Enzyme and Metabolic Inhibitors

Volume I

General Principles of Inhibition

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FOREWORD

The past fifty years have seen an astounding increase in our understanding of the nature of the physical world. Only fifty years ago was it discovered that an atom consists of a small heavy nucleus and some electrons surrounding the nucleus. A tremendous amount of knowledge has been gathered during this period of fifty years about the structure of atoms and of simple molecules.

It is now becoming possible to extend the experimental techniques that have been applied to simple molecules to the more complicated molecules that are found in living organisms. Some information has already been gathered about the structure of the simplest proteins, but the job of making a complete three-dimensional structure determination for a protein molecule has not yet been carried through to completion. Nevertheless, it is evident that this job will soon be done. During the past year great progress has been made by John Kendrew of Cambridge University in the determination of the positions of the atoms in the molecule of myoglobin, and the techniques of X-ray diffraction that he and other investigators in the field of protein structure are now applying can be expected to lead in another decade or two to the complete determination of the structure of many globular proteins.

The enzymes constitute a class of protein molecules of extraordinary significance. Just as deoxyribonucleic acid may be said to be the substance that we need to understand if we are to know in its atomic detail the mechanism by means of which organisms reproduce themselves, so are the enzymes the molecules that we must understand to know how organisms carry on their metabolic activities.

No one knows the precise molecular structure of any enzyme and the exact way in which the enzyme carries on its characteristic function of catalyzing chemical reactions. During the next fifty years we can be assured that this knowledge will be gained, in large part probably through structure determinations made by the X-ray diffraction technique. Nevertheless, even though detailed structural information is still lacking, a very great deal has

been discovered about enzymes, about the reactions that they catalyze, and about their interactions with inhibitors.

In this book, of which I have the honor to write the Foreword, John Webb has summarized our existing knowledge about enzymes and has contributed further to it by new methods of analysis. There are few men in the world who could have done this difficult job in such an effective way — few men with the background of knowledge and experience in the fields of mathematics, physics, and physical chemistry as well as biochemistry and biology sufficient to permit them to comprehend the great field of enzyme inhibition and to analyze it and discuss it in such a valuable way. In particular, the through discussion of intermolecular forces may be expected to be the basis for future discussion of enzyme inhibition for many years. I am confident that every scientist whose work brings him into contact with enzymes will find this book of value.

LINUS PAULING

PREFACE

Enzyme inhibitors have become valuable tools in many of the biological sciences and are used widely to study isolated enzymes and the various aspects of cellular metabolism, growth, and function in organisms ranging from protozoa to mammals and from bacteria to the higher plants, as the voluminous and disseminated modern literature shows. However, inhibitors can be more than tools. The study of the manifold actions of all substances that significantly distort cellular metabolism is one of the major provinces of molecular biology and its ramifications extend throughout all levels of biological investigation. My simple aims in writing these volumes are to present concisely the basic principles of inhibition, to describe the actions and mechanisms of the most important and interesting inhibitors, to correlate the actions at the enzyme level with the changes observed in cellular function, and to provide practical information on the uses of these inhibitors. It is hoped that these books will aid investigators in the various disciplines in discovering the fundamental relationships that occur ubiquitously in living matter and thus will advance in a small way the search for the elusive laws of cellular organization involved in the flow of energy that characterizes vital processes. There is another aim which is in one sense more personal. I believe that the subject of enzyme inhibition is fascinating in itself and I would like to think that my presentation here will inspire interest in others so that they will come to share my enthusiasm.

Every attempt has been made to place inhibition on a quantitative basis, inasmuch as I am convinced that only in this way can work with inhibitors develop significantly. Indeed, some of the theoretical and quantitative aspects have been developed here beyond our present experimental knowledge in the hope that this will stimulate both accurate experiments and better theories. I have not restricted the treatment to the enzyme and cellular metabolic levels but wherever possible have considered the actions of inhibitors on functioning organs and whole animals. Attention has not been primarily focused on any form of life — microorganisms, plants, or animals — but instead the attempt has been made to treat all equally on the

principle that the fundamental mechanisms and problems are the same and that workers in one field can learn a great deal from results obtained in other fields.

The writing of the first volume and the preparation for the succeeding volumes have involved the accumulation of approximately 16,000 references, and several thousand more will be added before the work is completed. A rigorous selection of the material and the examples to be presented is thus necessary. At the present time over 1,400 publications on enzyme and metabolic inhibition appear each year, and this figure must be increased if one includes the work on drugs and chemotherapeutic agents that act in some manner by altering metabolism. The use of inhibitors has increased markedly since 1950. Not only are the older inhibitors applied more widely and intensively, but each year one sees with interest the development of new inhibitors. It is interesting to survey by decades the number of publications on the two common inhibitors, malonate and iodoacetate. The accompanying

	Number of publications	
Decade	Malonate	Iodoacetate
1880–1890	1	0
1890–1900	1	0
1900–1910	1	0
1910–1920	3	0
1920–1930	9	4
1930–1940	47	227
1940–1950	87	145
1950–1960	485	764

table shows these figures taking into account only biologically oriented work. The large number of papers on iodoacetate between 1930 and 1940 was due to the demonstration of its interesting effects on muscle and yeast, and subsequently on the glycolytic pathway. Most enzyme inhibitors would provide similar histories. The marked upsurge in reports since 150 is evident and it is also clear that this acceleration has by no means ceased. Considering the tremendous amount of work that has been done with inhibitors and their importance in many fields, it is quite remarkable that no book on them has yet appeared; indeed, it is uncommon to find reviews or monographs on the individual inhibitors.

This first volume will be concerned with the general principles of enzyme inhibition and specific inhibitors will be mentioned only for illustration of these principles. The second and third volumes will deal with the individual inhibitors and inhibitor groups, and there much that is discussed in the present volume will be applied.

Inasmuch as the Preface is the only place in a book where one can attend to personal questions, one may perhaps be anticipated. Some may justifiably question my qualifications for writing on such a subject and for so often suggesting the ways in which inhibition studies be done. I can only answer this by saying that I believe I realize my inadequacies more keenly than the reader does, having lived with them daily, but desire to contribute a little to the formulation of this field of inhibition, and perhaps stimulate others to more penetrating analyses and to better presentations in the future.

I would here like to express my gratitude to Professor Linus Pauling for perusing the manuscript and writing the Foreword, and also for what he has contributed to my understanding in the past.

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CONVENTIONS

The effects of inhibitors on enzyme activity, metabolism, or tissue function are expressed as *fractional inhibitions*, designated by the symbol i. This is the fraction of the total control activity that is inhibited under the conditions specified and thus may vary from zero to unity. The various ways in the literature of expressing inhibition have all been reduced to this convention and liberty has frequently been taken to calculate fractional inhibitions from untreated data. It is hoped that such a standard system will allow comparisons to be made readily. The kinetic equations are also expressed in terms of the fractional inhibition.

Rate constants are always expressed by the lower case k and *equilibrium constants* by the upper case K. The subscripts for the rate constants are positive or negative depending on the direction of the reaction; thus, for the reaction $A \rightleftharpoons B$, k_1 is the rate constant for the reaction of A to B and k_{-1} is the rate constant for the reverse reaction. The subscript number indicates the step in a sequence of reactions. K always refers to a dissociation constant and thus is the inverse of the corresponding association constant; for example, for the reaction $A + B \rightleftharpoons AB$, the value of K is given by (A) (B)/(AB).

Concentrations of substrates and inhibitors are generally given as millimolar (mM), as are the dissociation constants, for the purpose of establishing a uniform system.

The *enzyme nomenclature* attempts to follow Table V. 1 of Dixon and Webb ("Enzymes," 1958) wherever possible.

SYMBOLS

The most commonly used symbols are defined in the following list. Other symbols of more restricted use are described in the text where they occur. Where the same symbol is applied to two or more different functions, there should be no confusion in the contexts in which the symbols appear.

a	fractional activity (enzymic)	A	constant for attraction energy
a	activity (thermodynamic)	AR	agonist-receptor complex
A	activator	B	constant for repulsion energy
A	agonist	d	distance between interacting particles
A	antibody		

d_e	equilibrium distance between interacting particles
d_0	thermal equilibrium distance between interacting particles
D	dielectric constant
e	electronic charge (4.802×10^{-10} esu)
E	enzyme (often apoenzyme)
E_a	catalytically active enzyme
E_{act}	Arrhenius activation energy
E_d	catalytically inactive enzyme (denatured)
E_t	total enzyme
EA	enzyme-activator complex
EH	protonated enzyme (active site)
EI	enzyme-inhibitor complex
EP	enzyme-product complex
ES	enzyme-substrate complex
EX*	activated complex of enzyme with substance X
f	force exerted on charged particle in electric field
f_b	pH function for base
f_{hb}	pH function for acid
f_{h_2b}	pH function for dibasic acid
f_r	resistant fraction of rate
f_s	sensitive fraction of rate
F	electrical field strength
ΔF	change in free energy
ΔF^*	activation free energy
ΔF_{es}	change in electrostatic free energy
ΔF_{nes}	change in nonelectrostatic free energy
g	factor by which rate of branched reaction is changed
h	Planck's constant (6.624×10^{-27} erg-sec)
H	hapten
ΔH	change in heat content (enthalpy)
ΔH^*	activation enthalpy
i	fractional inhibition
$\bar{i}$	mean fractional inhibition
i_L	inhibition required for lethal action
I	inhibitor
I_i	inhibitor inside a compartment
I_o	inhibitor outside a compartment
I_1, I_2	ionization potentials
$(I)_L$	lethal concentration
$(I)_m$	minimal inhibitor concentration to produce effect
$(I)_{50}$	concentration for 50% inhibition
k	rate or permeability constant
k	Boltzmann constant (1.380×10^{-16} erg/deg-molecule)
k_m	salting coefficient
k_n	rate constant for a particular step n
k_{-n}	rate constant for reverse reaction of step n
K	equilibrium (dissociation) constant [= (A)(B)/(AB)]
K_a	activator constant
K_a	ionization constant
K_{as}	dissociation constant for substrate-activator complex
K_i	inhibitor constant
K_i'	experimental or apparent inhibitor constant
K_i^o	inhibitor constant for isoelectric enzyme
K_m	Michaelis constant
K_o	equilibrium constant between S and P
K_p	Michaelis constant for reverse reaction (product reaction)
K_r	dissociation constant for inhibitor-reversor complex
K_s	dissociation constant for enzyme-substrate complex (substrate constant)
K_s'	experimental or apparent substrate constant
K_{si}	dissociation constant for substrate-inhibitor complex
K_{sp}	equilibrium constant between ES and EP
l	length of dipole
L	limiting rate coefficient
n	number of molecules of substrate or inhibitor binding to an active site

n	number of doses of an inhibitor
N	Avogadro's number (6.024×10^{23} molecules/mole)
pH_i	pH inside a compartment
pH_o	pH outside a compartment
pH_{opt}	optimal pH for enzyme reaction
pI	$-\log$ (I)
pK_i	$-\log K_i$
pS	$-\log$ (S)
P	product
P	phosphate group
P_i	inorganic orthophosphate
P	pressure
P	total polarization
P_a	atomic polarization
P_e	electronic polarization
P_i	induced polarization
P_m	orientation polarization
q	magnitude of charge
q_d	charge on a dipole
r	ratios in general (e. g., of rates in branches of convergent chain)
r_o	distance of closest approach of ionic atmosphere
R	receptor
R	reversor
R	gas constant (8.31×10^7 ergs/deg-mole = 1.99 cal/deg-mole)
R_o	molar refraction
ΔR_o	refraction exaltation
s	slope of an inhibition plot
s	ionic strength
S	substrate
S_e	endogenous substrate
S_i	substrate inside a compartment
S_o	substrate outside a compartment
S_t	total substrate
ΔS	change in entropy
ΔS^*	activation entropy
t	time
t_m	minimal time in which effect is produced
Δt	transition time between steady states
T	absolute temperature
TN	turnover number
v	rate of reaction
v_f	rate of forward reaction
v_f	final rate of reaction
v_i	rate of inhibited reaction
v_i	initial rate of reaction
v_n	rate of the n^{th} step in multienzyme system
v_r	rate of reverse reaction
v_r	rate of resistant reaction
v_s	rate of sensitive reaction
v_{st}	steady-state rate of reaction
v_t	total rate of reaction
w, W	water
w	electrostatic factor
X	transferred group
(X′)	specific concentration of X
z	valence of ion
Z	number of electrons in outer shell
$\alpha, \beta, \gamma, \ldots$	interaction constants giving the effects of the binding of one substance on the binding of another to the active site
α_0	molecular polarizability
β	constant characterizing the change in the rate of breakdown of the ES complex
δ	molar depression of the dielectric constant
ζ	electrical potential between a particle and the medium
ϑ	angle between dipoles
$\varkappa$	Debye-Hückel constant
μ	temperature characteristic
μ	dipole moment
$\bar{\mu}$	mean (statistical) dipole moment
ν	frequency of electrical oscillator
σ	heterogeneity index
σ	specificity of inhibition
σ_{max}	maximal specificity of inhibition
φ	inhibition index (of Kistiakowsky and Shaw)
φ	potential energy
φ_e	equilibrium potential energy of interacting particles

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CHAPTER 1

PERSPECTIVES OF METABOLIC INHIBITION

Living cells are characterized by a complex and beautifully organized pattern of chemical reactions mediated and directed by enzyme systems. These reactions provide the energy necessary for the various functions of the cell and support the synthesis of material upon which the cell depends for maintenance, growth, and multiplication. One basic way of expressing this pattern is to consider the flow of energy through the cell: the intake of the potential energy resident in prospective substrates, regulated by membrane permeability and occasionally by active processes; the release of this energy into utilizable forms during the oxidative degradation of these substances, wherein the energy is fractionated and distributed in smaller units; the transfer of this energy, perhaps through several steps, until it is manifested in terms of some evident cellular activity, such as secretion, active transport, impulse conduction, movement or emission of radiation; the partial flow of the energy into the complex compounds synthesized either for primarily structural purposes or for the metabolic and functional systems of the cell, for even the enzymes themselves must be assembled using the energy derived from their catalyzed reactions; and, finally, the inevitable release of a certain fraction of the energy as heat, since energy transfer and utilization cannot be completely efficient. If one were acquainted with the full pattern of this energy flow and the means by which it is organized, one could be said to understand the nature of life.

The application of enzyme inhibitors to cells or their aggregates is one of the ways in which the scientist attempts to penetrate into the nature of living material and its transformations. This approach pertains to that class of investigations in which a perturbation is induced in a complex system for the purpose of understanding better the initial normal state. Such a procedure must be quantitative to be fully effective. Also the primary perturbation produced should be as small as possible, consistent with the sensitivity of the methods of measurement, in order to reduce secondary phenomena to a minimum. The normal state of a biological system should not be conceived as a vague ensemble of simultaneous processes, but as a perfect balance of complex chemical and functional activities superimposed

on an intricate structure, the possibility of life depending upon a proper and accurate coordination of the rates of these various processes. The introduction of an inhibitor into the system leads to an alteration of certain rates and a disturbance of this balance. The extent to which the imbalance will affect the total system will depend upon many factors. One can usually measure only the results of the imbalance and, hence, must always be aware of the factors that stand between the metabolic dislocation and what is measured. If the inhibitor shifts the balance to the detriment of the organism, it is generally classed as a poison; when the balance is shifted in favor of the survival of the total system, the inhibitor may be considered as a drug.

The actions of inhibitors upon living systems can often be most profitably visualized by interpreting the results in terms of a modification of the energy flow (Fig. 1-1). There are many ways of deviating the course of the flow

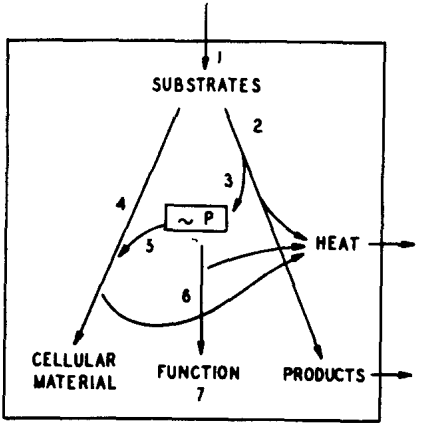

FIG. 1-1. Scheme of the basic energy flow through a cell.

of energy through cells and, therefore, in some manner altering the biochemical morphology and the functional phenomena necessary for the life of the cell and for the cell's role in the life of the total organism. The principal pathways of energy flow are indicated in the diagram and it is possible in this scheme to designate some of the general loci at which inhibitors may act. An inhibitor may interfere with the entrance of the substrates into the cell (1) by altering permeability or depressing transport mechanisms; in this way the inhibitor can reduce the total potential energy available to the cell. The action may also be exerted on any of the metabolic pathways whereby energy and matter are distributed, especially on the degradative reactions in which the substrates are broken down oxidatively to products

(2), the formation of high-energy phosphate (3), the synthetic reactions for the formation of the protoplasmic components (4), or the utilization of the high-energy substances by these synthetic processes (5). With respect to the functional activity of the cell, the inhibitor may act either on the reactions involved in the utilization of energy (6) or directly on the functional systems themselves (7). It is necessary to realize that these intracellular processes are closely related so that, for example, a primary inhibition on the oxidation of substrate will depress to varying degrees the formation of high-energy substances, synthesis, function, and the appearance of products. Also it must always be borne in mind that the actions of many inhibitors are not localized to a single pathway inasmuch as inhibitors are seldom completely specific.

Directly distorting the pathways of enzymically directed reactions by the introduction of a chemical substance — blocking a pathway or diverting a sequence into a new channel, interfering with coupled processes or hindering the accumulation of energy — is one approach amongst others. It is an interesting and a useful way because it often provides a rather specific attack upon some particular reaction or phase of metabolism, and often one is able to relate certain aspects of cellular activity to this susceptible region in the metabolic network. In fact, it is often possible to isolate from the cell the special enzyme or enzyme system acted upon and to study the detailed mechanism of the interference, so that it becomes possible to postulate more accurately what occurs within the cell when the inhibitor exerts its effects. Many other ways of altering metabolic activity — for example, by changing the temperature or the pH, by irradiation or high pressure — are nonspecific and seldom does one have any ideas as to exactly what is occurring in the complex protoplasmic matrix. If one had to choose the most interesting and important characteristic of enzyme inhibitors, what it is that makes them one of the most powerful tools in so many fields of biological investigation, it would be their relative specificity, or at least the possibility of attaining greater and greater specificity as new inhibitors are discovered for attack upon reactions of interest. The question of specificity cannot be overemphasized. The more we know about the exact nature of the perturbation produced and the more selective this action can be made, the more likely it is that clear interrelationships will emerge and the goal of understanding the energetics of the cell be achieved.

Sometimes in using inhibitors one does not begin initially with a normal cellular system, but one in which something has gone astray in the complex pattern, in other words a group of pathological cells. If this aberration is basically metabolic, there is the possibility of restoring the cells towards the normal pattern by the use of substances effective in specifically altering appropriate reactions. It is probable that many of the most useful drugs act exactly in this manner. On the other hand, if the cells have become