Principles of IMMUNODIAGNOSTICS

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Preface

For decades, the specific and sensitive reaction of antigen with antibody has been utilized clinically as a diagnostic aid for the patient with infectious diseases. As researchers explored this miraculous reaction, it became evident that a powerful analytical tool was at hand. During the last decade, clinical tests based on the immune reaction have grown at a phenomenal rate. No longer is serology hidden in dark, crowded corners of the clinical microbiology laboratory. A new discipline of laboratory medicine is rapidly passing from infancy to adolescence. In most major laboratories this departmental section is designated "Immunodiagnostics" or "Clinical Immunology." This analytical laboratory traverses the classical boundaries within the department of pathology and is currently utilized in research, diagnostics, and for the management of patients with a wide variety of disorders.

Currently available immunology books fall into several categories: the research-oriented techniques books, the encyclopedia volumes of procedures books, the comprehensive immunology texts, and references on immunopathology. The time has come for a book to deal with the basic principles of immunochemistry applied to the diagnostic field. The purpose is not to provide step-by-step prpcedures but rather to isolate and lay down fundamental concepts. This book attempts to provide a logical and relevant approach to this most fascinating field. The major objective is to elucidate and apply basic concepts so that the student of laboratory medicine can better deal with the magic of the immunoassay kits that now flood the marketplace. Clearly, a thorough understanding of fundamental principles provides the laboratorian with a means for keeping abreast of new assays and better controlling and understanding of the classical immunologic tests.

The text is divided into two major sections. The first section isolates fundamental concepts of protein chemistry and immunology. Those characteristics that govern protein behavior are discussed to provide better understanding of

antibody activity. The immunology reviewed in this section is that portion of this immense field that is necessary to understand clinical applications. Section two of the text classifies and categorizes a selection of currently used immunodiagnostic techniques. The generalizations developed are applicable to both present and future immunoassays. The clinical procedures reviewed not only provide interest and relevancy but are critical examples of the category of tests. Clinical examples briefly describe the principle, methodology, interferences, clinical correlations, and interpretations. At the end of each chapter "Readings and Resources" direct the student, instructor, and laboratorian to current and classical procedure references.

While not primarily designed to be a reference book, this text aids in the interpretation of tests, in the choice of methodologies, and in developing quality control techniques as well as identifying common pitfalls or sources of error. The primary audience for this book is the student of laboratory medicine who wishes to better utilize the powerful analytical capabilities of immunologic tests. No attempt is made to review the rapidly expanding area of cellular immunology and its clinical applications, for this area is beyond the scope and "basic" premise of this text.

The diligent and systematic research of scientists, such as Berson and Yallow, has provided modern medicine with techniques of immense value. It is the clinical laboratorian who must make valid use of these analytical techniques.

I wish to thank Drs. M. Sochard, G. Roberts, and E. Kennedy for their guidance. Dr. Eleanor Griffith offered helpful suggestions in her review of portions of the manuscript. The contributions by Millipore Biomedica, Hyland Laboratories, Kallestad Laboratories, Ames Company, and Helena Laboratories were extremely helpful. The credit for converting the handwritten manuscript to legible text goes to the fine secretarial assistance of Debra Becker. Finally, I wish to thank my family for their patience and encouragement during this endeavor.

Ralph Michael Aloisi

Contents

SECTION ONE

FUNDAMENTAL CONCEPTS

The clinical laboratory practitioner must be aware of the factors that may influence an assay. A review of the basic concepts of protein chemistry as well as the modern tenets of immunology is a necessary requisite to a complete understanding of the fundamental principles of immunodiagnostics. The clinical laboratorian must be prepared to maximally utilize the powerful tools of modern clinical immunochemistry. This section includes protein structure and function, immunoglobulins, antigens and antigenicity, and the interaction of antigen-antibody.

CHAPTER 1

Proteins

Proteins are the expression of DNA and one of the products of evolution. These molecules are physiologically active and perform a variety of functions. The term "operational molecule" has been used to describe the biologically specific function of proteins. Proteins are most frequently classified structurally. A classification based on the common functional characteristic, specific binding of molecules or ligands, helps emphasize the operational nature of proteins. The common functional characteristic of the proteins in Table 1 is recognition and specific binding of other molecules. What is it that enables proteins such as enzymes and antibodies to specifically bind their appropriate ligands, substrates, and antigens? Clearly, ligand binding is the common denominator. A review of those factors involved in binding in reality is a review of protein interaction. An understanding of protein interactions provides insight into the binding of all ligands by these operational molecules.

What is it that determines binding activity of proteins? Enzymes are catalytic proteins with readily measurable activity. The reaction of enzyme with substrate produces a decrease in substrate and an increase in product. Simultaneous advances in instrumentation and in enzymology have made enzymes excellent models for the study of protein structure and function. Christian Anfinsen carefully studied the activity of the enzyme ribonuclease in an altered and native

Table 1. Functional classification of proteins

Type	Function	Type example
I	Catalytic	Enzymes
II	Immune	Antibodies
III	Transport	Ceruloplasmin
IV	Regulatory	Insulin
V	Contractive	Myosin

state. The term "native state" refers to the three-dimensional shape the enzyme would have in its natural physiologic environment. In the altered state ribonuclease lost activity (denaturation). If the altered molecule was exposed to certain conditions, it would twist, bend, and fold until it regained its natural shape or conformation. As the enzyme regained its native state, it regained its activity (renaturation). One hypothesis to account for this phenomenon is that the primary structure (the sequence of amino acids) dictates the final conformation of the protein molecule. Anfinsen's study is similar to the following somewhat bizarre hypothetical experiment. A finely tooled Swiss watch is taken apart and the pieces placed in a bag. The bag is shaken. Given enough time, the watch parts would find their proper positions and the watch would resume its shape and finally its activity. There is something more than reality in this animated hypothetical study, but it does serve to clarify Anfinson's problem. It is now known that structure dictates function! Is it the fine chemical detail of molecules or the gross shape that determines specificity and activity? What energy is used to drive his altered enzyme to its native state? The concepts involved are fundamental to molecular biology and indeed to any form of protein chemistry such as enzymology or immunology.

PROTEIN CHEMISTRY

Amino acid bondings and interactions

Most proteins when treated with acids, bases, or proteolytic enzymes are degraded to alpha amino acids. The approximately 20 amino acids usually derived from this hydrolytic process all have a similar core structure and differ primarily by their side groups or R-groups (Fig. 1-1). The varied structure of R-groups provides each amino acid core with a differing chemical behavior. The in vitro combination of amino acids to form chains occurs via a condensation process with the exclusion of water (Fig. 1-2).

The in vivo process is by far more complex but the end product is the same, a linear molecule of polyamino acid. When this polyamino acid reaches a length of 40 amino acids, it is considered, by some, to be a protein. The line between polyamino acid and protein is arbitrary and frequently disputed. The peptide bonds formed between the amino acids account for the use of the term "polypeptide" as a synonym for polyamino acid.

Peptide bond. The peptide bond, seen in Fig. 1-2, is formed between the amino group of one amino acid and the carboxyl group of another amino acid. The peptide bond, referred to by some scientists as an amide bond, has a resonating character (Fig. 1-3). The bond between the carbon and nitrogen would be expected to have a bond length of 1.49 Å, but due to the partial double-bonded nature of this bond, the actual length is 1.32 Å. The resonance on the peptide bond gives it some rigid features and partially limits its free rotation. The rigid or planar features of the peptide bond alternating with bonds that contain free rotation would tend to allow the molecule to fall into a randomly coiling conformation. As

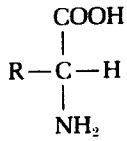


Fig. 1-1. Typical alpha-amino acid demonstrating common core and site for variations in side group or R-group attachment.

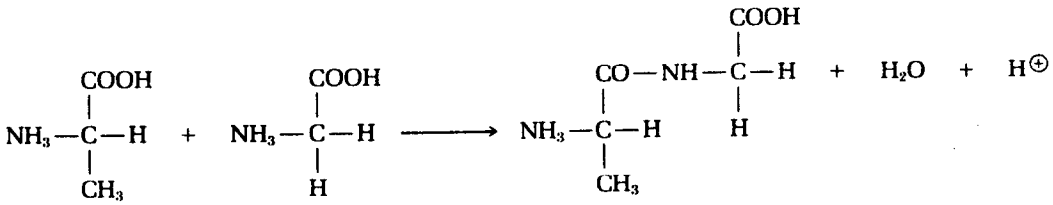


Fig. 1-2. Condensation reaction of first amino acid, alanine, with second amino acid, glycine, results in formation of alanylglycine. Formation of a two-amino acid chain is the result of peptide-bond formation and water exclusion.

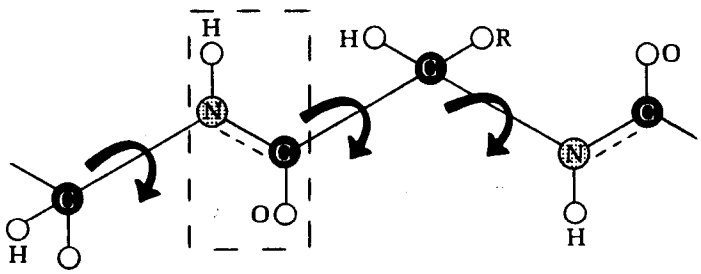


Fig. 1-3. Segment of protein molecule. Arrows indicate bonds with free rotation and dotted enclosures indicate peptide bonds. Note free rotation does not exist at the peptide bond.

the molecule begins to take on this randomly coiling shape, the amino acids within the structure apply restrictions on the developing structure. For example, an amino acid with an enormous bulky side group could inhibit this coiling by competing for available space. This type of interference is referred to as steric hindrance. A variety of forces such as intrachain bonding can thrust the molecule into its final shape. Therefore peptide bonds and steric hindrance can effect the interactions of amino acids and the conformation of proteins. However, there are other significant interactions that affect amino acid bonding and protein conformation.

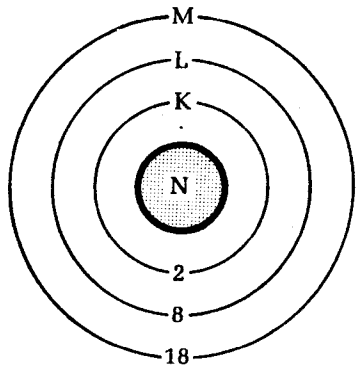


Fig. 1-4. Diagrammatic representation of an atom showing two electrons are required to fill K orbit, eight are required to fill L orbit, and eighteen are needed to fill M orbit.

Ionic bonding. Stability is the final goal of every atom and molecule. We are well aware of the decay of radioactive isotopes. Decay is a means for the isotope to become a stable molecule. A fundamental concept in chemistry is that the filling of electron orbits tends to yield inertness or stability. Fig. 1-4 shows the number of electrons required to fill the K, L, and M orbits of any atom.

The greater the distance from the outermost orbiting, negatively charged electrons to the positively charged nucleus, the more readily that electron can be removed. An atom with one electron in its M orbit can be considered to have a low ionization potential, and therefore little energy is required to remove that electron. An atom such as chloride has seven electrons in its L orbit; this atom would be more apt to gain one electron in order to fill that orbit than to loose its seven outer electrons. Chloride has a high ionization potential and a high affinity for electrons. Electron transfer will occur when an atom with a low ionization potential comes into proximity of an atom with a high electron affinity. The transfer of the outermost electron of sodium to chloride leaves both atoms oppositely charged. The positively charged sodium ion will now be attracted to the negatively charged chloride ion. The formation of an ionic bond, actually an interaction or an electrostatic force, leaves a net charge of zero. Any marked increase in the free ion concentration in the solution that contains the sodium and chloride ions would reduce the probability of bonding between the sodium ion and the chloride ion.

Covalent bond. In ionic bonding the key concept was the complete transfer of an electron. In covalent bonding the crucial point is an incomplete electron transfer. Covalent bonds are incomplete transfers of electrons between two or more atoms. Hydrogen has a high ionization potential and a high electron affinity. The reaction of one hydrogen atom with another hydrogen atom can occur by sharing each others' one electron. This sharing process acts to fill the K orbit of both atoms (Fig. 1-5).

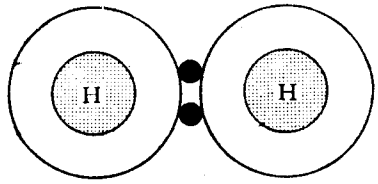


Fig. 1-5. Hydrogen gas; hydrogen atoms are covalently linked. Each atom shares the other one's electron.

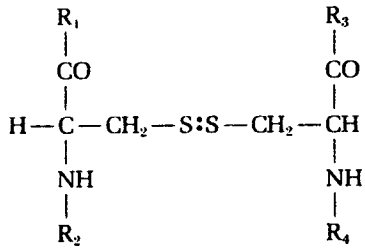


Fig. 1-6. Cysteine residues on two separate polypeptide chains covalently bonded to form cystine.

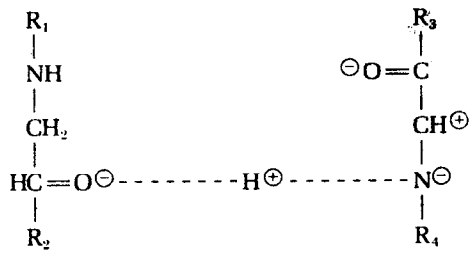


Fig. 1-7. Interchain hydrogen bonding between polypeptides R_1 to R_2 and R_3 to R_4.

Covalent bonds are relatively stable, strong bonds. It requires extremely harsh treatment to disrupt this type of bond. Covalent bonding frequently occurs between particular amino acids in proteins. As shown in Fig. 1-6, this type of bonding can be seen in the bridging of two cysteine residues on two separate chains to form cystine.

Hydrogen bond. The hydrogen atom tends to share its electron with other atoms, hence it is predominantly a positively charged proton. As such, the hydrogen may be mutually attracted by negatively charged atoms or ions. Under certain circumstances, hydrogen can find itself suspended between two negatively charged ions, forming the hydrogen bond. As shown in Fig. 1-7, hydrogen bonding frequently occurs between polypeptide chains but can also occur within a single chain (intrachain). Although each hydrogen bond is relatively weak,

the great number of hydrogen bonds in any one molecule can add up to a substantial effect. This type of bonding can be readily altered by pH changes and/or changes in ionic strength.

Hydrophobic interactions. The R-groups of certain amino acids are hydrophobic or "water-hating." Amino acids such as valine, leucine, and phenylalanine are hydrophobic, and these amino acids are unable to form hydrogen bonds with water. Hydrophobic amino acids are electrically neutral; their positive and negative centers coincide, hence they are referred to as nonpolar. The lack of polarity on these amino acids is responsible for their inability to hydrogen bond with water. Hydrophobic amino acids in an aqueous solution are driven together as are oil droplets on water. Hydrophobic interaction often is the driving force that folds and bends polypeptides, forming globular proteins excluding water. As shown in Fig. 1-8, hydrophobic amino acids form nucleation sites or points of folding, and hydrophilic amino acids are driven to the surface of the molecule to interact with water.

Van der Waals forces. Atoms and their electron clouds interact with other atoms and their electron clouds. As the two atoms approach each other, the electron cloud of one is attracted to the nucleus of the other. At a certain critical dis-

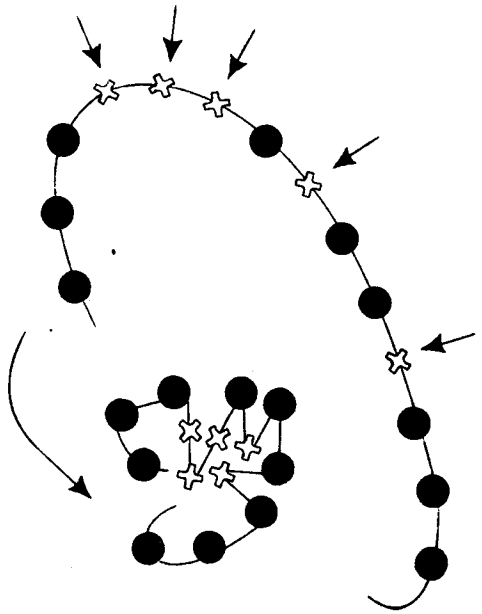


Fig. 1-8. Polar amino acids that readily interact with water (hydrophilic) are indicated by —●—. Nonpolar (hydrophobic) amino acids are indicated by —⊠—. Note that polypeptide tends to form nucleation sites at nonpolar amino acid, resulting in conformation that allows hydrophilic amino acids to remain at surface of molecule.

tance, as the two atoms approach each other, the clouds repel each other. The actual force can be mathematically derived. Van der Waals interactions are nonspecific and are a measure of attraction and repulsion of any two atoms. The strength and direction of this force are critically dependent on distance.

Amino acid chemistry

Classification of amino acids. Each amino acid has unique physical and chemical characteristics. Classifying amino acids by their polarity provides a functional approach to amino acids and a more useful approach to protein structure and function (note Tables 2 to 4).

Acid-base properties of amino acids. All amino acids have at least two ionizable groups ($R-NH_3^+$ and $R-COOH$). Like other weak acids, amino acids do not completely dissociate. The term "protonic equilibria" refers to the association and dissociation of proton donors and proton acceptors. In solution this equilibria can be expressed as seen in equations 1-1 and 1-2.

$$R-COOH \rightleftharpoons RCOO^- + H^+ \qquad \textbf{(Eq. 1-1)}$$

$$R-NH_3^+ \rightleftharpoons H^+ + R-NH_2 \qquad \textbf{(Eq. 1-2)}$$

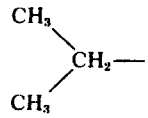
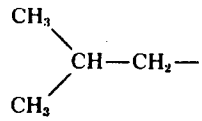
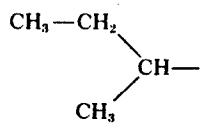
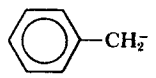
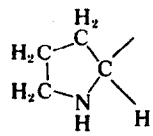
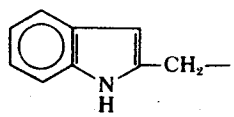
The $R-COOH$ and $R-NH_3^+$ are both acid groups capable of donating a hydrogen ion. The $R-COO^-$ and $R-NH_2$ groups are the conjugate bases of the above weak acids. The $R-COOH$ is a far better acid than the $R-NH_3^+$ group. At a pH of 6.0 to 7.0 $R-COOH$ readily donates its hydrogen ion. Even at a pH as low as 4 the $R-COOH$ group may donate its hydrogen ion. $R-NH_3^+$ tends not to give up its hydrogen ion or proton below a pH of 9.0. As is seen in Tables 2 to 4, each amino acid group has slightly varying pK values. pK is the negative log of the dissociation constant; the larger the dissociation constant, the smaller the pK and the stronger the acid. The pK of an amino or carboxyl group is the pH at which 50% of these groups would be dissociated and 50% would be associated.

Although most students are put on the defensive at the sight of pH, pK, and pI, it is important to understand the implications in their use. For example, if we were to look at glycine, the pK of $R-COOH$ group is 2.34, the pK_2 of $R-NH_3^+$ is 9.6. At a pH of 2.34, 50% of the $R-COOH$ would be protonated and 50% dissociated. At a pH of 9.6, 50% of $R-NH_3^+$ groups would be protonated and 50% dissociated. As can be seen in Fig. 1-9, glycine at a physiologic pH of 6 to 7 is partially protonated and partially dissociated.

The isoelectric point (pI) of an amino acid is that pH where the net charge per amino acid is equal to zero. With a net charge of zero, amino acids or proteins are at their pI and would not migrate in an electric field. For a diprotonic molecule such as glycine the pI would be calculated using equation 1-3.

$$pI = \frac{pK_1 + pK_2}{2} \qquad \textbf{(Eq. 1-3)}$$

Table 2. Hydrophobic amino acids with nonpolar R-groups

Amino acid	Three-letter code	One-letter code	R-group	R—COOH pK	R—NH_3^+ pK_2
Alanine	Ala	A	CH_3	2.34	9.69
Valine	Val	V		2.32	9.62
Leucine	Leu	L		2.36	9.60
Isoleucine	Ile	I		2.36	9.68
Phenylalanine	Phe	F		1.83	9.13
Proline	Pro	P		1.99	10.60
Tryptophan	Trp	W		2.38	9.39
Methionine	Met	M	$CH_3—S—CH_2—CH_2—$	2.28	9.73

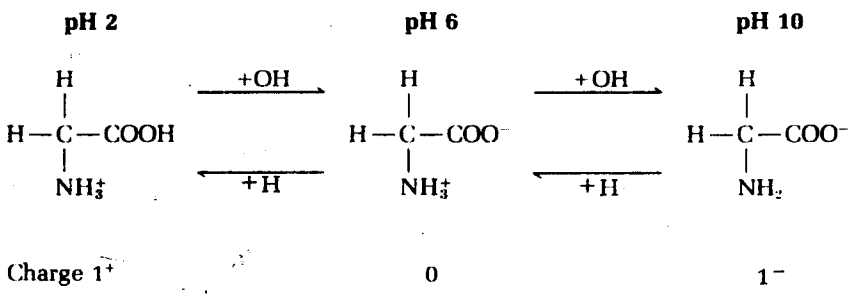


Fig. 1-9. pH titration of amino acid glycine showing ionization and charge distribution at varied pH ranges.