may acquire hydrophobic surfaces which will allow them to self-associate and acquire secondary structure. Aggregation and association are potentially major problems for the experimenter working with highly purified apolipoproteins. Association is dependent upon protein concentration (increasing with increasing concentration) and, for individual apolipoproteins, upon solvent characteristics such as pH, temperature, and the ionic strength and composition of the medium. The subject is covered in considerable detail in a review by Osborne and Brewer (O7).

3.1.2. *Interactions with Enzymes*

Apolipoproteins serve to direct metabolism of particular lipoproteins by acting as cofactors or perhaps inhibitors for enzymes. Examples are apoA-I and apoC-I, each of which may activate lecithin:cholesterol acyltransferase

(LCAT), which catalyzes the synthesis of cholesterol esters (F14, S46, S59); apoA-II, which activates hepatic triglyceride lipase (J2); and apoC-II, which activates lipoprotein lipase, responsible for the hydrolysis of triglycerides in chylomicrons and VLDL (H20, L5). Their mode of action is considered in Section 4 when the individual apolipoproteins are discussed.

3.1.3. *Receptor Interactions*

Apolipoproteins may interact with specific receptors, either to initiate uptake of a particle (e.g., apoE in remnant lipoproteins, apoB in LDL) or to inhibit uptake (e.g., apoC appears to inhibit hepatic uptake of triglyceride-rich apoE-containing particles). Apolipoprotein–receptor interactions are considered when the individual apolipoprotein is discussed.

4. The Apolipoproteins

4.1. Apolipoprotein A-I (ApoA-I)

Human apoA-I is a major constituent of HDL, with an M_r of approximately 28,300, calculated from the known primary structure (B1, B43). ApoA-I is initially synthesized as a 267-amino-acid precursor protein, pre-pro-apoA-I (G25, G26), containing an 18-amino-acid prepeptide and a 6-amino-acid propeptide [determined by nucleic acid sequence analysis of cloned apoA-I (L6), and by isolating the primary translation product of human intestinal apoA-I mRNA (G25)].

The cDNA and genomic clones for human apoA-I have been isolated and characterized, and shown to be located on chromosome 11 within 3 kb of the apoC-III gene (B57, K5, K7, S30, S31). DNA polymorphisms associated with apoA-I abnormalities have been described, and are discussed briefly in Section 4.1.4.

It had been known for some years that there is more than one form of apoA-I in plasma (E1, L23, O7), when Nestruck *et al.* (N5) reported that four forms of apoA-I could be isolated by preparative flat bed isoelectric focusing. The two major forms in human plasma (referred to as apoA-I_1 and apoA-I_2 by Nestruck *et al.*, but as isoforms or isoproteins 4 and 5 in this review (following references S9, Z1, and Z6), focus at p*I* 5.62 and 5.53, respectively, and contain 71 and 19%, respectively, of total apoA-I. All forms had an identical apparent M_r and common antigenicity to antisera against apoA-I. The amino acid analyses of isoforms 4, 5, and 6 resembled previously published apoA-I analyses (B1, B43) and these isoforms activated purified lecithin:cholesterol acyltransferase.

Zannis *et al.* (Z1) separated six plasma apoA-I isoproteins, each of M_r

28,000, by two-dimensional gel electrophoresis of plasma apoA-I. Isoproteins 2–6 were separated from each other by one charge unit, so that the p*I* values of isoproteins 1–6 were >6.50, 5.85, 5.74, 5.64, 5.52, and 5.40, respectively. (The identity of isoprotein 1 is uncertain.) Incubation with phosphatase or neuraminidase did not affect the pattern. Further culture studies clarified the problem. Normal human intestine or liver in organ culture releases mostly isoproteins 2 (78%) and 3 (21%) of apoA-I but very little of isoproteins 4 and 5 into the medium. Plasma from normal subjects has isoproteins 4 (79%), and 5 and 6 (19%) as the major forms of apoA-I. Isoproteins 2 and 3 form only 2% of apoA-I isoforms in normal plasma. The findings suggest that posttranslational processing of apoA-I isoprotein 2, involving charge modification, yields isoprotein 4.

Gordon *et al.* (G25) isolated the primary translation product of human intestinal apoA-I mRNA from wheat germ and ascites cell-free translation systems. This proved to be pre-pro-apoA-I, with an 18-amino-acid prepeptide and a 6-amino-acid propeptide. Intracellular pro-apoA-I (in a human hepatoma cell line) was secreted without further cleavage, and corresponded to isoproteins 2 and 3 (isoprotein 2 being the major form) of Zannis *et al.* (Z1).

To summarize, pre-pro-apoA-I undergoes intracellular cotranslational cleavage to pro-apoA-I (G25). Pro-apoA-I is secreted from the cell and undergoes posttranslational proteolytic cleavage to mature apoA-I in plasma by a pro-apoA-I peptidase (G1, G25). Pro-apoA-I is isoprotein 2 in plasma and apoA-I is isoprotein 4. Isoprotein 3 seems likely to be a variant of pro-apoA-I, and isoprotein 5 a variant of isoprotein 4. A postulated converting enzyme is responsible for the conversion of pro-apoA-I to mature apoA-I in the plasma compartment.

The primary amino acid sequence of apoA-I has been published by Brewer and colleagues (B43) and, with a few minor differences, by Baker *et al.* (B1). As with other apolipoproteins that have been sequenced, there are no long sequences of hydrophobic or hydrophilic amino acids, but there is evidence that amphipathic regions of α-helices are formed during combination with lipid, with one face of each helical region more hydrophobic than its opposite face (O7).

There have been extensive analyses of segments of the 243–245 amino acids of apoA-I by Sparrow, Gotto *et al.* and by several other groups. Analysis of the amino acid sequence reported by Baker *et al.* shows that there are 13 sequences that, on Chou–Fasman analysis (C13), have a high probability of helix formation (S52). There is experimental evidence, using synthesized sequences of apoA-I in lipid recombination studies, to support the Chou–Fasman predictions of helicity [summarized by Sparrow and Gotto (S52)].

Sparrow and Gotto (S51) have synthesized peptide apoA-I (amino acids

148–185) (i.e., the peptide containing amino acids 148–185 of apoA-I) and have shown that it activates LCAT, but the segment of apoA-I (amino acids 164–185) contains a lipid-binding region which does not activate LCAT. Fukushima *et al.* (F22) find that apoA-I (amino acids 147–168), expected perhaps to be a very potent LCAT activator, does not bind under assay conditions to phosphatidylcholine–cholesterol vesicles, and does not activate LCAT. It seems therefore that LCAT activation requires a specific LCAT-binding activating segment [probably apoA-I (148–167) or similar] together with an adjacent amphipathic lipid-binding segment. The molecular basis for LCAT activation is unknown, but the data suggest that apoA-I stabilizes LCAT in a particular position with respect to its phospholipid substrate to ensure maximum activity.

4.1.1. *Apolipoprotein A-I Synthesis and Distribution in Plasma*

Large triglyceride-rich chylomicrons in human mesenteric lymph contain apoA-I, together with apoA-II, apoA-IV, apoB-48, apoC, and apoE (B33). It appears that all except apoE are synthesized in the intestine (B33). Studies in the rat show that about 130–140 mg/hour of apoA-I is transported in the mesenteric lymph of saline or glucose-fed animals (I5, R12), and that this value rises twofold during triglyceride absorption (G7), so that the rat intestine contributes over 50% of the total daily synthesis of apoA-I (W22). Studies in man indicate that a similar proportion, at least 30–40%, of total daily synthesis of apoA-I occurs in the intestine (A23, G28). After fat feeding in man the content of apoA-I in the intestinal epithelium increases (G8), and plasma levels are also increased (G8). Glucose infusion increases rat intestinal apoA-I secretion (W19), and it may be that a number of factors other than dietary fat influence intestinal apoA-I secretion in man.

While chylomicrons in lymph contain apoA-I as a major apolipoprotein, plasma chylomicrons do not normally contain apoA-I, and apoA-I appears to transfer from chylomicrons to HDL in plasma (A23, P4, S8, T5). Parks and Rudel compared the kinetic fates of labeled apoA-I and apoA-II from lymph chylomicrons in monkeys (P4). The two apolipoproteins behaved differently when injected into plasma. ApoA-II appeared to be almost instantaneously transferred from injected chylomicrons into HDL, while the tracer apoA-I specific activity rose in HDL for 1–3 hours after chylomicron injection, before falling at a rate identical to that of autologous HDL apoA-I tracer. These and other findings suggest that some chylomicron apoA-I transferred to chylomicron remnants or to disks or vesicles of redundant surface material released from chylomicrons, or remained free in solution, rather than immediately transferring to HDL.

There is also evidence from the rat that HDL particles, rich in apoA-I and containing little apoE, are secreted from the intestine into mesenteric lymph

(B20, B33, G29, S16). These include both spherical and discoidal HDL particles, and production of the discoidal particles at least is independent of intestinal triglyceride absorption. [Most of the spherical HDL particles are thought to reach the lymph from the blood plasma. Anderson *et al.* have calculated that a considerable proportion (perhaps more than 80%) of thoracic duct apoA-I represents recirculating apoA-I derived originally from the plasma (A23)]. Most apoA-I in human thoracic duct lymph (81 ± 8%) is in HDL particles (mostly HDL_{2b} and HDL_{2a}, rich in triglyceride and poor in cholesteryl ester, relative to mature plasma HDL) (A23).

ApoA-I [or rather isoprotein 2, now known to be pro-apoA-I (B44, S12), and also isoprotein 3] is secreted in organ culture by human liver as well as intestine (Z1, Z5). The quantitative contribution of the liver to plasma apoA-I is unknown.

ApoA-I is also said to be synthesized in the kidney (Williams, quoted as a personal communication in P4), though the magnitude of this contribution to plasma apoA-I concentration is also unknown.

4.1.2. *Apolipoprotein A-I Catabolism*

Little is known about apoA-I catabolism or even HDL catabolism. In the rat, HDL can deliver cholesterol to steroidogenic tissues; although this is thought to be receptor mediated, the mechanism of such binding is not clear (A20, C12, G37, K27). High-affinity specific binding sites for HDL (and LDL) have been demonstrated in rat liver cells (C9, O9, V2) and in rat intestinal mucosal cells (S63).

Glass *et al.* have studied the tissue uptake in the rat of homologous plasma HDL containing [^{3}H]cholesterol ether (as a tracer for cholesterol ester) and HDL apoA-I (labeled with [^{125}I]tyramine cellobiose), two tracers which are not hydrolyzed in cells after uptake. About 39% of apoA-I was taken up in the kidney and 26% in the liver (almost all of which was in hepatocytes). On a wet-weight basis (uptake of apoA-I per gram wet weight) the kidney was the most active, and ovary and adrenal next most active. The site of apoA-I deposition in the kidney seemed to be on the brush border and in apical granules of proximal tubular epithelial cells. The high renal uptake of apoA-I was associated with a low (<1%) uptake of cholesteryl ether, and the authors speculated that the kidney filtered free apoA-I and reabsorbed it in the tubules (G5). The liver was the major organ of cholesteryl ether uptake (65% of total), but the addrenal gland and ovary were the most active organs per gram wet weight. Again, uptake of cholesteryl ether and apoA-I were dissociated; uptake of cholesteryl ether was sevenfold greater than apoA-I in the adrenal, fourfold greater in the ovary, and over twofold greater in the liver (G6). The mechanisms underlying these observations are unknown.

4.1.3. *Apolipoprotein A-I Deficiency: Tangier Disease*

Tangier disease is a rare autosomal recessive disorder characterized by the near absence of plasma HDL and the storage of esterified cholesterol in foam cells in many tissues. Clinical features include enlarged orange-colored tonsils, splenomegaly and a relapsing sensory-motor neuropathy. The clincial aspects of Tangier disease have been reviewed elsewhere (H24, S10).

It has been known for some years that HDL in Tangier disease is not only quantitatively deficient but is of abnormal composition (A27). Homozygotes have HDL cholesterol levels 2% of normal and apoA-I and A-II levels 1% and 7%, respectively, of normal (A7). There is evidence that apoA-I in Tangier disease does not adequately bind lipid, as on ultracentrifugation of plasma from patients with Tangier disease, apoA-I is found in the very high-density lipoprotein fraction (or the $d = 1.21$ g/ml infranatant) and the $d = 1.063$ g/ml supernatant, but is almost absent in the $d = 1.063$–1.21 g/ml range (A28, H23, S6). Schaefer *et al.* (S6) confirmed this apoA-I distribution; after ultracentrifugation 30% of plasma apoA-I in Tangier disease is associated with the $d = 1.063$ g/ml supernatant and 70% or more with the $d = 1.21$ g/ml infranatant. In a later study, Schaefer *et al.* confirmed defective binding *in vivo* of Tangier apoA-I to HDL (S9).

Schaefer *et al.* performed kinetic studies with radioiodinated apoA-I and apoA-II in two patients with homozygous Tangier disease (S5). ApoA-I was removed from the circulation faster than apoA-II, but both were catabolized much faster than in normal subjects. After the plasma HDL concentration was increased by HDL infusion in one of the Tangier patients, a repeat kinetic study showed that increasing HDL concentration may have influenced the initial removal of HDL tracer from the circulation, but not the final slope of the plasma radioactivity removal curve (S5).

ApoA-I (Tangier) has a different amino acid composition from normal apoA-I, and includes a component of molecular weight slightly greater than normal apoA-I (K10). Evidence that patients with Tangier disease have an abnormal apoA-I was reinforced by studies from Zannis *et al.* (Z6). Intestinal organ culture from a patient with Tangier disease synthesized and secreted isoproteins 2 and 3 in normal amounts, but much of the plasma apoA-I from three patients with homozygous Tangier disease was isoprotein 2 (49%). Brewer's laboratory confirmed that an increased proportion of plasma apoA-I in Tangier disease is isoform 2, or pro-apoA-I.[2] The apparent defect in processing pro-apoA-I in plasma might have been the result of an abnormal

[2]Brewer *et al.* (B44) refer to isoforms 1 and 3 whereas others (S12, Z6) refer to the same proteins as isoforms 2 and 4. We have used the latter convention.

propeptide sequence, but the propeptide sequence of pro-apoA-I (Tangier) proved to be identical to that of normal pro-apoA-I (B44).

Schmitz *et al.* (S12) also found that serum from Tangier subjects contains apoA-I isoproteins 2 and 4 in roughly equivalent amounts, with isoprotein 2 corresponding to pro-apoA-I. Very little Tangier isoprotein 2 (pro-apoA-I) associated with HDL in recombination experiments, while the recombination of Tangier isoprotein 4 was almost normal.

The consensus of these studies is that the underlying defect in Tangier disease is faulty conversion of pro-apoA-I to mature apoA-I, either because of a defect in converting enzyme activity or a specific structural defect in Tangier apoA-I, although this hypothesis still awaits direct confirmation.

4.1.4. *Other Conditions in which Plasma Apolipoprotein A-I Is Deficient*

Subjects with hypoalphalipoproteinemia may have a variety of clinical manifestations or metabolic defects. Fish-eye disease is characterized by very low HDL levels, severe corneal opacity, visual impairment, and hypertriglyceridemia (C4, F18); the metabolic basis is unknown. A lipid disorder in which very low plasma levels of apoA-I and HDL cholesterol is associated with hypertriglyceridemia but not with corneal opacities or evidence of atherosclerosis (F20) has been shown to be associated with a cysteine-containing variant of apoA-I (W8). A patient has been described with very low HDL, apoA-I and apoA-II levels, high apoB levels, advanced atherosclerosis, and no clinical signs of Tangier disease (T9). A family has been described in which a moderate familial deficiency in HDL [HDL cholesterol in affected subjects (mean ± SD) was 0.66 ± 0.11 mmol/liter; in related controls it was 1.31 ± 0.23 mmol/liter] is associated with a high prevalence of premature coronary events (V5). Other cases with very low apoA-I and HDL cholesterol levels, with corneal clouding, and with premature coronary artery disease have been reported (G36, N13, S7), in one case with an associated apoC-III deficiency (N13) and an apparently homozygous DNA polymorphism in or near the apoA-I/C-III gene complex on chromosome 11 (B57, K6). Not all lipid disorders in which a genetic abnormality has been demonstrated in or near the apoA-I gene are associated with very low HDL levels. A group of severely hypertriglyceridemic patients have been shown to have an increased incidence of a restriction fragment length polymorphism associated with the apoA-I gene (R7). Further studies on such patients may help to explain the role of apoA-I in the prevention of atherosclerosis, and the interrelationships between low apoA-I levels, hypertriglyceridemia, and atherosclerosis.

4.1.5. *Apolipoprotein A-I and Coronary Artery Disease*

Although a large body of literature documents the well-known inverse relationship between plasma HDL cholesterol and coronary heart disease risk, there is little evidence to allow us to assess the relationship between plasma apoA-I concentration and coronary heart disease. The apoA-I/A-II ratio is higher in HDL_2 than in HDL_3. De Backer *et al.* (D4) reported a decreased apoA-I/A-II ratio in persons with coronary artery disease, suggesting that HDL subpopulations differ in cases and controls. On the other hand, others (A32, R10) found no overall difference in apoA-I/A-II ratios between subjects with coronary artery disease and controls.

In a study from the Mayo Clinic (M4), a group of male patients undergoing diagnostic coronary angiography for chest pain or suspected coronary artery disease had plasma cholesterol and triglyceride, HDL cholesterol, and apoA-I concentrations measured. Whereas HDL cholesterol discriminated to some extent between those with and those without important coronary artery disease (and total cholesterol and triglyceride did not discriminate at all), apoA-I levels provided an almost perfect prediction of obstructive coronary artery disease. Some caveats on the interpretation of apoA-I levels in this and other studies have been noted by Blackburn (B34).

ApoA-I concentration may prove to be an excellent predictor of coronary artery disease within a high-risk population with generally high LDL levels. However, much work must be done before there is agreement on the place of apoA-I estimations in assessing coronary heart disease risk.

Some of the relevant literature has been summarized by Brunzell *et al.* (B59).

4.2. Apolipoprotein A-II (ApoA-II)

ApoA-II is a major constituent of human HDL, M_r 17,380. Its primary amino acid sequence is known. It is composed of two chains of 77 amino acid residues each, linked with a disulfide bond at residue 6, and it contains no carbohydrate (B45, J4, L24, L26). It interacts with the other major apoprotein of HDL, apoA-I, and seems to have a stronger affinity for HDL than apoA-I, in that 2 mol apoA-II can displace 1 mol apoA-I from HDL when free apoA-II is added to HDL containing apoA-I (L2, R19). ApoA-II may interact with apoE or a rare mutant cysteine-containing form of apoA-I, apoA-I Milano (also referred to as apoA-I_{cys}), in forming a disulfide-linked complex, apo(E-A-II) or apo(A-I_{cys}-A-II) (W8, W11).

Schmitz *et al.* (S13) have recently shown that apoA-II isoproteins can be

separated by isoelectric focusing as follows: apoA-II_1 (p*I* 5.16), apoA-II_2 (p*I* 4.89), apoA-II_3 (p*I* 4.58) and apoA-II_4 (p*I* 4.31). The amino acid compositions of these isoforms appear to be virtually identical. The isoforms do not seem to be affected by treatment with neuraminidase, acid phosphatase, or alkaline phosphatase. They are not an artefact of purification, as their presence in native serum can be demonstrated. Both the nature of these charge modifications (perhaps sequential deamidations or point mutations affecting the primary amino acid sequence) and their physiological significance are unknown.

ApoA-II has served as a model protein for studies on the lipid-binding properties of peptides. Each 77-amino-acid peptide chain has three segments which have been proposed as amphipathic helices. There is evidence, summarized by Sparrow and Gotto (S52), that suggests that these segments form helices in the presence of phospholipid (with or without cholesterol), that is, that the proposed α-helices are in fact the lipid-binding segments.

4.2.1. *Apolipoprotein A-II Metabolism*

Purified apoA-II was shown by Jahn *et al.* (J2) to increase hepatic triglyceride lipase activity by threefold *in vitro*. Human plasma also activates hepatic triglyceride lipase activity, and it is a reasonable assumption that this activation is due to apoA-II. The physiological importance of this effect is not yet clear.

There is immunochemical evidence that in man apoA-II is produced in intestinal epithelial cells (B33, S16, S20). Anderson *et al.* estimated that 28–82% of total body apoA-II synthesis takes place in the intestine (A23). Most lymph apoA-II (90 ± 11%) is associated with HDL particles. As with apoA-I, a high proportion (perhaps more than 70%) of thoracic duct apoA-II is calculated to be derived originally from plasma and to be recirculating back into the plasma (A23).

Parks and Rudel (P4) showed in African green vervet monkeys that the kinetic fate of apoA-II on lymph chylomicrons introduced into plasma differed from that of apoA-I. ApoA-I metabolism has been discussed (Sections 4.2 and 4.3). ApoA-II was transferred immediately from chylomicrons to HDL particles. It is possible that in so doing it may displace apoA-I from HDL (L2, R19); the data of Parks and Rudel are consistent with this possibility.

The means whereby apoA-II is finally cleared from the circulation is unknown. Because the affinity of apoA-II for HDL particles appears to be greater than that of apoA-I, it is possible that apoA-II is cleared from the circulation only as part of an HDL particle.

4.3. Apolipoprotein A-IV (ApoA-IV)

ApoA-IV is an immunologically distinct apolipoprotein of M_r 46,000 (B22, G28, W7). It has been demonstrated in intestinal epithelial cells from fasting subjects and a marked increase has been shown during lipid absorption (G27). About 10–13% of chylomicron apolipoprotein and 24–30% of intestinal VLDL apolipoprotein is apoA-IV. In fasting plasma, 98% of apoA-IV is in the $d > 1.21$ g/ml fraction and in lipemic plasma 90% is in this fraction, while 10% is associated with triglyceride-rich lipoproteins (G27). Gel permeation chromatography confirmed that in plasma most apoA-IV is free, unassociated with lipoproteins (B22, G27).

Normal plasma apoA-IV in 14 subjects was 157 ± 9 mg/liter, and values in four subjects with abetalipoproteinemia were reduced. Lipid feeding in normal volunteers resulted in a rise in plasma apoA-IV (161 ± 7 mg/liter rising to 185 ± 7 mg/liter) (G27).

When rats were fed a high olive oil diet the apoA-IV concentration in lipoprotein-free plasma was increased (compared with rats on a control diet). When cholesterol was added to the diet the lipoprotein-free plasma apoA-IV concentration was higher still, but the HDL apoA-IV was decreased (D9). It is possible, therefore, that apoA-IV metabolism is related to chylomicron formation or metabolism, or cholesterol metabolism, but its significance in man is unknown.

4.4. Apolipoprotein B (ApoB)

Apolipoprotein B is the major protein component of LDL, and it appears to be an essential component of chylomicrons, VLDL, and LDL. Unlike other apolipoproteins, apoB is insoluble in aqueous buffers after delipidation with organic solvents, and it does not exchange between lipoprotein particles. This may be because of its molecular weight and insolubility; it is by far the largest of the apolipoproteins, although, as noted in Section 4.4.1, estimates of molecular weight vary.

There are two original forms of apoB in human plasma, one produced by the liver and characteristic of VLDL, VLDL remnants, and LDL, and the other produced by the intestine and characteristic of chylomicrons and chylomicron remnants. Because of some controversy over their molecular weight, Kane and co-workers have proposed that a system based on relative molecular weight be used to designate them (K4). Thus in man the larger original form, of hepatic origin, is arbitrarily called B-100 and the smaller, produced by the intestine [with apparent molecular weight 48% of the larger on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis], is

called B-48. In the rat the liver synthesizes both apoB-100 and apoB-48 (B23, S50, W23) and at least 95% of apoB synthesized by the rat intestine is apoB-48 (W23).

4.4.1. *Characterization of Apolipoprotein B-100*

Almost all studies on the characterization of apoB in terms of molecular weight, amino acid composition, etc., have been carried out on apoB-100 from LDL.

ApoB-100 is a glycoprotein with about 8–10% of its protein mass as carbohydrate, including neuraminic acid, galactose, mannose, and glucosamine (L9, M29, S64). The predominant apolipoprotein in LDL and VLDL, apoB-100 can most easily be differentiated from apoB-48 by SDS–polyacrylamide gel electrophoresis according to Kane *et al.* (K4). The major obstacles in estimating its molecular weight are its extreme insolubility in water and its tendency to self-associate or aggregate after lipid removal. Efforts have been made to solubilize apoB-100 in SDS, guanidine hydrochloride, or urea. Steele and Reynolds reported the isolation of LDL apoB in SDS (after the reduction of disulfide bonds) with changes suggesting α-helix formation near the critical micellar concentration (S54). Replacement of detergent with 7 *M* guanidine hydrochloride produced a soluble polypeptide without evidence of ordered structure in the circular dichroic spectrum. Steele and Reynolds used sedimentation velocity and sedimentation equilibrium studies to determine that the molecular weight of the apoB monomer (in guanidine hydrochloride) was 250,000 and that apoB formed a dimer in excess SDS (S55). These results are similar to those obtained some years previously by Smith and co-workers, who demonstrated that delipidated, reduced, carboxymethylated apoB is a random coil with molecular weight 255,000 in concentrated guanidine hydrochloride solution (S40). Presumably Kane *et al.* are measuring apoB dimers when they report the M_r of apoB-100 in excess SDS (on polyacrylamide gel elecrophoresis) as 549,000, and that of apoB-48 in the same system as 264,000 (K4).

Although apoB-100 is virtually the sole form of apoB in VLDL, many speciments of LDL also contain two additional forms of apoB which Kane *et al.* have called B-74 and B-26 (as their mobility on SDS-polyacrylamide gel electrophoresis suggests proteins of apparent molecular weight 74% and 26% of B-100 (K4). Kane *et al.* suggest that the B-74 and B-26 forms are fragments of the original B-100 form. As evidence they note that equimolar amounts of B-74 and B-26 are found in LDL samples, and that while the amino acid compositions of B-74 and B-26 differ from each other and from B-100, the calculated amino acid composition of an equimolar mixture of B-74 and B-26 is similar or identical to that of B-100 (H8).

As a practical point, it is worth noting that apoB seems particularly suscep-

tible to the action of proteases *in vitro*. Bacterial proteases can be obviated by aseptic techniques and storage in the presence of appropriate antibiotics, and the addition of EDTA inhibits the cleavage of apoB by azide (D7, S32). Lipid peroxidation may also affect apoB, and Lee *et al.* have increased apoB solubility by purification in the presence of glutathione and the absence of oxygen (L8). The mechanism whereby B-74 and B-26 are formed is unclear. A number of studies [summarized by Kane (K2) in a recent review] have suggested that B-100 contains either smaller subunits or repeating sequences, but in view of the tendency of apoB-100 to self-aggregate and to undergo proteolysis these claims must be treated with some reserve.

4.4.2. *Apolipoprotein B-100 Metabolism*

VLDL containing apoB-100, apoE, and apoC is secreted by the liver into the space of Disse. Metabolism of this triglyceride-rich particle by lipoprotein lipase leads to shrinkage of the core triglyceride component. As Eisenberg and others have shown experimentally (E4), other VLDL components must be removed before the mature LDL particle is formed. These changes include removal of about 75% of the phospholipid, 85% of the unesterified cholesterol, and most of the apoC and apoE from the VLDL surface (E3). The mass of apoB per particle stays constant during metabolism of VLDL to LDL, but all other surface and core materials are diminished.

The size of the VLDL particle in plasma diminishes and its density increases as triglyceride is hydrolyzed by endothelial lipoprotein lipase, and the particles are thus converted to intermediate-density lipoproteins (IDL) (B32, S35). The IDL detach from the endothelium, and some are taken up by hepatic B-100,E receptors. The remaining particles in the circulation are further depleted of some cholesteryl ester (by an unknown mechanism), and most of the remaining triglyceride (probably by hepatic triglyceride lipase, in the liver sinusoids) (D5). The resulting LDL particles are largely composed of cholesteryl ester as the core lipid and apoB-100 as the apolipoprotein.

The metabolism of apoB-100-containing lipoproteins is controlled by receptors for apoB on many cell types including the liver. The apoB receptors were first clearly demonstrated in cultured fibroblasts by Goldstein and Brown, and these workers have headed an extremely productive drive to elucidate the role of the apoB receptor in lipoprotein metabolism. Reviews by these and other authors are available (B54, B55, G19, G20, G22, H16, K23).

Before discussing the uptake of LDL by B-100,E receptors, it is appropriate to discuss briefly an animal model, the WHHL rabbit, which is deficient in B-100,E receptors. It has proved to be very useful in working out the

mechanisms by which LDL is metabolized, and the association between LDL metabolism and atherosclerosis.

4.4.3. *The Watanabe Hereditable Hyperlipidemic (WHHL) Rabbit*

Watanabe, in 1973, found one of a colony of experimental rabbits to be markedly hypercholesterolemic (11.6 mmol/liter) (W5). From this he bred a strain of rabbits with hypercholesterolemia expressed as an autosomal dominant trait, so that homozygotes had severe hypercholesterolemia and heterozygotes moderate hypercholesterolemia. WHHL homozygotes develop severe aortic atherosclerosis within the first few months of life followed by coronary atherosclerosis. The histological appearance and progression of atherosclerotic lesions resembles that in humans (B60). First, there is a deposition of esterified cholesterol in smooth muscle cells and in macrophage foam cells of the intima and media. The lesion develops into full-blown atherosclerosis, with the formation of plaques containing a necrotic cholesteryl-ester-filled core and a fibrous cap. Within 2 to 3 years, half the WHHL rabbits have severe coronary disease (>50% narrowing). In addition to widespread atherosclerotic lesions, there are deposits of cholesterol in tendon sheaths (as there are in familial hypercholesterolemia).

The WHHL model has stimulated major advances in our understanding of apoB receptors (G22). In particular, it has allowed a clear differentiation of two kinds of hepatic receptors: one involved in the uptake of chylomicron remnants, recognizing (it is thought) apoE when in a particle containing apoB-48, and the other involved in the hepatic uptake of apoB-100-containing VLDL, IDL, and LDL particles. The apoB-100 receptors, which also have an affinity for apoE and are referred to in this review as apoB-100,E receptors, are found in many extrahepatic cells. The WHHL rabbit is deficient in apoB-100,E receptors, but not in those receptors responsible for chylomicron clearance.

4.4.4. *Metabolism of Low-Density Lipoproteins*

Kinetic studies in normal human subjects show that 70–100% of the apoB of VLDL is converted to LDL apoB, and alll LDL apoB is derived from VLDL (B31, P1, R5, S35). When radiolabeled chylomicrons were reinfused into a subject with failure of apoB-100 production, the plasma half-life of the apoB-48 was 50 minutes, with no conversion to LDL (M20, M21). Studies on subjects with hypertriglyceridemia have suggested that up to two-thirds VLDL-apoB is removed from the circulation as IDL-sized particles and not metabolized to form LDL (F16, R5). However, VLDL may be heterogeneous in several respects. The VLDL fraction of fasted individuals with hypertriglyceridemia may contain both apoB-100 and apoB-48 (K2). VLDL

also contains at least two groups of particles which can be separated by heparin-affinity chromatography; those cholesterol-rich particles containing more apoE (a higher apoE:apoC ratio), which bind to heparin, and a class poor in apoE, not bound to heparin (N2, S24). These two groups of VLDL particles clearly have different metabolic characteristics (N2). Final assessment of the metabolism of apoB in VLDL will need to await precise definition of VLDL composition, including separation of the two major forms of apoB.

The kinetics of LDL catabolism in normal subjects have been examined in a number of studies. Miller has recently summarized data from 29 studies (M33). When considered together, these studies on 94 men and 22 women give a mean fractional catabolic rate for LDL in men of 0.352 ± 0.098 per day ($\pm$ SD) and in women 0.339 ± 0.101 per day.

B-100,E receptors have now been purified. They are glycoproteins, synthesized as a precursor of M_r 120,000 (T8), then converted to the mature protein of M_r 164,000 (S14) and inserted into the plasma membrane of cells. Human fibroblasts contain up to about 20,000 such receptors per cell, depending on cellular cholesterol requirements. It is thought that at any one time less than 1% of such receptors are newly synthesized, and that most have already been internalized by the cell and have then been recycled back to the surface. Once bound to the receptor, LDL is rapidly internalized (half-time less than 10 minutes). The ligand–receptor complex moves to particular areas on the surface of the cell, "coated pits," where internalization occurs as the coated pit invaginates to form an endocytic vesicle (G15). The endocytic vesicle migrates through the cytoplasm until it fuses with a primary lysosome to form a secondary lysosome.

The LDL-derived cholesterol, released from the lysosomes into the cytoplasm of the cell, sets in train a number of effects designed to stabilize intracellular cholesterol content (G16). These include suppression of the rate-controlling enzyme active in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) (thus turning off cholesterol synthesis), activating the cholesterol-esterifying enzyme acyl-CoA:cholesterol acyltransferase (ACAT), which re-esterifies excess cholesterol that can then be stored in droplets in the cytoplasm, and supressing the synthesis of receptors for LDL (B-100,E receptors), allowing the cell to control the receptor-mediated entry of LDL cholesterol into the cell. The work leading to the elucidation of these processes has been reviewed by Goldstein, Brown and co-workers (B54, B55, G15, G16, G18–G20, G22).

The degree to which the B-100,E receptors are expressed in various tissues appears to be a function of the requirement of that tissue for cholesterol. Thus, cell membranes from the adrenal cortex and gonads, organs that require cholesterol for the production of steroid hormones, contain many

B-100,E receptors and bind a large amount of LDL with high affinity [as shown in the cow (K24) and the human fetus (B53)]. Other cell types (e.g., liver, kidney, lung, and heart) have cell membranes with fewer B-100,E receptors which bind a smaller amount of LDL (B53, K24). The numbers of B-100,E receptors are controlled by a cellular feedback mechanism, so that no cell becomes overloaded with LDL cholesterol entering the cell by way of these receptors.

Reichl *et al.* have measured the concentration of LDL cholesterol in lymph, and compared it with that in plasma (R9). Knowing the concentration of LDL cholesterol above which maximum suppression of B-100,E receptors occurs in cells in tissue culture, it has been calculated that LDL levels in interstitial fluids would be sufficient to occupy LDL receptor sites in body cells if the plasma LDL-cholesterol concentration was only 25 mg/100 ml (R9). LDL cholesterol in industrialized man is at least four times that level, that is, well above the level at which maximum suppression of most LDL receptors would be expected to occur.

It is possible to block the ability of LDL to bind to B-100,E receptors in fibroblasts (M14) and liver (C9, K26, W18) by modifying the arginine residues of LDL with cyclohexanedione. *In vivo* turnover studies using cyclohexanedione-modified and unmodified LDL have suggested that about two-thirds of LDL removed from the circulation in normal man is taken up by B-100,E receptors, and one-third by another mechanism (S26).

In the B-100,E receptor-deficient WHHL rabbit, LDL is taken up by a low-affinity mechanism whose net activity increases as the concentration of LDL increases (i.e., the pathway is not subject to feedback control from intracellular cholesterol). In normal rabbits the B-100,E receptor is calculated to account for 63, 92, and 83% of LDL degraded by the liver, adrenal gland, and intestine, respectively (P18). Whether the low-affinity mechanism alone is responsible for the localized accumulations of cholesterol in artery walls or tendons, seen in human familial hypercholesterolemia and also in the WHHL rabbit, is uncertain; perhaps local trauma, or some other additional factor is also required.

There appears to be yet another pathway for cellular LDL uptake which is not subject to feedback control. High-affinity binding sites which are not apparently B-100,E receptors have been demonstrated on macrophages and other scavenger cells, and not on cultured fibroblasts, smooth muscle cells, lymphocytes, or adrenal cells (B49). These binding sites recognize LDL in which the positive charges on the ϵ-amino groups of lysine have been removed or neutralized by chemical modification, e.g., by malondialdehyde. Macrophages bind, internalize, and hydrolyze negatively charged LDL by this method, but the steady accumulation of intracellular cholesterol exerts no feedback effect on the receptor for negatively changed LDL (B49). The

relevance of this pathway to the development of atherosclerosis, with large amounts of cholesterol deposited in tissues, including foam cells which may be of macrophage origin in atherosclerotic plaques, is not yet settled. It is unlikely that malondialdehyde, produced *in vivo* by platelets, is present in plasma in sufficient concentration to modify LDL lysine residues (B49), though it is possible that LDL apoB within the artery wall might be exposed to high local concentrations of reagents such as malondialdehyde. This pathway may turn out to play a part in the deposition of cholesterol in scavenger cells in atherosclerotic plaques.

4.4.5. *Familial Hypercholesterolemia*

Patients who are heterozygotes for familial hypercholesterolemia (FH) have plasma LDL levels that are two- to threefold that of the "normal" population, so that they are in the upper fifth percentile of LDL cholesterol for their age. About 50% of men and 15% of women with this trait have at least one myocardial infarct by the age of 60. Approximately 1 in 500 persons has inherited this abnormality, which is inherited as an autosomal dominant characteristic (G20).

Homozygous FH is a rare disease, occurring in only about 1 in 1 million persons. The LDL concentration can reach six to eight times normal, and myocardial infarction before the age of 20 is almost inevitable. The clinical aspects of FH have been covered very well in extensive reviews (G20).

More than one genetic abnormality affecting B-100,E receptors may be responsible for FH. The most frequent mutation results in absence of the B-100,E receptors. As the result of another mutation the M_r 120,000 receptor precursor is not converted to the functional M_r 160,000 form (T8). Yet another genetic abnormality results in the production of a receptor which reaches the plasma membrane, binds LDL, but does not localize in coated pits for ingestion (G15, G17).

Analysis of the abnormalities in the WHHL rabbit has cast new light on the pathogenesis of FH in man (G22). In normal subjects most IDL containing apoB-100 is converted into LDL, but a little is taken up by the liver. A deficiency of hepatic B-100,E receptors leads to LDL overproduction (because the normal hepatic IDL uptake is defective). In homozygotes for FH after the intravenous injection of VLDL tracer, the expected delay in IDL clearance and increase in conversion of tracer to LDL can be demonstrated (S48). Defective clearance of LDL in the liver and in extrahepatic tissues means that the removal of LDL from the plasma is slow (about two-thirds the normal rate in the FH heterozygote and one-third in the FH homozygote). Because of these twin abnormalities, i.e., LDL overproduction and impaired clearance, the plasma LDL concentration rises to two or three times normal in FH heterozygotes and six to eight times normal in homozygotes

(G20, G22). Evidence from the WHHL rabbit suggests that the focal deposition of cholesterol in the artery wall and in tendons (seen in the WHHL rabbit and in humans with FH) is a direct response to increased uptake of LDL, perhaps into traumatized tissues, by pathways other than B-100,E receptors. This is in contradistinction to the cholesterol-fed rabbit with a normal LDL receptor system, which develops an entirely different pattern of cholesterol deposition in liver, spleen, and other tissues (B60).

4.4.6. *Hypercholesterolemia in "Normal" Industrialized Man*

Newborn babies have a low plasma LDL cholesterol, between 25 and 50 mg/100 ml (K36), a level similar to that found in many animal species (C2, M35). Goldstein and Brown have hypothesized that the marked rise which occurs in industrialized man, with LDL-cholesterol levels of over 100 mg/100 ml, may be attributed to suppression of LDL receptors as a result of environmental factors (B55, G19). They adduce evidence from studies on LDL turnover performed by Bilheimer and others that indicates that dogs, baboons, and humans each produce about 15 mg LDL cholesterol per kilogram body weight per day, but the very marked differences in plasma LDL-cholesterol levels in these species is the result of a fractional catabolic rate lower in the baboon than in the dog and much lower in man (G19). Miller also summarizes evidence from others suggesting that the slow rise in plasma LDL concentration seen with age in men and women is associated with a corresponding fall in the fractional catabolic rate of LDL, and he hypothesizes that the decrease in efficiency of LDL clearance with advancing age is a consequence of a decrease in either the number or the function of LDL (B-100,E) receptors (M33).

The development of polyclonal and monoclonal antibodies to B-100,E receptors suggests that we will be able to measure receptor numbers in tissue samples directly. If so, clinical studies on the effects of dietary and other factors on receptors will become possible.

4.4.7. *Drug Treatment of Hypercholesterolemia*

Receptor numbers have been increased by the administration of cholestyramine or colestipol, bile acid sequestrants that diminish the bile acid pool, force the liver to convert more cholesterol into bile acids (D11), lower the intracellular cholesterol in hepatic cells, and thus increase the number of hepatic B-100,E receptors (K25, S27).

Bile acid binding resins have been the mainstay of treatment for heterozygous FH for many years. Unfortunately they are not as effective as one might hope, because the liver partially compensates for the drain on cholesterol by increasing its own production of cholesterol from acetyl-CoA (B51, D11). Two recently developed drugs, compactin (B50, E7) and mevinolin

(A18), are competitive inhibitors of the rate-limiting enzyme for cholesterol synthesis, 3-hydroxy-3-methylglutaryl-CoA reductasė, and are thus potent inhibitors of cholesterol synthesis. Clinical and animal testing has shown that bile acid binding resins and drugs inhibiting cholesterol synthesis have a synergistic effect, lowering plasma LDL cholesterol dramatically with no change in HDL cholesterol levels (G19, K25, M1). Such manipulations have not yet been shown to have long-term efficacy and safety, but they give promise of exciting advances in the treatment of hypercholesterolemia.

4.4.8. *Apolipoprotein B-48 (ApoB-48)*

ApoB-48 serves as a convenient marker for apoB-containing lipoproteins of intestinal origin. Chylomicrons and VLDL in thoracic duct lymph in African green monkeys and cynomolgus monkeys both contain apoB-48 (K16), and the cholesteryl esters of these particles are similar and characteristic of those that are synthesized by ACAT, known to be active in intestinal mucosa (H11). By contrast, LDL, also found in intestinal lymph, contained mostly apoB-100 and a little apoB-48. The evidence suggests that, in these primate species, the intestine secretes only apoB-48; most of the minor LDL fraction in lymph probably comes from the plasma compartment (K16). Studies in the rat, in which apoB-48 of rat chylomicrons was labeled as an *in vivo* tracer, rule out the possibility that chylomicrons are LDL precursors (V4). Evidence that in man chylomicron apoB-48 is not converted into LDL apoB-100 is provided by a report of a form of abetalipoproteinemia associated with normotriglyceridemia, in which apoB-100 containing LDL and VLDL is absent but apoB-48 containing chylomicrons appear to be produced and metabolized normally (M21).

4.4.9. *Chylomicron Metabolism*

Chylomicrons are large (75–600 nm in diameter), spherical triglyceride-rich particles which are formed in the intestinal wall during the absorption of dietary fat. Dietary triglyceride is hydrolyzed in the intestinal lumen and absorbed into the enterocyte, and together with a complement of apolipoproteins, esterified and unesterified cholesterol, and phospholipid, is formed into chylomicrons that are released into the intestinal lymph (B33). Between meals, smaller triglyceride-rich VLDL particles are secreted by the intestine (O1, T13). The smaller size and higher density of VLDL of intestinal origin is presumably a consequence of the decreased requirement for triglyceride transport in the fasting state; they may represent the absorptive route for endogenous biliary and intestinal lipids (O1).

Chylomicrons in human lymph contain apoB-48, apoA-I, apoA-II, apoA-IV, and a little apoC-II (considered to be synthesized in the intestine) [evidence summarized in (B33)], and apoE and apoC (thought to originate

from the plasma compartment) (I4). Lymph VLDL particles have apolipoproteins similar to those in chylomicrons, and are in this respect quite different from plasma VLDL, which are secreted by the liver (G28). All of these apolipoproteins may transfer from one lipoprotein particle to another, except apoB.

As chylomicron triglyceride is hydrolyzed by lipoprotein lipase in the plasma compartment, the chylomicron shrinks and loses redundant surface material, including phospholipid and apolipoproteins. ApoC is lost, the particle apoE:apoC ratio rises, and the apoB-48 remains with the particle. The remnant finally is taken up by a specific hepatic receptor, in a process that appears to be dependent upon the content of both apoE and apoC of the remnant particle. The uptake of apoE-containing remnant particles is discussed in Section 4.7.4.

It has been shown that monoclonal antibodies to apoB-100 that inhibit LDL binding to the B-100,E receptor failed to react with apoB-48, suggesting that apoB-48 is not a ligand for the B-100,E receptor (M20). Also, chylomicrons and chylomicron remnants containing apoB-48 are cleared from the circulation rapidly and normally in WHHL rabbits, a strain of rabbit which are homozygous for a deficiency of B-100,E receptors in the liver and extrahepatic tissues (K14). Chylomicronemia or elevated plasma remnant concentrations are not features of homozygous familial hypercholesterolemia in man, suggesting that the clearance of these particles is normal in the absence of B-100,E receptors.

4.4.10. *Immunoassay of Apolipoprotein B*

Although most epidemiological studies on the relationships between plasma lipids and coronary heart disease have examined total plasma cholesterol (most of which is apoB-associated cholesterol) as a major risk factor, some case-control studies have shown that apoB-100 (i.e., apoB in LDL and VLDL) is higher in subjects with coronary heart disease than controls (A31, A32, F2, M34, O6, R10, S43, V6, W14). It may be that apoB is itself a risk factor, and that a raised apoB concentration but normal LDL cholesterol will be a marker for a previously unsuspected group of people at risk from coronary heart disease (S43). An increasing body of evidence suggests that hyper-B-apoproteinemia may be an important risk factor for coronary heart disease. This evidence, and the possibility that apolipoprotein assays (in particular apoB and apoA-I assays) may serve as a better marker of risk for atherosclerosis than the standard lipid measurements, have been discussed by Brunzell *et al.* (B59).

An excellent review of the methodology and standardization of apoB immunoassays has been published (R20), and it is not necessary to cover this ground again. Problems in setting up and validating an apoB immunoassay

include masking of antigenic sites by lipid, differing sizes of apoB-containing lipoproteins, and the presence in some samples of at least two forms of apoB, apoB-100 and apoB-48. In addition there are problems peculiar to particular methods, e.g., the effect of particle size on results obtained by radial immunodiffusion and immunonephelometry. Purified apoB preparations seem to be unstable on prolonged storage. Although apoB immunoassays appear simple, there are many problems to be solved if interlaboratory comparability is to be assured.

Mean values (± SD) for plasma apoB reference ranges obtained by various workers (summarized in R20) range from 0.81 ± 0.20 g/liter to 0.94 ± 0.33 g/liter using radioimmunoassays, 0.63 ± 0.16 g/liter to 1.32 ± 0.05 g/liter using electroimmunoassays, and 0.82 ± 0.23 g/liter to 1.59 ± 0.69 g/liter (most were between 0.82 and 0.97 ± 0.22 g/liter) using radial immunodiffusion or immunonephelometric assays. In neonates, mean plasma apoB (±SD) was 0.25 ± 0.02 g/liter; at 7 days, 0.57 ± 0.03 g/liter; and at 30 days, 0.62 ± 0.02 g/liter, using an immunonephelometric assay (V3). Age and country of residence are major variables affecting LDL-cholesterol concentration.

4.5. Apolipoprotein C (ApoC)

The C apolipoproteins are commonly considered as a group, although they are quite distinct small proteins with different functions.

1. *Apolipoprotein C-I* can be isolated from VLDL (B56, S52) or HDL (O8) by repeated chromatography. It has been sequenced and found to contain 57 residues (J8, S33). It appears to be highly helical on lipid binding (J7), with three lipid-binding helical sequences predicted by Chou–Fasman analysis (S22). Both native and synthetic apoC-I bind lipid, and also activate LCAT (H7, S34, S46). This dual activity is probably due to the peptide which contains amino acids 17–57; this sequence appears to activate LCAT, and the sequence 32–57 appears to bind lipid (S49). ApoC-I readily self-associates in aqueous solution (O7).

2. *Apolipoprotein C-II* can also be isolated from VLDL or HDL (H20, L5, N3). It contains 78 residues (J3) and has been shown by Chou–Fasman analysis to bind phospholipids (M26, M40), with three predicted helical sequences (M26). ApoC-II has attracted a great deal of attention because it activates one of the most important enzymes in plasma lipid metabolism, lipoprotein lipase, responsible for the hydrolysis of triglyceride in chylomicrons and VLDL. Sparrow and Gotto have summarized a number of studies on structure–function relationships (S52). These, taken together, indicate that there are separate functional domains in apoC-II, in that lipoprotein lipase activation is mediated by residues 55–78 and phospholipid binding by

residues 43–51. It seems that phospholipid binding is probably important in lipoprotein lipase activation (K13, S41). Bengtsson and Olivecrona have shown that lipoprotein lipase binds to lipid substrates and suggests apoC-II orients lipoprotein lipase and/or the lipid for effective hydrolysis (B28).

A disorder of lipid metabolism, in which absence of lipoprotein lipase activity due to an absolute apoC-II deficiency results in marked hypertriglyceridemia (Type I phenotype), has been reviewed elsewhere (N8). There are some unexplained differences in the clinical picture and plasma lipoprotein pattern between apoC-II deficiency and primary lipoprotein lipase deficiency. In apoC-II deficiency, symptoms appear to be milder (but recurrent abdominal pain, caused apparently by acute pancreatitis, is a frequently reported symptom). Patients do not show xanthomas or hepatomegaly, and few have splenomegaly (all features of lipoprotein lipase deficiency). Diagnosis is by electrophoresis of the C apolipoproteins, and a plasma triglyceride concentration usually 1000–3000 mg/dl (N8). There may be an increase in plasma VLDL concentration, whereas in classical lipoprotein lipase deficiency plasma VLDL concentration is nearly normal (N8).

In functional lipoprotein lipase deficiency there appears to be a normal removal rate for VLDL from the plasma (B31, F19, N7), and an unimpaired rate for the conversion of VLDL apoB to LDL apoB (N7). It may be that VLDL is hydrolyzed by hepatic triglyceride lipase (unaffected in lipoprotein lipase deficiency) (N7). Hepatic triglyceride lipase does not require apoC-II as a cofactor (E2).

3. *Apolipoprotein C-III* may be isolated from chylomicrons and VLDL (B56, N3) or HDL (N3). The apolipoprotein contains 79 residues with a carbohydrate chain attached to threonine-74 (B46). Studies with segments of the primary sequence indicate that residues 41–79 contain the phospholipid binding site of apoC-III (S22). Three main isoforms of apoC-III are recognized: apoC-III_0, apoC-III_1, and apoC-III_2, which have, respectively, no sialic acid, or 1 or 2 moles of sialic acid residues per mole of apoC-III (thus imparting a different charge to each isoform and allowing separation by isoelectric focusing or ion-exchange chromatography). In addition, each mole of apoC-III contains 1 mole of galactosamine and 1 mole of galactose (V1). The functional significance of the degree of sialylation is uncertain. Two groups have reported that the increase of apoC-III_1 with hypertriglyceridemia is more pronounced than that of apoC-III_0 and apoC-III_2 (K9, L3), and another has shown that a high-carbohydrate fat-free diet for 7 days leads to an increase of apoC-III_0 relative to the other C peptides in VLDL (F6). Nestel *et al.* have reported that a short-term high-carbohydrate diet leads to a relative decrease in apoC-III_2, though total apoC-III is increased (H34), while a high-cholesterol diet increases the proportion of apoC-III_2 (N4). The biochemical basis for these changes, and their effects, is unknown.

Stocks *et al.* (S57) reported that plasma from two hypertriglyceridemic subjects contained a triglyceride-rich lipoprotein which was abnormal, in that it contained excess apoC-III_2, associated with an impaired interaction of the abnormal lipoprotein with lipoprotein lipase. This group has reported a further study on 4 hypertriglyceridemic subjects with the abnormal lipoprotein (found after screening some 200 hypertriglyceridemic patients) and 4 patients with hypertriglyceridemia secondary to chronic renal failure (H28). The abnormal triglyceride-rich lipoproteins, containing excess apoC-III_2, were poor substrates for bovine milk lipoprotein lipase. When *in vitro* treatment of the lipoproteins with neuraminidase restored the proportions of apoC-III isoforms to normal, they interacted normally with lipoprotein lipase, but a severe reduction in apoC-III_2 by prolonged neuraminidase treatment impaired the ability of the lipoproteins to act as a lipoprotein lipase substrate. When three hypertriglyceridemic patients were successfully treated, their lipoprotein apoC isoform compositions reverted toward normal and the *in vitro* reaction of triglyceride-rich lipoproteins with lipoprotein lipase improved. *In vitro* tests suggested that apoC-III_2 inhibited hydrolysis by lipoprotein lipase, but that apoC-III_1 may have increased the degree of hydrolysis. These findings, taken all together, suggest that the different isoforms of apoC-III have identifiable functions with regard to lipolysis, but the details are still unclear.

4. *Other ApoC peptides.* Other C peptides that have been reported include apoC-IV and apoC-V, which make up less than 2% of apoC in plasma (C5), and a VLDL apoC-II variant (H18).

4.5.1. *Plasma Concentrations of Apolipoprotein C*

Although it is possible to estimate apoC concentrations in lipoprotein fractions by delipidation, electrophoresis or isoelectric focusing, staining, and densitometry [e.g., (C5, N3)], or by high-pressure liquid chromatography (H6), most reported measurements of plasma apoC concentration have been by immunological means. These include radioimmunoassay (K8, K9, S17), electroimmunoassay (A4, A5, C27), radial immunodiffusion (P21), and enzyme immunoassay (H29).

There seems to be little information available on plasma apoC-I concentrations. Alaupovic *et al.* (A5) report that the mean ($\pm$ SD) concentration of apoC-I in HDL from five normolipidemic subjects was 48 ± 17 mg/liter, i.e., about twice that of apoC-II (22 ± 8 mg/liter). Herbert *et al.* give the plasma concentration of apoC-I as 40–70 mg/liter (H24).

The reported mean plasma concentrations of apoC-II in normal subjects range from 30 to 52 mg/liter, and of apoC-III from 100 to 154 mg/liter (A4, C27, F6, K8, K9, S17, summarized in N3). The mean concentrations of apoC-II and apoC-III rise with hypertriglyceridemia, so that the mean plas-

ma concentration of apoC-II in Type IV hyperlipidemia is 85–100 mg/liter and that of apoC-III 230–330 mg/liter (A4, C27, F6, K9, S17). In Type I hyperlipidemia the apoC-II concentration is high (230 mg/liter) (K9). In Type V hyperlipidemia the mean apoC-II concentration was about 130 mg/liter (A4, S17) and the mean apoC-III concentration 540 mg/liter (C27, K9).

4.5.2. *Origin of C Apolipoproteins*

Studies in man are limited. Biopsies have shown detectable apoC in human intestine after fat has been eaten (S18). In the rat there is evidence that the intestine is not a significant source of apoC (K28), while the liver is (B23, H2). It is thought that, in man, the liver is the main source of apoC, but there appear to be few data casting a direct light on this.

4.6. Apolipoprotein D (ApoD)

Apolipoprotein D, a glycoprotein referred to originally as "thin-line" polypeptide, is an apolipoprotein of M_r 22,700 found in HDL and VLDL (A6, L7, M2, M3). Kostner (K18) has described "thin-line" polypeptide, which he termed apoA-III. The amino acid composition of apoA-III differs from that described for apoD; nevertheless, many workers have assumed that apoA-III is apoD. ApoA-III has been reported to be a potent activator of lecithin:cholesterol acyltransferase (K17, O6), but apoD, though associated with LCAT (*vide infra*) appears not to activate the enzyme (A16). It appears quite possible, therefore, that apoD and apoA-III are different apolipoproteins.

ApoD is found in association with LCAT and with apoA-I in the HDL fraction. Albers *et al.* used a specific antibody to apoD to remove all apoD by immunoadsorption chromatography from plasma; about 64% of LCAT activity and 11% of apoA-I were also removed from plasma (A14). Purified apoD has an apparent M_r of 32,500, and appears as three isoforms on isoelectric focusing (p*I* 5.20, 5.08, and 5.00) (A14). An HDL apolipoprotein, M_r 35,000, has been thought to be apoD, and to be a cholesteryl ester transfer protein (i.e., to transfer newly synthesized esterified cholesterol from HDL to LDL) (C8). Cholesteryl ester transfer activity in plasma was removed by polyclonal immunoglobulin to "apoD" (C8, F10). However, Morton and Zilversmit (M41) were able to separate apoD and lipid transfer protein (i.e., the cholesteryl ester transfer protein, or lipid transfer protein I) by chromatography, and they showed that the removal of apoD from plasma by precipitation with specific antisera did not remove any lipid transfer activity. Albers *et al.* (A14) also showed that immunoadsorption with antibody specific for apoD removed all the apoD from plasma without removing any cholesteryl ester transfer activity.

The concentration of apoD in human serum has been reported as 10–12 mg/dl by electroimmunoassay and radial immunodiffusion (C25) and a little less (6.2 ± 1.0 mg/dl for males, 5.6 ± 1.4 mg/dl for females) by radial immunodiffusion (A14). In the latter study, apoD was significantly correlated with HDL cholesterol levels in both sexes (A14).

4.7. Apolipoprotein E (ApoE)

Apolipoprotein E is a glycoprotein found in several classes of plasma lipoproteins, in chylomicrons, VLDL, remnant particles derived from partial metabolism of the triglyceride-rich lipoproteins, and in a subclass of HDL (B39, G3). The M_r calculated from the primary amino acid sequence of apoE is 34,145 (R4), in agreement with previous estimates of 33,000 to 39,500 (M8, M17, S23, U1, U5, W11). Analysis of the primary sequence predicts that 62% of the amino acids are part of an α-helical structure, including five segments that satisfy the requirements for an amphipathic helix (R4) and are thus implicated by analogy with other apolipoproteins as likely sites for lipid binding by apoE (P26). Rall *et al.* (R4) suggest that the site involved in the interaction of apoE with cell receptors occurs in the region bounded by amino acids 118–162, numbering from the *N*-terminal end. This region includes a predicted amphipathic α-helical structure with a segment of β structure at either end. Substitution of a cysteine residue for arginine at position 158 in one of these segments of β structure impairs apoE binding to apoB,E cell surface receptors (W10) (as in the naturally occurring apoE-2 isoform—see later) and chemical modification of a number of arginine or lysine residues prevents apoE binding to apoB,E receptors (M14, W9).

The isoforms of apoE were first clearly demonstrated by Utermann *et al.*, who showed on one-dimensional isoelectric focusing of VLDL apolipoproteins that there were four major isoforms of apoE (U3–U5). These were named, from acidic to basic, apoE-1, apoE-2, apoE-3, and apoE-4. Patients with Type III hyperlipoproteinemia (dysbetalipoproteinemia) were, in virtually all cases, deficient in apoE-3 (U3–U6). Only 27% of subjects had apoeE-4, but this band was not associated with any particular abnormality. However, individuals deficient in apoE-3 were also deficient in apoE-4 (U6). Utermann suggested that the genetic pattern of apoE isoforms could be explained if there was a single genetic locus for apoE which could produce three apoE phenotypes, i.e., apoE-N (normal), apoE-D (deficient, associated particularly with the Type III disorder), and apoE-ND (U4, U6).

Zannis and Breslow extended this analysis with two-dimensional polyacrylamide gel electrophoresis of apoE (isoelectric focusing in the first dimension followed by SDS–polyacrylamide gel electrophoresis in the second dimension). They numbered the isoprotein positions 1 to 7 from basic to

acidic (cf. Utermann *et al.*, who numbered from acidic to basic) and carried out a genetic analysis (Z3). Their model suggests that three alleles produce three forms of apoE in man (Z3). Using Utermann's terminology, these are apoE-2 (focusing at p*I* 5.89), apoE-3 (p*I* 6.02) and apoE-4 (p*I* 6.18). (Zannis and Breslow refer to these three forms, respectively, as $apoE_{\beta IV}$, $apoE_{\beta III}$, and $apoE_{\beta II}$). The phenotype will be a product of two alleles, either homozygous (apoE-2/E-2, apoE-3/E-3, or apoE-4/E-4) or heterozygous (apoE-2/E-3, apoE-3/E-4 or apoE-2/E-4). Each form of apoE may be sialylated in a posttranscriptional process. The sialo derivatives of apoE-2, apoE-3, and apoE-4 with 1 mole of sialic acid per mole of protein focus at p*I* 5.78, p*I* 5.89, and p*I* 6.02, respectively. Further sialo derivatives have a p*I* approximately 0.1 unit less than the parent isoform for each additional sialic acid residue. The sialylated forms are less prominent on two-dimensional electrophoresis than the nonsialylated forms (Z2). "ApoE-1" appears to be produced by a different gene locus and is not involved in the pathogenesis of Type III hyperlipoproteinemia.

Amino acid sequence analysis has shown that these three isoforms, apoE-2, apoE-3, and apoE-4, have different amino acid compositions (W13). ApoE-2 has two cysteine residues per mole, at positions 112 and 158, while apoE-3 has one cysteine at position 112 and an arginine at 158 (R4), and apoE-4 has no cysteine and is thought to have two arginine residues at positions 112 and 158 (R4, W13). These point substitutions account for the charge differences noted on isoelectric focusing on polyacrylamide gels. These findings are consistent with the genetic model proposed by Zannis *et al.* (Z4) that there are three independent alleles at a single genetic locus, with each allele coding for one major isoform leading to three homozygous and three heterozygous states. They establish that the influence on apoE is at the level of the structural gene.

4.7.1. *Apolipoprotein E Synthesis*

Evidence from rats suggests that apoE is synthesized almost exclusively in the liver (M31, W19). Perfusion experiments show that the liver produces discoid nascent HDL particles which are rich in apoE, and which also contain apoA-I (D1, D2, F8, H3, H5, K11, M31). ApoE is not found in chylomicrons of intestinal lymph, and its presence in chylomicrons in blood suggests that a transfer from nascent HDL to chylomicrons occurs (G28, I4).

Zannis *et al.* have examined the synthesis of apoE by organ cultures of fetal liver (Z5). They find that the apoE secreted by liver cells is mainly sialylated. Since only 10–20% of plasma apoE is sialylated, these authors conclude that apoE is first synthesized as sialo-apoE and that most is desialylated in plasma. Zannis and Breslow have speculated (Z3) that asialo-apoE may be taken up by the liver more rapidly than sialo-apoE isoproteins.

There is some evidence that asialo-glycoproteins in general may be taken up by the liver more rapidly than sialylated glycoproteins (F15, M24, M39).

4.7.2. *Apolipoprotein E Metabolism*

ApoE is thought to reach the plasma from the liver in nascent HDL, disk-shaped particles about 4.6-nm thick and with a mean diameter of about 19 nm which have clearly been shown to be of hepatic origin in the rat (H5). In plasma from human subjects with lecithin:cholesterol acyltransferase deficiency a similar population of apoE-rich HDL particles, 14–40 nm in diameter and 4.4–4.5 nm thick, is seen (M37, S47).

4.7.3. *Apolipoprotein E-Containing HDL*

Incubation of LCAT with plasma from patients with hereditary LCAT deficiency allows the esterification of cholesterol in nascent HDL particles. Thus, disk-shaped nascent HDL is converted to spherical HDL particles and apoE is redistributed from HDL to VLDL (G12, N12). In nonfasting normal plasma, this distribution extends to chylomicrons after they enter the blood from the intestinal lymph (G28, I4) so that apoE is associated with chylomicrons and VLDL, their remnant particles, and a fraction of HDL which is a little larger than non-apoE-containing HDL (G3). Heparin administration *in vivo* results in hydrolysis of triglyceride in the triglyceride-rich lipoproteins by lipoprotein lipase together with a fall in apoE associated with these lipoproteins and a rise in HDL-associated apoE (B38). It may be that apoE distributes itself in these fractions in such a way as to achieve a steady-state mass equilibrium with continuing exchange of apoE particles. The reasons for this particular distribution pattern are quite unclear.

Plasma apoE concentrations are elevated in various types of hyperlipoproteinemia, especially in hypertriglyceridemia. In all animal species studied in detail, high levels of dietary fat and cholesterol cause hypercholesterolemia, with an increase in HDL apoE (M8, M10, M16, M17, M19), which Mahley *et al.* have referred to as HDL_c (i.e., HDL increased in concentration by cholesterol feeding). These apoE-containing HDL_c particles are larger (11- to 15-nm diameter versus 8–11 nm diameter for normal HDL), have a slower electrophoretic mobility, lower density (HDL_c particles contain about 30% more cholesteryl ester than normal HDL), and different apolipoprotein composition from typical HDL (which lacks apoE). They can be separated from typical HDL completely or partially (depending on the species of animal) by preparative electrophoresis in Geon–Pevikon, (partially) by zonal ultracentrifugation (density 1.04–1.09 g/ml), or completely by heparin-affinity chromatography (M18).

Man also responds to a high-fat high-cholesterol diet with the production of HDL_c particles (C17, M8, M12). Mahley *et al.* (M12) fed eggs to normal

volunteers, resulting in the production of an HDL fraction which migrated more slowly than typical HDL on electrophoresis and interacted avidly with cultured fibroblasts. The production of an HDL_c-like particle could be observed regardless of whether the total plasma cholesterol was increased. In other studies by the same group the production of apoE-containing HDL by a high-fat high-cholesterol diet was demonstrated directly, by isolating apoE-containing HDL by heparin–Sepharose chromatography (M9). A study by Cole *et al.* (C17) showed that an increase in dietary cholesterol and fat did not affect the amount of apoE in VLDL–IDL, but increased the amount of apoE in the region of HDL_c (as shown by heparin–Sepharose chromatography) in most subjects. Others have performed similar dietary studies, and not shown a rise in plasma apoE (A24, T6). [It is noteworthy that a study on the cholesterol- and fat-fed rat showed that this diet led to a *fall* in plasma apoE. There was evidence that, in this animal, the rate of apoE synthesis increased but the rate of removal of apoE in chylomicron remnants was even greater (D8)].

ApoE-containing HDL, obtained by heparin–Sepharose affinity chromatography, contains apoA-I, apoA-II, and apoC, as well as apoE (W12, W18). Clearance from the plasma appears to be dependent on a specific hepatic receptor for apoE, which binds apoE-containing HDL and chylomicron remnants, but not other lipoproteins (H35, M11, S28). Canine apoE-HDL_c, with apoE as the only detectable apoprotein, is cleared from the plasma very rapidly by the liver (more than 90% in the first 20 minutes after intravenous injection) (M15). In adult man, dogs, and swine the apoE receptor numbers do not seem to be significantly reduced by cholesterol feeding (H35, M11).

ApoB-100,E receptors present in the liver of immature dogs and swine are suppressed by cholesterol feeding and are not apparently active in the liver of mature animals (H35, M11). The hepatic apoB-100,E receptors are thought to be identical to the apoB-100 receptors demonstrated in many extrahepatic cell types (B21). ApoE-containing HDL *in vitro* have a 20- to 25-fold greater affinity for apoB-100,E receptors than LDL (I6, P15) it seems, because there are four receptor binding sites for each molecule of HDL_c as opposed to one binding site for LDL (P15, P16).

In summary, it seems likely that, in the adult animal including man, apoE-containing HDL is taken up rapidly by the apoE receptors in the liver. Some apoE-containing HDL may also be taken up by apoB-100,E receptors in extrahepatic tissues. The significance of apoE-containing HDL in cholesterol metabolism in man is unknown, but this pathway may allow HDL cholesterol to be taken up by the liver. An apoE-enriched HDL fraction, larger than typical HDL but smaller than LDL, has been demonstrated in the plasma of normolipidemic subjects (G3). The cholesterol content of apoE-associated

HDL in normal human plasma is probably only about 5–10 mg cholesterol/100 ml, but the rapid rate of removal of apoE-associated HDL from plasma (at least in the dog) suggests its metabolism may nevertheless be a quantitatively important pathway in man.

4.7.4. *Apolipoprotein E-Containing VLDL and LDL*

In normolipidemic subjects, apoE is found not only in HDL_c-like particles but also in two other fractions associated with triglyceride-rich lipoproteins. These are VLDL, and a lipoprotein class intermediate in size between VLDL and LDL (G3). The latter may be the normal counterpart of the β-VLDL which accumulates in Type III hyperlipoproteinemia and in cholesterol-fed animals.

There is good evidence that nascent chylomicrons acquire apoC and apoE from HDL present in lymph and blood plasma (G28, I4). The fate of apoE during the hydrolysis of chylomicron triglyceride by lipoprotein lipase is unknown. Perhaps some apoE is lost to the HDL fraction during lipolysis, in the same way that redundant phospholipid and apoC are lost (H17, M38).

ApoE-containing VLDL in hyperlipidemic subjects has been shown to be both the product of particles less rich in apoE (as judged by the apoE:apoC ratio) and the precursor of apoE-rich intermediate-density lipoprotein (N2). In cholesterol-fed dogs (F5) and humans with Type III hyperlipoproteinemia (F5, K3) there is evidence (based on the form of apoB, B-48, or B-100, and the response to fasting) that apoE-rich β-VLDL contains remnants of both VLDL and chylomicrons.

It is likely that the major site of uptake of apoE-containing remnants of the triglyceride-rich lipoproteins is the liver. As apoC is removed and the apoE:apoC ratio rises, so the remnant lipoprotein becomes more amenable to hepatic uptake by specific receptors (S25, S28, W16, W17). VLDL remnants and IDL also experience apoE-mediated binding by apoB,E receptors in hepatic cell membrane preparations (H35, M11). The smallest apoE-rich VLDL subfractions from normolipidemic human plasma compete with LDL for fibroblast (apoB-100,E) receptors *in vitro* (T10) and in cultured fibroblasts (F17, G2, I7).

ApoE-containing β-VLDL will also bind with a specific high-affinity receptor, thought to be an apoE receptor, on the surface of cultured peritoneal macrophages. Binding is followed by uptake of the ingested lipoproteins and accumulation of cholesteryl ester in the macrophages. Whereas in cultured fibroblasts, suppression of apoB-100,E receptors limits the amount of cholesterol that can be accumulated by the cell, the macrophages appear to have no such regulatory mechanism. They continue to bind and take up apoE-containing β-VLDL, resulting in a 20- to 160-fold increase in their cholesterol content (G21, M13). The macrophage binding site appears to be quite

different from the apoB-100,E receptors that have been characterized in many cell types, in that it recognizes apoE-containing β-VLDL but not LDL. Normal VLDL, apoE-containing HDL_c, and other lipoprotein fractions have little or no effect on cholesteryl ester accumulation in macrophages (M13).

Fainaru *et al.* (F5) have subfractionated apoE-containing β-VLDL from cholesterol-fed dogs and from two patients with Type III hyperlipoproteinemia. The β-VLDL contains a population of larger particles (90–300 nm in diameter, of irregular shape, containing equal amounts of high- and low-molecular-weight forms of apoB) and smaller particles (20- to 70-nm-diameter spherical particles, containing predominantly the high-molecular-weight form of apoB). The larger particles, thought to be cholesteryl ester-rich chylomicron remnants, were 3- to 15-fold more active than the smaller particles (cholesteryl ester-rich lipoproteins probably of liver origin) in stimulating cholesteryl ester formation in mouse peritoneal macrophages.

Macrophages appear to do more than simply bind and take up remnant particles. Cultured macrophages secrete lipoprotein lipase into the culture medium (C6, K12, M7, W3), and the consequent depletion of chylomicron triglyceride appears to accelerate the uptake of chylomicron remnants and the accumulation of cholesteryl esters by macrophages (O10). Macrophages also can produce apoE (B17); perhaps this may be a mechanism for removing cholesterol from a cholesterol-loaded cell.

It has been established, therefore, that apoE-containing β-VLDL obtained from several animal species by cholesterol feeding is taken up *in vitro* by a specific receptor mechanism in macrophages, which thus accumulate cholesteryl ester. It is thought that these are the *in vitro* equivalent of the macrophage-derived foam cells found in atherosclerotic lesions produced in animals by cholesterol feeding (P17). It remains to be shown that this is an important process in the genesis of atherosclerosis in man. ApoE-containing particles midway in size between VLDL and LDL have been demonstrated in normolipidemic man (G3), and it is possible that a very low plasma concentration of apoE-containing β-VLDL may play an important part in transporting cholesterol into macrophages and in the accumulation of cholesterol in the artery wall. The studies of Fainaru *et al.* (F5) suggest that chylomicron remnants may be most important in this respect. They may be the atherogenic remnant particles proposed by Zilversmit (Z7).

4.7.5. *Type III Hyperlipoproteinemia (Dysbetalipoproteinemia)*

While practically all subjects with Type III hyperlipoproteinemia appear to be homozygous for apoE-2 (i.e., to have the *E-2/E-2* genotype), most subjects who are homozygotes for apoE-2 do not develop Type III hyperlipoproteinemia. Indeed, about 1% of the population is an E-2/E-2 homo-

zygote and at most only 1 in 50 of these appears to develop Type III hyperlipoproteinemia (U2).[3]

Because most homozygotes for apoE-2 do not have hyperlipidemia, and in fact have rather a low plasma LDL cholesterol (U9), Utermann *et al.* proposed that Type III hyperlipoproteinemia results from the simultaneous inheritance of the *apoE-2/E-2* genotype and some other independent genotype or factor acting synergistically with the *apoE-2/E-2* genotype (U8). Examples of independent genotypes that have been documented are familial combined hyperlipoproteinemia (H21) and perhaps familial hypercholesterolemia (H21). Thyroid deficiency has also been implicated as the synergistic second factor, and it is possible that other causes of secondary hyperlipidemia may precipitate the Type III phenotype in an apoE-2/E-2 homozygote.

The biochemical basis for the frequent finding of a low plasma LDL cholesterol in apoE-2/E-2 homozygotes is unclear. The basis for the development of the Type III hyperlipoproteinemia phenotype is also not entirely clear. Particles containing only apoE-2 or apoE-containing particles from an apoE-2/E-2 homozygote have a diminished ability to bind to hepatic lipoprotein receptors (H14). It seems likely, therefore, that the low binding of apoE-2/E-2 to hepatic receptors is responsible for the retarded clearance of lipoproteins containing apoE-2 from plasma in normal and dysbetalipoproteinemic man (G30). Further studies on the binding of phospholipid vesicles or disks containing apoE by LDL receptors in four different assay systems have confirmed some expectations but raised new problems (S15). In these latter studies, apoE from six patients with Type III hyperlipoproteinemia did not bind to LDL receptors. However, apoE from three other patients bound normally to receptors *in vitro* and was taken up *in vivo* by the perfused liver of the 17α-ethinylestradiol-treated rat (with high LDL receptor activity). These findings suggest that the Type III phenotype is heterogeneous, involving more than one pathogenetic mechanism.

4.7.6. *Assay of Apolipoprotein E and Demonstration of Phenotype*

Radioimmunoassays of human apoE have been reported by Havel *et al.* (H19), Blum *et al.* (B39, B40), Falko *et al.* (F7) and Gibson *et al.* (G3), a

[3]We have used the nomenclature of Zannis *et al.* (Z2, Z4). An alternative nomenclature has been used (B52, U2); the E-2/E-2 homozygote has been said to have the apoE-D phenotype controlled by two alleles $apoE^d/E^d$ at the *apoE–N/D* locus; the E-2 heterozygote has the apoE-ND phenotype controlled by alleles $apoE^n/E^d$ or $apoE^d/E^4$, and others have the apoE-N phenotype, with alleles $apoE^n/E^n$, $apoE^n/E^4$, or $apoE^4/E^4$. Although this nomenclature has its attractions, it was based on a two-gene model for apoE inheritance; it is now accepted that apoE primary polymorphism results from the action of three alleles at a single locus, and we have used what is in our opinion a more appropriate nomenclature.

radial immunodiffusion assay by Kushwaha *et al.* (K33), and an electroimmunoassay by Curry *et al.* (C26). The difficulties in developing an immunoassay for apoE include a tendency for apoE to aggregate, and detergents are usually included in radioimmunoassays. The problems of apoE measurement have been discussed at an NIH Workshop on Apolipoprotein Quantification (P27), summarized by Lippel *et al.* (L18). For instance, apoE associated with VLDL may be immunochemically different from the apoE standard. ApoE is sensitive to cleavage by serine protease degradation.

Plasma apoE concentrations in normal subjects range from about 30–60 mg/liter. The apoE levels in men and women are reported by two groups to be similar (B39, C26), though another group reported slightly higher apoE levels in women (H19). There is a clear correlation between plasma apoE concentration and plasma triglyceride (B39, H19) and, in one study, plasma cholesterol concentration (B39). Plasma apoE concentrations in severe hypertriglyceridemia are elevated to about 200–300 mg/dl (B39, C26, H19).

The apoE phenotype may be assigned after isoelectric focusing of proteins obtained by delipidating VLDL, obtained either by ultracentrifugation (H19, P2, U5, W4, W6) or heparin–Mn^{2+} precipitation (U4) of serum. The assignment of a phenotype is usually straightforward (H13); if not, the elimination of sialylated components with neuraminidase may be helpful (H13).

4.8. Apolipoprotein SAA (ApoSAA, or Threonine-Poor Apolipoprotein)

Apolipoprotein SAA is one of several proteins which may be associated with plasma lipid, but which do not, as far as we know, have any clear functional role in lipid metabolism.

ApoSAA, normally a trace component of plasma, is an acute-phase plasma protein, that is, one that is elevated in a variety of disease states (R18). Its identification is interesting. A small protein of 76 residues, now called protein AA, was identified during the study of the proteins present in extracellular amyloid deposits in the type of amyloidosis particularly associated with inflammation (B24, H36, L11, S38). Antibodies to protein AA reacted with two AA-related proteins in plasma, one of approximate M_r 180,000 (SAA) and the other found in HDL of M_r 14,000–15,000 or 12,000 (apoSAA) (A19, B25, B26, L12, L15). The N-terminal 76-amino-acid portion of apoSAA is identical to that of amyloid protein AA (E8). Human apoSAA has now been sequenced and has been shown to consist of 104 amino acid residues (B27). Further studies in man have demonstrated microheterogeneity in apoSAA (B18, B19, M30) and Benditt *et al.* describe specific amino acid substitutions (B27, P6). Shore *et al.* have described a second similar "threonine-poor" apolipoprotein, apparently a dimer of M_r 40,000

reduced by treatment with mercaptoethanol to the monomer (S29). Malmendier *et al.* have described "threonine-poor" apolipoproteins of M_r 8,000 to 11,000 in the plasma of sick patients (M23, M25).

ApoSAA is secreted by mouse hepatocytes *in vitro* apparently independently of lipid and of apoA-I. However, when HDL is added the bulk of the apoSAA is recovered in the HDL fraction, with apoA-I. In mouse plasma, apoSAA and apoA-I appear to reside together on a population of HDL_3 particles (H26, H27).

ApoSAA, an acute-phase protein, is produced quickly in mice and men in response to a stress (e.g., endotoxin administration, etiocholanolone injection). The apoSAA concentrations rise from less than 1% to more than 25% of the total HDL protein content, depending on the degree of stress (B25, B26). In man, major changes in plasma concentration with disease have been reported, e.g., 100-fold or 1000-fold decreases in concentration with resolution of an acute illness (R18). There are reports that glucose infusion in a normal subject (M22) and in hospital patients (M23) may modify HDL composition and increase plasma apoSAA in HDL and, in vervet monkeys, chair restraint rapidly induces apoSAA production (P3, P5). In cynomolgus monkeys, apoSAA is cleared rapidly from the circulation, more rapidly than apoC-III_2 and much more rapidly than apoA-I (B19).

Another acute-phase protein, C-reactive protein, may interact with VLDL. Purified C-reactive protein is composed of five noncovalently-associated subunits (M_r each about 21,000). The complex with VLDL appears to be destroyed by ultracentrifugal isolation of lipoproteins, or chelation with EDTA (C1).

4.9. Apolipoprotein (a) [Apo(a)]

In 1963 Berg described the Lp antigenic system (i.e., the presence or absence of the Lp antigen) in human plasma. Lp(a+) and Lp(a−) states were characterized by precipitating antibodies from the rabbit (B29). Later studies showed that the Lp(a) antigen, apo(a), is demonstrable in plasma from practically all subjects [in one large study the only subject with no plasma apo(a) had abetalipoproteinemia (A9)]. Plasma apo(a) concentration ranges from about 2 to 76 mg/100 ml, with a highly skewed distribution (mean 14.0 mg/100 ml, median 8.0 mg/100 ml, no differences between sexes) (A9, A15).

Apo(a) is a carbohydrate-rich protein found in a lipoprotein with a slightly higher density than LDL (1.025–1.125 g/ml) (S19, S37). Lp(a) lipoprotein contains apo(a), together with apoB and small amounts of apoA-III (J10) [or perhaps small amounts of albumin and apoC (S21)]. Albers *et al.* found that apo(a) levels in plasma were not correlated with apoB, and changes in apoB concentration were not reflected in changes in apo(a) (A11). Lp(a) seems

likely to be the "sinking pre-β" fraction ($d > 1.006$, pre-β mobility on paper electrophoresis) previously described (A11). *In vivo* tracer studies suggest that Lp(a) is not a metabolic product of other lipoproteins containing apoB (K29), and it is not converted to other serum lipoproteins (K30).

A relationship between the serum concentration of Lp(a) and coronary heart disease has been postulated by several investigators (A9, B30). Kostner *et al.* (K20), in a case-control study of patients who had had myocardial infarction, suggested that plasma apo(a) concentration above 30 mg/100 ml represented a 1.75-fold relative risk and above 50 mg/100 ml represented a 2.3-fold relative risk for myocardial infarction. They suggested the risk threshold might be 30 mg/100 ml.

Krempler *et al.* measured the turnover of apo(a) in nine individuals with serum apo(a) levels ranging from 1 to 68 mg/100 ml. The fractional catabolic rate was similar in all, and elevated apo(a) levels seemed to be the consequence of increased apo(a) synthesis (K30). Lp(a), containing apo(a) and apoB, binds to the same receptor site on cultured fibroblasts as LDL, before being internalized and degraded (H12). It may be that Lp(a) is an atherogenic lipoprotein because it contains apoB, and is subject to similar degradative processes as LDL.

4.10. Other Apolipoproteins

Olofsson *et al.* (O5) have described apolipoprotein F from HDL. With a molecular weight of 26,000–32,000, and a p*I* of 3.7, amino acid analysis of apoF demonstrated all common amino acids except tryptophan. Olofsson *et al.* (O4) have also isolated a glycine- and serine-rich polypeptide from HDL, which appears to be a unique polypeptide of M_r 4900.

Another apolipoprotein, β_2-glycoprotein-1, or apoH, is a glycoprotein of M_r 54,000 found in all major lipoprotein density fractions, especially VLDL where it forms about 4% of the protein mass. Serum concentration is about 15–30 mg/100 ml. Two-thirds to three-quarters is found in the $d > 1.21$ g/ml fraction after ultracentrifugation (P19, P20). ApoH is taken up readily by a triglyceride–phospholipid emulsion (Intralipid) (P20) and it activates lipoprotein lipase (N1). Lipoprotein lipase activation by either apoH or apoC-II is inhibited by apoC-III (N1).

5. Plasma Lipid Transfer Proteins and Enzymes Concerned with Lipid Metabolism

5.1. Lipid Transfer Protein (LTP)

A protein, originally described as cholesteryl ester exchange or transfer protein, or triglyceride transfer protein, has been purified from human and

rabbit plasma. It is referred to as plasma lipid transfer protein I (LTP-I) to distinguish it from other less clearly defined lipid transfer proteins. Human LTP-I, which facilitates transfers of cholesteryl ester, triglyceride, and phosphatidylcholine between lipoproteins, is a hydrophobic glycoprotein with a p*I* of 5–5.2 (A17, A33, C3, I2, M43, P11). The purified protein has a molecular weight of 63,000–69,000 (A17, C3, I1, M43, T4). A similar protein has been purified from rabbit plasma (A1). Rabbit LTP has a molecular weight of 68,000 and, like human LTP-I, it facilitates transfers of cholesteryl ester, triglyceride, and phosphatidylcholine between lipoproteins. Several groups have reported that a protein of molecular weight 55,000–58,000 is often detected in highly purified preparations of LTP-I (A17, C3, M43), and it has been suggested that this protein is a molecular form of LTP-I. This smaller protein, designated LTP-II, has also been claimed to facilitate transfers of cholesteryl ester, triglyceride, and phosphatidylcholine between lipoproteins (C3). It is possible that LTP-II is an artefact, produced from LTP-I during the purification procedure, and may not be present in plasma.

LTP-I may be part of a 150,000-Da molecular complex that includes LCAT (I2). The presence of such a complex in plasma might account for the observation that in some cases lipid transfer activity on gel permeation chromatography elutes in a fraction characteristic of large-molecular-weight proteins ($M_r > 100{,}000$) (B47, R3, Z8).

Partially purified LTP-I is unstable when stored at pH 4.5 at 4°C, but more stable after adjustment to pH 7.4 (M42, M43, T2). If stored at 4°C in an ampholyte-containing solution (Polybuffer) with 4 *M* urea, LTP-I activity is stable for several months (A33). LTP-I is heat stable with >95% of its transfer activity retained after incubation at temperatures up to 62°C (A17, I1).

Another plasma LTP (LTP-2), which facilitates transfer of phosphatidylcholine but not cholesteryl ester or triglyceride (A17, T2), is heat labile, losing 90% of its activity after incubation at 58°C for 1 hour (A17). This latter transfer protein has not been characterized in detail.

5.1.1. Metabolism of Lipid Transfer Protein

Studies on the perfused rabbit liver suggest that rabbit LTP-I is produced by the liver (A2). Although models of esterified cholesterol transfers between lipoproteins (mediated by LTP) have been described (B7, I3) there is little information on LTP-I metabolism derived from direct studies.

LTP-I was originally referred to as cholesteryl ester exchange protein, as the transfer of cholesteryl ester (CE) tracer between plasma HDL and LDL by this protein did not result in mass changes to either lipoprotein (P11). However, it has since been shown not only to facilitate the transfer of triglyceride (TG) and phospholipid in addition to cholesteryl ester (A33), but also to facilitate net mass changes in lipoprotein fractions under appropriate condi-

tions (A1). The addition of rabbit LTP-I to plasma deficient in LTP-I activity had a marked effect on the composition of the plasma lipoprotein classes. VLDL was enriched with cholesteryl ester and depleted of triglyceride, and LDL and HDL were enriched with triglyceride. As LCAT activity was not inhibited, the toal mass of esterified cholesterol increased throughout the incubation. In addition to these changes there was some redistribution of apoE; an increased amount was found in the LDL fraction ($d = 1.019–1.063$ g/ml) (A1).

Studies on the physiological effect of LTP-I have been assisted by the recognition that there are marked species differences in activity. Rabbit plasma contains between two and three times the activity in human plasma, and plasma from rats, sheep, and pigs, for instance, contains less than 20% of the activity of human plasma (H1). Whereas rat plasma is deficient in cholesteryl ester and triglyceride transfer activity, facilitated phospholipid transfer activity is not impaired (I1, I2, T4). The reason is unknown; it is possible that rat plasma contains a different lipid transfer protein (T2), perhaps homologous to the LTP-2 reported in human plasma (A17).

The species differences observed in lipid transfer protein activity may, in part, be due to the presence of an inhibitor which markedly reduces CE and TG transfer and can be separated from LTP-I in human plasma (M42). Inhibitory activity has also been demonstrated in lipoprotein-free plasma from rat, pig, goat, chicken, and cow, but not in rabbit lipoprotein-free plasma. The levels of inhibitor in the species studied were not quantitated, but it seems possible that the level of inhibitor in the plasma of different species may be an important factor in determining LTP-I activity (M42).

The rate of bidirectional transfers of esterified cholesterol between human plasma LDL and HDL *in vitro* ranges from 100 to 300 nmol/hour per milliliter of plasma, representing an hourly exchange of 8–12% of LDL and 7–31% of HDL esterified cholesterol (B12). As the rates of catabolism *in vivo* of human LDL and HDL apoprotein are 1–2% and less than 1% per hour, respectively (B41, L4), it is apparent that the residence time in plasma of human LDL and HDL is such that these lipoproteins *in vivo* may achieve a virtually complete equilibration of esterified cholesterol. However, as pointed out in a review by Barter *et al.* (B7), the residence time in plasma of human VLDL is such that equilibration of esterified cholesterol with VLDL [which *in vitro* requires 5–8 hours of incubation at 37°C (H31)] is incomplete *in vivo*.

Determination of the rates of esterified cholesterol transfer between plasma lipoproteins has allowed the construction of a model of cholesteryl ester transfer (B7). According to this model, the probability of LTP-I picking up or depositing a molecule of esterified cholesterol in HDL or VLDL or LDL is in the proportion of approximately 30:7:1. Although the probability of in-

teraction with HDL may reflect no more than the large number of HDL particles present in plasma, the difference between VLDL and LDL cannot be so explained, and suggests preferential interaction of LTP-I with VLDL relative to LDL.

The major function of LTP-I in human plasma may be to distribute esterified cholesterol from the HDL fraction, where cholesterol is esterified, to other lipoprotein fractions. LCAT activity is responsible for the production of some 50–100 nmol esterified cholesterol per milliliter of plasma per hour (G11). The concentration of esterified cholesterol in human HDL is about 1000 nmol/ml of plasma. Only 0.5–1.0% of HDL apoprotein is removed from plasma per hour (B41), probably mainly in intact HDL particles. If so, then the uptake of HDL particles can account for the removal from plasma of only about 10–20% of the esterified cholesterol formed in HDL in the LCAT reaction.

The model for LTP-I activity in human plasma (B7) is such that it is possible that the other 80–90% of esterified cholesterol formed in HDL by the LCAT reaction is transferred to VLDL and thus almost quantitatively to LDL. In normal human plasma containing LDL esterified cholesterol at a concentration of 2000–3000 nmol/ml and an *in vivo* removal rate of LDL apoB of about 2% per hour (L4), the uptake of esterified cholesterol from plasma in whole LDL particles can account for 40–60 nmol per hour, or a large part of the esterified cholesterol formed by LCAT.

In this way, it is possible that in man LTP-I distributes a large proportion of the esterified cholesterol formed to VLDL and thus to LDL. If so, LTP-I might be considered atherogenic, in that its activity is responsible for much of the esterified cholesterol in the major atherogenic lipoprotein, LDL. By contrast, those species with low levels of plasma LTP-I activity, such as the dog, rat, pig, cow, and sheep (H1) have low plasma LDL esterified cholesterol concentrations (C2) and tend to be resistant to the development of atherosclerosis.

It is, of course, also possible that the esterified cholesterol formed in HDL may be removed from plasma by some process other than uptake of the whole HDL particle or LTP-I-mediated transfer to other lipoprotein particles, but this possibility has not been fully investigated. Some evidence that there may be other pathways than these for the removal from plasma of HDL esterified cholesterol comes from the studies of Glass *et al.* (G5, G6), who showed that cholesteryl ether incorporated in rat HDL as a tracer for cholesteryl ester was taken up *in vivo* by the rat liver (and by other organs) faster than apoA-I tracer (see Section 4.1.2). These studies are complicated by the relatively high concentration of apoE in rat HDL (compared, for instance, to man) and the unknown effect of apoE on HDL cholesteryl ester metabolism in the rat. Further studies on the removal of esterified cholester-

ol from the plasma compartment are required before these questions are answered.

Another area of plasma lipoprotein metabolism in which lipid transfer protein may have a part to play is the modeling process whereby VLDL remnants, depleted of much of their triglyceride by the action of lipoprotein lipase, are converted almost quantitatively into LDL (R5). It has often been assumed that some intrahepatic process, perhaps involving hepatic triglyceride lipase, is responsible for the final removal of excess triglyceride from the VLDL remnant (D5), but no convincing explanation for the removal of excess esterified cholesterol from VLDL remnants has been given (E3). It is possible that excess esterified cholesterol is transferred to VLDL by LTP-I [as has been demonstrated *in vitro* (B5)], thus allowing the formation of LDL. Again, further studies are required to clarify the role of LTP-I in this process.

5.2. Lecithin:Cholesterol Acyltransferase (LCAT; EC 2.3.1.43)

Human LCAT was first purified to homogeneity by Albers *et al.* (A10). Since then the original method, which included removal of apoD by immunoadsorption chromatography, has been modified by Albers *et al.* and by others (A8, A16, C11, C15, K15, M6).

The apparent M_r of LCAT is approximately 65,000 by SDS–polyacrylamide gel electrophoresis (A10, A16, C14, K15, M6, U7) or 59,000 by sedimentation equilibrium ultracentrifugation (C15). The difference in M_r obtained by these two methods may reflect the high carbohydrate content of LCAT (24% by weight), which would give an inaccurate estimate of M_r by SDS–polyacrylamide gel electrophoresis (C15).

LCAT has been reported to have five isoforms with p*I* values ranging from 5.1 to 5.5 (A16) and, by another group, three isoforms focusing between p*I* 4.1 and 4.4 (M6). In the absence of urea, Utermann *et al.* observed three isoforms with p*I* values 4.28, 4.33, and 4.37 (U7). The latter group considered that details of the method of isoelectric focusing influenced the p*I* value obtained.

Purified LCAT is unstable in the usual aqueous buffers. The stability has been increased, so that activity is retained for at least 4 weeks, by storing purified LCAT in a low-ionic-strength buffer at 4°C under nitrogen (F23, K15). A similar increase in stability by freezing the enzyme in liquid nitrogen has been reported (U7). A report by Jahani and Lacko (J1) suggests that the purified enzyme can be stored indefinitely as a precipitate in saturated ammonium sulfate solution.

5.2.1. *Lecithin:Cholesterol Acyltransferase Activity*

LCAT has three catalytic functions; it acts as an acyltransferase (G9), a phospholipase (A25, P14), and a lysolecithin acyltransferase (S60). In this review only the acyltransferase action of LCAT will be discussed in any detail. The enzyme transfers the acyl group from the number two position of lecithin to the 3-hydroxyl group of cholesterol, resulting in the formation of cholesteryl ester and lysolecithin.

The acyltransferase activity of LCAT is dependent on the presence of apoA-I (A10, A16, A25, C15, F14, K15, Y1) or, probably to a lesser extent, on the presence of apoC-I (A16, S46). Kostner (K17) reported that apoA-III (thought by some to be apoD) is a cofactor for the LCAT reaction, whereas Albers *et al.* were unable to demonstrate any effect of apoD (or apoC-II, apoC-III, or apoA-II) on the reaction (A16).

There are suggestions in the literature that plasma LCAT forms a complex with other proteins, though there is little agreement on the nature of these proteins. Fielding and Fielding (F10) bound antibodies specific to apoD to a gel support in an immunoadsorption chromatography column. They were able to remove all LCAT activity from plasma passed through the column by removing a complex containing LCAT, apoA-I, and apoD in a molar ratio of 1.0:0.9:1.8 (LCAT:apoA-I:apoD). Further work by Fielding *et al.* (F12) suggested that over 90% of plasma LCAT is complexed with apoD in plasma from normal and hyperlipidemic subjects. Albers *et al.* (A14) and Utermann *et al.* (U7) also removed LCAT activity from plasma with an antibody to apoD (64% of activity and 50% of activity, respectively). Ihm *et al.* (I1) suggest that LCAT exists in a complex with other proteins including lipid transfer protein I.

5.2.2. *Origin and Plasma Concentration of Lecithin:Cholesterol Acyltransferase*

LCAT in the rat appears to be produced by the liver (B48, N11, O11). Although there seems to be no direct evidence of production by the liver in man, the hepatic origin of LCAT is suggested by the marked reduction in LCAT activity associated with liver diseases (C23, S51) including viral hepatitis (B37, T11) and cirrhosis (B37, S2, S36). LCAT activity is also said to be reduced in uremia (S11) and pancreatic carcinoma (S36). The concentration of LCAT in normal human plasma (measured by radioimmunoassay) is about 6 mg/liter (A8).

5.2.3. *Substrates for Lecithin:Cholesterol Acyltransferase*

HDL is the preferred lipoprotein substrate for LCAT (G11). Fielding and Fielding (F9) showed that VHDL and HDL_3 are substrates for purified

LCAT, whereas LCAT activity with HDL_2 as a substrate is minimal. Others have confirmed that HDL_3 is a better substrate for LCAT than HDL_2 (B11, M28). Hamilton *et al.* (H5) noted that nascent disk-shaped HDL secreted by rat liver were better substrates for LCAT than mature spherical HDL isolated from plasma. Synthetic discoidal complexes of apoA-I, phosphatidylcholine, and cholesterol were better substrates for LCAT than unilamellar vesicles of phosphatidylcholine and cholesterol, incubated in the presence of apoA-I (M32).

It has been suggested that HDL_2 acts as an inhibitor of the LCAT reaction (K19, M27, P13), but under certain circumstances it may enhance LCAT esterification (E5). Barter *et al.* (B11) have shown that HDL_2 is a competitive but less efficient substrate than HDL_3 for LCAT. The addition of HDL_2 to an incubation mixture containing LCAT and HDL_3 will therefore enhance esterification if there is a substrate deficiency, and depress esterification if there is substrate excess. The same group has also shown that LDL and VLDL may act as substrates for LCAT (B2, B6, B8, B9). Earlier findings that VLDL and LDL were not effective substrates for LCAT (A3, F9) may have arisen because purified LCAT, not part of a complex, was used.

Barter *et al.* (B9) have calculated the apparent K_m for the esterification of HDL_3 and LDL cholesterol. The K_m for free cholesterol associated with HDL_3 is seven times less than for free cholesterol associated with LDL. However, if these calculations are expressed in terms of lipoprotein surface area, instead of free cholesterol concentration, the K_m for LDL and HDL as LCAT substrates may well be comparable.

5.2.4. *Regulation of Lecithin:Cholesterol Acyltransferase Activity*

The rate of cholesterol esterification in plasma is not correlated with HDL concentration (A12, R17, S45, S58, W1, W2) but is correlated with the concentration of VLDL or triglyceride (A12, P8, R17, S58, T7, W1, W2). Although HDL is the major substrate for LCAT, VLDL and indirectly LDL are the major recipients of the esterfied cholesterol, transferred (it is thought) by lipid transfer protein. Accumulation of esterified cholesterol in the recipient lipoproteins is associated with a decrease in LCAT activity (C7, F11, F13) that can be relieved by the addition of recipient lipoproteins but not by addition of LCAT substrate (F11). Hopkins and Barter (H32, H33) have explained these observations by showing that the depletion of HDL esterified cholesterol by transfer to VLDL enhances the capacity of HDL to act as a substrate for LCAT.

5.3. Lipoprotein Lipase (EC 3.1.1.34)

Lipoprotein lipase (EC 3.1.1.34) is an enzyme or group of enzymes which catalyze the hydrolysis of the 1(3) ester bond(s) of triacylglycerols and the 1 ester bond of phospholipids. The enzyme plays a central role in lipoprotein metabolism, being responsible in particular for the hydrolysis of chylomicron and VLDL triglycerides and the formation of remnant particles from these lipoproteins. There have been reviews of this enzyme [e.g., (N9, Q1)] and lipoprotein lipase will not be discussed in detail in this review. Familial lipoprotein lipase deficiency and related disorders of chylomicron metabolism have also been reviewed (B58, N8) and will not be discussed in detail.

5.4. Hepatic Triglyceride Lipase

Hepatic triglyceride lipase is synthesized and secreted primarily by hepatocytes (J9), and is released into the plasma, along with lipoprotein lipase, after an intravenous injection of heparin. While *in vitro* hepatic triglyceride lipase may hydrolyze a variety of substrates (e.g., monoglyceride, diglyceride, triglyceride, and phospholipid) (E2, G31, K34), it was described in 1980 as "an enzyme in search of a function with regard to lipoprotein metabolism *in vivo*" (N9). In an early study in rats, hepatic lipase activity did not seem to be a rate-limiting step in the removal of plasma triglyceride. Injection of an antibody against hepatic lipase resulted in an accumulation of cholesterol and phospholipid in plasma LDL and HDL, but no change in lipoprotein triglyceride concentrations (K35). However, further studies in rats (which normally have very little plasma IDL and LDL) have shown that the intravenous administration of antibody to hepatic triglyceride lipase results in the accumulation of plasma IDL (M46).

When antisera against human hepatic triglyceride lipase (which fully inhibited cynomolgus monkey hepatic triglyceride lipase activity *in vitro*) was infused over 3 hours into cynomolgus monkeys, quite marked changes were seen (G14). Lipoprotein lipase activity was unchanged. VLDL triglyceride increased 60–300%. The total mass of IDL doubled over the 3-hour infusion, while the mass of LDL decreased. Tracer studies supported the conclusion that hepatic triglyceride lipase in this primate species participates in the hydrolysis of the triglyceride in VLDL, IDL, and the larger LDL particles. It appears to function together with lipoprotein lipase in the conversion of VLDL and IDL to LDL.

As hepatic triglyceride lipase is released into the circulation by heparin, it might be thought that postheparin plasma hepatic triglyceride lipase activity

would be an index of the *in vivo* fractional catabolic rate of either VLDL or VLDL-remnant particles, as they are converted to LDL. Reardon *et al.* found no such correlation in man, suggesting that hepatic triglyceride lipase (as measured in postheparin plasma) does not play a rate-limiting role in the catabolism of these particles (R6).

6. High-Density Lipoproteins (HDL)

Plasma HDL constitute a heterogeneous group of lipoproteins; their common property is that they have a hydrated density in the 1.063–1.21 g/ml interval. HDL subfractions are usually described in terms of their hydrated density, but it should not be forgotten that a number of functions may be the property of particular subsets of HDL which can be defined better in terms of their apolipoprotein composition than their hydrated density. A preparation of HDL isolated by flotation in the ultracentrifuge, *d* between 1.063 and 1.21 g/ml, may contain almost all the major apolipoproteins (except perhaps apoA-IV and apoB-48), together with LCAT and lipid transfer protein.

Perhaps the clearest demonstration of HDL heterogeneity was that by Suenram *et al.* (S61). When specific antisera to apolipoproteins A-I, A-II, B, C-III, D, E, and F were set up in double diffusion analyses against HDL, reactions of nonidentity were observed between each possible combination of these antisera. The only exception was a reaction of partial identity between antisera to apoA-I and apoA-II, indicating two types of apoA-containing lipoproteins, a major class containing both proteins and a minor one containing only apoA-I. The other apolipoproteins in HDL therefore appear each to be largely peculiar to their own lipoprotein particles, and the nomenclature LpC, LpD, etc., has been used by Alaupovic and co-workers to describe lipoproteins characterized by a single apolipoprotein class (A5–A7).

Clearly, not all particles in the HDL density class contain apoA-I or apoA-II (often taken to be a hallmark of HDL). Conversely, not all particles containing apoA-I, for instance, are characteristic of HDL as obtained by ultracentrifugation. This has been well shown by McVicar *et al.* (M5). These workers selected a subpopulation of monospecific antibodies directed against apoA-I, which dissociated from apoA-I under mild elution conditions. The apoA-I-containing particles sequestered from serum by immunoadsorption were polydisperse in diameter, included a proportion of large particles 15 nm or more in diameter, and contained more triglyceride and more protein than HDL isolated ultracentrifugally. The apoA-I-containing particles contained a number of minor apolipoproteins that were not observed in ultracentrifugally prepared HDL. On electrophoresis under nondenaturing con-

ditions, the immunosorbed apoA-I containing particles appeared as a number of bands which were not seen in a centrifugally isolated sample of HDL similarly treated. However, electrophoresis of whole serum under similar conditions (and stained with a lipid stain) displayed rather similar bands, suggesting that HDL obtained by immunosorption chromatography contains subspecies of particles with relatively defined lipid and apoprotein compositions, and that ultracentrifugation disrupts these species.

6.1. Characterization of High-Density Lipoproteins

It should be remembered that sequential ultracentrifugation causes significant loss of apolipoproteins from HDL (C24, F3, F4, K32, M10), and a preliminary ultracentrifugation is necessary for the isolation of HDL by gel permeation chromatography (R21) or high-pressure liquid chromatography (O2, O3). HDL precipitation also causes a loss of apolipoproteins (R15). Although advances in studying the metabolism of HDL subfractions of HDL are being made, we should not necessarily assume that observations on purified HDL samples reflect changes in plasma.

On analytical ultracentrifugation at density 1.21 g/ml a spectrum of lipoprotein particles with *F* rates (Svedberg flotation rates) ranging from 0 to 9 is seen. Those in the range *F* 0–3.5 are called HDL_3, and the less dense subclass of *F* 3.5–9 are HDL_2 (L14). Preparative ultracentrifugation at density 1.125 g/ml has often been used to separate these subclasses. A minor subclass of HDL, HDL_1, may sometimes be identified as a shoulder on the LDL distribution curve on analytical ultracentrifugation, and may be isolated from some serum specimens at $d < 1.063$ g/ml (A13).

For studies on HDL metabolism, a variety of separation techniques have been used. Preparative ultracentrifugation, either with a regular ultracentrifuge (H15) or with a bench-top air-driven ultracentrifuge (Beckman Airfuge) (E9), can separate HDL_2 and HDL_3. HDL_2 is normally separated by isopycnic centrifugation in the density interval 1.063–1.125 g/ml. Anderson *et al.* (A21) found HDL_{2b} (defined by the analytical ultracentrifuge) in the 1.063–1.100 g/ml fraction and HDL_{2a} in the 1.100–1.125 g/ml fraction, as defined by isopycnic preparative ultracentrifugation. Whether we would benefit by changing our preparative ultracentrifuge habits to include these refinements is unclear.

Polyanion precipitation has been used for separating apo-B-containing lipoproteins from other lipoproteins. Gidez *et al.* (G4) have used a fractional precipitation procedure with dextran sulfate and Mn^{2+} to precipitate apoB-containing lipoproteins first, then HDL_2.

Analytical ultracentrifugation with computer analysis, in the Donner Lab-

oratory, University of California, Berkeley, has been used to identify and measure two subspecies within HDL_2 (HDL_{2a} and HDL_{2b}) and total HDL_3 (A21, A22), but this technique is costly and limited to one laboratory. Rate-zonal density-gradient ultracentrifugation, either in a zonal rotor (P7, P9) or a swinging bucket rotor (G32), may be used to separate HDL_2 and HDL_3.

Gradient polyacrylamide gel electrophoresis followed by densitometry has proved to be an extremely powerful and simple technique for characterizing HDL subfractions (B35). HDL patterns showed five maxima: two in the relative migration (R_f) range associated with the HDL_{2a} and HDL_{2b} subclasses (as determined by analytical ultracentrifugation) and three in the R_f range associated with HDL_3 (HDL_{3a}, HDL_{3b}, and HDL_{3c}). The mean hydrated densities of the HDL_{3a} and HDL_{3b} subpopulations were comparable to those of two HDL_3 subfractions isolated by zonal ultracentrifugation (P10).

A number of other techniques are available for separating subclasses of human HDL. Gel permeation chromatography yields a single very slightly skewed peak if freshly prepared serum (male and female) is used, but yields a peak containing a shoulder on the ascending side of the peak after sample storage (K22). Hydroxyapatite chromatography yields some eight peaks (K21, K22), but the physiological significance of this separation is unknown. Affinity chromatography of HDL on heparin–Sepharose yields two classes (M28). The unretained fraction has apoA-I as a major apoprotein, and also apoA-II, apoC-II, and apoC-III, but only traces of apoE and no apoB. The retained fraction has apoE as the major protein, but most of the retained fraction is in the HDL_2 fraction (on ultracentrifugation).

7. Concluding Remarks

The scale and pace of research on plasma lipoprotein metabolism is such that any review must perforce be incomplete and out of date at the time of publication. In this review we have focused on recent advances, and in particular on those aspects of recent advances which are likely to impinge on clinical chemistry.

The most dramatic advances, without doubt, have been in protein biochemistry. The genetic basis of lipoprotein disorders is being probed with the tools of molecular biology, proteins concerned with lipoprotein metabolism are being characterized with structural and physiological studies, lipoprotein receptors are being fully characterized, and competitive inhibitors of the rate-limiting enzyme for cholesterol synthesis, HMG-CoA reductase, have been produced.

We have also discussed the equally important advances that have been

made in clinical and epidemiological studies. The role of hypercholesterolemia in promoting atherosclerosis and coronary heart disease is now firmly established. The feasibility of halting or reversing the extent of atherosclerosis and lowering the risk of coronary heart disease by lowering plasma cholesterol is also established, and exciting studies on the ways in which these changes are mediated will surely follow.

Finally, perhaps, the most significant long-term trend in research on lipid metabolism, atherosclerosis, and coronary heart disease is for the structure and limitations of the old research disciplines to be broken down. Cell biologists, molecular biologists, basic and clincial biochemists, pathologists, pharmacologists, and clinical researchers are each using a range of techniques formerly considered esoteric or the preserve of those from another discipline. Collaboration between disciplines is not only fruitful but necessary, and this must be the way to progress in future.

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