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COMMENTARY

ASSAY OF PEPTIDASE AND PROTEASE ENZYMES *IN VIVO*

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Means are becoming available for measuring the instantaneous activities of cell-bound peptidase and protease enzymes whose catalytic sites have access to the vascular space. For example, it is now possible to measure the effective concentration of angiotensin converting enzyme in lungs of chronically cannulated, unanesthetized experimental animals, and it is feasible to make such measurements in man [1–3]. By analogy, it appears that similar assays can be performed to measure activities of cell-bound enzymes exposed to the urinary space and perhaps of cell-bound enzymes exposed to other body fluids in transit. Although the text that follows deals primarily with the assay of peptidase and protease enzyme activities, the approach and general principles to be described undoubtedly can be used to measure activity of virtually any accessible hydrolase enzyme for which a suitable substrate can be found. The latter clearly includes esterase and phosphatase enzymes.

To this point, only a small group of investigators have begun to assay peptidase and protease enzymes *in vivo*, and, in fairness, these investigators do not necessarily agree on what the results may mean. Technically, the territory may no longer be virgin, but it has lost little innocence. Thus, much of what follows should be understood to be my personal views of what the data may mean and where they may lead.

Historical notes

It is a time-honored practice to examine the pharmacokinetics of a radiolabeled drug or hormone following intravenous injection. Typically, tissues and body fluids are collected minutes or hours after injection and are examined by methods such as extraction and autoradiography. However, in the mid-1960s, it became evident that the metabolic fates of certain vasoactive substances were determined within seconds or fractions of a second following intravenous injection. Implied by the latter is that the distribution of radioactivity of, say, [^{3}H]Phe^8-bradykinin, 60 or even 2 min after injection, is more likely to reflect the fate of phenylalanine than that of bradykinin. For example, Ferreira and Vane [4] showed that bradykinin disappears almost completely during the 3–4 sec required for blood to pass from the right heart to the left. Shortly thereafter, Ng and Vane [5] showed that angiotensin I is somehow activated as it passes across the pulmonary vascular bed, an activation that we now understand, in part, as owing to the conversion of angiotensin I into its much more potent lower homolog, angiotensin II [6, 7]. We now know that the immediate fates of intravenously injected bradykinin and angiotensin I are, in large part, determined by a single enzyme, angiotensin converting enzyme, situated along the luminal surface of pulmonary endothelial cells [8–10].

The fates of bradykinin and angiotensin I are probably determined in a much shorter time than that suggested by the 3–4 sec mean transit time of blood across the pulmonary vascular bed. Given the disposition of angiotensin converting enzyme along the inner wall of blood vessels and considering the importance of blood vessel diameter to blood volume, hematocrit and velocity of flow, it seems virtually certain that interactions of circulating substrates with cell-bound enzyme occur primarily at the level of the capillary bed—in the case of the lungs, a capillary bed traversed by blood in about 1 sec at rest and in a small fraction of a second during maximum exercise. Bradykinin (BK) undergoes six to eight half-lives during passage through the lungs. If the pertinent reactions occur primarily within the pulmonary capillary bed, the half-life of BK is on the order of 1/6th–1/8th of a second.

Increasingly, we and others have begun to change the focus of our studies from the hormones themselves to the enzymes that catalyze their degradations [11, 12]. The change in focus, for some purposes, is based on the belief that by assaying the activities of vascular surface enzymes, we can gain some insight into diffuse vascular injury as may occur in, for example, anaphylaxis, hyperoxia, hypoxia and the various forms of the acute respiratory distress syndrome. In other cases, we simply want insight into questions such as: Do vascular surface enzymes react with their substrates in a manner understandable in terms of the Michaelis–Menten equation? Are results of *in vivo* assays understandable in terms of results obtained in a comparable *in vitro* assay? Can one more accurately evaluate the pharmacologic effects of an enzyme inhibitor by measuring the effects via

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an *in vivo* assay rather than *ex vivo* assay? What are the important variables in an *in vivo* assay, an assay system in which the common *ex vivo* variables of temperature, pH, buffer and ionic strength are very tightly controlled by homeostatic mechanisms and a system in which time of incubation not only can vary over several multiples but can seldom be measured with great accuracy? Is it possible that the rate of reaction of vascular enzyme and circulating substrate is determined almost exclusively by hemodynamic factors, alternative natural substrates, inhibitors and cellular and tissue events that affect the expression of enzyme activity? Will we, for these purposes, understand enzyme concentration not in terms of moles per unit volume but in terms of diameter of blood vessel, tone, surface area, affinity and density of enzyme per μm^2? Are the intravascular reactions in some way coordinated with performance of the tissue or organ? In the case of the lungs, is the extent of a reaction determined, in part, by ventilatory phase, greater in full inhalation than in maximum exhalation?

Substrates for in vivo *assays*

As part of a program to develop simple means of measuring angiotensin converting enzyme activity of endothelial cells in culture, we prepared a series of acyl-tripeptides, each labeled at high specific radioactivity (20–27 Ci/mmole) with an atom per molecule of ^3H in the acyl moiety [13, 14]. Each substrate was selected or designed such that the substrate itself was relatively hydrophilic and the acyl-amino acid leaving group was, at acid pH, relatively organophilic. Thus, the reaction of enzyme and substrate could be terminated by acidification, and substrate could be largely separated from its radioactive product by extraction with an organic solvent [15]. The substrates are listed in Table 1, a table that also shows the reaction constants of each substrate with highly purified human serum angiotensin converting enzyme provided by Drs. Lanzillo and Fanburg [16].

For purposes of a concurrent study of the role of the kallikrein-kinin system in anaphylaxis, we prepared the analogous radiolabeled substrates shown in Table 2 [17, 18]. The latter substrates were designed such that their radiolabeled leaving groups would be relatively organophilic at alkaline pH, a pH range in which each substrate remains strongly hydrophilic. Table 3 shows other radiolabeled substrates available at present and their known or presumed target enzymes. With only three exceptions (the enkephalinase substrates), radioactive product can be separated from its substrate by simple solvent extraction. In some highly favorable cases, the separation can be effected using a small liquid scintillation vial such that enzyme and substrate are incubated in the vial, the reaction is terminated, and then the organophilic radioactive product is extracted into the "counting phase" (organic solvent containing scintillants) while unhydrolyzed substrate remains in the aqueous ("non-counting") phase. Each of the substrates listed in Tables 1, 2 and 3 can be used in an *in vivo* assay. Given access to target enzyme, each is hydrolyzed to a degree apparently directly proportional to its respective K_c/K_m.

The importance of the so-called P_1, P_2 and P_3 subsites [20] is well illustrated for glandular kallikrein (see substrates in Table 2). Not only are the side chains important, the orientation of a given side chain can make a profound difference. In addition, for unknown reasons, some substrates bearing [^3H]benzylamide groups are highly reactive with their target enzymes and others are not. Hence, the P_1' subsite is not always well-satisfied by a nonspecific group such as the benzylamide group, and the K_c/K_m of reaction of a given substrate with its

Table 1. Radiolabeled substrates for angiotensin converting enzyme*

Substrate (abbrev.)	K_c (min^{-1})	K_m (M)	K_c/K_m ($M^{-1}\ min^{-1}$)
[^3H]benzoyl-Gly-Gly-Gly (hippuryl-Gly-Gly; HGG)	1.064×10^5	5.4×10^{-3}	1.97×10^7
[^3H]benzoyl-Gly-His-Leu (hippuryl-His-Leu; HHL)	3.7×10^3	1.06×10^{-4}	3.49×10^7
[^3H]benzoyl-Pro-Phe-Arg (BPPA)	6.86×10^3	1.4×10^{-4}	4.90×10^7
[^3H]benzoyl-Phe-His-Leu (BPHL)	6.36×10^2	5.3×10^{-6}	1.2×10^8
[^3H]benzoyl-Phe-Ala-Pro (BPAP)	1.054×10^3	3.4×10^{-6}	3.1×10^8

* Each substrate was prepared from its halogenated precursor, either 4-iodo-benzoyl- or 3-bromo-benzoyl-, by dehalogenation in 10 Ci of $^3H_2$ gas. Specific radioactivities of the substrates range from 20 to 27 Ci/mmole. The reaction constants were measured using highly purified human serum angiotensin converting enzyme. Each substrate required different buffer solutions for optimal activity. Thus, HGG (see abbreviations below) was used in 0.05 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) buffer, pH 8.0, containing 0.1 M NaCl and 0.6 M Na_2SO_4. HHL was used in 0.05 M Hepes buffer, pH 8.0, plus 0.1 M NaCl and 0.75 M Na_2SO_4, and BPHL required 0.05 M Hepes buffer, pH 8.0, plus 0.1 M NaCl and 0.95 M Na_2SO_4. BPAP and BPPA had less severe requirements for high ionic strength. BPPA was used in 0.05 M Hepes buffer, pH 8.0, plus 0.1 M NaCl, and BPAP was used in 0.05 M Hepes buffer, pH 7.5, containing 0.15 M NaCl.

Table 2. Radiolabeled substrates for glandular and plasma kallikreins*

Substrate	Glandular kallikrein K_c/K_m (M^{-1} min^{-1})	Plasma kallikrein K_c/K_m (M^{-1} min^{-1})
(D)Pro-Phe-Arg-[^{3}H]Bz	9.091×10^6	4.488×10^4
Ac-Phe-Arg-[^{3}H]Bz	4.546×10^6	5.110×10^4
(D)Val-Leu-Arg-[^{3}H]Bz	3.247×10^6	Not tested
Cpc-Phe-Arg-[^{3}H]Bz	2.525×10^6	6.530×10^4
Pro-Phe-Arg-[^{3}H]Bz	1.894×10^6	1.006×10^5
PhP-Phe-Arg-[^{3}H]Bz	1.623×10^6	6.852×10^5
<Glu-Phe-Arg-[^{3}H]Bz	1.136×10^6	1.191×10^6
Bzl-Phe-Arg-[^{3}H]Bz	1.109×10^6	7.467×10^3
Cbo-Phe-Arg-[^{3}H]Bz	5.612×10^5	8.393×10^4
4-keto-Pro-Phe-Arg-[^{3}H]Bz	1.878×10^5	6.625×10^5
4-OH-Pro-Phe-Arg-[^{3}H]Bz	1.871×10^5	Not tested
Phe-Arg-[^{3}H]Bz	1.225×10^5	No hydrolysis
(D)Phe-Phe-Arg-[^{3}H]Bz	Not tested	6.897×10^5

* Each substrate was prepared from its halogenated precursor, typically a 3-iodo-, 4-iodo- or 3-bromo-benzylamide, by dehalogenation in $^3H_2$ gas [17]. Near-theoretical specific radioactivity for one atom of ^{3}H was obtained in each case. The reaction constants were measured using pure human urinary (glandular) kallikrein [18, 19] and pure human plasma kallikrein provided by Drs. C. Kettner and E. Shaw. Glandular kallikrein was assayed using 0.2 M Tris buffer, pH 9.5. Even though Tris has very little buffering capacity at pH 9.5, the measured reaction rates in Tris were up to 40% higher than those in other buffers tested. Plasma kallikrein was assayed in 0.2 M Tris buffer, pH 8.0, containing 0.4 g/100 ml bovine serum albumin.

Abbreviations : Ac, acetyl; Cpc, cyclopentanecarbonyl; PhP, 3-phenylpropionyl; Bzl, benzoyl; Cbo, carbobenzoxycarbonyl; and Bz, benzylamide.

enzyme can occasionally be increased by 1000-fold by appropriate selection of the "non-specific" leaving group. Even greater increases in reactivity are obtainable with esters or thio esters [21, 22]. However, the relatively unstable nature of esters and thio esters favors spontaneous hydrolysis, a highly undesirable characteristic for *in vivo* assays.

Table 3. Other radiolabeled substrates prepared for assay of protease or peptidase enzymes *in vivo**

Substrate	Target enzyme(s)
(D)Phe-Pro-Arg-[^{3}H]Bz	Thrombin
Tos-Gly-Pro-Arg-[^{3}H]Bz	
(D)Phe-Pip-Arg-[^{3}H]Bz	
Tos-Gly-Pro-Lys-[^{3}H]Bz	Plasmin
(D)Val-Leu-Lys-[^{3}H]Bz	
Bzl-Ile-Glu-Gly-Arg-[^{3}H]Bz	Factor Xa
<Glu-Gly-Arg-[^{3}H]Bz	Plasminogen
Ile-Ile-Arg-[^{3}H]Bz	activators
Gly-Gly-Arg-[^{3}H]Bz	
Boc-Ile-Ile-Arg-[^{3}H]Bz	
Leu-Gly-Arg-[^{3}H]Bz	Complement
DNS-Leu-Gly-Arg-[^{3}H]Bz	convertase enzymes
Boc-Leu-Gly-Arg-[^{3}H]Bz	
Asp-[^{3}H]Bz	Aminopeptidase A
Tyr-[^{3}H]Bz	Enkephalinase
[^{3}H]Bzl-Gly-Phe-Met	enzymes
[^{3}H]Bzl-Gly-Phe-Leu	
[^{3}H]Bzl-Phe-Arg	Carboxypeptidase N
[^{3}H]Bzl-Ala-Arg	
Gly-Pro-[^{3}H]Bz	Dipeptidyl amino
Arg-Pro-[^{3}H]Bz	peptidase IV

* In addition to those substrates prepared for angiotensin converting enzyme (Table 1) and the kallikreins (Table 2), the above substrates have been synthesized.

Abbreviations: DNS, dansyl; Boc, *t*-butyloxycarbonyl; and Tos, tosyl. See Table 2 for other abbreviations.

Methods of assay of enzymes in vivo

In general, assays of vascular surface enzymes can be considered as extensions of indicator-dilution studies [23, 24]. Ideally, neither substrate nor product should leave the vascular space during a single pass through the vascular bed under study, and their volumes of dilution and mean transit times should be like those of a well-characterized intravascular marker such as [^{125}I]albumin. In principle, extravascular enzymes can be measured via indicator-diffusion techniques, and studies have been made of the metabolism of some biogenic amines, prostaglandins and adenosine during a single transit through a vascular bed [25–27]. However, the latter studies are dependent on rate-limiting cellular uptake systems and are more informative about transport than enzyme-catalyzed modifications of substrate. It should be noted, however, that studies of endothelial transport systems can yield data of similar and complementary nature to those obtainable by assay of vascular surface enzymes. Each approach has its merits and perhaps its separate uses.

Catravas and Gillis [1] have described in detail the use of [^{3}H]BPAP to measure angiotensin converting enzyme activities of lungs of rabbits and dogs (cf. Refs. 25 and 28). In brief, an injection cannula is placed with its tip in the right atrium. A collection cannula is passed along a common carotid artery such that its tip is near the aortic arch. [^{3}H]BPAP, e.g. 1–10 μCi (20 mCi/μmole), with or without carrier, and with or without indocyanine green as vascular indicator, is injected in a small volume as quickly as possible. Blood is withdrawn via the

arterial cannula, continuously and at a constant rate, for 20 sec following injection. Concurrently, the animal receives an infusion of saline at the same rate as that of blood withdrawal. Blood is collected into a fraction collector, each tube of which contains a measured volume of saline which in turn contains an inhibitor of angiotensin converting enzyme (e.g. Catravas and Gillis use captopril, 5×10^{-5} M; we use Na_2EDTA, 5×10^{-4} M).

The blood cells are packed by centrifugation, and a sample of each supernatant fraction is submitted for liquid scintillation counting. These counts, converted to dpm and corrected for volume of the blood–saline mixture, are total counts and can be taken to represent total substrate available. A second sample of each blood–saline supernatant fraction is acidified and extracted with a measured volume of toluene to separate product, [^{3}H]benzoyl-Phe, from unhydrolyzed substrate. A portion of the toluene phase is counted, and appropriate corrections are made for dpm, volume, and partition coefficients of substrate and product between acid aqueous and toluene phases. By this procedure, substrate utilization can be computed and expressed as a percentage (see Fig. 1) or as $\ln(S_0/S_0 - S_x)$. We prefer the latter

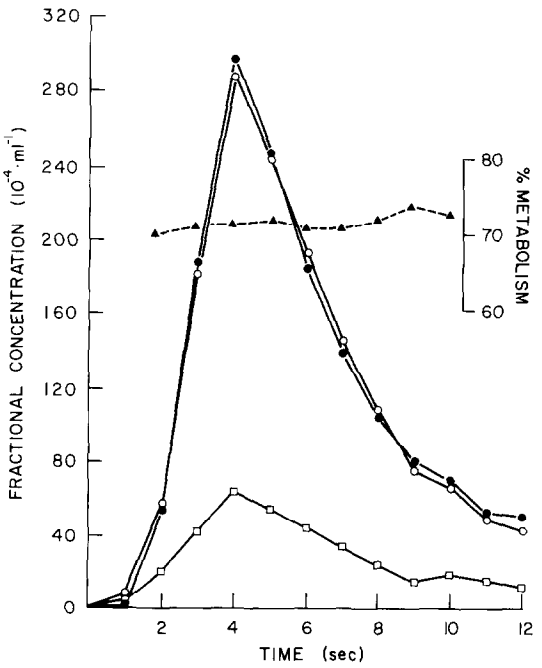

Fig. 1. Hydrolysis of [^{3}H]benz-Phe-Ala-Pro (BPAP) during a single passage through the lungs of an anesthetized rabbit. Following i.v. injection of a bolus containing indocyanine green (ICG) and [^{3}H]BPAP, aortic blood is collected and the fractional concentration of each is determined [1]. Notice that fractional concentration curves of ICG and total blood ^{3}H are coincident, implying similar volumes of distribution for each. Percent metabolism in each blood sample is calculated as [^{3}H]benz-Phe/([^{3}H]benz-Phe + unchanged [^{3}H]BPAP). Key: (●——●) indocyanine green; (○——○) total tritium; (□) unchanged [^{3}H]BPAP; and (▲---▲) percent metabolism of [^{3}H]BPAP. (Figure was provided by Dr. C. N. Gillis, Yale University, New Haven, CT.)

for reasons to be explained in later sections. Other data, obtained by the usual indicator dilution techniques, such as volume of distribution, mean transit time and cardiac output, can be computed. The natural log of fractional substrate utilization can be corrected for time (mean transit time), a maneuver that in principle yields apparent (V_{max}/K_m) (see below).

This assay approach has been used to measure activities of angiotensin converting enzyme, carboxypeptidase N, aminopeptidase A and 5′-nucleotidase [1–3, 26], and for each of these assays the pulmonary vascular bed has been used as enzyme source. However, as indicated by the substrates listed in Tables 2 and 3 and as is evident from the wide utility of indicator-dilution techniques, these and many other enzymes, if present, can be assayed in any vascular bed. With modern techniques of microvascular research, it is not inconceivable that, for example, angiotensin converting enzyme activity of a single capillary could be measured.

As may be evident, bradykinin or angiotensin I, either labeled intrinsically and at high specific radioactivity, could be used. However, the hormones are targets of attack by more than one enzyme and yield a variety of radioactive products not readily identified without long and expensive work-up. Further, either hormone exerts pharmacologic effects that can influence blood pressure, blood flow, cardiac output and mean transit time. Extrinsically labeled analogs of angiotensin I have been used [29] and are obtainable at specific radioactivities high enough to permit use of concentrations with few, if any, pharmacologic effects. However, the effort required for identification and quantification of products is not trivial.

Within the limits of studies performed to date, it appears that none of the substrates listed in Tables 1, 2 and 3 has pharmacologic effects. And, with the exception of the substrates for enkephalinase enzymes, each substrate is designed to provide simple means for isolation and quantification of product.

Enzyme kinetics in vivo

It is not known with certainty whether cell-bound enzymes *in vivo* interact with their soluble substrates in a manner understandable in terms of the Michaelis–Menten equation. However, such data as are available indicate that at least some of the enzymes (notably, angiotensin converting enzyme) are saturable. Further, substrate injected in amounts likely to achieve a concentration *in vivo* well-below its apparent K_m is hydrolyzed such that fractional substrate utilization per unit time is independent of substrate concentration itself. The foregoing is to say that tracer quantities of substrate are hydrolyzed *in vivo* as if first-order reaction kinetics were obeyed, and hydrolysis of tracer quantities of substrate can be inhibited competitively by adding increasing quantities of carrier substrate (or alternative substrate) to the injectate [1, 2, 30, 31]. Further, in the relatively few experiments in which enzyme inhibitors have been used, it appears that inhibitors that act competitively *in vitro* also act competitively *in vivo* [1]. Therefore, the remainder of the text is

based on the assumption that Michaelis–Menten kinetics are obeyed *in vivo*.

Many, if not all, reactions that occur between naturally occurring circulating substrates and cell-bound enzymes appear to proceed according to first-order enzyme kinetics. For example, angiotensin I seldom achieves concentrations in plasma much above 1×10^{-10} M [32], and bradykinin seems seldom to reach concentrations above 2×10^{-11} M [33]. Assuming that the K_m values for the reactions of bradykinin and angiotensin I with angiotensin converting enzyme are of the same magnitude *in vivo* as those measured *in vitro* (8×10^{-7} M and 3.3×10^{-5} M respectively; see Refs. 34 and 35), it seems highly likely that conditions of mixed first- and zero-order enzyme kinetics are never achieved, indeed, are never approached. In fact, the concentration of enzyme itself exceeds the concentrations of circulating bradykinin and angiotensin I. Human plasma contains the enzyme at a concentration of approximately 1×10^{-8} M, a concentration exceeded by almost 100-fold by the apparent concentration of enzyme in, for example, rat or guinea pig pulmonary capillary bed (see below).

If it is assumed that the reactions of bradykinin and angiotensin I with angiotensin converting enzyme achieve a steady state *in vivo* such that

$$K_m = [E][S]/[E:S] \tag{1}$$

(where $[E]$ is the concentration of free enzyme, $[S]$ is the concentration of unbound substrate, and $[E:S]$ is the concentration of the enzyme:substrate complex), the angiotensin I:enzyme complex should not, even in the lungs, exceed about 3×10^{-12} M, and the bradykinin:enzyme complex should not exceed about 2.5×10^{-11} M (in either case, less than 1/10,000th of available enzyme). An obvious corollary is that there should never be a physiologic competition between bradykinin and angiotensin I for a limited concentration of enzyme.

Thus, if one wishes to simulate, using a synthetic substrate, the reaction of bradykinin or angiotensin I with angiotensin converting enzyme *in vivo*, one should use the synthetic substrate at concentrations as far below its K_m as feasible. It is worth emphasizing that angiotensin converting enzyme may well have naturally occurring substrates not yet recognized and that such substrates may occur in concentrations significant in terms of their K_m values (see below for the importance of the function, $[1 + S/K_m]$). Nonetheless, for the purposes of this simulation, reaction conditions favoring first-order enzyme kinetics are to be preferred. Enzymes known to have significant concentrations of natural substrates, such as thrombin (fibrinogen), plasma kallikrein (high molecular mass kininogen), and plasmin [fibrin(ogen)], are likely to require a different approach.

Advantages of first-order enzyme kinetics. Standard textbooks of biochemistry seldom deal with first-order enzyme kinetics beyond the admonition that, for assay purposes, conditions of first-order kinetics should be avoided if avoidable. The conventional wisdom is that mixed first- and zero-order reaction kinetics should be avoided if zero-order kinetics are obtainable, and this advice is offered even with the understanding that conditions for true zero-order kinetics are not obtainable under the best of circumstances [36]. For example, when $[S]$ is ten times K_m, true initial velocity, v_0, cannot exceed $0.9\,V_{max}$. Setting aside the problem of cost of substrate, it should be noted that few enzymes can be reacted with substrate at ten times K_m without showing evidence of substrate or product inhibition. Further, when the substrate is a hormone, the pharmacologic consequences of an *in vivo* assay become severe as the concentration of the hormone is increased from, say, 1/300,000th of K_m to 1/10,000th of K_m (see above for plasma concentration of angiotensin I and its K_m of reaction with angiotensin converting enzyme).

A priori, the use of substrate at concentrations well-below K_m ($S \ll K_m$) must have the advantages of economy and elimination or marked reduction of substrate or product inhibition. There are some less widely recognized advantages. First, the first-order rate constant is V_{max}/K_m and, if enzyme is taken as a reactant, the second-order rate constant is K_c/K_m [37]. These are two values usually estimated by rather tedious graphical procedures using data obtained from several separate assays. V_{max}/K_m is, under conditions of first-order kinetics, simply a measure of fractional substrate utilization per unit time:

$$V_{max}/K_m = t^{-1}(\ln S_0/S_0 - S_x) \tag{2}$$

(where t is time of incubation, S_0 is the initial substrate concentration and S_x is the concentration of substrate used in time t). If $[E]$ is known, K_c/K_m can be computed

$$V_{max}/K_m = K_c[E]/K_m \tag{3}$$

and, if K_c/K_m is known, $[E]$ can be computed. Second, when a competitive, mixed or non-competitive inhibitor (or alternative substrate) is present, the measurement of fractional substrate utilization per unit time becomes a measure of apparent (V_{max}/K_m), which in turn is equal, under these conditions, to $V_{max}/K_m(1 + i/K_i)$ or $V_{max}/K_m(1 + S/K_m)$. Indeed, when a tracer concentration of radioactive substrate is used, with carrier substrate, to measure K_m, one in effect measures the ability of carrier substrate to inhibit the hydrolysis of radioactive substrate.

Third, when $S \ll K_m$, very little error is introduced by simplifying the Michaelis–Menten equation from

$$v_0 = V_{max}[S_0]/[S_0] + K_m \tag{4}$$

to

$$v_0 = V_{max}[S_0]/K_m \tag{5}$$

Thus, $v_0/[S_0]$ is essentially equal to V_{max}/K_m, the first-order rate constant.

Fourth, effects of competitive inhibitors are best measured under conditions of first-order enzyme kinetics, where the ratio of the velocities for the uninhibited (v_c) and inhibited (v_i) reactions becomes

$$v_c/v_i = 1 + i/K_i \tag{6}$$

Equation 6 can, by substituting $[S]$ for i and K_m for K_i, be seen as

$$v_c/v_s = 1 + [S]/K_m \tag{7}$$

where v_s represents the fractional utilization of radioactive substrate in the presence of carrier or alternative substrate, $[S]$. Thus, once one decides to use a radiolabeled substrate in tracer concentrations, virtually all further manipulations, including the use of inhibitors, carrier substrate and/or alternative substrate, can be treated in terms of first-order enzyme kinetics.

Do results of the in vivo *assays meet theory?* As is evident from Tables 1, 2 and 3, each substrate is designed and labeled such that its radioactive leaving group has the same specific radioactivity as does the substrate itself. Thus, one can express a combination of equations 2 and 3 by substituting the concentration of product (P) for the concentration of substrate used (S_x)

$$K_c[E]/K_m = t^{-1}(\ln S_0/S_0 - P) \quad (8)$$

In fact, plasma and some other body fluids contain competitive inhibitors of angiotensin converting enzyme [38], and it is probably prudent to consider

$$K_c[E]/K_m(1 + i/K_i) = t^{-1}(\ln S_0/S_0 - P) \quad (9)$$

as being the more general equation for *in vivo* assays.

It is a characteristic of these assays that the absolute concentrations of S_0 and P need not be known so long as P can be measured accurately and precisely in terms of S_0 and so long as S_0 is known to be far below K_m. Thus, there is no discernible difference if S_0 is precisely 1/100th of K_m or, for example, 1/10,078.3 of K_m if the concentration of P can be measured as a ratio with that of substrate. S_0 can be taken as 1 or 100 and P as a fraction of 1 or a percentage of 100 [39]. This is a condition seldom obtainable with chromogenic or fluorogenic substrates, substrates that could in theory be used for *in vivo* assays.

The relation of P to S_0 is highly favorable for *in vivo* assays. Once a substrate has been injected into, say, the right ventricle, it begins to undergo dilution. The injectate is transported across the lungs as a bolus of increasing length. The bolus maintains a relatively high concentration of substrate in its center and increasingly lower concentrations at either end. Assuming that the pulmonary capillary bed is the reaction vessel, a vessel whose contents are not readily sampled, and knowing that further mixing occurs in the left atrium and ventricle before blood sampling, it follows that enzyme of the reaction vessel does not react with a single concentration of substrate and that we cannot (in any event, not easily nor with certainty) know any of the substrate concentrations that were actually achieved in the reaction vessel(s). We can, however, for each collected blood sample, know P in terms of S_0, a critical ratio for first-order enzyme kinetics. Generally, efforts to approximate zero-order enzyme kinetics are more likely to confuse than elucidate.

Within limits, it appears that the theory of first-order kinetics is met when rats, guinea pigs, rabbits and dogs are injected intravenously with tracer quantities of substrates for angiotensin converting enzyme and carboxypeptidase N (a second enzyme known now to occur in abundance on the luminal surface of pulmonary endothelial cells [40, 41]). Catravas and Gillis [1] have shown that the percent utilization of [^{3}H]benzoyl-Phe-Ala-Pro during passage across the pulmonary vascular beds of rabbits and dogs is remarkably constant from the front end of the substrate bolus through its center (from first appearance of radioactivity in aortic blood through the peak of radioactivity).

Expression of substrate utilization as a percentage carries the assumption that the reaction is linear when, in fact, the relation between time of reaction and fractional substrate utilization is linear only when the latter is expressed as $\ln(S_0/S_0 - S_x)$ (e.g. see equation 2). However, even using the latter expression, our results [2, 3] tend to agree with those of Catravas and Gillis. Using anesthetized rats and guinea pigs breathing spontaneously, we see a slight increase of $\ln(S_0/S_0 - S_x)$ from the front of the bolus through its center. Thereafter, recirculation of radioactivity (both substrate and product) obscures the results. The slight increase is consistent with the known heterogeneity of blood flow through the lungs [42, 43], such that the front of a given bolus is likely to have had a shorter reaction time than subsequent portions of the bolus.

Thus, it appears that first-order reaction kinetics are obeyed and that measurement of $\ln(S_0/S_0 - S_x)$ is easily accomplished no matter what the actual concentration of substrate (when $S_0 \ll K_m$). However, as implied by the term "heterogeneity of blood flow", we cannot measure nor control time of incubation with the same accuracy and precision obtainable in *in vitro* assays. Under physiological conditions, time of incubation may well be the most important variable.

Considering again equation 9, there is the potential for at least three variables, namely time, $[E]$, and concentration of inhibitor(s) in respect to K_i. Conceivably, there are circumstances in which K_c and/or K_m may change. The enzyme activity of carboxypeptidase N, *in vitro*, is known to be enhanced by limited proteolysis by trypsin and plasmin [44]. The question arises, then: which potential variables actually vary? It may well be a long time before we can answer the question, but I suspect that not all potential variables vary all of the time (with the possible exception of time of incubation) and that a major variable in, say, diffuse lung inflammatory disease may be a trivial variable in another condition such as acute hypoxia. The low molecular mass inhibitors may vary most prominently and abruptly among those patients undergoing hemodialysis. Obviously, there is a broad scope for both basic and clinically-oriented studies.

For the short-term, there are maneuvers that one can use to eliminate or greatly reduce the effects of several variables at once. For example, one can measure relative K_c/K_m values of two substrates for one enzyme by using one substrate labeled with one isotope and the other substrate labeled with a second isotope. By injecting a mixture of the differently labeled substrates (each in tracer quantity), the variables of equation 9, namely t, $[E]$ and $(1 + i/K_i)$, can be taken as constants such that K_c/K_m of the first substrate can be ranked in terms of the K_c/K_m of the second substrate simply by comparing the respective values of $\ln(S_0/S_0 - S_x)$. We have performed a few

such experiments. Five anesthetized rats were injected intravenously with mixtures of [^{3}H]benzoyl-Phe-Ala-Pro and benzoyl-[^{14}C]Gly-His-Leu. The ratio of $\ln(S_0/S_0 - S_x)$ for BPAP and that for HHL was 10.14 ± 0.30 (S.E.M.), a value close to the ratio (8.88) of their respective K_c/K_m values measured *in vitro* (human enzyme, see Table 1). The data support the concept that angiotensin converting enzyme situated on pulmonary endothelial cells reacts with its circulating substrates according to Michaelis–Menten kinetics and appear to emphasize that the extent of hydrolysis of a given substrate during passage through the pulmonary vascular bed depends not only on enzyme abundance (effective concentration) by also on affinity. In general, whether an enzyme acts on a circulating substrate depends on what I call the rule of three A's (access, affinity and abundance): The enzyme must have access and adequate affinity for the substrate and must occur in an abundance sufficient to capitalize on affinity.

Another maneuver that can be performed to eliminate one, but no other, variable of equation 9 is to inject simultaneously two substrates for two different enzymes. This perhaps is one of the best ways of minimizing the effects of time of incubation. Sometimes this approach requires different radiolabels and sometimes not. One can inject two ^{3}H-labeled substrates, e.g. [^{3}H]BPAP and Asp-[^{3}H]benzylamide, the first for angiotensin converting enzyme and the second for aminopeptidase A, and then rely on their markedly different partition coefficients (and those of their products) between acid and alkaline aqueous solution and two carefully selected organic solvents. However, to assay converting enzyme and carboxypeptidase N activities simultaneously, each radioactive product requires extraction from acid aqueous solution; thus the respective substrates should bear different labels.

Presumably, measuring the activities of two (or more) different enzymes simultaneously would facilitate studies *in vivo* of the selectivity or specificity of drugs that act as enzyme inhibitors [12]. In addition, the procedure may well be of interest to explore for differential effects of, for example, hyperoxia or oleic acid on two or more surface enzymes. Other uses are implied by the variables that remain in equation 9 as it applies to each enzyme:substrate reaction. If, as will be discussed below, vasoconstriction and vasodilation are major determinants of effective enzyme concentrations, the phenomenon of varying enzyme concentration by varying tone might be revealed by studies of the immediate metabolic fates of two or more substrates, each targeted for a different enzyme, injected simultaneously. Vascular tone could be varied by physiologic or pharmacologic means. Because simultaneous injection of substrates has the effect of making time of incubation a constant, physiologic or pharmacologic effects on cardiac output and blood flow should be offsetting. At this time, however, there is little point in predicting whether new variables might be introduced or whether a given pharmacologic agent might cause active changes in K_c/K_m or the function of $(1 + i/K_i)$ for one enzyme but not another. Whether one can gain insight into differential effects of torque, reflection coefficients, or relative times required for enzyme:substrate reactions to achieve steady states is beyond predicting at present.

Mathematics of cylinders: Relevance to in vivo *assays*

While acknowledging that blood vessels are not perfect cylinders, it is worth considering what the mathematics of cylinders may mean to an enzyme-catalyzed reaction in which the enzyme is, perhaps statistically, uniformly distributed along the inner wall of the cylinder, and the substrate is dissolved in the fluid contained by, or flowing through, the cylinder. For true cylinders, the ratio of surface area (A) to volume (V) is

$$A/V = 2/r \tag{10}$$

where r is radius. It may be obvious but nonetheless has some instructive value to consider what the A/V ratio may mean in terms of cell-bound enzyme:soluble substrate reactions at the level of a 10 μm segment of pulmonary capillary 10 μm in diameter and an equivalent length of aorta 2.5 cm in diameter. The A/V for the capillary segment is about 2500 times greater than that of the aortic segment. In terms of potential for enzyme:substrate reactions, the disadvantage of aorta (or other vessels larger than capillaries) is made worse by considering relative rates of blood flow (about 750-fold greater in aorta). The laminar flow of blood in aorta may increase the disadvantage. Nonetheless, restricting considerations to ratios of A/V and rates of blood flow, the advantage of the capillary segment over an equivalent length of aorta is almost 2 million-fold. It could be argued that the aorta of man is many magnitudes longer than 10 μm, the segment length used for the above computations, but the counter-argument can be made that the capillary bed of adult human lung is even more extensive and is not likely to be less that 1500 miles in length. Further, there is ample reason to believe that the A/V ratio for a capillary is very much greater than 2500 times that of an equivalent length of aorta. The inner surface of a capillary segment is not smooth but is thrown up in a series of 300 to 3000 nm cylindrical projections and is pocketed by numerous caveolae that communicate with the vascular lumen. Both projections and caveolae appear to possess angiotensin converting enzyme [8, 10, 45].

As one begins to compare arterioles and venules 40 μm in diameter and 10 μm in length, with capillaries 10 μm in diameter and length, there still remain significant differences in terms of A/V and rates of blood flow. Where the rate of blood flow in the arteriole or venule is four times that of the capillary, the capillary has a 16-fold advantage over the larger vessel. And a capillary 8 μm in diameter has an A/V ratio 20% higher than that of a capillary 10 μm in diameter. Reduced rates of blood flow, if they occur in the smaller capillary, would increase the likelihood of enhanced enzyme:substrate reaction. Thus, one can appreciate the possibility that effective concentrations of vascular surface enzymes and incubation times may be varied dramatically by vessel diameter and indirectly by hormones, drugs, nerve impulses and other factors capable of influencing vessel diameter and/or rates of blood flow.

Within this context, one can readily imagine that an arteriole or venule segment having an internal diameter of 40 μm at rest plays an insignificant part in the bulk metabolism of bradykinin, angiotensin I or one of the synthetic substrates. Yet, the same vessel, contracted such that its internal diameter becomes 10–12 μm, may become an important extension of the enzyme : substrate reaction vessel. If such occurs, it would appear to have the consequence of decreasing the availability of the vasodilator bradykinin and of increasing the availability of the vasoconstrictor angiotensin II, unless there are off-setting reactions such as release of natural inhibitors or rapid cellular changes that influence effective enzyme density.

What is the concentration of angiotensin converting enzyme in a capillary?

A point generally known, but sometimes not acknowledged, is that the rate of a given enzyme-catalyzed reaction is determined not by the quantity of enzyme present but by its concentration. The common practice of expressing enzyme specific activities in, for example, units of activity per unit time per mg of protein may be time-honored but is deceptive in that it implies quantity of enzyme without specifying whether the mg of protein is dissolved in a rain drop or an ocean.

Once focus is on the importance of enzyme concentration as opposed to quantity, it becomes clear that a capillary segment need not possess much quantity of enzyme in order to have an effective enzyme concentration of 10^{-7} M or even 10^{-6} M. It also follows that one could assay many, many optimally prepared lung homogenates and still not be able to compute enzyme concentration of the intact capillary bed. Setting aside the impossible hydrodynamic requirements, it is otherwise conceivable that if the entire cardiac output were put through one pulmonary capillary segment in 1 sec, the rate of hydrolysis of substrate would be precisely the same as that in normal lungs supplied with 5×10^{11} identical capillary segments.

The lungs are remarkable in their ability to lose vascular volume without seriously affecting pulmonary artery pressure or blood flow. As much as 75% of the lungs may be removed before pulmonary blood pressure undergoes a sustained increase [46]. Thus, if it is true that one capillary segment has the same concentration of angiotensin converting enzyme as does any other capillary *and* all other capillaries, progressive embolization with, say, 40 μm spheres should cause pulmonary blood volume to fall without affecting the ability of the lungs to convert angiotensin I into angiotensin II or to inactivate bradykinin. Within the limits of our studies, this appears to be true: anesthetized rats and guinea pigs embolized with pecan pollen (~40 μm in diameter), such that their pulmonary blood volumes were reduced by about 50%, did not lose their abilities to hydrolyze [^{3}H]BPAP nor [^{3}H]benzoyl-Ala-Arg (see Table 3). Indeed, in the few experiments (three of twelve) in which embolization changed apparent rates of hydrolysis, the rates were invariably increased, possibly because of reflex vasoconstriction. In these experiments, the activities of plasma angiotensin converting enzyme and carboxypeptidase N were unaffected by embolization.

While it is not known that a capillary endothelial cell possesses the same number of molecules of angiotensin converting enzyme as does an endothelial cell of mainstem pulmonary artery, certain computations can be made if such an assumption is made. Typically, we find that post-confluent cultures of bovine pulmonary artery endothelial cells possess angiotensin converting enzyme equivalent to about 4×10^{-19} moles/cell [47]. Rabbit and rat endothelial cells appear to have about 1×10^{-18} moles/cell. If it is then assumed that a capillary segment 10 μm in diameter and 10 μm long represents one endothelial cell, an estimation can be made of the enzyme concentration. The volume of blood of the segment should be approximately 785 μm^3; hence, for rat, the estimated enzyme concentration of a pulmonary capillary segment may be estimated as 1×10^{-18} moles/785 μm^3 or about 1.3×10^{-6} M. Interestingly, this concentration is not far from that estimated from results of *in vivo* assays computed on the bases of two assumptions: (1) that the K_c/K_m for the reaction of rat endothelial cell converting enzyme and [^{3}H]BPAP is the same as that for BPAP and human serum enzyme (see Table 1), and (2) that the mean transit time of substrate through rat pulmonary capillary bed, the incubation time, is 1 sec. Using equation 8, it appears that the effective enzyme concentrations of anesthetized Sprague–Dawley rats vary from about 2×10^{-7} M to 7×10^{-7} M.

Given the assumptions, coincidence cannot be ruled out. Further, the estimations are based on computations suitable for enzymes and substrates in their soluble forms, computations that may need extensive modifications for assessing a reaction in which enzyme is in solid phase and is washed by substrate in liquid phase. Nonetheless, it is important to keep in mind that enzyme concentration, not quantity, is, of the two, the determinant of rate of catalysis. In addition, the concept that one capillary has a concentration of enzyme equal to that of all other capillaries bears importantly on the question of whether measurement of vascular surface enzymes, or transport systems, can or cannot be used to detect changes in surface area. Our experience with progressive microembolization of lungs suggests that substrate hydrolysis is virtually independent of vascular surface area. Changes in vascular surface quality may be another matter. There may be conditions of mild or chronic injury or inappropriate vascular repair in which affected capillaries do not express one or more surface enzyme activities.

Clinical implications of in vivo *assays*

Mention has already been made of possible uses of *in vivo* assays to monitor the effectiveness and selectivity of drugs that exert their effects by inhibiting enzymes. If efforts are made to avoid recirculation of drug and radioactive substrate and product, it should be possible to estimate the off-time or dissociation constant for the enzyme : inhibitor complex. Presumably, the latter might readily be accomplished using isolated lungs perfused continuously with substrate and pulsed with inhibitor. The coun-

terpart *in vivo* assay may be very much more difficult.

Gillis and colleagues [48] have reported that the pulmonary metabolism of low concentrations of [^{3}H]BPAP (and the uptake of ^{14}C-labeled serotonin) is impaired among unanesthetized rabbits exposed to normobaric hyperoxia, and impairment is evident before morphological changes become detectable by electron microscopy. Thus, the assay of vascular surface peptidase enzymes *in vivo* may have uses as a predictor of impending tissue damage.

In on-going studies of aggregate anaphylaxis in guinea pigs, a condition characterized by the formation of leukocyte and platelet emboli, deposition of intravascular and extravascular fibrin and accumulation of edema fluid (all manifest within 2 min of antigenic challenge), we examined for the rate of loss of ^{3}H-labeled substrates during a single pass through the lungs. We assumed, erroneously, that the recovery of radioactivity (and indocyanine green) in aortic blood would be decreased markedly because of intravascular fluid and solute loss and, perhaps, entrapment of segments of blood by vessels undergoing occlusion. We found, however, that there was no easily discernible loss of indocyanine green nor of ^{3}H of [^{3}H]BPAP, [^{3}H]benzoyl-Ala-Arg (for carboxypeptidase N), (D)Pro-Phe-Arg-[^{3}H]benzylamide (kallikrein) or (D)Phe-Pro-Arg-[^{3}H]benzylamide (thrombin). Five minutes after antigenic challenge, the mean transit times of substrate and intravascular marker often changed but decreased as often as they increased. Volumes of dilution fell as much as 70% and cardiac outputs invariably decreased (see Table 4). As might have been predicted from results of the embolization studies (see above), hydrolysis of substrates for angiotensin converting enzyme and carboxypeptidase N tended to rise, possibly because of vasoconstriction but in any event not because of increased activities of plasma enzymes. The plasma enzyme activities were remarkably constant. Substrates for kallikrein and thrombin were not metabolized when injected either before or 5 min after antigenic challenge. The lack of hydrolysis of the thrombin substrate after induction of anaphylaxis was surprising, especially in view of the extensive intravascular coagulation evident by both light and electron microscopy. The substrate used in our studies is highly reactive with thrombin and thrombin bound to α_2-macroglobulin.

Taken together, the data suggest that the extensive microembolism and intravascular coagulation of anaphylaxis very quickly close off substantial areas of the pulmonary vascular bed, and intravascular indicators and substrates injected early in the course of the disorder are directed through what remains of the normal vascular bed. Thus, in conditions such as anaphylaxis, the *in vivo* enzyme assay approach may be more informative in what it does not show in terms of altered substrate hydrolysis than in terms of what it does show. The failure of substrate and indocyanine green to leak through "permeability gaps" raises interesting questions about formation of edema fluid. Intense pulmonary edema occurs quickly, and there is a concomitant rise of hematocrit of aortic blood. Given that the hematocrit of blood in microvascular beds is believed to be markedly less than that of blood of large vessels [49], it is conceivable that plasma loss and enhanced vascular permeability occur largely in those occluded vessels that have lost their abilities to maintain blood flow. Similarly, it would appear that coagulation occurs without benefit of thrombin (unlikely) or occurs in vessels no longer accessible to the injected substrates. Further, either thrombin does not leak into the remaining circulation or does so at a slow rate or bound to some inhibitor other than α_2-macroglobulin.

The foregoing suggestions are based on preliminary and incomplete data and are subject to revision. More work is needed to know if an enzyme such as

Table 4. Assay of pulmonary carboxypeptidase N before and during anaphylaxis*

Measurement	Control	Anaphylaxis	P value
$\ln S_0/S_0 - S_x$	0.684 ± 0.33†	0.951 ± 0.075	<0.01
App. V_{max}/K_m‡ (min^{-1})	17.72 ± 0.78	39.25 ± 3.37	<0.001
Lung wt/kg body wt (%)	0.64 ± 0.02	0.90 ± 0.04	<0.001
Blood vol. of dilution (ml)	28.48 ± 2.28	10.64 ± 1.06	<0.001
Plasma vol. of dilution (ml)	15.89 ± 1.27	4.33 ± 0.43	<0.001
Cardiac output (ml/sec)	3.01 ± 0.27	0.745 ± 0.10	<0.001
Hematocrit (%)	44.2 ± 1.1	59.3 ± 3.2	<0.001

* [^{3}H]benzoyl-Ala-Arg, 10 μCi (25 Ci/mmole) in 25 μl of saline containing indocyanine green, was injected as a bolus into the right atrium of sensitized guinea pigs (N = 11) 30 min before and 5 min after intravenous challenge with antigen (egg albumin). Arterial blood samples, 25 μl/sample, were collected continuously for 20 sec after antigenic challenge. Differences were tested by Student's *t*-test for paired samples except for lung wt/kg body wt. In the latter case, lungs collected 10 min after induction of anaphylaxis were compared to lungs collected from sensitized guinea pigs not challenged with antigen. In these experiments, plasma carboxypeptidase N was essentially unchanged by anaphylaxis (app. V_{max}/K_m 13.1 min^{-1} before and 11.81 min^{-1} after induction of anaphylaxis).

† Means ± S.E.M.

‡ App. V_{max}/K_m is $\ln S_0/S_0 - S_x$ corrected for mean transit time.

thrombin or plasma kallikrein, unmeasurable by the *in vivo* assay procedure performed 5 min after induction of anaphylaxis, becomes measurable at later time intervals, perhaps in the form of its complex with α_2-macroglobulin. The answer may well bear on the potential, or lack of potential, of the *in vivo* assay approach for the diagnosis of conditions such as disseminated intravascular coagulation or extending venous thrombi.

What the *in vivo* assay approach may do is to allow distinction between conditions in which vascular damage leads to or is followed by vascular occlusion and those in which vascular damage is sustained but does not impede blood flow. Conceivably, the ability to distinguish one from the other would provide early assistance in the differential diagnosis of cardiogenic and non-cardiogenic pulmonary edema. And, as discussed above, the data of Dobuler *et al.* [48] on the effects of normobaric hyperoxia appear to indicate that there is a period in which functional injury is evident and morphologic injury is not.

Until studies are begun to examine for the immediate fates of radiolabeled substrates in vascular beds other than that of lung, one cannot be certain that there are clinical uses of sufficient importance to justify the catheterization procedures necessary for *in vivo* assays. For research purposes, however, it may be worthwhile to examine for the different means of processing of a substrate for angiotensin converting enzyme or glandular kallikrein by enzymes of the renal vascular bed and enzymes of the proximal tubule [50, 51]. Other experiments that come to mind are those in which, for example, kallikrein activity is measured in pancreas and salivary gland to explore for correlations of enzyme secretion and functional vasodilation. It would appear that further exploration of potential uses of the assay of enzymes *in vivo* is limited primarily by imagination and effort.

Finally, the concept of assays of total body enzyme activity deserves comment. The urine of a rat injected intravenously with [^{3}H]BPAP contains [^{3}H]benzoyl-Phe and no other ^{3}H-labeled product. Given that tracer concentrations of [^{3}H]BPAP are metabolized almost completely during a single pass through the lungs, there is little reason to believe that the quantity or concentration of [^{3}H]benzoyl-Phe in urine provides any useful information on enzyme activity of other organs. However, the urine of a rat injected with an angiotensin converting enzyme substrate of good selectivity but low affinity, e.g. [^{3}H]hippuryl-Gly-Gly, contains both substrate and product ([^{3}H]hippuric acid). The product can be assumed to be excreted by the organic acid transport system and, if the substrate is filtered but not excreted via the active transport system, it is likely that the relative concentrations of product (P) and substrate (S_0) in voided urine cannot be used in equation 9 to compute total body enzyme activity. However, the fact that both substrate and product appear in voided urine argues in favor of the development of a non-invasive means of monitoring the effectiveness of inhibitors of angiotensin converting enzyme, say in phase I clinical trials. Further, by changing the radioisotope from ^{3}H to ^{123}I, one could, in principle, make the indicator-dilution measurements enabled by, for example, ^{123}I- or ^{99}Tc-labeled albumin, measure "total body" enzyme activity and, in the control study, perform a renogram. If, in fact, the substrate is filtered and not excreted by the organic acid active transport system, the rate of development of the renogram, in hydrated patients, may be indicative of the degree of inhibition of angiotensin converting enzyme. Uses are implied for the diagnostic work-up of patients suspected of having renal or renovascular hypertension, patients who tend to respond well to treatment with inhibitors of angiotensin converting enzyme [52].

Concluding comments

We still know little of what kinds of information can be obtained by assaying enzyme activities *in vivo*. Yet, it is difficult to avoid the conclusion that this assay technology provides a basis for understanding aspects of biochemistry in physiological terms and aspects of physiology in biochemical terms. The technology may help define ordered and disordered intravascular events, presumably at the cellular and molecular levels. The simplicity of the assay technology is such that it can be used clinically with equipment already in place in most intensive care units and departments of nuclear medicine.

Drugs that exert their effects, at least in part, by inhibiting enzymes have been available for many years. Notable among these are inhibitors of acetylcholine esterase, carbonic anhydrase, cyclooxygenase and dihydrofolate reductase. The new orally effective inhibitors of angiotensin converting enzyme represent a new advance in drug design [53], an advance that has quickened the pace to find other enzyme inhibitors with therapeutic potential. There is every prospect that many of the drugs that will be marketed in the 1980s and 1990s will be drugs deliberately designed to inhibit one or more enzymes. Prototype renin inhibitors have promise as antihypertensives [54], and synthetic oligopeptides capable of inhibiting thrombin have been tested successfully as anticoagulants [55]. If, in fact, the metastatic spread of certain cancers requires special expression of plasminogen activator activity [56], one can envision wide use of inhibitors of certain protease enzymes among patients with the susceptible malignancies. The implied role of proteases in tumor angiogenesis provides yet another worthy target [57].

Given the outlook for the further development of drugs that act as enzyme inhibitors, it does not seem too early to begin to find out what the *in vivo* enzyme activity assay technology might mean to future pharmacological practice. And given the ability of the technology to monitor certain events moment-to-moment, it seems not too early to ask: What can we gain by defining pathogenesis of a given disorder in terms of seconds and minutes as opposed to hours and days? And finally, can the assay of enzymes *in vivo* give us new insights into the modes of action of drugs already in use?

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