Clinical Enzymology

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Introduction

Clinical enzymology is not a new sphere of medical science. It is, rather, an old one dusted off and shined up by the biochemist's purification and crystallization of enzymes and legitimized by double-blind clinical research. Until quite recently it would have been thought impossible to give parenterally a proteolytic enzyme. The textbooks turned over in their intellectual graves; the reactionary medical scientist ascended the pulpit and expounded on the evils of such practice; the prophets of the past reascended Sinai in the hope of receiving a revelation that would destroy the new direction. There were, of course, false prophets on the side of clinical enzymology and these were soon classified and rendered comparatively innocuous by the passage of time. The contest is by no means terminated but it is abating and, as the dust of the battlefield settles, the true structure of the new world of clinical enzymology takes form. It is not a fairyland castle of the tribe of panacea but rather a solid brick, two-story detached building of real but limited utility.

In this small volume, an effort will be made to outline the basic knowledge essential to an understanding of the field, to review the biochemical basis, to survey the current status of clinical observations, and finally to offer a concept essential to the extension of efforts in this immediate sphere of medical endeavor. No consideration is given to the topical application of enzymes as this has been completely reviewed in other volumes of recent date.

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The theory is frequently offered that with maturity comes intellectual impotence: that radical modifications, brilliant new concepts, ideas of significance come exclusively from the younger scientist, the man less bound by history, the student verging on discovery. The concept is then supported by historical statistics but no analysis is made. Explanation actually relates to the fact that the older person is so restricted, so restrained, so conditioned as to be unable to deviate from chapter and verse. Escape from this intellectual impasse for mankind relates to curiosity.

Assuming physiological normality, the brain should be quite capable of creativity for several hundred years. It is intellectual atheromatosis, intellectual sclerosis, and not anatomical modification that prevents advancement; it is intellectual hibernation and not organic brain deterioration that closes the door to new and profitable dreams. The general idea of clinical enzymology is not new. It is in fact more than fifty years old, but traditionalism — the shackles of chapter and verse, the reluctance to tread new pathways — has kept it in sheltered infancy. Presently, the clinical use of enzymes is maturing, acceptance is in the offing, and for this reason some assembly of current knowledge in the field seems in order.

The evolution of medical practice from art to science saw first the use of a modified clinical enzymology, as immunology is no more than indirect enzymology. The antigen is a biocatalytic agent. Therefore, when antigens in any form were administered for the purpose of inducing antibody formation, a type of clinical enzymology was being used. When antibodies were given to create a state of immunity, an indirect type of clinical enzymology was employed. It is most probable that a parallel system will be utilized

in what might be designated modern clinical enzymology. Protein material, specifically antienzymes, will doubtless be used to reverse those clinical states characterized by excessive activity of any given enzyme.

With advancement of knowledge, simplification of detail occurs and simultaneously complexity of understanding develops. Where once the antigen, the enzyme, the gene, the virus, the peptide hormone all stood as distinct entities, distinct in all aspects, now modern biology classifies all these as biocatalysts with a relative specificity, a relative directional modifying power, a relative target effect. Sevag (1951) has indicated that all proteins in the proper environment can function as biocatalysts. Stated inversely, all biocatalytic phenomena are a characteristic property of the protein molecule. This does not relate solely to the idea that the biocatalytic quality is inherent in the protein molecule, but also to the fact that this complex organic moiety possesses the chemical power to coordinate those reactions essential to enzymatic activity. Some review of protein biology seems necessary in order to develop the general concept of clinical enzymology.

ENZYMATIC DIMENSION – ASPECTS WITHIN THE CELL

Knowledge of cell structure is far from complete, but recent investigations have gone far toward disclosing the geometry of these units of life. The cell is now thought to contain a continuous aqueous gel-like phase, the cytoplasmic ground substance, in which a tremendous number of granules having various enzymatic functions are dispersed. The precise significance of the granules in terms of the grosser aspect of cell structure is not determined, but it hardly seems possible that the cytoplasmic granules are as free as marbles in a bag while the remainder of the living structure is such a marvelously organized system.

The cytoplasmic granules occur over a considerable range of particle sizes; the larger granules are called mitochondria and the smaller, microsomes. Here again, the relativity of all things is operative as no sharp gradations in size occur, and it is possible that the microsome is the building stone for the mitochondria. In any event, these cytoplasmic granules are the key to the enzymatic geometry of the cell. It is the mitochondria which are the enzyme carriers *par excellence.* Another manifestation of relativity in

these structures is the fact that different mitochondria do not possess identical enzymatic spectra. Phosphatase, for example, is found on a different fraction of granules than is cytochrome oxidase.

This latter enzyme, cytochrome oxidase, acts in a system consisting of cytochrome b, Slater's factor, and cytochromes c, a, and a_3 — all of which are attached to the mitochondria. Glass (1954) has proposed that these are arranged in assemblies containing a single unit of each of the essential enzymes. This conjecture can be logically extended to assume that each enzymatic unit is held in a fixed position with respect to the others, thus permitting proper relative adjustment of the differential of diffusion and kinetics, which form an inevitable correlative of any enzymatic system. One is even led to speculate that each plastic enzyme moiety, once formed, will function as a template for the creation of a series of molecular units, similar in structure, and fixed in relative position by secondary valences as a consequence of their very origin. It is logical that such a system would function in the modification of a given substrate through a series of transformations as each subsequent member of the system would vary from the preceding form by only a small degree.

STRUCTURE OF PROTEINS

The ultimate magic of nature is in the largest sense revealed in the microcosm and in the macrocosm. Of all the magical treasures of the microcosm, none exceeds the seven-strand cables of seven-strand cables of peptide sequences of amino acids. Considering the significance of the number seven, one is, out of the beauty of the protein helix, compelled to view it with renewed respect.

Into the warp of the classic pattern of the peptide union of amino acids, constituting as it does the basis of the protein molecule, many scientists, and particularly Linus Pauling, have woven the woof of the compound helix structure. X-ray diffraction techniques provided the methodology for these investigations, an early phase of which related to the coplanar characteristics of the amide groups. The six atoms of the amide grouping, lying as they do in a common plane, are comparatively rigid and susceptible to no more than slight distortion. From this point, there were potential structures related to trans or cis configuration of these groups and the trans seemed to offer the greatest thermodynamic stability.

This conclusion led Pauling and his associates to the next major concept, namely, the probability that the amide groups form so-called hydrogen bonds with other amide groups. Considering these basic observations and conclusions, the geometric pattern most probable from all standpoints was that of the alpha-helix. In this masterpiece of natural design, there are 3.6 amino acid units per turn of the helix and 5.4 Angstrom units between turns, and the entire helix has a diameter of 10.5 Angstrom units. The complex design of this unit would seem to stagger even the ingenuity of nature, but she extends herself to twist helix forms about one another. The resultant compound helix forms take the shape of seven-strand cables with diameters of 30 Angstrom units. In this marvel of geometric pattern, there are six strands wound about a central core strand. Finally, seven of these seven-membered strands are woven into a macro-design and ultimately, as in a bacterial flagellum, three of the seven-membered units comprising seven compound helix forms are seen. Furthermore, this tapestry of threads is a logical outcome of the laws of symmetry.

This is atomic and molecular biology in one of its finest manifestations. Complexity is beautifully blended with simplicity in the creation of a master plan of living matter. The implication is not intended that all of the approximately 100,000 different kinds of proteins in the human body or the astronomical numbers of proteins constituting other living systems are of this particular design, but it seems to be the common pattern of all the fibrous proteins, such as those that occur in hair, fingernail, horn, epidermis, fibrinogen, and bacterial flagella.

One sees here how dimensional complexity is conferred on protein structures not only by the differences in the alpha-carbon substituents other than the amino and carboxyl groups, and by the sequential arrangement of these substituents, but also by the geometrical pattern and design of the macro-form. In view of this complexity it is not difficult to comprehend the confusion that is likely to ensue in a consideration of the reaction of any unit, such as a pharmacological agent, intended to react at some specific site on the surface of such a structure. To add even greater complexity, the protein macromolecular and compound macromolecular units are dynamic not only in interchange of amino acids, but in modification of the gross geometric pattern which is assumed. The environment determines the macro-form and possibly even in some

measure the amino acid sequence. The environment also determines the surface pattern exposed to pharmacological agents, as it will determine the gross geometric pattern of the seven-strand seven-strand coil of protein units. The final conclusion is inescapable that the action of pharmacological agents cannot be predicted unless the precise nature of the environment is known. Form and structure, essential elements of symmetry, determine pharmacological action and these are created by the environment from the plasticity of protein moieties. This, then, is the dilemma of the modern pharmacologist.

When the rigidity of the Newtonian universe was displaced by the brilliant elasticity of Einstein's relativity, the physicist was at one and the same time freed and chained. Relativity, and the general concept of mass-energy equivalence, combined with the Heisenberg principle of indeterminability created the "physicist's dilemma." The electron was a wave and a particle. Certainty gave way to probability.

There seems some reason to believe that the physicist's dilemma is reflected in a parallel Mayan veil for the biologist. In view of the basic complexity of the protein molecule, it has been comforting for the biochemist to consider a crystalline protein as being homogeneous, as being composed of macromolecular units each exactly similar to the others. According to the general concept of biological relativity, such precise duplication should be improbable. In any given population of protein macromolecules, the number precisely duplicating one another would be some probable mathematical formulation. The homogeneous should in fact be heterogeneous. Certainty should give way to probability. And this is indeed the case. Microheterogeneity of protein (Colvin *et al.*, 1954) has been demonstrated. Molecules of the most carefully prepared protein from a single source are found to differ in molecular shape, weight, configuration, solubility, and even in biological activity.

The biologist's dilemma, then, arises from the fact that he must deal not with specific protein chemical structures but with probabilities. If he examines a given molecular protein population, he cannot hope to arrive at other than the most statistically probable configuration and structure. Molecular biology thus becomes a probability phenomenon and acquires indeterminability.

Misshapen molecules of protein

As previously mentioned, the molecular tridimensional structure in protein is a Jekyll-Hyde mystery and can be the basis of pathology. Pauling and his associates (1949) first observed this in a study of the hemoglobin molecule as it occurs in the red cells of patients afflicted by sickle-cell anemia. The shape, or rather distortion, of the hemoglobin molecule is an aspect of the twist and turn, the geometric design, of the macro-unit in this disease. The red cell assumes its sickle form as a reflection of the hemoglobin structure. Such abnormal design is inherent in the genetic composition and might relate to proteosynthetic systems by virtue of abnormalities in nucleic acid structure or to some abnormality in the proteosynthetic enzymes themselves. There is even a third potentiality, that of abnormal environmental (for example, ion composition) aspects for the protein unit during its formative phases. Little is known concerning the chemical forces which underlie the macro-geometric pattern of the protein molecule. Only speculations can be advanced, but abnormality in protein pattern necessarily can be equated to environment, whether that environment relates to other macromolecules, possibly even those related to its own formation, or to smaller molecules and ions which exert no less marked an effect. Relativity is at the basis of all biological phenomena and it is only to be anticipated that abnormality in protein structures should occur.

Sickle-cell anemia can be regarded as an inborn error in the molecular biology of the afflicted. Beyond doubt, such errors in molecular biology due to geometric design will be found to be manifestations of many disease states. It is my belief that aging and many other chronic diseases as well as malignancy will eventually be shown to reflect a change in geometric design as the qualitative defect.

BIOLOGICAL EXCLUSION PRINCIPLE

The exclusion principle of Pauli, stated in a qualitative manner, is that the presence of the first electron in an atomic structure so modifies the physical state as to force the second into a different state of motion than would be called for by the quantum laws of motion. Margenau (1950) has related this principle of exclusion to biological systems and proposes that it offers a way of under-

standing why entities in a system show laws of behavior distinct from the laws governing them as individuals.

Schubert (1954) has indicated the importance of forced configuration (Martell and Calvin, 1952) in necessitating abandonment of the lock and key analogy of Emil Fischer, in which the key relates to substrate and the lock to enzymatic protein moiety. Schubert states that "the key does not have to fit the lock exactly, because the forces exerted by the spatial and directional requirements of valence bonds enable the lock or the key to change dimensions so as to accommodate each other." Forced configuration is a stress condition in chelate units, reflecting a state in which distortion of metal bonds occurs as a result of environment. This is certainly a biological exclusion principle and may, as Schubert indicates, be a requirement for enzymatic activity. Furthermore, if the deforming properties are sufficiently powerful (a likely probability), the relative specificity of the enzyme could be altered. The medicinal chemist is faced with the necessity of considering the "altogetherness" of any system which it is desired to modify by means of any molecular unit. Protein microstructure is not enough; the macro-form must come into consideration, and the microheterogeneity of proteins forces a realization that structures of this degree of complexity are statistical probabilities and not fixed, rigid homogeneous molecular populations. Biological indeterminability is a direct extrapolation of microheterogeneity of proteins. Finally, the existence of a biological exclusion principle must be considered. Any factor added to a system modifies the totality of the system, which is never simply the sum of its parts; each molecular species added to the system modifies the behavior of all other molecular units in the system.

There is little question but that our general approach to medicinal chemistry is being modified with staggering rapidity. What seemed a movement toward rigid clarity must be supplanted by the comparative confusion of relativity. Biology can be said to be in only a primitive state, but it is emerging and the next few years will startle the world of classical biology.

ANTIGEN–ANTIBODY REACTIONS AND EXTRAPOLATION THEREOF

The multiple biological activities of proteins must be considered as a function of their environment. In other words, it is not

something inherent in the protein molecule that conveys enzymatic activity; rather it is something inherent in the environment which creates the protein enzyme. The plastic protein moiety has the capacity to react to its environment, and specifically to a potential substrate, thus becoming relatively specific in its enzymatic activity. The dawn of physical relativity rendered essential the consideration of the world of nature, as created by measurement, as symbolic rather than as a real thing with inherent qualities called measurements. So the biologist must consider that his measurements create an entity rather than that the entity exists with specific measurements. Enzymatic activity is not something preexisting in a protein molecule; it is rather a measurement which creates a symbol, namely, the enzyme. Or, put another way, the measurements carried out under specific conditions which indicate enzymatic activity create in the mind of the biologist a statistical probability of which the enzyme is the symbol.

As noted earlier, the classification of enzyme, antigen, virus, gene, protein hormone, etc., as manifestations of the biocatalytic power of a protein moiety destroys the general concept of specificity. The term "biocatalytic power" must be considered as referring to that state which represents or symbolizes the statistical probability state of the protein moiety in relationship to its environment. Elimination of the boundaries of fixed and absolute biological specificities should carry with it the implication that interactions of protein units, once considered specific, must have features in common. And this is the case.

Tyler (1948) and his associates consider the antigen-antibody reaction as paralleling in all essential respects the fertilization of egg by sperm, the invasion of bacterium by virus, a white cell phagocytizing a bacterium, or the establishment of the parasite in the host cell. The initial phase held in common by all of these phenomena is that of some aspect of complementariness which causes adherence of two units. There is relative specificity in this force of adherence which, of course, could well be a function of the basic molecular units involved as modified by a comparatively selective environment. It might be that once again we are viewing a phenomenon reflecting specificity related to environment rather than to some inherent quality of the interacting units. In other words, these important biological phenomena should be considered from the standpoint of the "wholeness" of the system involved

and not from the standpoint of the individual molecules which seem specifically to interact.

From the concepts developed primarily by Linus Pauling and others (1949), the complementariness is due to the possession of two or more reactive sites. It is interesting to reflect that with respect to some enzymes, the reactive sites involved in enzymatic activity are the same as those related to antigenicity (Sevag, 1954). Thus, the reactive sites of enzyme, of antigen, of gene, of virus, of protein hormone may well be alike in basic structure and differ only in their spatial relationship. The general contention of lack of necessary pre-existence of the biologically important molecule has been supported by Lwoff (1951), who contends that a virus particle is formed by modification of a normal constituent of the bacterium itself. He modifies the environment of the constituent by means of ultraviolet irradiation of the bacterium and thus creates a "virus."

CONCEPTS OF ENZYME ACTION

Enzymes, long regarded as the basic ingredient of the mystery of life (as indeed they are), are slowly revealing their innermost secrets to the ingenious devices of the chemist and the physicist. The mystery is no longer a mystery but rather a complex biophysical concept. Its significance is essential for the story of clinical enzymology, but no more than the barest outline can be provided.

For many years, fundamental knowledge of the action mechanism of enzymes related to the fact that the activation energy of certain reactions was lowered by these biological catalysts and that the substrate and the enzymes formed an intermediate complex. All chemical reactions, whether catalyzed by enzymes or not, occur because the reactants are activated and from this activated state pass to the final products. The energy of activation bears a relationship to the heat or energy of the reaction. The function of the enzyme is simply that of creating an environment in which a given chemical reaction can occur with greater ease. A great deal of information is available on the mechanism of these actions; one of the most important aspects is that of protein configuration.

No enzyme can be considered as a static protein molecular aggregate, each molecule of which precisely parallels every other. The aspect of protein microheterogeneity establishes the necessity for considering any population of protein molecules as being a sta-

tistical probability problem — comparatively few of the enormous numbers of molecules of a given protein enzyme will be the same. Further, in any given system, the total enzyme present will be the sum of the active enzyme present, the denatured or inactive enzyme present, the substrate combinations with both active and inactive enzyme forms, and the enzyme inhibitor combinations. The environment of the instant will determine the relative concentrations of each form. To this complexity must be added the three basic aspects of protein structure. First, the differential amino acid composition and sequential arrangement of the protein probably determine the relative potentialities for substrate specificity; second, the spiral coiling — macro-form — of the protein determines the availability of surface sites for substrate interaction; and third, the twisting or twining of spiral forms is a factor in availability and reactivity of surface sites.

The reactive surface site will vary within itself, as the proton population of any given molecule of protein will shift with time. Therefore, even in the event of a relatively stable amino acid composition at that site, and assuming no stress modification in configuration, the proton situation may vary and create a state in which the site will not be reactive. The structural features of the surface site are dual — provision must be made for the fixation of the substrate and for the mobility of charges of an electrostatic nature which are responsible for the interaction. While the macrostructure of the protein is controlled by five different kinds of chemical bonds, fixation of substrate is a function of van der Waals forces and these forces establish fixation of the substrate at two points.

While there are many proposed mechanisms for the immediate breaking of the reactive bond, Eyring *et al.* (1954) calls one "the rack." In this system, the substrate molecule is fixed at two points; a strain is put on the molecule which tends to disrupt it almost as if it were being pulled apart; and, as a third factor, electrons decisive in the adherence of the two parts of the substrate unit are drawn away by adjacent molecular configurations. In more technical language, these three factors are: (1) secondary electrostatic bonds which provide the stretch on the primary bond, (2) groups which make the bond labile by pulling electrons out of it, and (3) a direct mechanical strain due to steric types of bond distortion. An analogy might be made to the pulling of an elastic

band to a point of maximum extension without rupture and then cutting part way through the band. The result would be that the band will snap, and so it is with the chemical bond and the enzyme.

Another aspect of enzyme action, currently receiving detailed study, is that of metals and the chelation of these metals onto protein surfaces. Many enzymes function only with a metal as a cofactor; in general, the metal ion forms a coordination complex between the substrate and the enzyme surface. In other words, it forms a bridge for the establishment of a substrate fixation on the protein surface and provides the electronic system essential to activation. One clear example of this is given by Smith (1951) and relates to the enzyme prolidase, which splits glycyl-L-proline. The enzyme is specifically activated by manganese, and Smith suggests that the metal combines with the nitrogen atom of the peptide bond and with the protein surface partially via a sulfhydryl grouping. This structure displaces electrons from the peptide carbonyl group to the nitrogen. As a result the carbonyl carbon is rendered more electropositive and attracts hydroxyl ions which then catalyze rupture of the peptide bond in the classic manner.

Still another branch of study has considered group transfer by enzymes. In such reactions, acyl radicals (for example, acetyl) or phosphate groups are transferred as intact units. An example of such interactions is the formation of acyl phosphates, proceeding via the formation of an acyl enzyme complex, followed by phosphorylytic action.

In this brief outline of the current status of enzyme biology, it is hoped that some concept of the complexity of the systems under consideration has been given. The principle of biological indeterminability applies to these systems which can only be considered in the light of statistical laws of probability; however, with this indeterminability has come the simplification of atomic and molecular biology, which considers the basic mechanisms of enzymatic action as manifestations of steric stress, electron withdrawal, and bond rupture by the pull of double points of substrate fixation. The fascination of the field comes with full recognition of the significance of the organismic whole; a cell is not a "bag full of enzymes"; it is rather a totality in which the total exceeds the sum of its parts. While study of enzymes in isolated systems is essential, the true meaning of enzymes can come only from study