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DESIGN OF ENZYMATICALLY RECONVERTIBLE PRODRUGS: AMINO ACID DERIVATIVES OF ASPIRIN

BY

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DESIGN OF ENZYMATICALLY RECONVERTIBLE PRODRUGS:
AMINO ACID DERIVATIVES OF ASPIRIN

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Gordon L. Amidon)

The objective of this research project was to develop and test a rationale for prodrug design utilizing the digestive enzymes of the gastrointestinal tract as the reconversion sites. Central to the prodrug design is the requirement that the prodrug be reconverted *in vivo* rapidly or at a controlled rate to the parent compound. Most of the prodrugs designed and tested so far depended on solution hydrolysis for reconversion. The enormous potential of the hydrolase enzymes serving as very efficient reconversion sites has not yet been explored. From the knowledge of substrate specificities of the hydrolase enzymes one can suggest specific amino acid derivatives of drugs with free functional groups, and thus ensure *in vivo* reconversion. These derivatives may be very stable towards solution hydrolysis. Thus a stable dosage form may be formulated and still a rapid enzymatic reconversion *in vivo* may be achieved.

The prodrugs synthesized were aspirin-phenylalanine ethyl ester, aspirin-phenylalanine amide and aspirin-phenyllactic ethyl ester. They were so designed that after the initial cleavage of the terminal ethyl ester or amide group by α-chymotrypsin, carboxypeptidase will hydrolyze the

remaining amide or ester linkage to regenerate aspirin *in vivo*. The free carboxyl group of aspirin has been indicated to be responsible for very low half life in solution and for gastric irritation and bleeding. Since, in these prodrugs the carboxyl groups remain masked until they reach the intestine, the stability problem was expected to be overcome and the gastric irritation problem minimized. Because of the expected low solubility of these derivatives suspension formulation could be made.

In vitro enzymatic hydrolysis kinetics were measured by pH-stat titration method. Aspirin-phenylalanine ethyl ester showed highest rate of hydrolysis in presence of α-chymotrypsin and aspirin-phenyllactic acid was most rapidly hydrolyzed by carboxypeptidase A. Aspirin-phenyllactic acid showed significant competitive product inhibition towards carboxypeptidase A. The enzyme kinetic parameters obtained were comparable to other substrates of the enzymes considered. Thus, one of the ideal conditions of the prodrug design that the rate of reconversion be independent of the exact structure of the drug molecule itself was closely met and the reconversion rates were within the range of expectation based on prior knowledge about the catalytic behavior of the enzymes considered.

Hydrolytic pathway of aspirin-phenylalanine ethyl ester in presence of simulated intestinal fluid was determined and

the regeneration of aspirin demonstrated by TLC experiments. Shelf life of a 600 mg/5 ml (equivalent of 300 mg/5 ml of aspirin suspension) suspension ofthe prodrug at 5.6 was calculated to be approximately four years. The prodrug was found to be adequately absorbed from rat intestine *in situ* at pH 7.5 as evidenced by the loss of the prodrug on perfusion through the intestine. A laminar flow model was found to best fit the data. Dissolution rate of the prodrug was significantly enhanced by the presence of α-chymotrypsin in the medium. A simple diffusion film model was found to fit the data at low enzyme concentrations, but showed significant deviation at high enzyme concentrations.

The results show the viability of this approach of making prodrugs. The pharmaceutical property of drugs can be altered in almost any direction and obtain very high *in vitro* stability and *in vivo* lability by using this approach. The reconversion rates may be predicted beforehand and since the amino acids are indigenous to the body, these pro-groups can be expected to impart no new toxicity to the drug. This approach would enable pharmaceutical scientists to obtain improved biopharmaceutical properties of drugs by making appropriate selection of the amino acid residues.

APPROVED: Gordon L. Amidon

Gordon L. Amidon, Assoc. Prof.

DATE: 5/1/79

To my parents

without whose love and sacrifices

this work would not have been possible.

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I. INTRODUCTION

A. General Considerations

Nearly all therapeutic agents possess various physicochemical and biochemical properties, some of which are desirable and others undesirable. The objective of pharmaceutical research is to provide safe, effective and convenient dosage forms for the administration of therapeutic agents. Typically this starts with a given therapeutic agent and then the numerous formulation, manufacturing, physical and chemical stability, and convenience variables are taken into consideration.

Improvement of efficacy of a given drug can be accomplished by biological, physical or chemical means. The biological approach entails varying the route of administration. Examples include the injectable route to optimize onset of action, maximize bioavailability and eliminate gastric irritation and stomach acid catalyzed drug degradation. Versatility is severely limited when utilizing the biological approach, alternative routes of administration are frequently unavailable and are always less convenient than oral administration.

A greater degree of flexibility of drug modification is offered by the physical approach, commonly referred to as dosage form design. The elements and philosophy of this

approach have been discussed (1-8). However, the highest degree of flexibility in altering drug efficacy is offered by the chemical approach.

Drug derivatization has been long recognized as an important means of producing better pharmaceuticals. Bayer, as far back as 1899, synthesized the drug aspirin in an attempt to improve the therapeutic activity of salicylic acid. Since then, literally thousands of drug derivatives have been synthesized and tested. These derivatives can be broadly classified into two categories: analogs or pro-drugs. Irreversible derivatives or analogs are usually synthesized for the purpose of finding a similar, new, biologically active entity possessing increased potency, a broader spectrum of activity, less side effects or some other desirable property not possessed by the parent compound. A reversible drug derivative utilizes a chemical moiety of proven biological activity (the parent molecule) and attempts to obtain increased efficacy of the drug entity by overcoming some of the undesirable aspects of the parent drug while maintaining the activity of the parent compound. The derivatives can be added at any functionality as long as reconversion (chemical and/or enzymatic) occurs in the body.

Any number of inherent disadvantages may preclude the use of the parent drug molecule in clinical practice. Among those properties considered disadvantageous in a drug

molecule are bitterness or tartness, offensive odor, gastric or intestinal upset and irritation, pain on injection, poor absorption, slow or rapid metabolism, poor stability in the bulk state, the dosage form, or in vivo (i.e., gastric instability). In many cases undesirable properties in a drug molecule cannot be overcome by conventional pharmaceutical formulation or route of administration changes. So the method of choice becomes reversible derivative formation. Reversible derivatives have also been termed prodrugs (9-13). In the intelligent design of prodrugs it is necessary to consider two questions:

1. What structural modifications of the parent molecule are necessary to reduce or eliminate the particular undesirable effect?
2. What conditions are available in vivo (enzymes, pH, etc.) to regenerate the parent molecule from the prodrug?

The first question requires knowledge of structure-activity relationships as they apply to elimination of these undesirable properties. The second question is dependent on a rather extensive chemical or biochemical knowledge.

B. Enzymes as Reconversion Sites

Central to prodrug design is in vivo reconversion. The drug derivative must rapidly or at a controlled rate be

reconverted to the active therapeutic agent *in vivo* while at the same time be sufficiently stable *in vitro* such that a stable pharmaceutical product can be developed. It has been suggested (37) that the hydrolytic enzymes could serve as an important class of reconversion sites such that *in vitro* stability and *in vivo* lability could be effected. The extensive scientific knowledge obtained through biochemical, physical organic and more recently x-ray crystallographic studies on the hydrolase enzymes is such that one can now suggest that specific functionalities be introduced into therapeutic agents to obtain *in vitro* stability and *in vivo* lability utilizing one or more enzymes of the hydrolase class as reconversion sites. Since this research project was focussed on chymotrypsin and carboxypeptidase A, these two enzymes are reviewed below. Other enzymes which can be potentially used as reconversion sites have been reviewed (37).

1. Chymotrypsin

Chymotrypsin is an endopeptidase involved in protein digestion. Its inactive precursor, chymotrypsinogen, is produced in the pancreas and converted to α-chymotrypsin (α-CT) in the duodenum by tryptic and subsequently by chymotryptic action.

The molecular weight of α-CT is approximately 25,000 g/

mole. The molecule consists of three peptide chains and a total of 241 amino acid residues whose sequence is completely known (14-18). Based on x-ray diffraction studies (17,19,20), atomic coordinates for the entire molecule have been published (21). Blow (18) has reviewed the numerous x-ray studies which focussed on the active site region of the native enzyme as well as enzyme-virtual substrate and enzyme-inhibitor complexes.

α-Chymotrypsin catalyzes the hydrolysis of a rather broad spectrum of peptides, amides and esters. An absolute requirement of substrate candidates is that the amino acid residue be of L- configuration. Substrates having an aromatic residue (e.g., tryptophan, phenylalanine, tyrosine) are hydrolyzed at appreciable rates (22), but the hydrolysis of substrates having hydrophobic residues (e.g., leucine, methionine) is also catalyzed (23). Simple esters are often very good substrates. The enzyme is inhibited by a variety of aromatic compounds, the aromatic amino acids, and the D-configuration of many substrates.

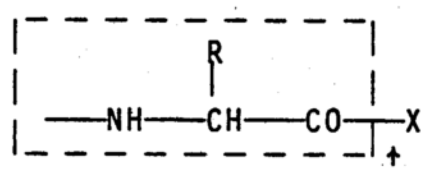
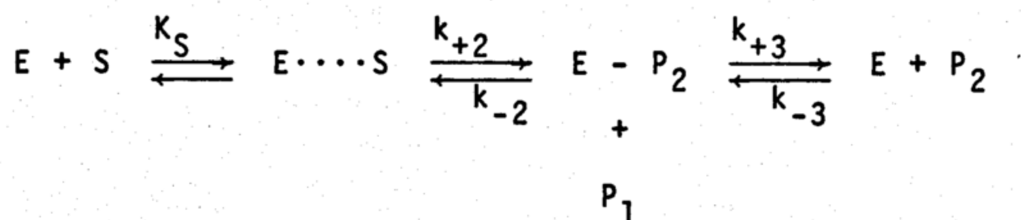


FIGURE 1. α-CT catalyzes the hydrolysis of the indicated bond. -X- represents either -O- or -NH.

Kinetic studies have revealed a bell-shaped relationship between the overall reaction rate and pH, maximum activity occurring near pH 8 (24). The following kinetic scheme (25,26) seems to apply to α-CT catalyzed hydrolysis.



$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} E\cdots S \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E - P_2 + P_1 \underset{k_{-3}}{\overset{k_{+3}}{\rightleftharpoons}} E + P_2$$

The three reaction steps represent the formation of the non-covalent (Michaelis) enzyme-substrate complex, the formation of an acyl enzyme with the loss of the leaving group, and the deacylation step. For nonspecific substrates with good leaving groups and for specific ester substrates, the deacylation step is rate limiting, whereas, for specific amides the rate is determined by the acylation step.

2. Carboxypeptidase A

Carboxypeptidase A (CPA) is a pancreatic hydrolase whose primary function is in protein digestion. It is produced as an inactive precursor which is converted to the active form by the action of trypsin.

Having a molecular weight of approximately 34,600 g/mole the active enzyme consists of a single chain of 307 amino acid residues and one zinc ion. The amino acid

sequence is almost completely known (27). The zinc ion has been shown to be essential for catalytic activity even though its removal does not greatly affect the structure of the enzyme (28). The enzyme remains active after the Zn^{++} ion has been replaced by Fe^{++}, Mn^{++}, Co^{++}, or Ni^{++} (29,30) and under certain conditions, The Co-CPA is a more efficient catalyst than Zn-CPA (30).

The primary action of CPA is, as shown in Figure 2, that of a C-terminal exopeptidase. The enzyme is also a good esterase. There are two absolute requirements for potential CPA substrates (31,32); the terminal carboxyl group must be free and the C-terminal residue must be of L-configuration. CPA will catalyze the hydrolysis of almost any peptide meeting the above requirements although substrates wherein R is a branched aliphatic or, optimally, an

$$-NH-\underset{}{\overset{R_2}{\overset{|}{C}H}}-CO-NH-\overset{R_1}{\overset{|}{C}H}-CO\overset{}{\underset{\uparrow}{-}}NH-\overset{R}{\overset{|}{C}H}-C\langle^{O}_{O}\ -$$

FIGURE 2. CPA catalyzes the hydrolysis of the indicated peptide bond.

aromatic group are significantly favored. The penultimate residue may be merely an acyl- group, although the rate of catalysis is enhanced if R_1 is an aromatic group.

Kinetic studies on CPA have revealed remarkable

complexities in its behavior towards dipeptides and ester substrates. CPA exhibits substrate and product activation and inhibition and is also inhibited by certain buffer ions (33). Typical pH-rate profiles exhibit maxima around pH 7.3 (32). However, the overall rate of reaction increases markedly and the aforementioned pH dependence disappears as the ionic strength of the reaction mixture is increased to 0.5 M (34).

It is interesting to contrast the catalytic mechanism of α-chymotrypsin with that of carboxypeptidase A. In the latter case, the carbonyl bond of the scissile peptide bond is polarized by a metal ion, making the carbon atom more susceptible to nucleophilic attack. In α-chymotrypsin the complementary approach is taken; the negative charge, and hence, the nucleophilicity of the attacking Ser 195 oxygen is significantly increased by the charge relay system (17).

3. Kinetic considerations

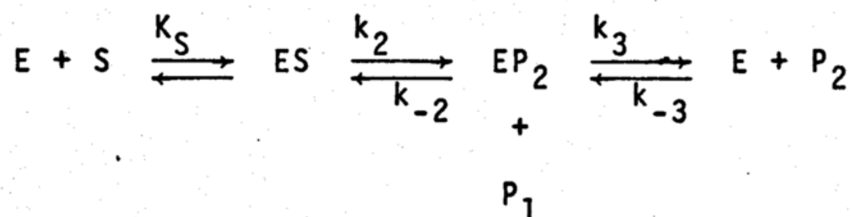
The general form of the Henri-Michaelis-Menten equation (35) is

$$v = \frac{V_{max}}{1 + K_m/[S]}$$

where v is the velocity at a given substrate concentration, [S], and V_{max} and K_m are complex kinetic constants which

depend on the reaction mechanism, pH, temperature, and concentration of other species such as metal ions, inhibitors, etc.

For the kinetic scheme shown below



we can write

$$v = \frac{k_{cat}E_o}{1 + K_m/[S]}$$

assuming $E_o << [S]$, where

$$k_{cat} = \frac{k_2k_3}{k_2 + k_3}$$

$$K_m = \frac{k_3}{k_2 + k_3}K_S$$

and E_o = enzyme concentration. Under saturation conditions (i.e., when $[S] >> K_m$)

$$v = V_{max} = k_{cat}E_o.$$

Hence, k_{cat}, often referred to as the turnover number, is the maximal number of moles of substrate hydrolyzed per mole of enzyme per unit time.

A very rough estimate of possible reconversion rates

can be made as follows. A reasonable range for enzyme concentrations *in vivo* is 10^{-4} M to 10^{-8} M (36). A reasonable dose range is 10^{-2} to 10^{-5} moles of drug and a reasonable serum or tissue concentration is 10^{-3} to 10^{-8} M. Table 1 (37) presents the estimated range of reconversion rates for the two cases, i) [S] >> K_m and ii) K_m >> [S]. From the table it is clear that very rapid reconversion rates are possible.

As a second point of comparison we can qualitatively compare an enzymatic rate with the corresponding rate of reaction in an aqueous solution. Bender *et al*. (38) have given the estimated rate constant for the hydroxide ion catalyzed hydrolysis of N-acetyl-L-tryptophan amide as 3×10^{-4} $M^{-1}sec^{-1}$. Converting this to an intramolecular imidazole catalyzed reaction results in an estimated first order rate constant of 4.8×10^{-9} sec^{-1}. The enzyme (α-CT) catalyzed rate constant is 4.4×10^{-2} sec^{-1} (38). Hence, the enzyme increased the hydrolysis rate by about seven orders of magnitude.

These comparisons emphasize the obvious interest in designing prodrugs with rapid enzymatic reconversion rates. Drug derivatives can be prepared which exhibit long shelf-lives in normal dosage forms, but are rapidly converted to the active drug *in vivo*.

TABLE 1. Estimated range of *in vivo* reconversion rates ($E_o = 10^{-6}$ M, $k_{cat} = 100\ sec^{-1}$, $K_m = 1$ M).

Maximal Rate (zero order)	Minimal Rate (first order)
$v = k_{cat}E_o$	$v = \frac{k_{cat}E_oS}{K_m}$
$= 100 \cdot 10^{-6}$	$= 10^2 \cdot 10^{-6} \cdot S$
$= 10^{-4}$ M/sec	
$k_0 = 10^{-4}$ M/sec	$k_1 = 10^{-4}\ sec^{-1}$
$t^{1/2} = 5 \times 10^{-5}$ sec ($S_o = 10^{-8}$ M)	$t^{1/2} = 6.93 \times 10^3$ sec
$= 5$ sec ($S_o = 10^{-3}$ M)	$= 2$ hrs.

C. Prodrug Considerations

On the basis of the known specificities of the enzymes reviewed (37) a general rationale for modification of drug physical properties can be developed. Given that the drug, D-X, has a free carboxyl, amino or hydroxyl group (X), corresponding esters or amides of amino acids can be made so as to alter the physical properties in almost any desired direction from that of the parent drug, with one or more of the hydrolase enzymes serving as the in vivo reconversion site(s).

The diverse properties of the various amino acid residues combined with the fact that they are nontoxic, except possibly in extreme cases, gives this approach wide applicability. Classifying the amino acids as non-polar, polar, acidic and basic, a given drug molecule may be made more or less polar, more or less soluble in a given solvent, the acid/base properties may be altered or completely changed (i.e., converted from acidic to basic drug or vice versa), may be converted from a neutral compound to an ionic (acidic or basic) compound or converted from an ionic to a neutral compound. Thus, great flexibility is afforded the pharmaceutical scientist in modifying physical properties of drugs using this rationale. Tables 2-4 briefly summarize some of these possibilities. In general, for compounds in Tables 2 and 3, α-chymotrypsin, trypsin, elastase or an amino

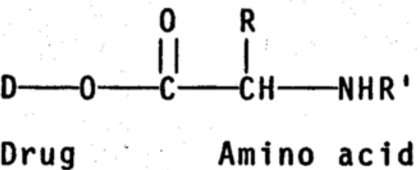
peptidase could serve as reconversion site. For compounds in Table 4, a carboxypeptidase or an initial cleavage of the ester group ($-OC_2H_5$) by α-chymotrypsin/trypsin/elastase followed by a carboxypeptidase would effect the reconversion.

A recent example of a prodrug approach utilizing lipases as target sites for reconversion is the study by Baugess *et al*. (39). The acetate and dodecanoate esters of acetaminophen were prepared and shown to be substrates for lipases. The acetate ester was completely hydrolyzed in 15 minutes *in vitro* and its pharmacokinetics in dogs were indistinguishable from that of the parent drug.

D. Intestinal Cell Wall Metabolism and Absorption

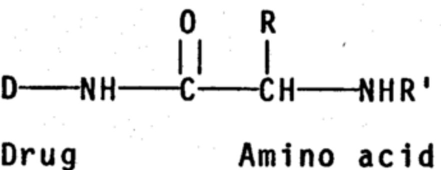
While for all practical purposes only amino acids enter the portal circulation following protein digestion and absorption, it has been demonstrated (45-47) that intraluminal hydrolysis accounts for only a fraction of the total protein hydrolyzed. Extensive recent literature (40-44) indicates that in addition to the luminal enzymes, enzymes localized on the brush border of the intestinal wall play an important role in the protein digestion and absorption processes. Chemical and histochemical studies have identified a number of brush border enzymes, including alkaline phosphatase, amino and carboxy- peptidases and serine proteinases. In one suggested mechanism for the digestion and

TABLE 2. Possible changes in physical properties of drugs with free -OH groups.



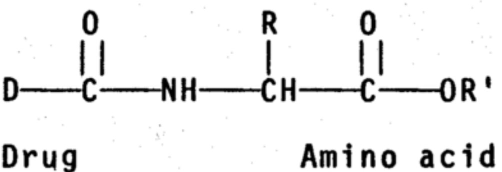
Drug	Amino Acid	R'	Change in Physical Properties
Non-polar	polar/acidic/basic	-H, $-COCH_3$	more polar/ionic
Polar	non-polar	$-COCH_3$	less polar
Liquid at room temp.	polar/non-polar	-H	to a salt
Low aqueous solubility	acidic/basic	-H, $-COCH_3$	to acid or base (salt)

TABLE 3. Possible changes in physical properties of drugs with free $-NH_2$ groups.



Drug	Amino Acid	R'	Change in Physical Properties
Weak base	non-polar	$-COCH_3$	to a neutral compound
Weak base	acidic	$-COCH_3$	to an acidic compound
Zwitterionic	polar/non-polar	$-COCH_3$	to an ionic (acidic) compound
Weak base	polar/non-polar	-H	change in pK_a

TABLE 4. Possible changes in physical properties of drugs with free -COOH groups.



Drug	Amino Acid	R'	Change in Physical Properties
Weak acid	polar/non-polar	-H	change in pK_a
Weak acid	basic	-H	Zwitterionic compound
Weak acid	basic	$-OC_2H_5$	to a basic compound
Zwitterionic	polar/non-polar	$-OC_2H_5$	to a basic compound

absorption, the proteins are broken down into oligopeptides by the gastric and intestinal luminal enzymes. The small peptides then diffuse to the brush border region where hydrolysis into amino acids are completed along with uptake into the mucosal cells.

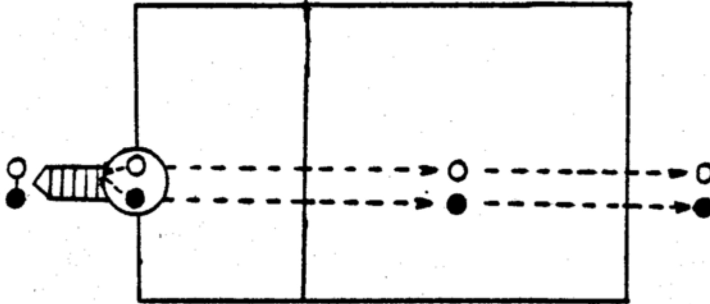
Available evidence suggests that in addition to the mechanism that invokes surface hydrolysis followed by absorption of liberated free amino acids by conventional amino acid carrier, dipeptides and tripeptides are transported across the brush border membrane, bypassing the brush border peptidases, followed by hydrolysis by the cytoplasmic enzymes. Still, a third suggested mechanism postulates that membrane hydrolysis of dipeptides and tripeptides is coupled with the uptake of the amino acids by carrier mechanisms. These mechanisms are schematically represented in Figure 3.

Therefore, in addition to the luminal enzymes, the brush border enzymes may serve as additional reconversion sites for amino acid derivatized prodrugs. The possible steps involved in reconversion and absorption of these prodrugs are shown in Figure 4.

E. Effect of Enzymatic Reactions on the Dissolution Rate of Prodrugs

After ingestion, a solid dosage form disintegrates into primary particles which then dissolves in the fluid in the

I



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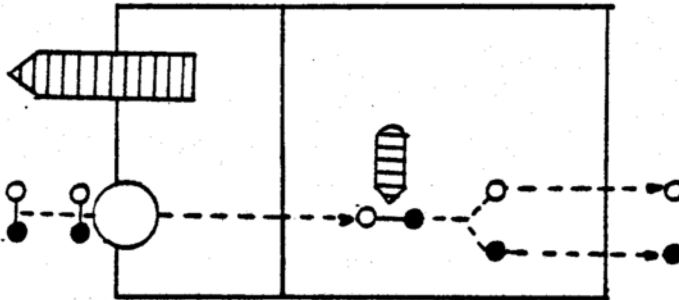

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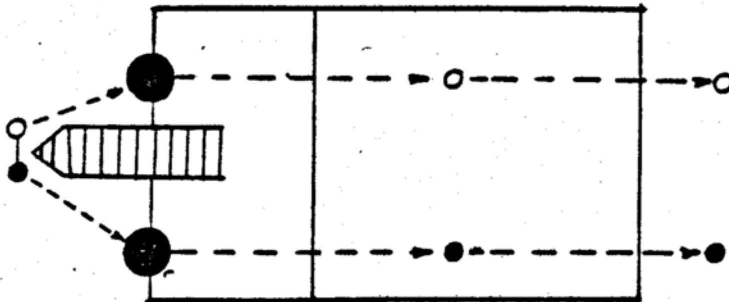
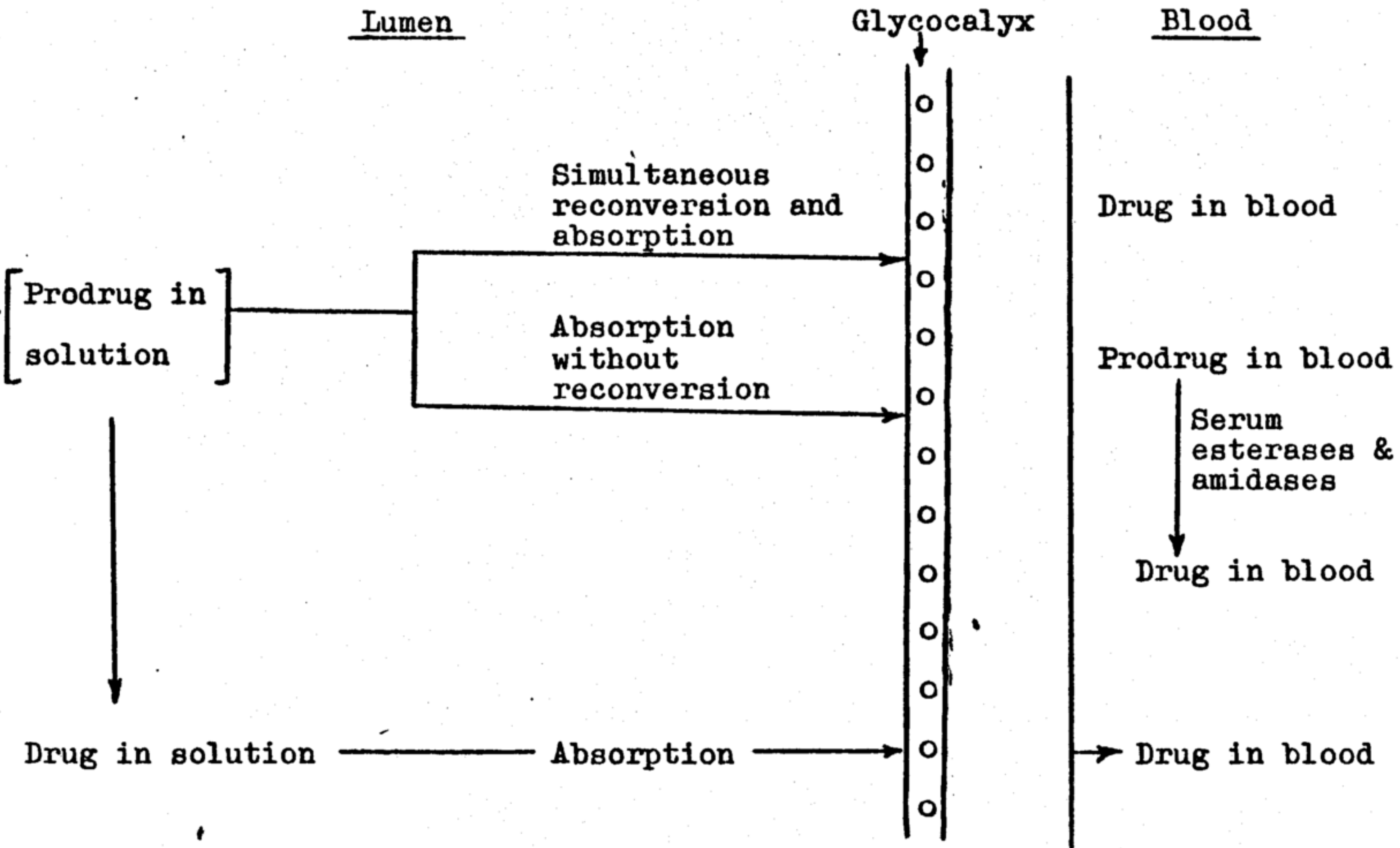


Lumen Membrane Cytoplasm Portal vein


Lumen
Glycocalyx
Blood
Prodrug in solution
Simultaneous reconversion and absorption
Absorption without reconversion
Drug in blood
Prodrug in blood
Serum esterases & amidases
Drug in blood
Drug in solution
Absorption
Drug in blood

intestinal tract before the drug can be absorbed into the blood to elicit the therapeutic activity. More and more importance is being placed on the dissolution rate because of its correlation with bioavailability (49,50).

While a number of apparatus are available for dissolution (51), the rotating disc method has some advantages. The surface area of the disc remains constant during the experiment and a theoretical expression for the intrinsic dissolution rate constant as a function of angular velocity, viscosity and diffusivity of the solute in the medium, has been developed by Levich (52). In the rotating disc method the solid is compressed into a pellet and is contained in a die which is rotated in the fluid and the bulk solute concentration is determined as a function of time.

Based on the principles of convective diffusion, Levich (52) obtained the fundamental equation

$$\frac{\partial c}{\partial t} = -\mathrm{div}\ J$$

At steady state and under appropriate boundary conditions this equation leads to the Levich equation (52);

$$J = 0.62D^{2/3}\nu^{-1/6}\omega^{1/2}c_o$$

where J = flux/area

D = diffusion coefficient of the solute

ν = Stokes viscosity

ω = angular velocity

C_o = solubility

If the dissolved solute undergoes any homogeneous chemical reaction in the dissolution medium, the equation for convective diffusion must be supplemented by a term which accounts for the disappearance of the particles within the bulk of the liquid. The convective diffusion equation can now be written as

$$\frac{\partial c}{\partial t} = -\text{div } J - Q_o$$

If the reaction is enzymatic then

$$Q_o = \frac{k_{cat}E_oC}{K_m + C}$$

This is a nonlinear, second order differential equation which can only be solved numerically. Alternately, a pseudofirst order rate constant, k_{obs}, can be calculated for the enzymatic reaction based on the pH stat recordings for the *in vitro* enzyme kinetic experiments. Then, $Q_o = k_{obs}C_o$ and an analytical solution can be obtained.

It has been shown (78,79) that for a homogeneous

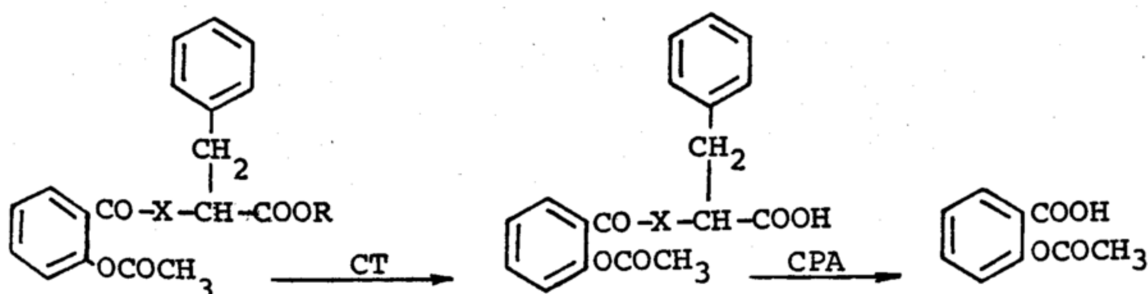
chemical reaction to have a significant effect on dissolution rate, the chemical reaction rate has to be very rapid. The inherent advantage of making enzyme directed prodrugs becomes evident here. The prodrug may be stable in the formulation, but still may have the dissolution rate enhancement effect of the enzymatic reactions in the intestine, whereas, the prodrugs which depend on the solution hydrolysis for the reconversion are likely to be much less stable in the dosage form.

II. STATEMENT OF THE PROBLEM

The objective of this research project was to develop and test a rationale for prodrug design using current knowledge of the hydrolase enzyme specificities. By combining the current understanding of the enzyme specificities with the need for drugs with more desirable pharmaceutical properties, new and novel drug derivatives can be suggested.

The specific problem addressed in this project was to make prodrugs of aspirin such that a stable suspension can be made and potentially gastric irritation and bleeding problems can be avoided.

The -COOH group has been shown to be responsible for very short half-life of aspirin in solution (55-57). The prodrugs whose structures are given in Figure 5 were designed such that the -COOH groups are masked and that after an initial cleavage of the terminal ester or amide group by chymotrypsin, carboxypeptidase A would effect the reconversion to aspirin.



where X = -NH, or -O- and R = $-OC_2H_5$ or $-NH_2$. These prodrugs would be less soluble in water than aspirin and because of the blocking of the -COOH groups, would be expected to be more stable in solution. Consequently, it may be possible to formulate pharmaceutically stable suspension of these aspirin prodrugs.

Since aspirin induced gastric irritation and bleeding has been attributed to the -COOH group (53,54) and to the adherence of insoluble particles of aspirin to the gastric mucosa. The proposed prodrugs may be effective in overcoming the side effects since the -COOH group remains masked until the prodrug reaches the intestine.

Specific aims of this project were to:

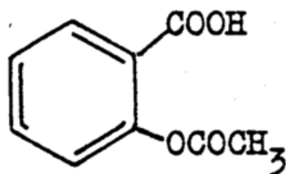
i) Synthesize L-phenylalanine ethyl ester, L-phenylalanine amide and L-phenyl lactic ethyl ester derivatives of aspirin. The structures are shown in Figure 5.

ii) Determine *in vitro* enzymatic hydrolysis rates of the synthesized prodrugs in presence of α-chymotrypsin and carboxypeptidase A.

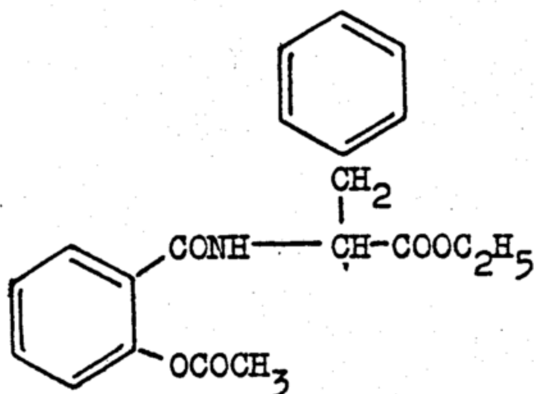
iii) Determine the pathway of the prodrug reconversion in simulated intestinal fluid, USP.

iv) Determine stability of the prodrugs in aqueous solution.

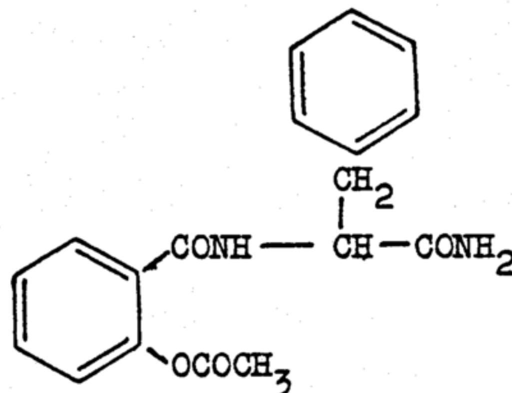
v) Study the absorption *in situ* of the prodrugs from



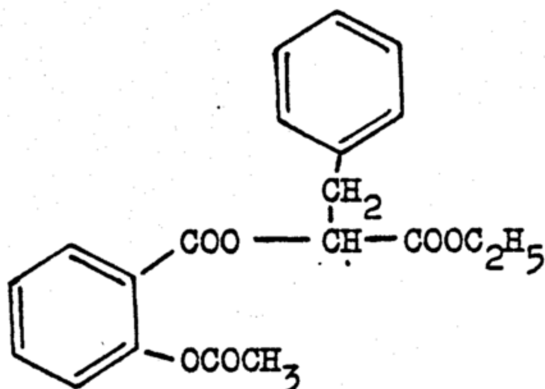
ASPIRIN



Aspirin phenylalanine ethyl ester



Aspirin phenylalanine amide



Aspirin phenyllactic ethyl ester

Fig.5. Structures of aspirin and the prodrugs.

rat intestine.

vi) Study the effect of enzymatic reaction on the dissolution rate of prodrugs using rotating disc apparatus and to develop a mathematical model to describe the intrinsic dissolution in the presence of a homogeneous enzymatic reaction.

III. METHODS

A. Synthesis

1. Materials

Commercial reagents of the highest available quality were obtained from the following sources: L(-)phenylalanine, Boc-ON, dicyclohexylcarbodiimide, triethylamine, ethyl chloroformate, L(-)-phenyllactic acid, from Aldrich Chemical Company; aspirin was obtained from Monsanto Company.

2. Phenylalanine ethyl ester, hydrochloride (I)

Thionyl chloride (10 ml) was added to ethanol (40 ml) portionwise in cold. Phenylalanine (2.3 g) was added to the mixture and refluxed for two hours. A clear solution is formed after about one hour, but in order to assure the completion of the reaction refluxing was continued for one more hour. Then the reaction mixture was cooled, the solvent evaporated under reduced pressure. The flaky solid residue was washed with cold anhydrous ether and was then purified by selective precipitation from ethanol ether mixture. m.p. 154-156°. Yield 2.9 gm.

3. Aspirin-phenylalanine ethyl ester

Aspirin (II) (1.8 g, 10 mM) was dissolved in 35 ml of dry methylene chloride and dicyclohexylcarbodiimide

(1 gm, 5 mM) was added to it at room temperature. The mixture was kept under stirring for two hours. The precipitated dicyclohexylurea was filtered off.

To the filtrate was added a solution of a mixture of phenylalanine ethyl ester, HCl (1.15 g, 5 mM) and triethylamine (0.5 gm, 5 mM) dissolved in 10 ml of methylene chloride. The mixture was kept under stirring overnight at room temperature, the solvent removed under reduced pressure. To the residue 20 ml dry ethyl acetate was added. A white precipitate appeared, which was filtered out (this was found to be dicyclohexylurea). The filtrate was washed three times with 10% sodium bicarbonate, followed by washing three times with water to make it free from alkali. The ethylacetate extract was dried over anhydrous $MgSO_4$, filtered, solvent removed from the filtrate under reduced pressure. The residue was dissolved in ethanol and water was added to it until turbidity appeared. It was then kept in the freezer overnight. The oily precipitate solidified. It was filtered and further purified by selective precipitation from ethanol-water mixtures. m.p. 72-74°C, yield 1.6 gm. Structure was confirmed by NMR (Appendix A) and mass spectrum. This is schematically represented by Scheme I.

4. Phenylalanine amide (III)

To a solution of L-phenylalanine (0.83 g, 5 mM) in

H_2O:dioxane (1:1, 8 ml) and triethylamine (1.05 ml, 7.5 mM) was added Boc-ON (1.36 g, 5.5 mM) at room temperature. It goes into solution in about one hour. It was then kept stirring overnight at room temperature. After adding 15 ml of water to the reaction mixture it was washed twice with 15 ml portions of ethyl acetate and the ethyl acetate washings were discarded. The aqueous layer was acidified to pH 2.0 by 5% citric acid solution and saturated with sodium chloride. It was then extracted with three 20 ml portions of ethyl acetate followed by three washings with saturated sodium chloride solution to make the organic extract acid free. Ethyl acetate extract was dried over anhydrous $MgSO_4$, filtered and the solvent removed under reduced pressure to obtain an oily residue of t-Boc-phenylalanine. Yield 1.2 gm.

Ethyl chloroformate was added dropwise over 10 minute period at -10°C to a well stirred solution of t-Boc-phenylalanine and triethylamine in dry tetrahydrofuran. The mixture was stirred for 30 minutes at -10°C and then saturated with dry ammonia by bubbling ammonia gas through the solution. The reaction mixture was kept under stirring overnight at room temperature. The solvent evaporated under reduced pressure. The residue stirred with 20 ml of ice-cold water, filtered, dried at 40-60°C. It was then treated with 4 N HCl-dioxane for 30 minutes at room temperature.

The solvent removed to obtain a solid residue of L-phenylalanine amide, hydrochloride. This is schematically represented by Scheme II.

5. Aspirin phenylalanine amide (IV)

To a solution of aspirin (1.8 g, 10 mM) in 35 ml dry methylene chloride was added dicyclohexylcarbodiimide (1.1 g, 5 mM). The reaction mixture was kept under stirring for two hours at room temperature, whereby a white precipitate of dicyclohexylurea was formed which was removed by filtration. To the filtrate was added a solution of a mixture of L-phenylalanine amide (1.1 g, 5 mM) and triethylamine (0.7 ml, 5 mM) in 15 ml methylene chloride. The reaction mixture was kept under stirring overnight at room temperature and the solvent was removed under reduced pressure. The residue was dissolved in chloroform, washed with water, three times with 15 ml portions of 10% sodium bicarbonate solutions, three times with 10 ml portions of 2 N HCl followed by washing with water to make it acid free. The organic extract was evaporated to dryness under reduced pressure. The residue washed with ether and filtered and recrystallized from chloroform. m.p. 176-178°, yield 1.4 g. The structure was identified by NMR (Appendix A) and mass spectrum. Synthetic scheme is given in Scheme III. The compound was found to be insoluble in water and ether, and sparingly soluble in

ethanol.

6. <u>Aspirin-L-phenyllactic ethyl ester (V)</u>

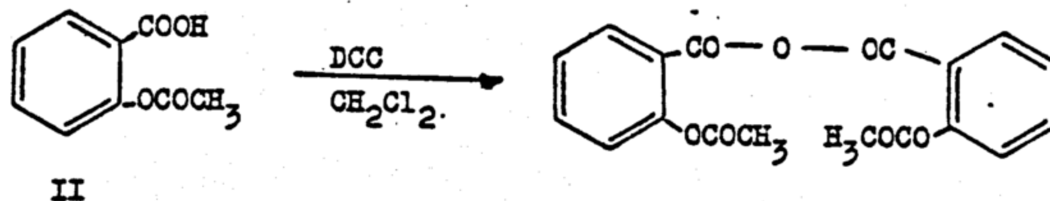
A solution of aspirin chloride (0.4 g, 2 mM) dissolved in 10 ml tetrahydrofuran was chilled to 5° and to it was added a cooled solution of L-phenyllactic acid (0.33 g, 2 mM) in 4 ml tetrahydrofuran. Over a 5 minute period, aliquots of pyridine totalling 0.5 ml were added to the reaction mixture and the system was stirred at 5° for one hour. After an additional hour of stirring at room temperature the solvent was removed on a rotary evaporator and the clear colorless oil dissolved in chloroform. The chloroform solution was extracted with water, dried over anhydrous $MgSO_4$ and evaporated under reduced pressure to yield a colorless oil which was purified by selective precipitation from chloroform by n-hexane. The oil did not solidify. Its purity was checked by TLC and, NMR and MS was used to confirm its identity as L-phenyllactic acid ester of aspirin (VI).

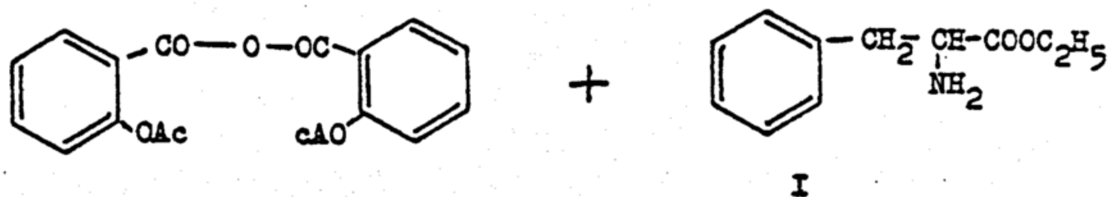
Aspirin-phenyllactic acid (0.23 g, 0.7 mM) was dissolved in dry methylene chloride and the solution chilled to 0°C. To it was added magnesium ethoxide dried ethanol (0.35 ml) followed by dicyclohexyl carbodiimide (0.13 g, 0.7 mM). After stirring at o°C for one hour it was kept under stirring for an additional hour at room temperature. The

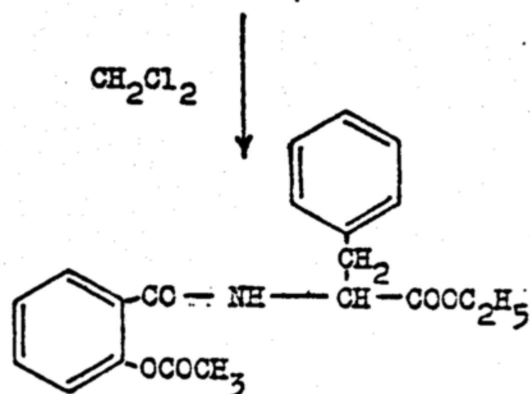
solvent was removed under reduced pressure to obtain a clear colorless oil which was further purified by selective precipitation from ethanol by water. The oil could not be solidified. TLC showed only one spot and NMR and mass spectrum confirmed it to be aspirin-L-phenyllactic ethyl ester (V). Schematic representation of steps involved is given in Scheme IV.

7. Aspirin-phenylalanine

Aspirin phenylalanine ethyl ester (60 mg) was dissolved in 10 ml of ethanol and added to 100 ml of a pH 7.5 phosphate buffer solution containing α-chymotrypsin (100 mg), mixed well and allowed to stand for about 2 minutes and transferred to a 250 ml separating funnel. It was then acidified with 2 N HCl to pH 1.0 and extracted with 100 ml of chloroform. A very thick emulsion resulted upon shaking and the layer had to be separated by centrifuging at 2000 RPM for 20 minutes. The upper aqueous layer was aspirated out, the lower layer was dried over anhydrous $MgSO_4$ and the solvent removed in a rotary evaporator to obtain a clear colorless oily residue which was further purified by selective precipitation from ethanol by water. NMR and mass spectrum confirmed it to be aspirin-phenylalanine.

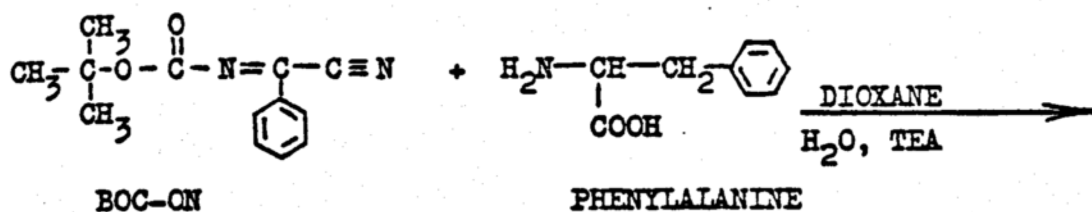


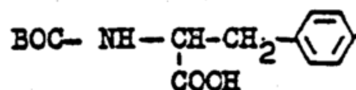


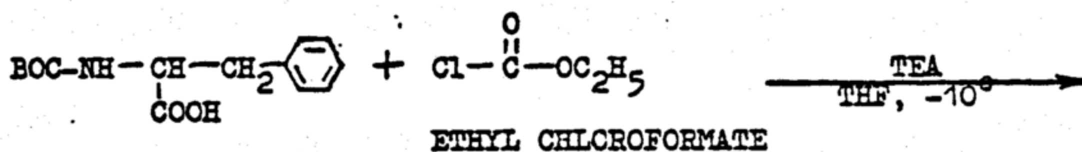


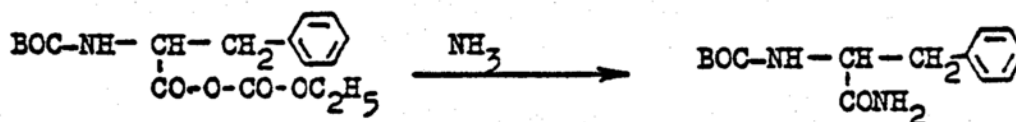
Aspirin phenylalanine ethyl ester

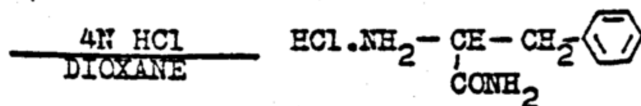
Scheme I. Synthesis of aspirin phenylalanine ethyl ester.



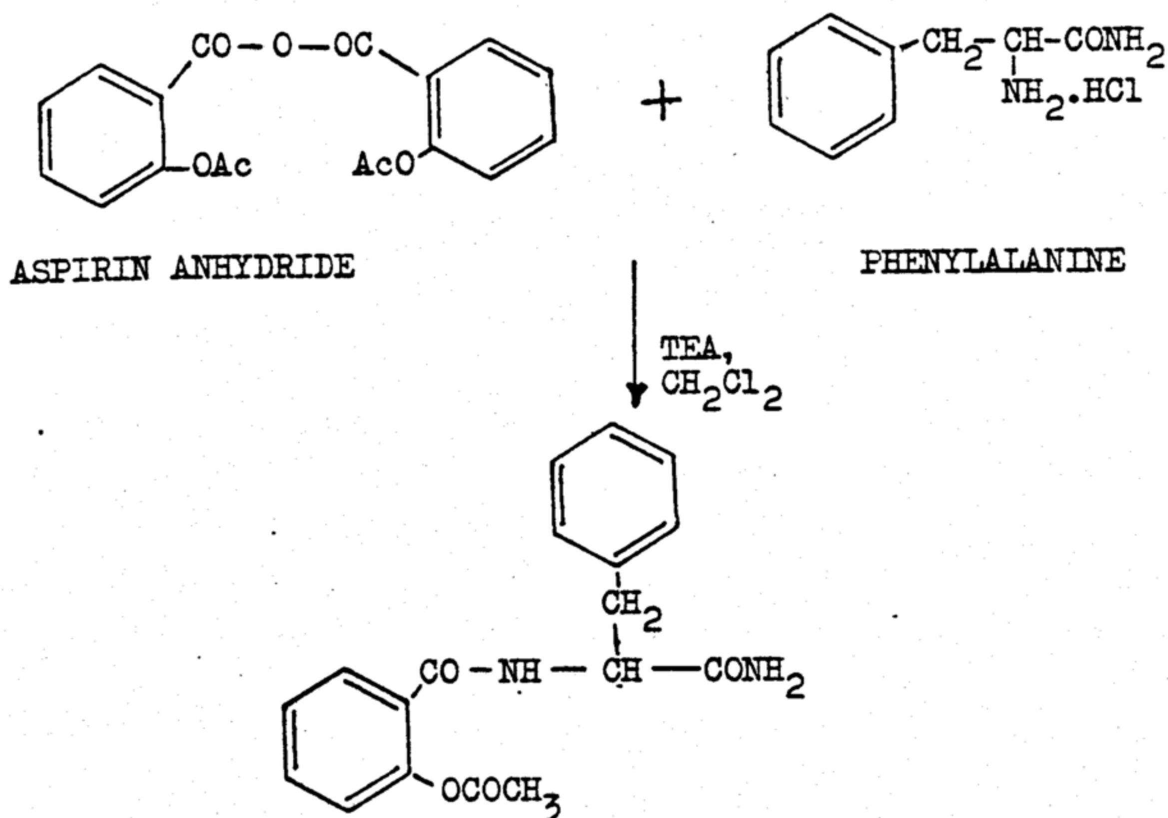




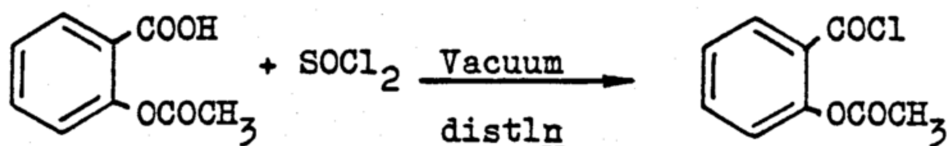




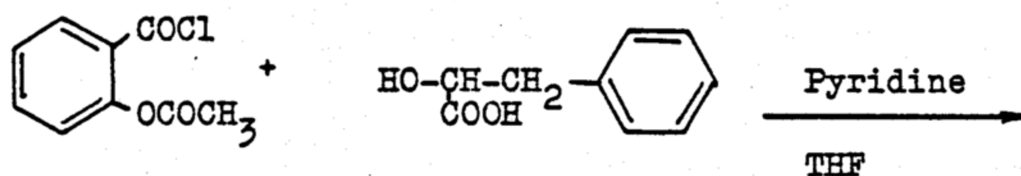
Scheme II. Synthesis of phenylalanine amide.

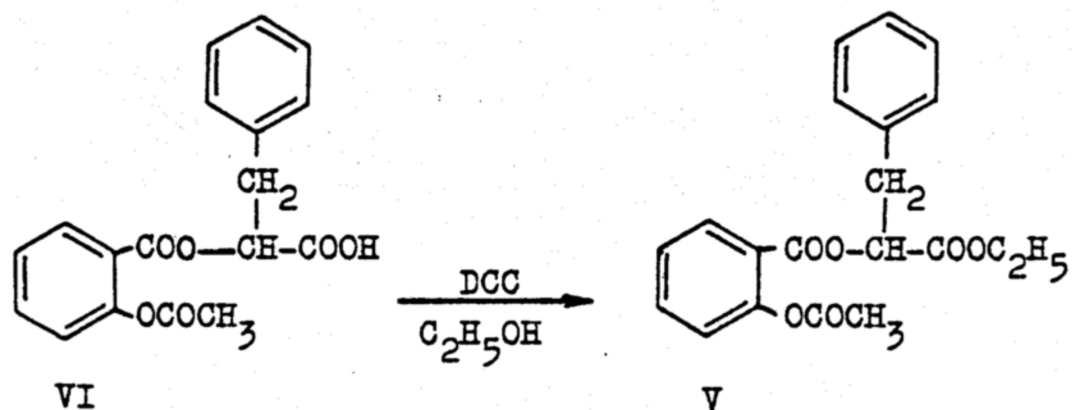


Scheme III. Synthesis of aspirin phenylalanine amide.



Aspirin





Aspirin phenyl-
lactic acid

Aspirin phenyllactic
ethyl ester

Scheme IV. Synthesis of aspirin phenyllactic ethyl ester.

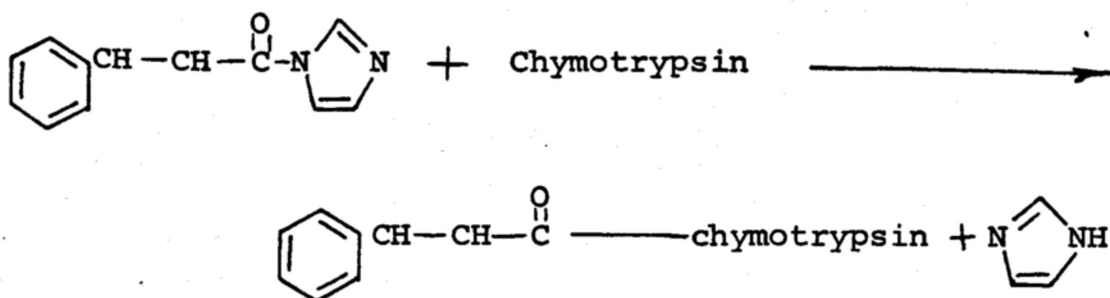
B. Enzyme Kinetic Studies In Vitro

1. Assay of enzymes - Materials

α-Chymotrypsin was obtained as a dialyzed, salt free, lyophilized powder from Aldrich Chemical Company. Carboxypeptidase A was obtained as an aqueous suspension with toluol preservative from Worthington Biochemical Corp. The specific substrates, cinnamoyl imidazole for α-chymotrypsin and hippuryl-L-phenylalanine for carboxypeptidase A were obtained from Eastman Kodak and Aldrich Chemical Company respectively.

a. Assay of α-chymotrypsin

A direct spectrophotometric titration, as described by Schonbaum _et al_. (58), which determines simply, rapidly and accurately the operational normality of α-chymotrypsin solutions, was utilized. The titration exploits the observation that a relatively stable acyl-α-chymotrypsin compound, trans-cinnamoyl-α-chymotrypsin, can be formed at pH 5 (59-61). N-trans-cinnamoylimidazole is employed as the acylating agent.



Since it is known that α-chymotrypsin contains one active site per molecule, the operational normality determined in this titration can be translated directly into molarity. The acylation takes place rapidly and quantitatively. Inactive protein does not interfere and the titration may be carried out in presence of trypsin.

The spectrophotometer[a] was set at 335 mμ and the chart speed was adjusted to 2 inches/min. 3 ml of pH 5 acetate buffer (0.1 M) are pipetted into a cuvette and 100 μl of a suitable stock solution of N-trans cinnamoylimidazole are introduced on the tip of a smooth flat ended stirring rod. A slow (virtually linear) decrease in absorbancy was observed owing to the spontaneous hydrolysis of the reagent. 100 μl of the enzyme test solution are added, such that there is a suitable excess of N-trans cinnamoylimidazole after acylation of enzyme is complete. A very rapid drop in absorbancy occurred which was complete in 90 seconds. The end point was obtained by extrapolation to the time of addition of enzyme. The absorbance of a solution of 100 μl enzyme in 3 ml of buffer was determined. Normality was calculated by using the formula:

$$N = \frac{0.969(A_2 + A_4) - A_3}{279}$$

[a]Cary 14 recording spectrophotometer was used.

where A_2 and A_3 are the initial and extrapolated final values of absorbancy. A_4 is the absorbancy of the 100 μl enzyme solution in 3 ml of buffer and N is the molar concentration of active sites of α-chymotrypsin in the enzyme test solution.

b. Assay of carboxypeptidase A

Before use, crystals of enzyme were washed twice with distilled water and dissolved in 3.0 M NaCl. The solutions were centrifuged and stored at 4° in concentrations of about 30 mg/ml. Such solutions were shown (62) to exhibit no change in activity over periods of several months. Working solutions were prepared daily by diluting the stock solutions to the desired concentrations with 1.0 M NaCl. Enzyme concentrations were measured by the absorbance at 278 mμ and using a molar extinction coefficient of 6.49 x 10^4 liter/mole·cm (63).

For measuring the activity of the enzyme, a dilution of stock enzyme to a concentration of 10 to 25 μg/ml in 10% LiCl were made immediately before use. Freshly prepared 0.001 M hippuryl-L-phenylalanine in 0.025 M Tris buffer, pH 7.5, containing 0.5 M NaCl, was taken. Spectrophotometer was set at 254 mμ. In the control cuvette was taken 2.9 ml of substrate solutions and 0.1 ml of 10% LiCl and in the test cuvette 0.1 ml enzyme solution was added at zero time

to 2.9 ml of substrate solution. The increase in absorbancy was recorded. Activity of the enzyme was calculated using the following formula:

$$\text{units/mg protein} = \frac{\Delta A_{254}/\text{min}}{0.36 \times \frac{\text{mg enzyme}}{\text{ml of reaction mixture}}}$$

where, A unit of activity is defined to be equal to one micromole of substrate hydrolyzed per minute under the conditions specified, 0.36 is the molar absorbancy index of hippuric acid which is formed stoichiometrically.

2. Kinetic measurements

The hydrolysis of the prodrugs were followed by automatic titration at constant pH employing a Radiometer Type TTI 60 titrator in conjunction with Type SBR 3 Titrigraph. The titration assembly used Radiometer ABU 12 Autoburette and PHM61 Laboratory pH meter. The consumption of basic titratant recorded by the pH-stat is a direct measure of the amount of substrate hydrolyzed provided the products do not buffer the reaction mixture.

All solvents and chemicals were of reagent grade quality, triple distilled water was used in preparing solutions, and the reaction vessel was thermostated at 25.00 ± 0.02° and dry nitrogen gas was gently blown into the reaction vessel in order to exclude atmospheric carbon dioxide

from the system.

In a typical run the instrument was first standardized against buffers of pH 7.00 ± 0.02 and pH 10.00 ± 0.02. The titrating mechanism was set at the desired pH and sufficient volume of solution of the prodrugs containing 0.1 M molar potassium chloride was pipetted into the reaction vessel and allowed to equilibrate for 10 minutes, while being magnetically stirred. The solution was then brought to the desired pH and when a stable baseline was obtained 10 μl of suitable concentration of enzyme was added. The automatic titrating mechanism maintained the set pH during the hydrolytic reaction by adding increments of base as small as 0.1 μl and also continuously recorded the amount of base consumed vs. time.

In the runs with aspirin phenyllactic ethyl ester and α-chymotrypsin, aspirin phenyllactic acid and carboxypeptidase A, and aspirin phenylalanine and carboxypeptidase, the substrate stock solutions were made in acetonitrile. For these cases, an aliquot of the stock solutions was added to the reaction vessel containing a predetermined volume of 0.1 M potassium chloride solution and then the titration carried out in the same manner as before. The initial substrate concentrations were determined by an assay method described in the next section.

Figure 6 shows a typical recording of the pH-stat

experiments. The recordings were fitted to a polynomial of the form

$$y = a_0 + a_1 t + a_2 t^2 + \dots$$

where y is the moles of alkali consumed and t is the time. The initial rate of the enzymatic hydrolysis were determined by evaluating the first derivative of 'y' with respect to time at t = 0, which is the coefficient "a_1". Thus 'a_1' represents the initial rate of hydrolysis.

C. Hydrolytic Pathways of Aspirin-Phenylalanine Ethyl Ester in Simulated Intestinal Fluid, USP

1. Intestinal fluid (USP XIX)

6.8 g of monobasic potassium phosphate was dissolved in 250 ml of water and to it was added 190 ml of 0.2 N sodium hydroxide solution and 400 ml water. 10.0 gm of pancreatin was mixed with it and the pH of the resulting mixture was adjusted to pH 7.5 ± 0.1 by addition of 0.2 N sodium hydroxide, and the volume was made up to 1000 ml with water.

2. Kinetic studies

35 mg of aspirin phenylalanine ethyl ester was dissolved in 10 ml ethanol. 95 ml of simulated intestinal

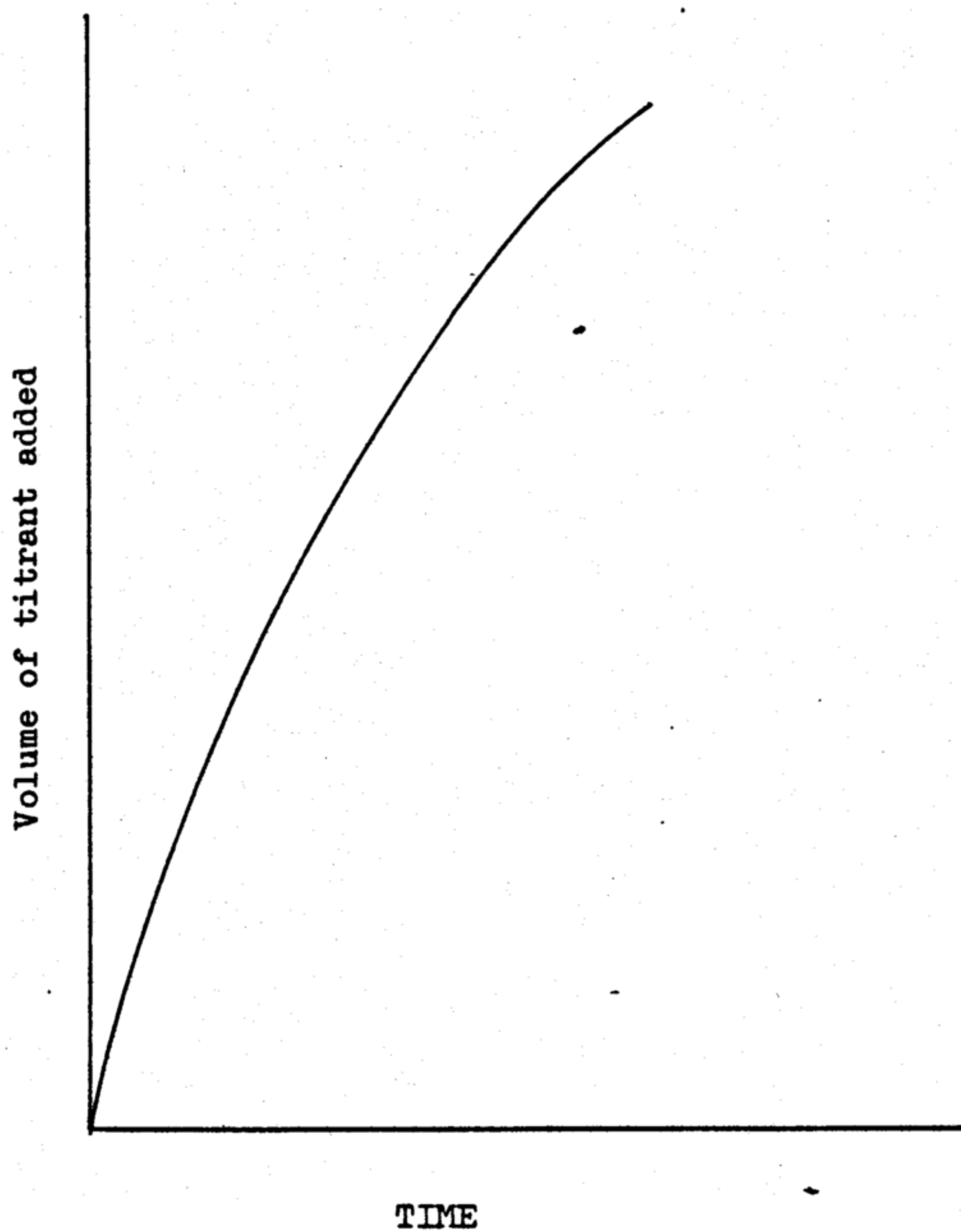


Figure 6. A typical pH-stat titration recording.

fluid was taken in a volumetric flask and allowed to equilibrate in a water bath kept at 25.00 ± 0.02°. 5 ml of the simulated intestinal fluid were withdrawn at zero time and poured into a 15 ml centrifuge tube containing 1 ml of 2 N HCl. The alcoholic solution of aspirin-phenylalanine ethyl ester was then mixed with the simulated intestinal fluid. 5 ml aliquot of this mixture were withdrawn at timed interval and poured into centrifuge tubes containing 1 ml of 2 N HCl. It was centrifuged at 1500 RPM for 15 minutes when the denatured proteins accumulate at the bottom of the tubes. 5 ml of the clear supernatant fluid were withdrawn with the aid of glass wool plugs on the tip of the pipettes and transferred to another 15 ml centrifuge tube. 5 ml of chloroform was added to it and vigorously shaken for 15 minutes whereby a very thick emulsion was formed. It was again centrifuged at 2000 RPM for 15 minutes when the layers separated. The upper aqueous layer was aspirated out. 1 ml aliquot of the lower chloroform layer was taken in 10 ml volumetric flask and the solvent evaporated by blowing gentle stream of nitrogen over it. The residue was dissolved in 10 ml of ethanol and the uv absorption spectrum recorded from 220 nm to 330 nm.

3. TLC studies

In order to be able to follow the interaction of

aspirin phenylalanine ethyl ester qualitatively and to identify the products a TLC system was sought which would separate the reactants and the products. The results are summarized in Table 5. Out of a number of solvent systems tried, methanol:acetic acid:ether:benzene (1:18:60:20) was able to resolve aspirin and salicylic acid (appears as a dense blue spot under short wavelength UV). The support was silica gel with UV_{254} fluorescent background. This solvent system could not differentiate between aspirin and aspirin-phenylalanine ethyl ester. Cyclohexane:chloroform:glacial acetic acid (4:5:1) was on the other hand able to distinguish between aspirin and aspirin phenylalanine ethyl ester, but could not differentiate between aspirin and salicylic acid.

Therefore, TLC plates were run in both of the solvents simultaneously in order to be able to identify all the degradation products of aspirin-phenylalanine ethyl ester in simulated intestinal fluid. All the chloroform extracts were spotted on silica gel plates (Macherey-Nagel Company), 0.25 mm thick, and developed for 15 cm in both the solvents. The spots were detected under short wavelength UV detector.

In order to determine the mechanism of degradation, experiments were also run with α-chymotrypsin (100 mg/100 ml) replacing pancreatin, but otherwise maintaining the same conditions as with simulated intestinal fluid.

TABLE 5. R_f values of Asp-Phe et and its degradation products.

Compounds	I	II	III	IV
Aspirin	0.73	0.46	0.53	0.58
Asp-Phe et	0.80	0.64	0.61	0.58
Asp-Phe	--	0.20	--	0.36
Sal-Phe	--	--	--	0.50
Phe-Ala	0.39	0	0	0
Sal-Acid	--	--	0.53	0.61
Phe-et, HCl	0.56	0	0	0

Support - 0.25 mm plate of silica gel with F254.

Solvent I - n-butanol:acetone:acetic acid:5% NH_4OH:H_2O [4.5:1.5:1:1:2].

Solvent II - cyclohexane:chloroform:acetic acid [4:5:1].

Solvent III - benzene:water:acetic acid [2:2:1].

Solvent IV - methanol:acetic acid:ether:benzene [1:18:60:120].

D. Shelf Life Studies of Aspirin Phenylalanine Ethyl Ester in Aqueous Solution and Solubility Determination

5 mg of aspirin phenylalanine ethyl ester were dissolved in 1 ml of ethanol, transferred quantitatively to a 100 ml volumetric flask and the volume made up with 1/15 M phosphate buffer of pH 7.5. 5 ml aliquots were withdrawn at timed interval, taken in 15 ml centrifuge tubes and extracted with 5 ml portions of chloroform. The aqueous layer was aspirated out. Two 0.5 ml portions of the chloroform extracts were taken in 10 ml volumetric flasks and the solvent evaporated by blowing gentle stream of nitrogen over it. To one of the flasks were added 0.5 ml ethanol and 1 ml of 5 N sodium hydroxide solution and the mixture heated in a boiling water bath for 45 minutes, cooled to room temperature and the volume made up with distilled water. To the other flask was added 0.5 ml ethanol and the volume made up with distilled water. Fluorescence of both the samples were determined at excitation wavelength of 328 nm and emission wavelength of 400 nm.

Solubility Determination

About 300 mg of aspirin phenylalanine ethyl ester was stirred with 100 ml of distilled water at 25.00 ± 0.02°. Aliquots of the solution were withdrawn until no change in concentration was observed. Equilibrium was reached in 20

hours. After diluting the aliquots 10 times, 1 ml of it was taken in a 10 ml volumetric flask, 1 ml 5 N sodium hydroxide was added to it, heated in a boiling water bath for 45 minutes, cooled, volume made up and fluorescent measurements taken at excitation wavelength of 328 nm and emission wavelength of 400 nm, using a 10^{-6} M solution of aspirin-phenylalanine ethyl ester, taken through all the steps mentioned above, as standard.

E. Absorption In Situ from Rat Intestine

Male, Holtzmann rats, weighing 200-250 grams and fasted overnight were anesthetized with 35 mg/kg sodium pentobarbital intraperitoneally. The rats were placed on a heating pad and the abdominal contents are exposed by a midline abdominal incision. The ligament of Treitz was identified and the jejunum ligated and cannulated approximately 10 cm and 2 cm distal to it with glass tubing (Fig. 7). Solution at ambient temperature was perfused through the section of the intestine at 0.4 ml/min rate with a Harvard infusion/ withdrawal pump and was collected at 8 minutes intervals. The intestine was perfused first with Sorenson phosphate buffer, pH 7.5 and isoosmotic, for 30 minutes then the solutions were collected for 8 minutes. The same 30 minute perfusion and 8 minutes collection sequence was repeated for

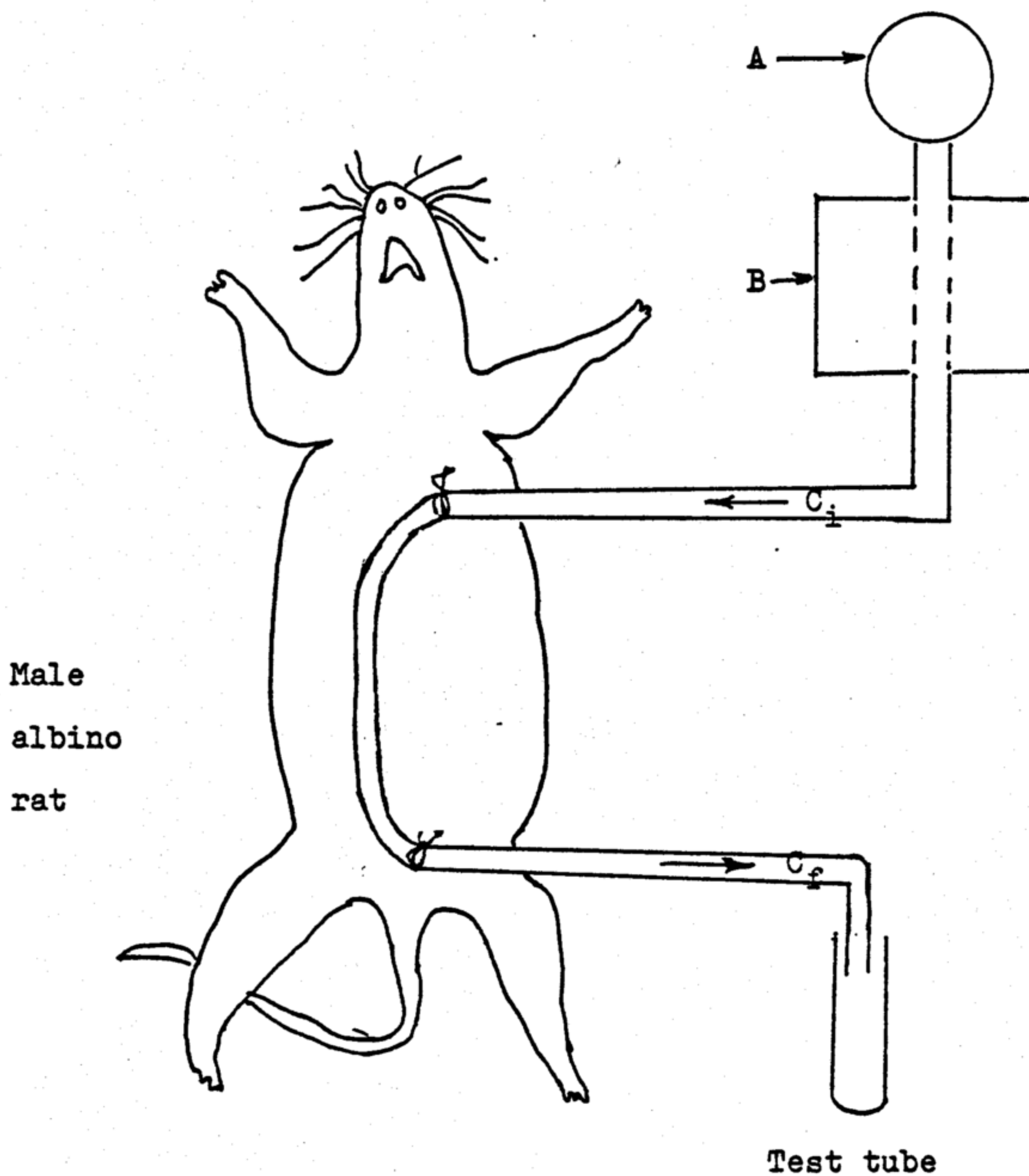


Figure 7. Schematic diagram of experimental set-up for intestinal absorption . Key : A: Syringe pump, B; water bath at 37° C, C_i; initial concentration , C_f; final concentration .

aspirin, aspirin phenylalanine ethyl ester and buffer again, done in that order. The perfusing solutions contained 0.01 μCi of ^{14}C-PEG/ml of solution, as a volume marker. After the final collection, the segment of bowel was excised, laid flat and the length measured.

Initial and final concentrations of aspirin and aspirin phenylalanine ethyl ester were determined by the fluorimetric method as described in the last section. For aspirin excitation wavelength of 308 nm and emission wavelength of 390 nm were used. Scintillation counting of the marker was done on a Packard scintillation counter using 200 λ of solutions in 10 ml of Aquasol. Counts were obtained over 10 minutes.

After making corrections for any volume change during the passage through the intestine, the final concentrations were calculated as:

$$C_{final} = \frac{\text{Final Fluorescence}}{\text{Initial Fluorescence}} \times \frac{\text{Initial Counts}}{\text{Final Counts}} \times C_{initial}$$

F. <u>Dissolution Studies</u>

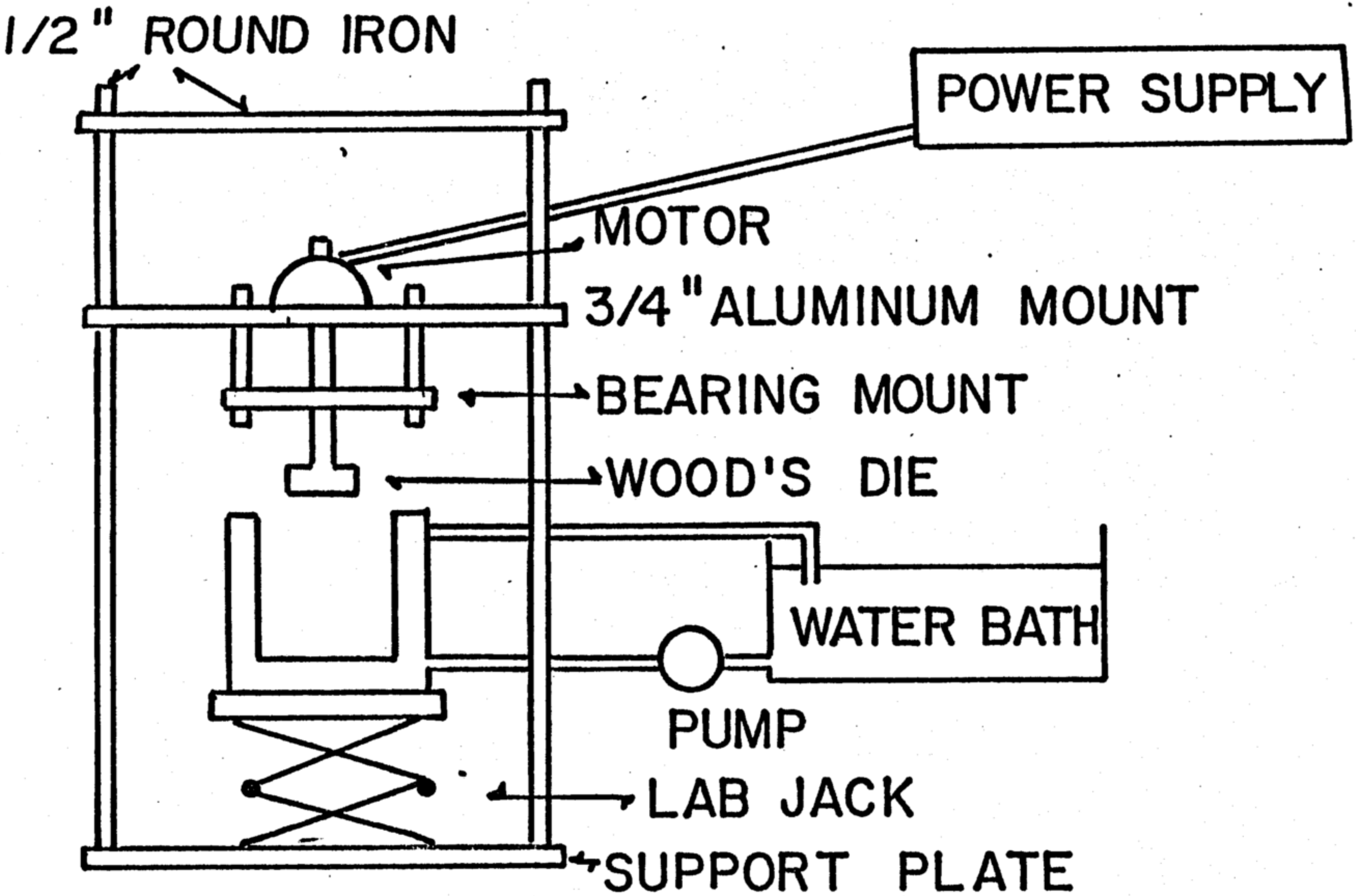
Dissolution rates were measured by the rotating disc method previously described by Wood *et al*. (64). 250 mg powder was introduced into the die of a Wood's apparatus and this was compressed at 2000 lbs force using Carver Press. The diameter of the opening in the die was 1.10 cm.

The die was attached to a constant RPM motor (Indiana General Motor, Philadelphia, PA) which was attached to a solid aluminum platform such as shown in Figure 8. The motor was started at the desired number of rotations per minute, and this checked by way of a strobe light (Strobette, Model 964, Power Instruments Inc., Skokie, Ill.). The precision of the rotations per minute is, in general, 5 rotations per minute. The stroboscope was calibrated against a precision timer. The power supply for the motor was assembled from Heathkit components (Heathkit Model No. IP2720, Heathkit Co., Benton Harbor, MI).

Once 500 ml of either Sorenson phosphate buffer, pH 7.5 or the same buffer containing α-chymotrypsin had been equilibrated at 25.0 ± 0.2°, it was poured into a beaker suspended in a 25.0 ± 0.2° water bath, so that the rotating die was immersed 4 cm below the surface of the liquid. The rotational speed used ranged from 150 to 500 RPM. Samples were taken periodically and assayed by a fluorimetric method described below.

Assay

5 ml of the dissolution medium were poured into 15 ml centrifuge tubes containing 1 ml of 2 N HCl. 5 ml of chloroform was added to it and shaken vigorously for 15 minutes and then centrifuged at 1500 RPM for 15 minutes. The upper


1/2" ROUND IRON
POWER SUPPLY
MOTOR
3/4" ALUMINUM MOUNT
BEARING MOUNT
WOOD'S DIE
WATER BATH
PUMP
LAB JACK
SUPPORT PLATE

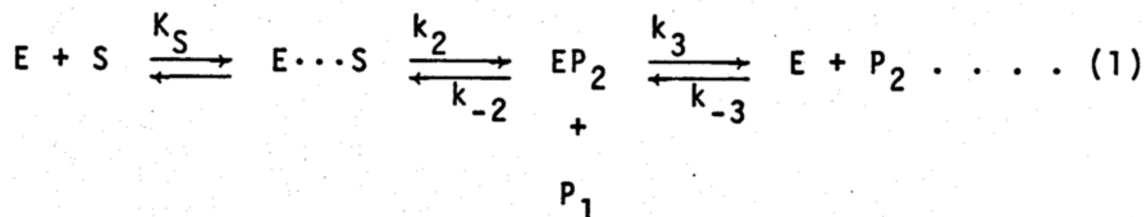
aqueous layer was aspirated out. 2 ml of the lower chloroform layer were taken in a 10 ml volumetric flask and the solvent evaporated by blowing a gentle stream of nitrogen over it. 0.5 ml ethanol and 1 ml of 5 N sodium hydroxide were added to it, heated in a boiling water bath for 45 minutes, cooled to room temperature, the volume made up with distilled water and the fluorescence intensity recorded with 328 nm excitation wavelength and 400 nm emission wavelength. A 10^{-6} M aspirin-phenylalanine ethyl ester solution taken through the same steps was used as a reference standard.

III. RESULTS AND DISCUSSION

A. Enzyme Kinetic Studies In Vitro

1. Studies with α-chymotrypsin

The results of the measurements of the initial rates of hydrolysis of aspirin phenylalanine ethyl ester, aspirin phenylalanine amide and aspirin phenyllactic ethyl ester catalyzed by α-chymotrypsin at pH 8.0 and 7.5 at 25° are shown in Tables 6 to 11. If α-chymotrypsin catalyzed hydrolysis follows the general kinetic scheme



$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} E\cdots S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP_2 + P_1 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P_2 \quad . \; . \; . \; . \quad (1)$$

then the initial rate would be given by the Michaelis-Menten equation

$$V_o = \frac{k_{cat}E_oS_o}{K_m + S_o} \quad . \; . \; . \; . \qquad (1a)$$

or by the Lineweaver-Burk equation

$$\frac{1}{V_o} = \frac{1}{k_{cat}E_o} + \frac{K_m}{k_{cat}E_o}\frac{1}{S_o} \quad . \; . \; . \; . \qquad (1b)$$

Plots of $1/V_o$ vs. $1/S_o$ for aspirin phenylalanine ethyl

ester and aspirin phenylalanine amide for pH 7.5 and 8.0 are shown in Figures 9 to 12. In all the cases good straight lines were obtained and from the slopes and intercepts k_{cat} and K_m were calculated. These results are summarized in Table 12.

It has been shown (65) that k_{cat}/K_m is the most meaningful kinetic parameter for the purpose of comparison of different reactions. K_m and K_S are combinations of individual binding and rate constants. If multiple binding is ignored and independent experimental proof exists that $k_2 >> k_3$ or $k_3 >> k_2$ then k_{cat} (= k_3 or k_2) may be used as a meaningful constant for the purpose of comparison.

It can be seen from Table 12 that for aspirin phenylalanine ethyl ester k_{cat}/K_m is smaller by a factor of six at pH 7.5 than at pH 8.0, while K_m does not change significantly. k_{cat} was eight times smaller at pH 7.5 than at pH 8.0. For N-acetyl phenylalanine ethyl ester k_{cat} is three times smaller at pH 7 than at pH 7.8 (74). K_m values for aspirin phenylalanine ethyl ester were found to be much smaller than those for the N-acetyl derivatives. These results are contrary to the belief that N-acyl groups generally do not contribute significantly to the binding of substrate (68). Nevertheless, the ratio k_{cat}/K_m was found to be of comparable magnitude with that for N-acetyl derivative. A similar but much less dramatic change in k_{cat}/K_m

TABLE 6. Initial rate data for the hydrolysis of aspirin-phenylalanine ethyl ester catalyzed by 2×10^{-10} M α-chymotrypsin at pH 8.0 and 25° in presence of 0.1 M KCl.

$[S_o] \times 10^6$ M	$V_o \times 10^8$ M/sec	$\frac{1}{[S_o]} \times 10^{-5}\ M^{-1}$	$\frac{1}{V_o} \times 10^{-8}\ sec \cdot M^{-1}$
0.845	0.1507	11.83	6.634
1.267	0.1835	7.892	5.449
2.1125	0.2294	4.733	4.359
4.225	0.2797	2.367	3.575
6.337	0.3154	1.578	3.17

SCHOOL OF PHARMACY

TABLE 7. Initial rate data for the hydrolysis of aspirin-phenylalanine ethyl ester catalyzed by 1.79×10^{-9} M α-chymotrypsin at pH 7.5 and 25° in presence of 0.1 M KCl.

$[S]_o \times 10^6$ M	$V_o \times 10^7$ M/sec	$\frac{1}{[S_o]} \times 10^{-5}$ M^{-1}	$\frac{1}{V_o} \times 10^{-8}$ sec·M^{-1}
0.8	0.0322	12.5	5.71
1.2675	0.0219	7.89	4.57
2.1125	0.0265	4.73	3.77
4.225	0.0301	2.37	3.32
5.4925	0.0345	1.82	2.9

TABLE 8. Initial rate data for the hydrolysis of aspirin-phenylalanine amide catalyzed by 4.4×10^{-6} M α-chymotrypsin at pH 8.0 and 25° in presence of 0.1 M KCl.

$[S]_0 \times 10^5$ M	$V_0 \times 10^7$ M/sec	$\frac{1}{[S]_0} \times 10^{-4}$ M^{-1}	$\frac{1}{V_0} \times 10^{-7}$ sec·M^{-1}
1.111	0.851	9.0	12.107
2.222	0.138	4.50	7.24
4.444	0.174	2.25	5.572
7.777	0.213	1.286	4.688
11.110	0.222	0.90	4.515

TABLE 9. Initial rate data for the hydrolysis of aspirin-phenylalanine amide catalyzed by 6.26 x 10^{-6} M α-chymotrypsin at pH 7.5 and 25° in presence of 0.1 M KCl.

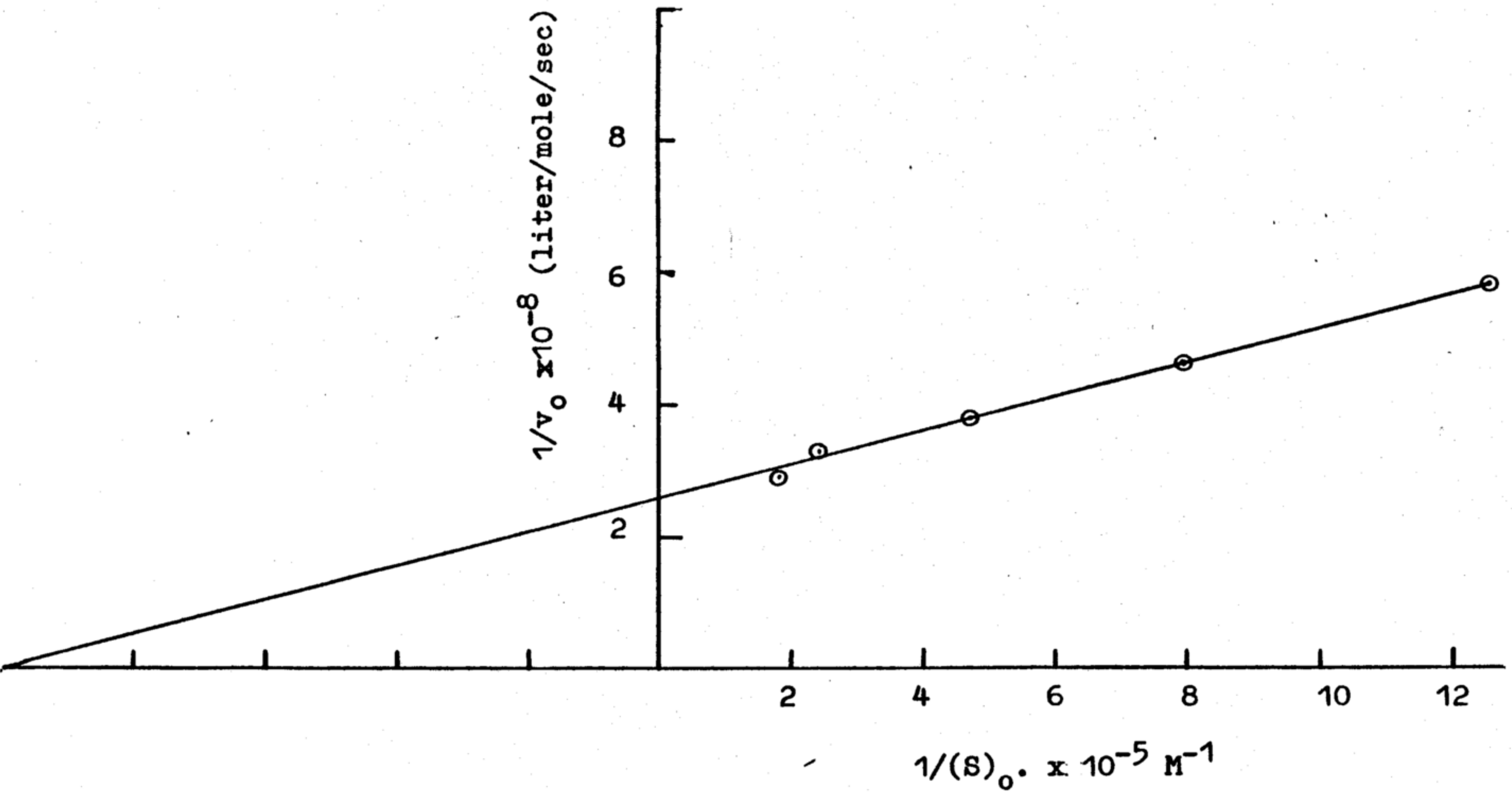
$[S]_o \times 10^4$ M	$V_o \times 10^7$ M·sec^{-1}	$\frac{1}{[S]_o} \times 10^{-3}$ M^{-1}	$\frac{1}{V_o} \times 10^{-7}$ sec·M^{-1}
1.226	0.0623	8.15	16.6
1.84	0.0827	5.43	12.1
2.454	0.104	4.075	9.615
3.067	0.116	3.26	8.62
6.3	0.174	1.58	5.75

TABLE 10. Initial rate data for the hydrolysis of aspirin-phenyllactic ethyl ester catalyzed by 1.5×10^{-5} M α-chymotrypsin at pH 8.0 and 25° in presence of 0.1 M KCl.

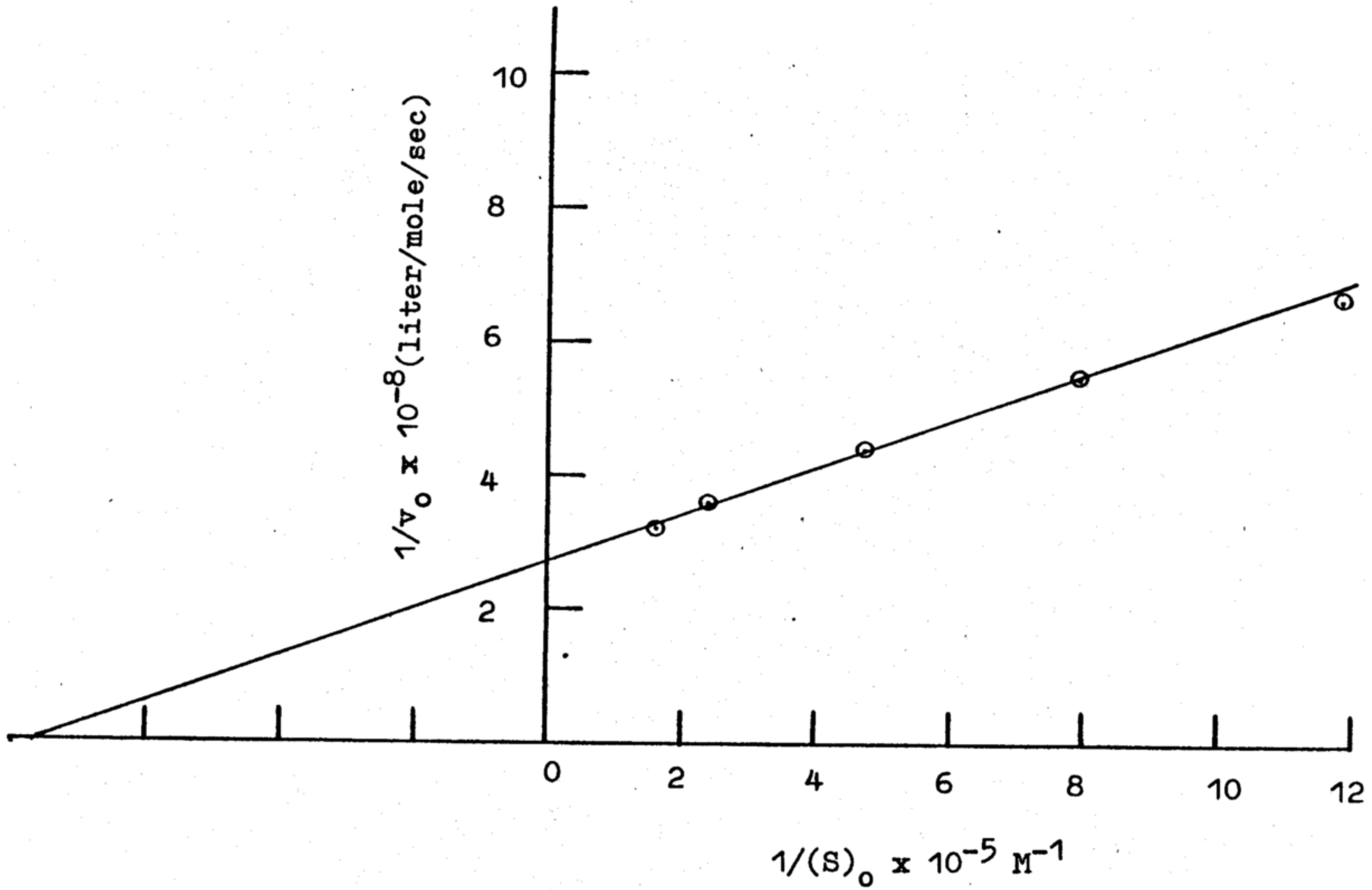
$[S]_o \times 10^5$ M	$V_o \times 10^8$ $M \cdot sec^{-1}$	$\frac{1}{[S]_o} \times 10^{-4}$ M^{-1}	$\frac{1}{V_o} \times 10^{-7}$ $sec \cdot M^{-1}$
1.053	3.27	9.49	3.06
2.106	5.43	4.75	1.84
2.95	6.36	3.39	1.57
4.21	8.16	2.37	1.26
8.43	8.83	1.19	1.13

TABLE 11. Initial rate data for the hydrolysis of aspirin-phenyllactic ethyl ester catalyzed by 3.02×10^{-5} M α-chymotrypsin at pH 7.5 and 25° in presence of 0.1 M KCl.

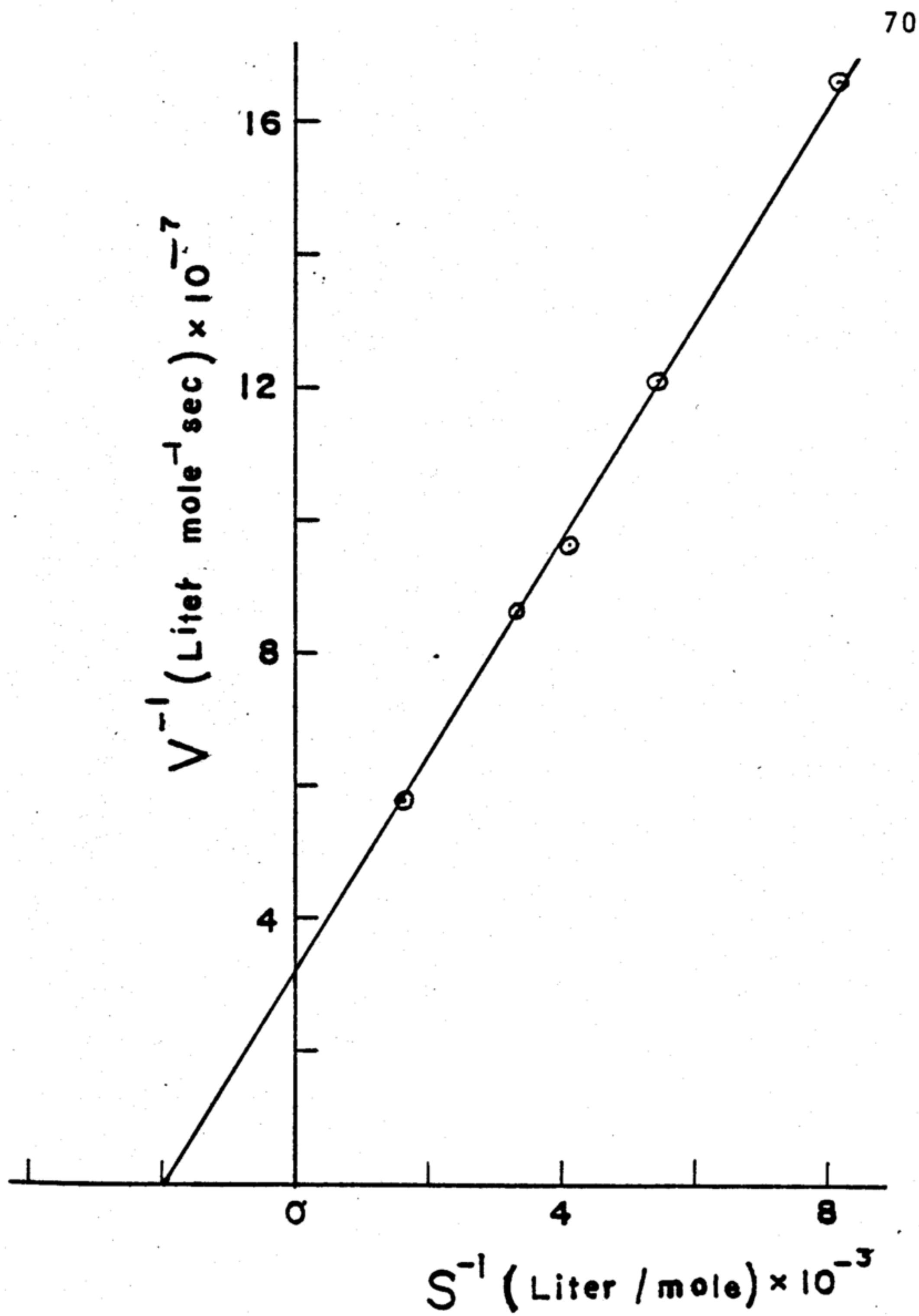
$[S]_o \times 10^5$ M	$V_o \times 10^8$ $M \cdot sec^{-1}$	$\frac{1}{[S]_o} \times 10^{-4}$ M^{-1}	$\frac{1}{V_o} \times 10^{-7}$ $sec \cdot M^{-1}$
2.106	3.8	4.75	2.63
4.213	6.3	2.37	1.56
6.212	7.35	1.61	1.36
8.43	8.1	1.19	1.23
16.85	9.1	5.93	1.10

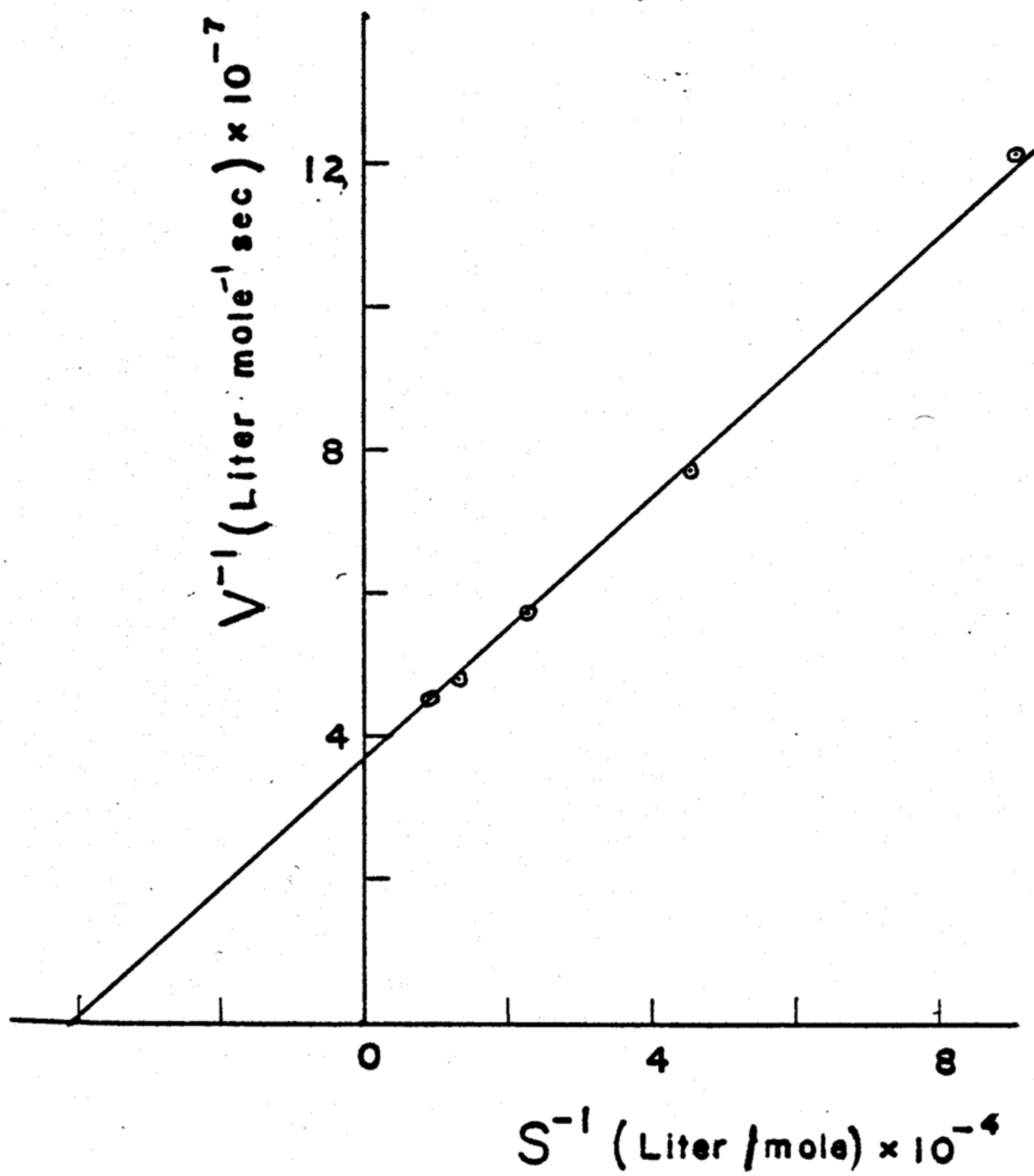


1/v$_o$ x10^{-8} (liter/mole/sec)
8
6
4
2
2
4
6
8
10
12
1/(S)$_o$. x 10^{-5} M^{-1}

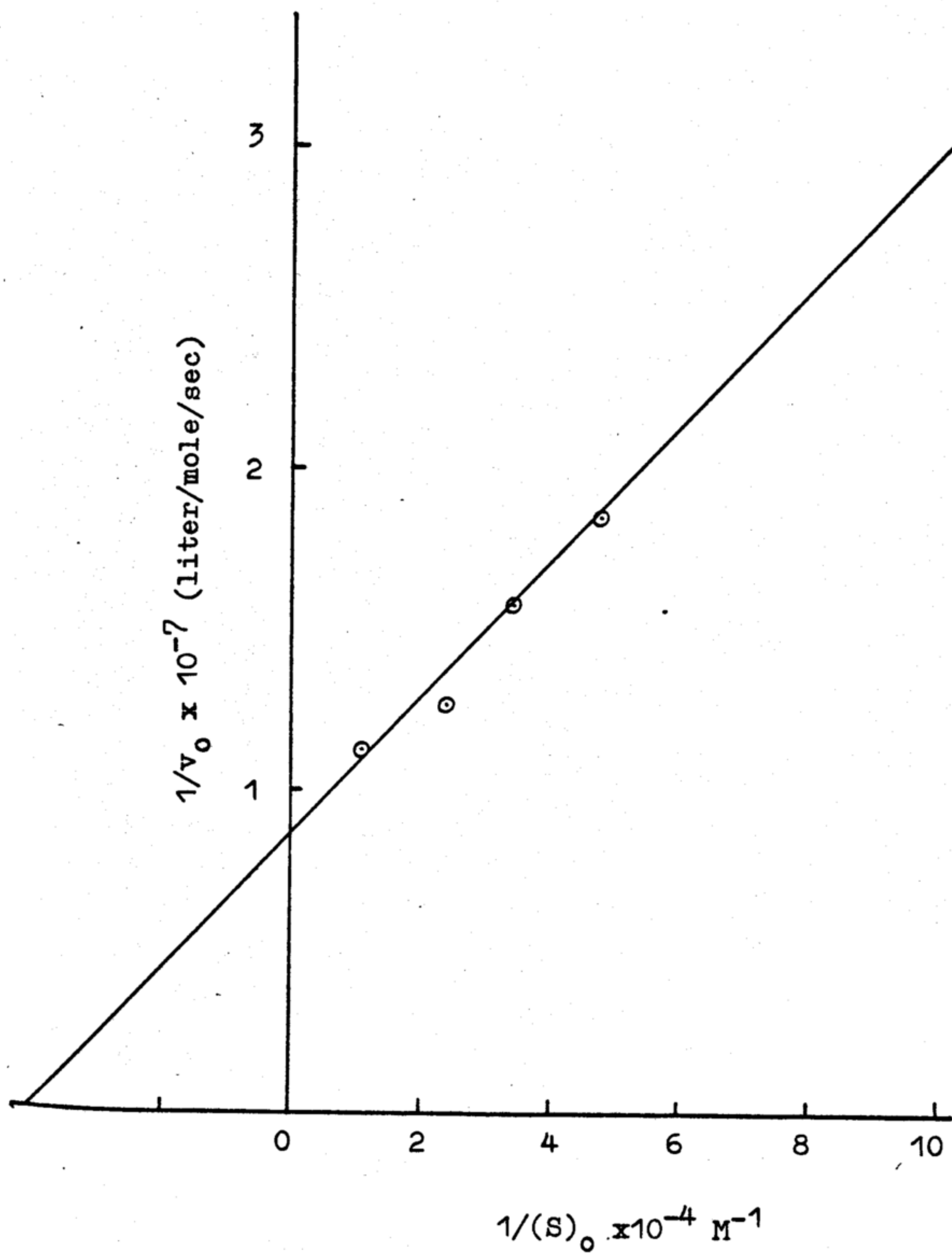


10
8
6
4
2
0
2
4
6
8
10
12
1/v_o x 10^-8 (liter/mole/sec)
1/(S)_o x 10^-5 M^-1

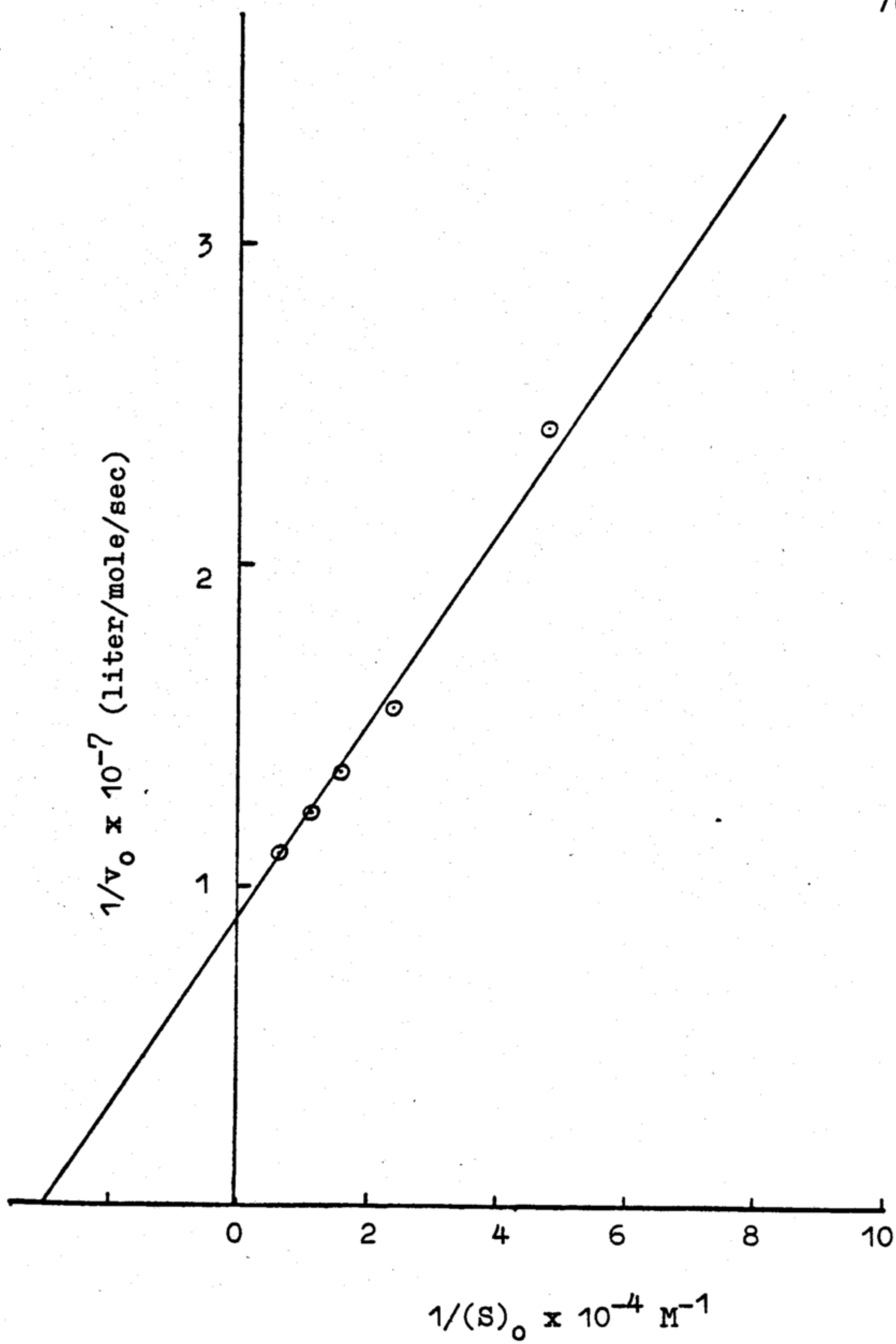

16
12
8
4
0
4
8
V^{-1} (Liter mole^{-1} sec) × 10^{-7}
S^{-1} (Liter / mole) × 10^{-3}



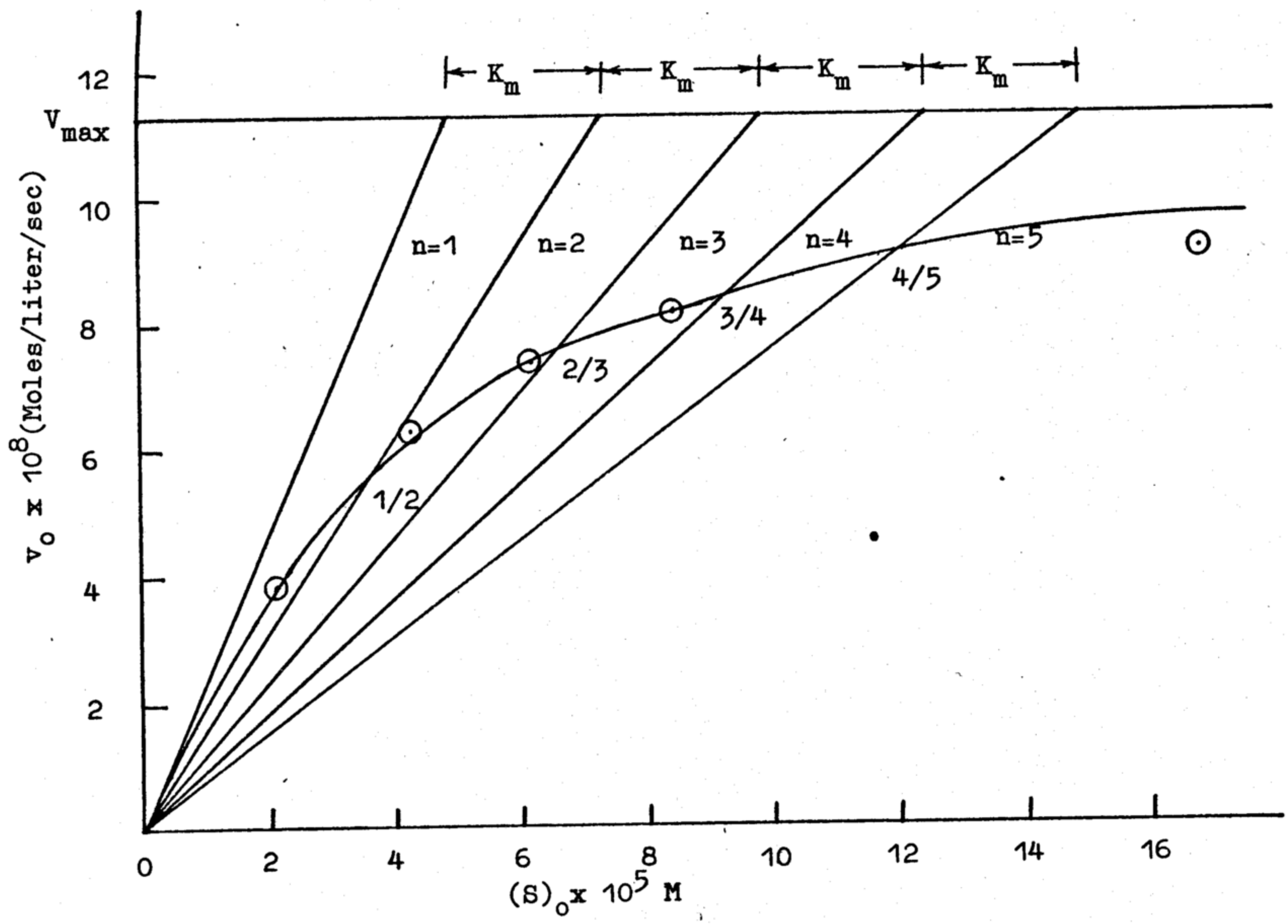
V^{-1} (Liter · mole^{-1} sec) × 10^{-7}
12
8
4
0
4
8
S^{-1} (Liter /mole) × 10^{-4}



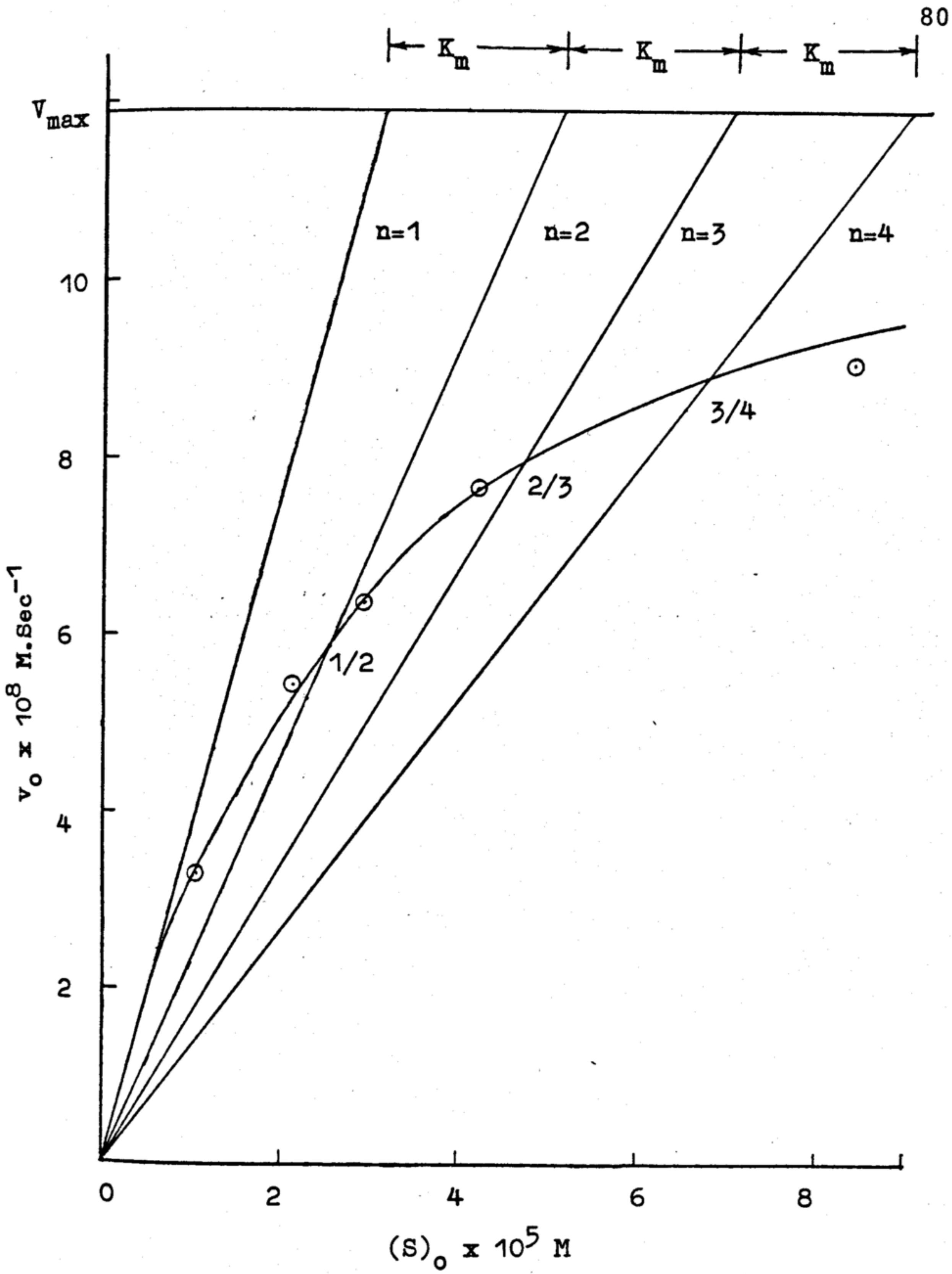
3
2
1
$1/v_o \times 10^{-7}$ (liter/mole/sec)
0
2
4
6
8
10
$1/(S)_o \times 10^{-4}$ M^{-1}



1/v_o x 10^-7 (liter/mole/sec)
3
2
1
0
2
4
6
8
10
1/(S)_o x 10^-4 M^-1



K_m
K_m
K_m
K_m
12
V_{max}
10
8
6
4
2
$v_o \times 10^8$(Moles/liter/sec)
n=1
n=2
n=3
n=4
n=5
1/2
2/3
3/4
4/5
0
2
4
6
8
10
12
14
16
$(S)_o \times 10^5$ M


K_m
K_m
K_m
V_max
n=1
n=2
n=3
n=4
1/2
2/3
3/4
10
8
6
4
2
0
2
4
6
8
v_o x 10^8 M.Sec^-1
(S)_o x 10^5 M

N-Acetyl phenylalanine amide	3×10^{-2}*	4.6×10^{-2}*	1.53*	7.9

*Taken from reference 74.

‡Taken from reference 67.

was also noticed for aspirin-phenylalanine amide hydrolysis. A change in structure of the prodrug from ethyl ester to an amide causes a reduction in the rate of hydrolysis by a factor of 10^5. This is consistent with the results reported by Zerner et al. (74) for N-acetyl- L-phenylalanine derivatives. Correlation between hydroxide ion catalyzed hydrolysis rate constants and the first order enzymatic hydrolysis rate constant k_{cat}/K_m has been shown (65). For aspirin-phenylalanine derivatives, the change in reactivity may be attributed to the electronic effect of the substituent, rather than to any change in binding with the active site. It may also be noticed from Table 12 that N-acetyl and N-aspirin derivatives have k_{cat}/K_m within an order of magnitude. Therefore, at least for the chymotrypsin catalyzed hydrolysis, the ideal condition sought for an enzyme directed prodrug that the rate of reconversion be independent of the drug molecule itself, was closely met. The hydrolysis rates obtained were within the range of expectation based on the reports in the literature.

Tables 10 and 11 show the effect of initial substrate concentration on the initial rate of α-chymotrypsin catalyzed hydrolysis of aspirin-phenyllactic ethyl ester. Experiments with aspirin-phenyllactic ethyl ester had to be carried out under conditions of approximately equal substrate and enzyme concentrations. This violates one of the

basic assumptions of the derivation of Michaelis-Menten equation ($S_o >> E_o$). Dixon (66) has described an elegant and simple direct plot for determining K_m when a substantial fraction of the substrate is bound.

The velocity at any [S] is given by $v = k_{cat}[ES]$. At some given point on the velocity curve we can write

$$v = V_{max}(\frac{n - 1}{n}) \quad . . . \quad (2)$$

where n is a whole number. Therefore, at the given point on the velocity curve

$$k_{cat}[ES] = V_{max}(\frac{n - 1}{n}) \quad . . . \quad (3)$$

$$k_m = \frac{[E][S]}{[ES]} = \frac{([E]_t - [ES])([S]_t - [ES])}{[ES]} \quad . . . \quad (4)$$

After making appropriate substitutions we obtain

$$K_m = \frac{[S]_t}{n - 1} - \frac{[E]_t}{n} \quad . . . \quad (5)$$

A series of lines are drawn from the origin through points on the velocity curve where $v = V_{max}(\frac{n - 1}{n})$. Since n is a whole number (2, 3, 4, etc.), the points correspond to $1/2(V_{max})$, $2/3(V_{max})$, $3/4(V_{max})$, etc. Each line intersects a horizontal line of height V_{max} at different $[S]_n$ values,

called $[S]_2$ (for the line through $1/2(V_{max})$), $[S]_3$ (for the line through $2/3(V_{max})$), $[S]_3$ (for the line through $2/3(V_{max})$), and so on. The value of each $[S]_n$ is given by

$$[S]_n = (\frac{n}{n-1})[S]_t \quad . . . \qquad (6)$$

where $[S]_t$ is the total substrate concentration required for a given n. Consequently,

$$[S]_t = (\frac{n-1}{n})[S]_n \quad . . . \qquad (7)$$

and from equation 5

$$K_m = \frac{[S]_n}{n} - \frac{[E]_t}{n} \quad . . . \qquad (8)$$

or,

$$[S]_n = nK_m + [E]_t \quad . . . \qquad (9)$$

The intercepts on the V_{max} line occur at increments of K_m; that is

$$[S]_n - [S]_{n-1} = K_m \quad . . . \qquad (10)$$

This procedure requires a knowledge of V_{max}. It has been shown (66) that if the V_{max} value chosen is too low, the distances between the intercepts (i.e., K_m) will decrease

toward the right. If V_{max} chosen is too high, the intervals will increase toward the right. Thus the method gives a check on the assumed value of V_{max}.

The data in Tables 10 and 11 were treated in the above mentioned way and the Dixon plots are shown in Figures 15 and 16. The V_{max} values used in the Dixon plots were obtained from the Lineweaver-Burk plots (Figs. 13 and 14) of the data. It can be seen from Figures 15 and 16 that the intercepts were at constant intervals on the V_{max} axis, indicating the validity of the V_{max} values used. As shown in Table 13, the values of K_m obtained by Dixon method are lower than those found by Lineweaver-Burk method. The K_m values shown in Table 12 are those obtained by the Dixon method.

Table 12 shows that the ratio k_{cat}/K_m is much lower for aspirin-phenyllactic ethyl ester than its phenylalanine analog. This may be explained by the specificity of α-chymotrypsin in terms of sites on the enzyme complementary to the four groups which are oriented tetrahedrally about the α-carbon atom of substrates (68-70). The substrate groups may fit into the corresponding sites or associate with them with varying effects. The α-acylamido group fits into its site and associates by hydrogen bonding (71,72). For the phenyllactic acid derivative the acylamido hydrogen capable of hydrogen bonding is lacking. This probably leads to less

Page 87 is missing.

favorable orientation and a concomitant reduction in the reaction rate (k_{cat}).

It has been shown (73) that all esters of the same acyl group give the same steady state rate of hydrolysis indicating that deacylation is the rate limiting step. For the amides, the rate depends on the amide group involved (i.e., acylation is rate limiting). Therefore, for ester hydrolysis with α-chymotrypsin $k_{cat} \simeq k_3$ and for amides, $k_{cat} \simeq k_2$. It may be shown from the enzyme kinetic scheme (equation 1) that K_m and the equilibrium constant K_s are related as follows:

$$K_m = \frac{k_3}{k_2 + k_3} K_s$$

if, $k_2 >> k_3$, $K_m \simeq \frac{k_3}{k_2} K_s$

if, $k_3 >> k_2$, $K_m \simeq K_s$

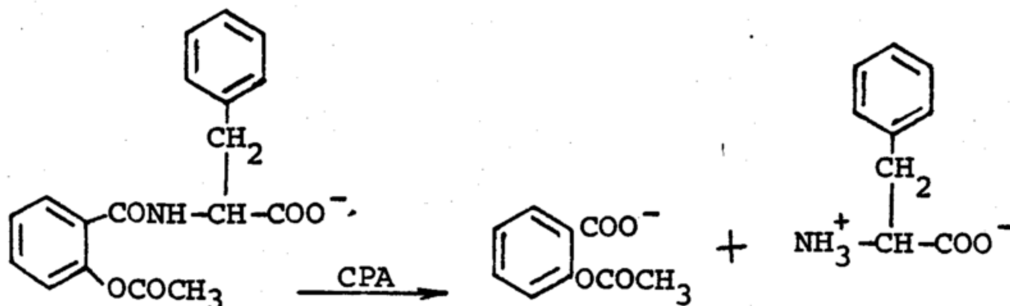
Bender and his coworkers (73,74) have outlined some consequences of equation 1 for two types of substrates, esters and amides. It has been shown (73,74) that the leaving groups (the ester or amide moieties) contribute very little, if anything, to the binding of the substrates to α-chymotrypsin. The values of K_s for amides and esters of the same acylamino acids should therefore be similar.

Because $K_s = K_m$ for the amide substrate, then this experimentally determined value may be used as K_s for the ester. In this way, all the rate constants for various substrates can be evaluated. At pH 8.0 the values obtained for aspirin phenylalanine ethyl ester are $K_s = 2.4 \times 10^{-4}$ M, $k_2 = 9.8 \times 10^3$ sec^{-1} and $k_3 = 25$ sec^{-1}. The values for aspirin phenylalanine amide are $K_s = 2.4 \times 10^{-4}$ M, $k_2 = 6.1 \times 10^{-3}$ sec^{-1}, and $k_3 = 25$ sec^{-1}.

Therefore, of the three prodrugs of aspirin synthesized, aspirin phenylalanine ethyl ester showed the highest rate of hydrolysis in presence of α-chymotrypsin.

2. Studies with carboxypeptidase A

The results of measuring the rates of hydrolysis of aspirin phenylalanine and aspirin phenyllactic acid at a variety of concentrations catalyzed by carboxypeptidase A at pH 8.5 and 7.5 respectively are shown in Tables 14 and 15. The products of carboxypeptidase A catalyzed hydrolysis of aspirin phenylalanine are aspirin and phenylalanine.



The pK_a of α-amino group of phenylalanine is 9.24 and at pH 7.5 it is present almost completely as NH_3^+. At pH 7.5, as soon as a molecule of aspirin is formed by the hydrolysis of aspirin-phenylalanine, its -COOH group is ionized to donate the proton to the α-amino group of phenylalanine, thereby not requiring any external alkali addition to maintain the pH. As such, the pH-stat apparatus could not be used to monitor the rate of hydrolysis of aspirin-phenylalanine at pH 7.5. At pH 8.5, using the Henderson-Hasselbalch relationship

$$pH = pK_a + \log \frac{[base]}{[acid]}$$

we find that in 18.19% of the phenylalanine molecules the α-amino group is present in the non-ionized form. At pH 8.5, the pH-stat recording of the alkali consumption represents only 18.19% of the total hydrolysis. For this reason the experiments had to be performed at pH 8.5 rather than at pH 7.5, the pH of maximum activity.

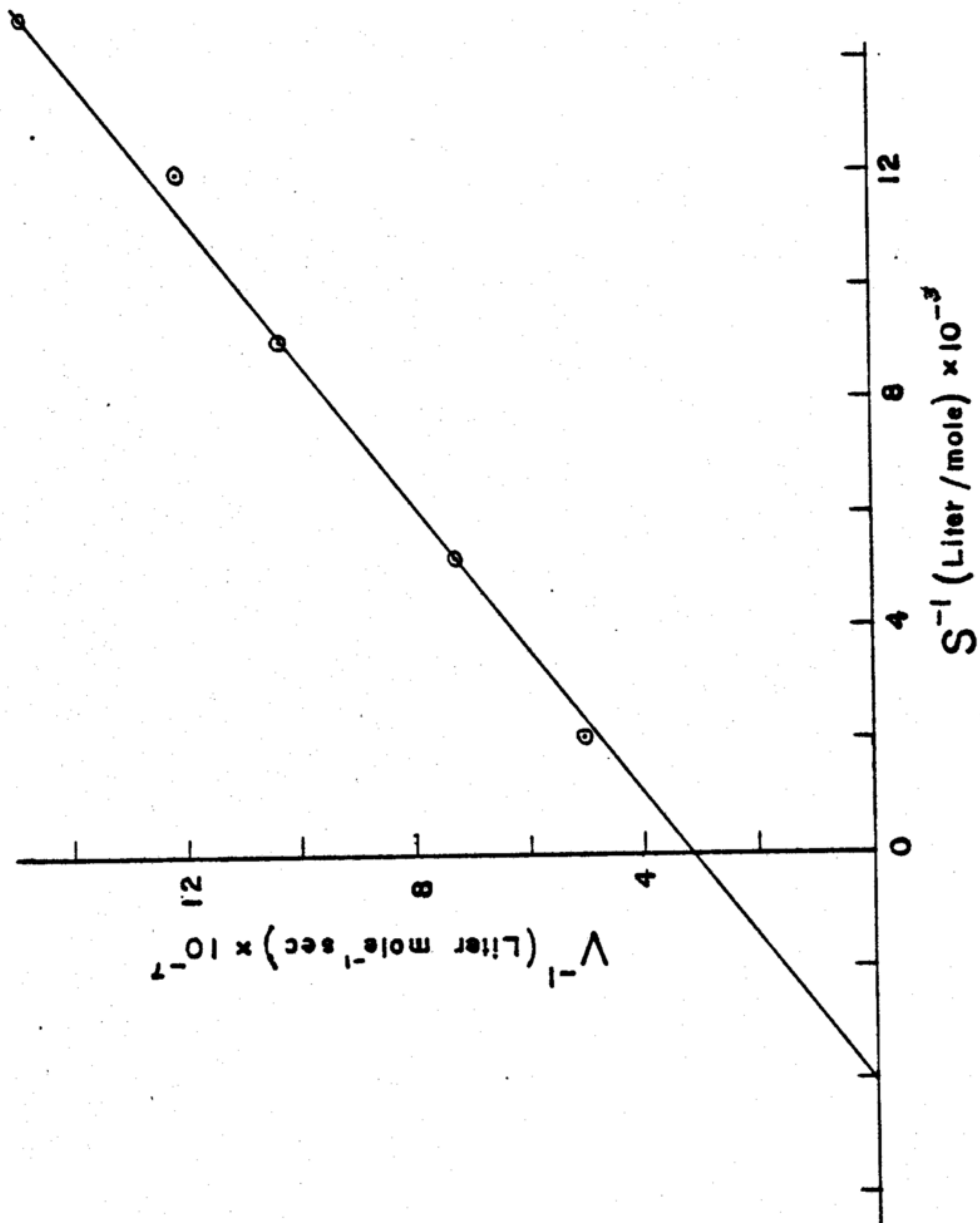
Here also, the Michaelis-Menten assumption of $S_0 >> E_0$ was not adhered to. Lineweaver-Burk plot (Fig. 17) was used to obtain V_{max}, which was then used in the Dixon plot (Fig. 18) to obtain K_m. K_m obtained by Dixon plot was found to be slightly lower than that obtained by Lineweaver-Burk plot. K_m value for aspirin-phenylalanine shown in Table 16 was

TABLE 14. Initial rate data for the hydrolysis of aspirin-phenylalanine catalyzed by 1.0×10^{-5} M carboxypeptidase A at pH 8.5 and 25° in presence of 0.1 M KCl.

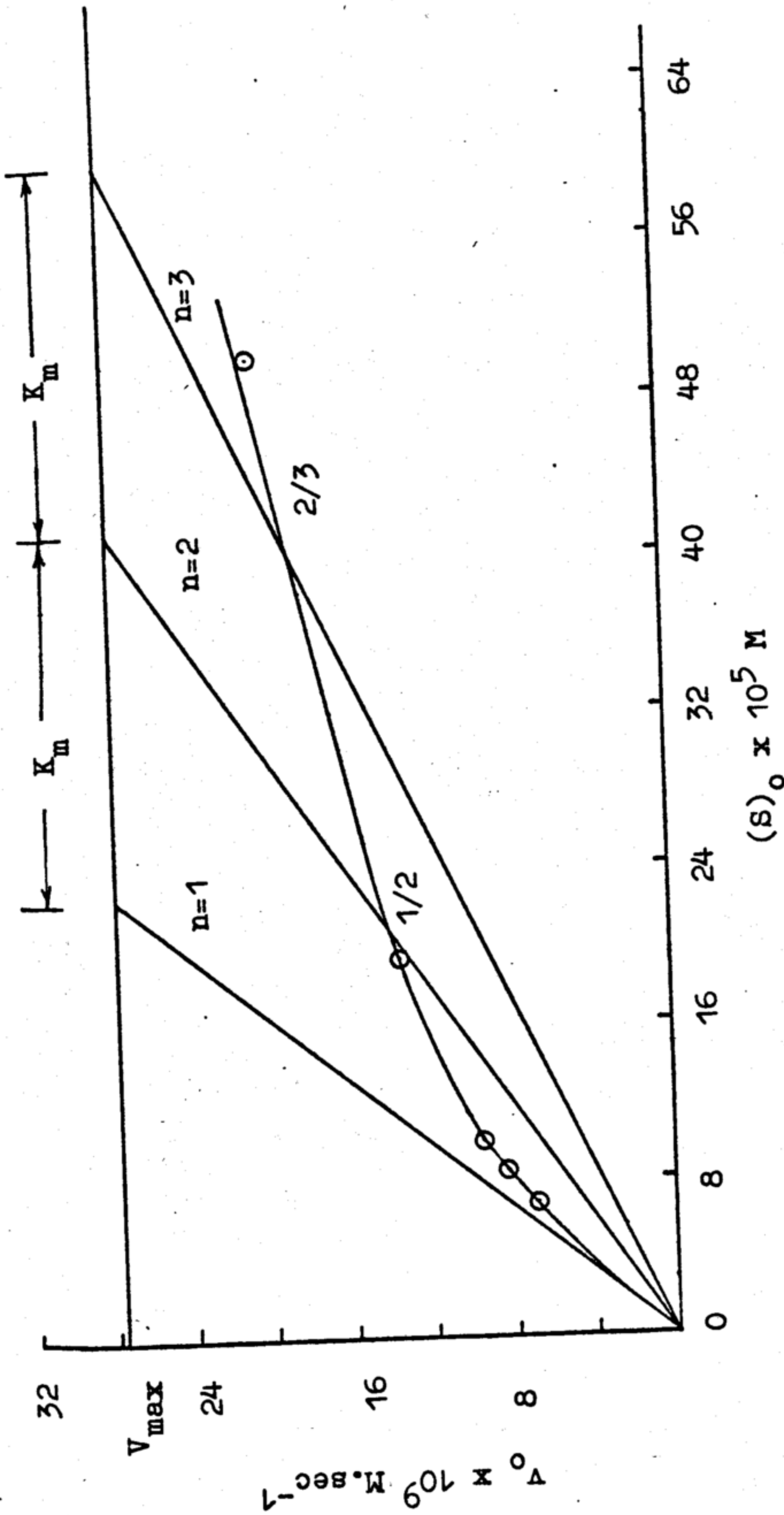
$[S]_o \times 10^5$ M	$V_o \times 10^9$ M·sec^{-1}	$\frac{1}{[S]_o} \times 10^{-3}$ M^{-1}	$\frac{1}{V_o} \times 10^{-7}$ sec·M^{-1}
6.757	6.8	14.8	14.7
8.333	8.265	12.0	12.1
10.0	9.708	10	10.3
19.23	113.51	5.2	7.4
50.0	20	2.0	5.0

TABLE 15. Initial rate data for the hydrolysis of aspirin-phenyllactic acid catalyzed by 2.45×10^{-10} M carboxypeptidase A at pH 7.5 and 25° in presence of 0.1 M KCl.

$[S]_0 \times 10^5$ M	$V_0 \times 10^7$ $M \cdot sec^{-1}$	$\frac{1}{[S]_0} \times 10^{-4}$ M^{-1}	$\frac{1}{V_0} \times 10^{-8}$ $sec \cdot M^{-1}$
1.41	0.0110	7.05	9.09
2.83	0.0195	3.53	5.13
4.25	0.0323	2.35	3.096
5.67	0.0367	1.76	2.725
8.49	0.038	1.17	2.63
11.3	0.045	0.884	2.22



0
4
8
12
S^{-1} (Liter / mole) x 10^{-3}
V^{-1} (Liter mole^{-1} sec) x 10^{-7}



$v_o \times 10^9$ M.sec^{-1}
32
V_{max}
24
16
8
0
8
16
24
32
40
48
56
64
$(S)_o \times 10^5$ M
K_m
K_m
n=1
n=2
n=3
1/2
2/3

TABLE 16. Kinetic parameters for carboxypeptidase hydrolysis at pH 7.5.

Substrate	K_m (moles/liter)	k_{cat} (sec^{-1})	K_i (moles/liter)	$\frac{k_{cat}}{K_m}$ (moles-sec/liter)
Cinnamoyl phenylalanine*	6×10^{-4}	2.1×10^{-2}	---	35
Indoleacryloylphenylalanine*	5.84×10^{-4}	1.4×10^{-3}	---	2.39
Aspirin phenylalanine (pH 8.5)‡	1.3×10^{-4}	8.47×10^{-2}	---	445
Cinnamoyl phenyllactic acid*	1.87×10^{-4}	67	5.78×10^{-5}	3.58×10^{5}
Furylacryloyl phenyllactic acid	1.32×10^{-4}	47	---	3.56×10^{5}
Aspirin phenyllactic acid	1×10^{-4}	25	4×10^{-6}	2.5×10^{5}

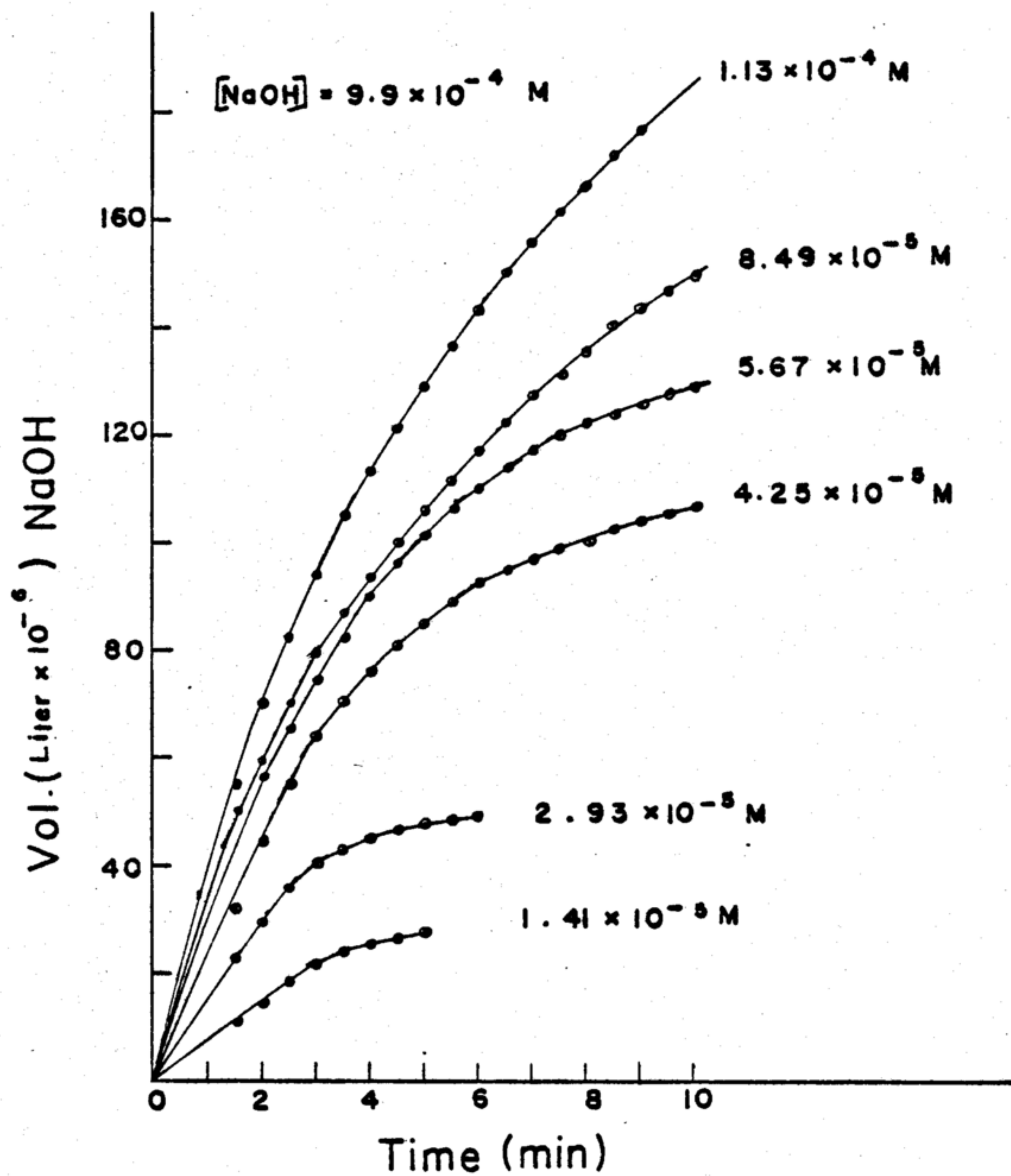
*Taken from reference 67.

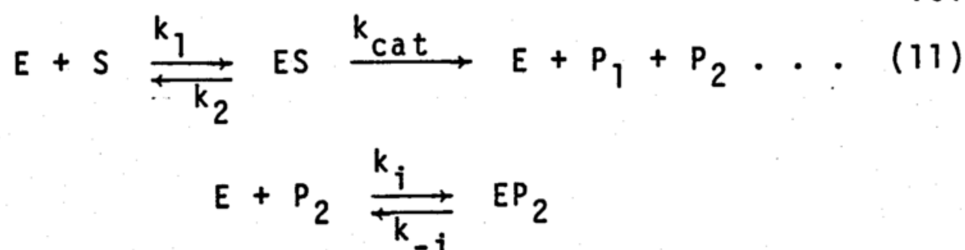
‡Done at pH 8.5.

obtained from the Dixon plot. Figure 17 shows a good straight line fit of the data when 1/v is plotted against 1/s, indicating the applicability of the classical equation even though the basic assumptions were not satisfied. It is highly likely that the experimental data are not precise enough to show the distinction between the two equations.

The results of six runs measuring the rates of hydrolysis of aspirin-phenyllactic acid at six different concentrations catalyzed by carboxypeptidase A at pH 7.5 are illustrated in Figure 19. The points shown were taken from the continuous record of the titrator for the purposes of calculation. The curves of this plot do not seem to follow a simple kinetic order, and sharp decrease of rates of hydrolysis with time suggests that product inhibition of the enzyme may be playing an important role. Significant product inhibition of carboxypeptidase A has been reported in the literature (75,76).

The scheme which fits the kinetics of the carboxypeptidase A catalyzed hydrolysis of aspirin-phenyllactic acid is shown below, together with the corresponding integrated Michaelis-Menten rate expression (E = carboxypeptidase A, S = aspirin phenyllactic acid, P_1 = aspirin, P_2 = L-phenyllactic acid). The L-phenyllactic acid (P_2) produced during the hydrolysis acts as a competitive inhibitor.


[NaOH] = 9.9 × 10⁻⁴ M
1.13 × 10⁻⁴ M
8.49 × 10⁻⁵ M
5.67 × 10⁻⁵ M
4.25 × 10⁻⁵ M
2.93 × 10⁻⁵ M
1.41 × 10⁻⁵ M
Vol. (Liter × 10⁻⁶) NaOH
160
120
80
40
0
2
4
6
8
10
Time (min)



Integrated Michaelis-Menten equation

$$\frac{[S]_o - [S]}{t} = \frac{K_m(K_i + [S]_o)}{(K_m - K_i)t} \ln([S]_o/[S]) + \frac{V_{max}}{1 - K_m/K_i} =$$

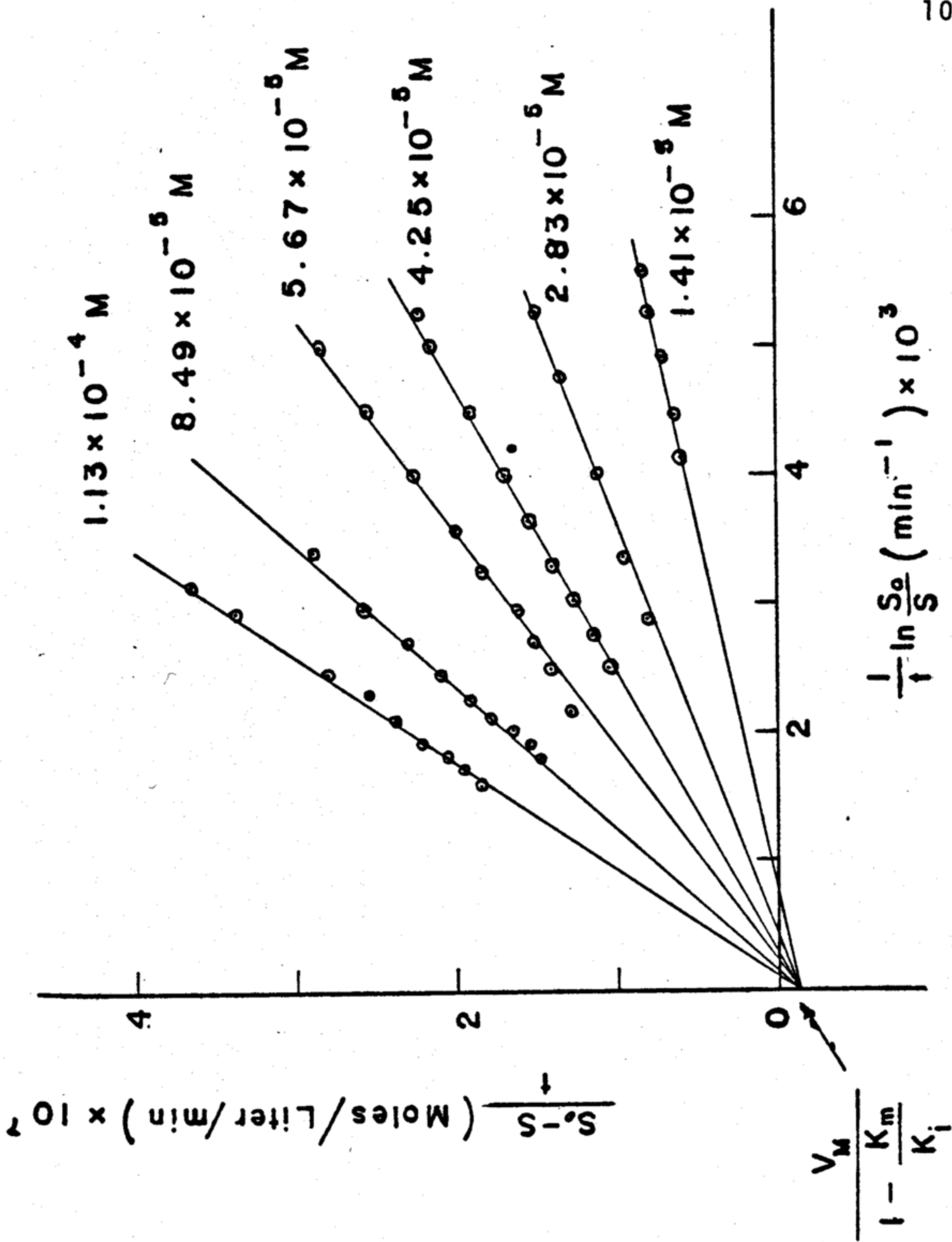
$$\frac{a}{t} \ln \frac{[S]_o}{[S]} + \frac{V_{max}}{1 - K_m/K_i} \quad . \; . \; . \qquad (12)$$

where, $K_m = \frac{k_{-1} + k_2}{k_1}$, $K_i = \frac{k_{-i}}{k_i}$

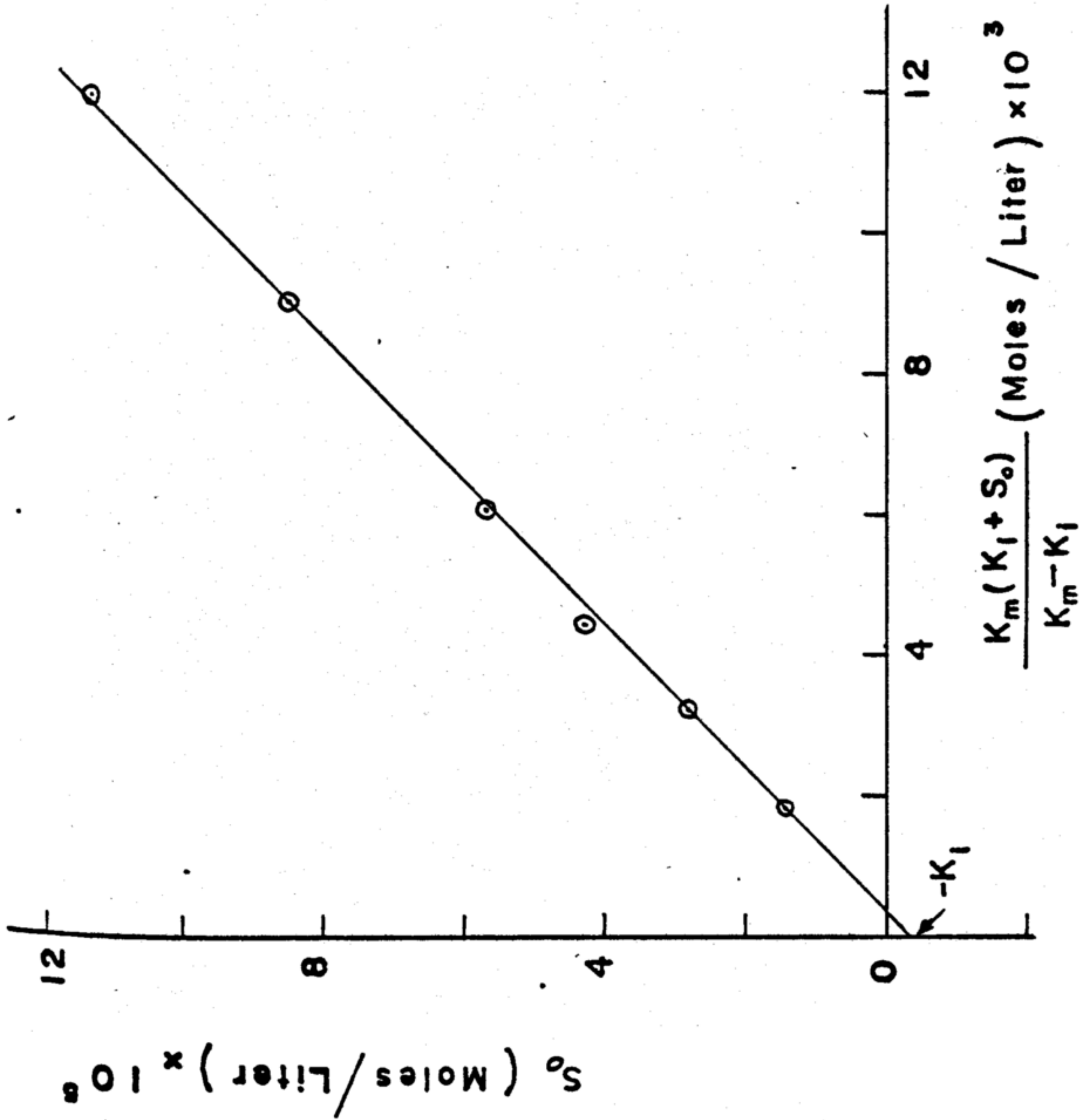
and $a = \frac{K_m(K_i + [S]_o)}{K_m - K_i}$

The integrated Michaelis-Menten equation for competitive product inhibition applies to the kinetic data obtained at pH 7.5 for aspirin-phenyllactic acid very satisfactorily. This is illustrated by Figure 20 in which $([S]_o - [S])/t$ is plotted on the ordinate against $1/t \ln ([S]_o/[S])$ on the abscissa. The linearity of the plots thus obtained is consistent with the proposed competitive product inhibition, since the lines would curve for other types of product inhibition.

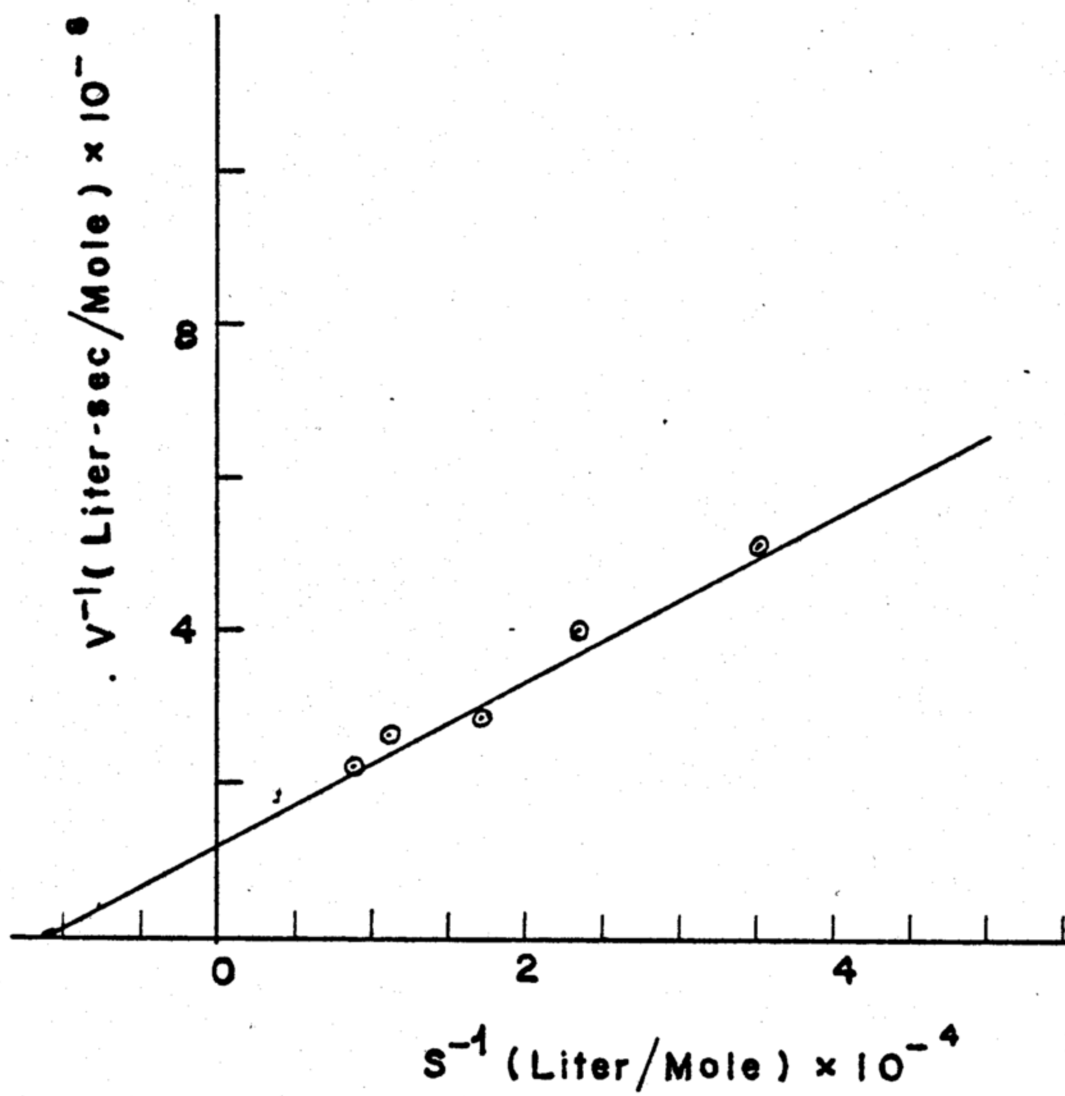
The intercept on the ordinate of Figure 20 is a function of V_{max}, K_m and K_i, and the slopes of the straight



1.13 × 10⁻⁴ M
8.49 × 10⁻⁵ M
5.67 × 10⁻⁵ M
4.25 × 10⁻⁵ M
2.83 × 10⁻⁵ M
1.41 × 10⁻⁵ M
0
2
4
6
$\frac{1}{t} \ln \frac{S_o}{S}$ (min⁻¹) × 10³
0
2
4
$\frac{S_o - S}{t}$ (Moles/Liter/min) × 10⁷
$\frac{V_M}{1 - \frac{K_m}{K_i}}$



S_o (Moles/Liter) x 10
12
8
4
0
$-K_I$
4
8
12
$\frac{K_m(K_I + S_o)}{K_m - K_I}$ (Moles / Liter) x 10^3



V⁻¹(Liter-sec/Mole) × 10⁻⁸
8
4
0
2
4
S⁻¹ (Liter/Mole) × 10⁻⁴

lines, a, are related to K_m, K_i and $[S]_o$ by the equation

$$[S]_o = a(1 - \frac{K_i}{K_m}) - K_i \quad . . . \qquad (13)$$

Thus, in order to separate the various kinetic parameters, in Figure 21 the values of $[S]_o$ have been plotted against the respective slopes, a, found from Figure 20. The intercept along $[S]_o$ axis is $-K_i$, and hence the values of K_m and V_{max} can be calculated. It is to be noted that K_i was found to be considerably smaller than K_m, indicating strong competitive inhibition by the product, L-phenyllactic acid.

As a check on the application of the integrated Michaelis-Menten equation and the computed kinetic constants K_m and V_{max} have also been determined by the Lineweaver-Burk procedure. From Figure 22, it can be seen that the double reciprocal plot of the kinetic results is linear. The agreement between kinetic parameters calculated by the two procedures at pH 7.5 is good, as indicated in Table 17, where the results are summarized.

An overall summary of the carboxypeptidase A catalyzed hydrolysis is given in Table 16 along with some results obtained from the literature for the purpose of comparison. Aspirin-phenyllactic acid was found to be a better substrate than the corresponding phenylalanine derivative. k_{cat}/K_m obtained were comparable to those of the substrates reported

TABLE 17. Kinetic parameters for carboxypeptidase A hydrolysis of aspirin-phenyllactic acid.

Kinetic Parameter	Calculated by Lineweaver-Burk method	Calculated by integrated Michaelis-Menten equation
K_m	8.74×10^{-5}	1×10^{-4} moles/liter
K_i	---	4×10^{-6} moles/liter
V_{max}	8.55×10^{-9}	6×10^{-9} moles/liter
k_{cat}	35	25 sec^{-1}

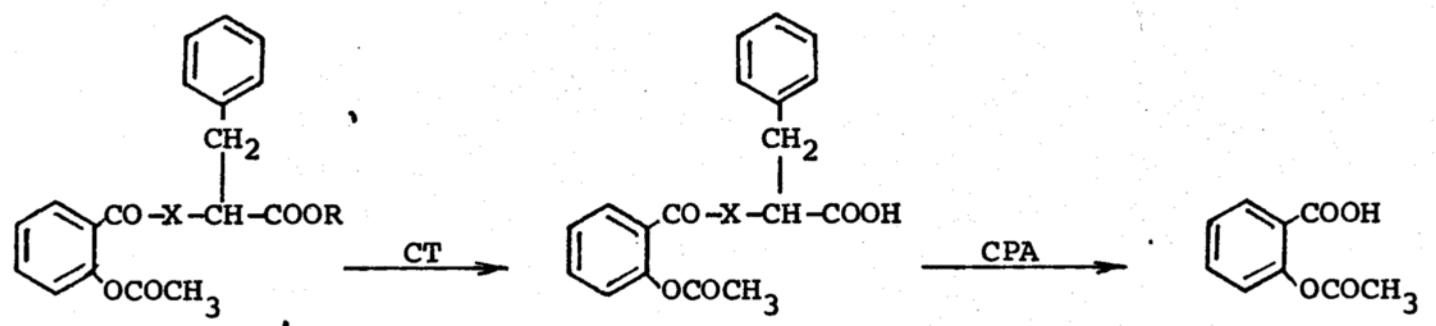
in the literature, indicating that our stated goal of making the reconversion rate independent of the structure of the drug molecule itself was closely met.

3. Conclusions

Table 18 gives the estimated range of *in vivo* reconversion rates using the kinetic parameters of the rate limiting steps. Table 18 shows the values obtained using 10^{-4} M and 10^{-6} M enzymes. For aspirin-phenylalanine ethyl ester the estimated ranges for half-life was from 5.9 sec to 16.8 sec (for $E_o = 10^{-4}$ M) and from 10 min to 28 min (for $E_o = 10^{-6}$ M). Those for aspirin=phenyllactic ethyl ester were from 13.5 sec to 61.9 sec (for $E_o = 10^{-4}$ M) and from 22.5 min to 103 min (for $E_o = 10^{-6}$ M).

The rate limiting step for aspirin-phenylalanine ethyl ester was that catalyzed by carboxypeptidase A and that for aspirin-phenyllactic ethyl ester was the reaction catalyzed by α-chymotrypsin. Since, aspirin-phenylalanine ethyl ester was found to have more favorable reconversion rate to aspirin and aspirin-phenyllactic ethyl ester was very expensive to make, it was decided at this point to carry out further studies with aspirin-phenylalanine ethyl ester.

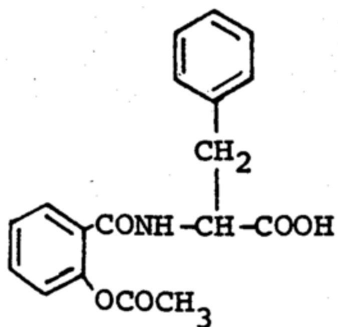
TABLE 18. Estimated range of *in vivo* reconversion rates ($S_o = 10^{-5}$ M).



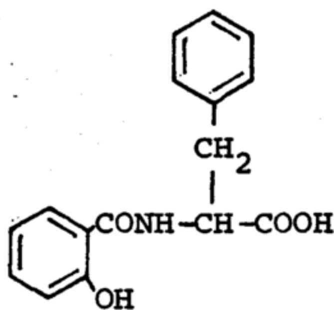
Structure	E_o	pH	Maximal rate (zero order) $v = k_{cat}E_o$	Minimal rate (first order) $v = (k_{cat}/K_m)E_oS$
X = NH R = $-C_2H_5$	10^{-4} M	8.5	$t^{½}$ = 5.9 sec	16.8 sec
X = -O- R = $-C_2H_5$	10^{-4} M	7.5	$t^{½}$ = 13.5 sec	61.9 sec
X = NH- R = $-C_2H_5$	10^{-6} M	8.5	$t^{½}$ = 10 min	28 min
X = -O- R = $-C_2H_5$	10^{-6} M	7.5	$t^{½}$ = 22.5 min	103 min

B. Hydrolytic Pathways of Aspirin-Phenylalanine Ethyl Ester in Simulated Intestinal Fluid

Aspirin-phenylalanine ethyl ester was not detectable on TLC plate in the very first sample drawn in less than one minute. Initially a dark spot with low R_f value, much lower than that of aspirin and salicylic acid, was found. After about six minutes this spot led to the formation of a leading spot (appearing dense blue under short wavelength UV source) with R_f value higher than that of the parent spot, but still lower than that of aspirin. After 15 hours, the lower spot disappears leaving only the upper dense blue spot behind. After running a thick layer plate, scraping off the respective areas, eluting in methanol, evaporating the solvent and taking NMR and mass spectra, the lower spot was identified as aspirin-phenylalanine (I) and the upper spot



I



II

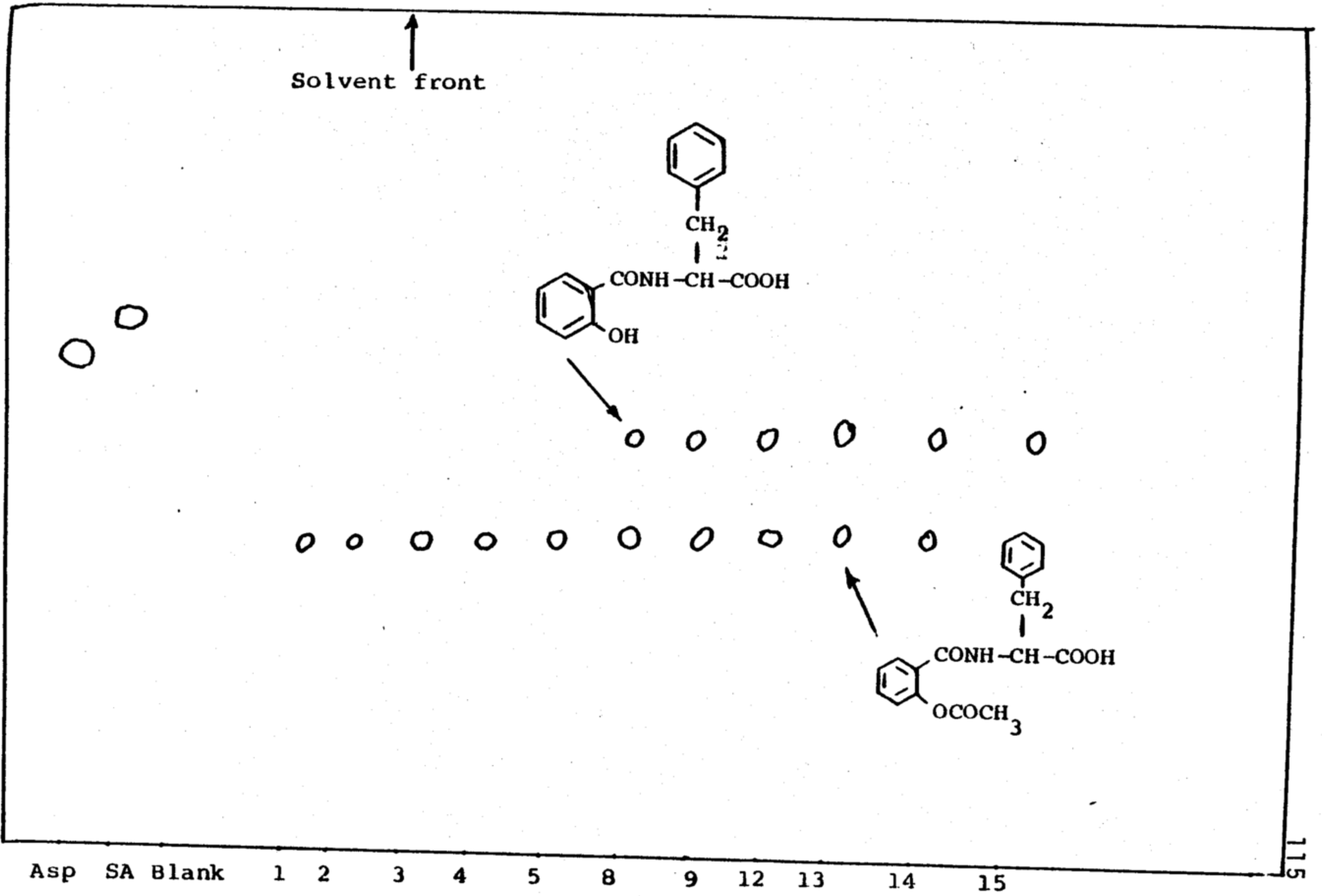
as salicylic acid-phenylalanine (II). The actual chromatogram using methanol:acetic acid:ether:benzene (1:18:60:20)

and silica gel as support material is shown in Figure 23.

A similar study in which α-chymotrypsin was used instead of pancreatin in the simulated intestinal fluid, also showed the exactly same chromatogram. This led to the suspicion that the pancreatin may be devoid of carboxypeptidase activity. An assay of pancreatin for carboxypeptidase A activity using hippuryl pehnylalanine as the substrate showed no detectable carboxypeptidase A activity.

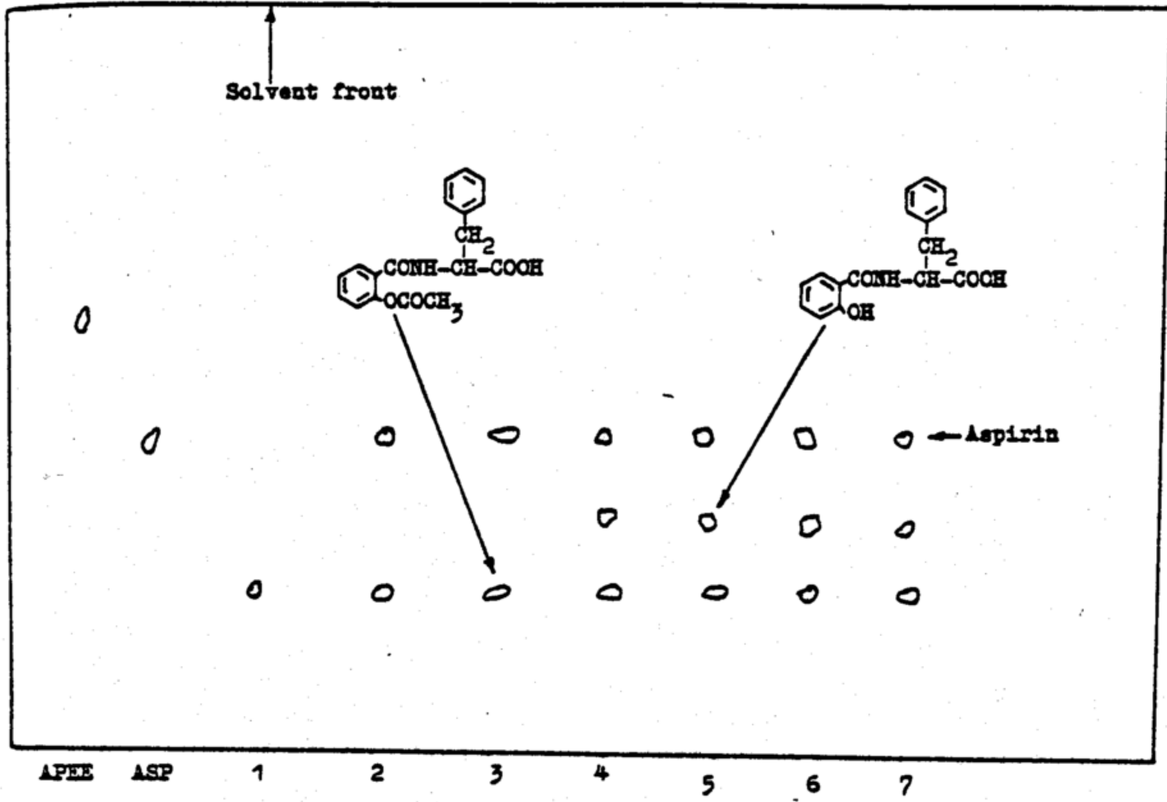
The objective of this experiment was to identify the pathway of degradation of aspirin-phenylalanine ethyl ester in presence of digestive enzymes of the intestinal tract. Scheme V shows the possible pathways of degradation. From TLC studies it was found that A is formed almost instantaneously. Therefore, the pathway through step I can be ruled out. In the absence of any detectable carboxypeptidase A activity, compound A was found to be converted to compound B via step II catalyzed by OH^- ion and possibly also by the protein side chains present in the solution acting as non-specific bases. When aspirin-phenylalanine ethyl ester was added to a mixture of α-chymotrypsin (50 mg/100 ml) and carboxypeptidase A (25 mg/100 ml) conversion of compound A to aspirin was observed. Thin layer chromatograms of the chloroform extracts of the acidified solutions are shown in Figure 24.

Quantitative studies were performed on aspirin-

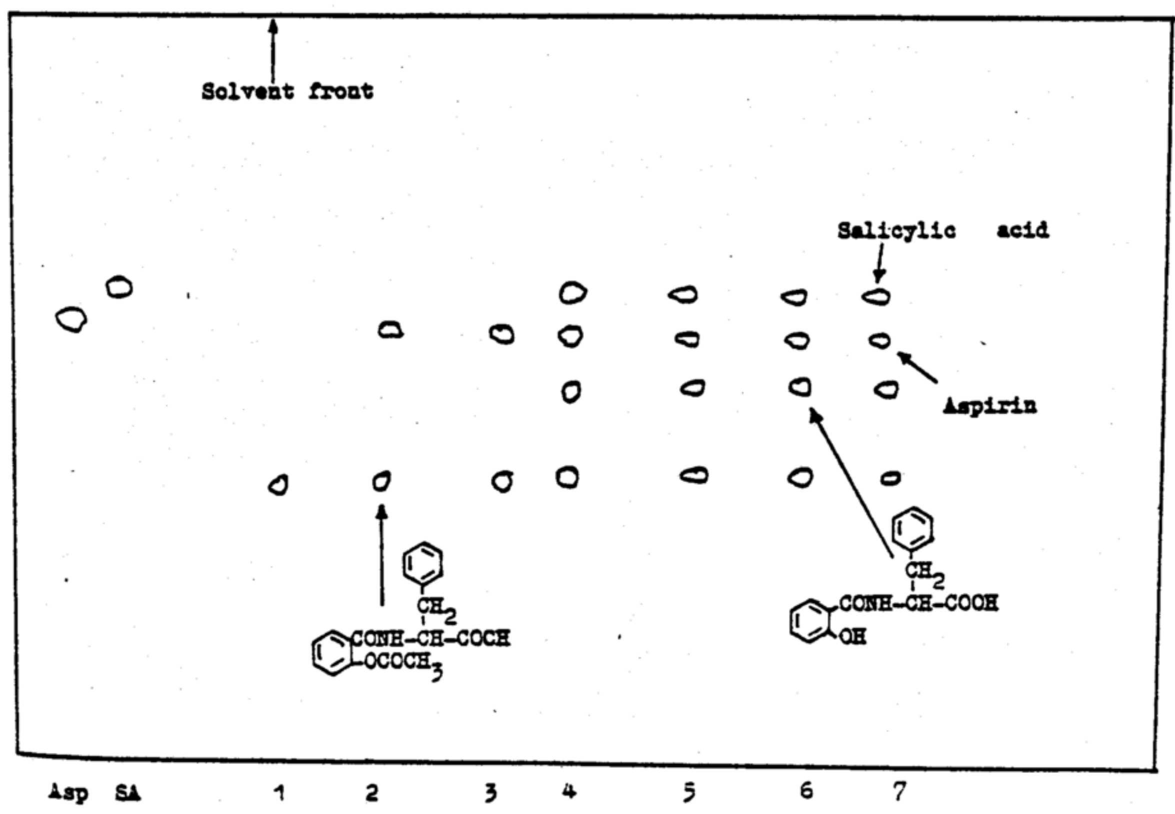


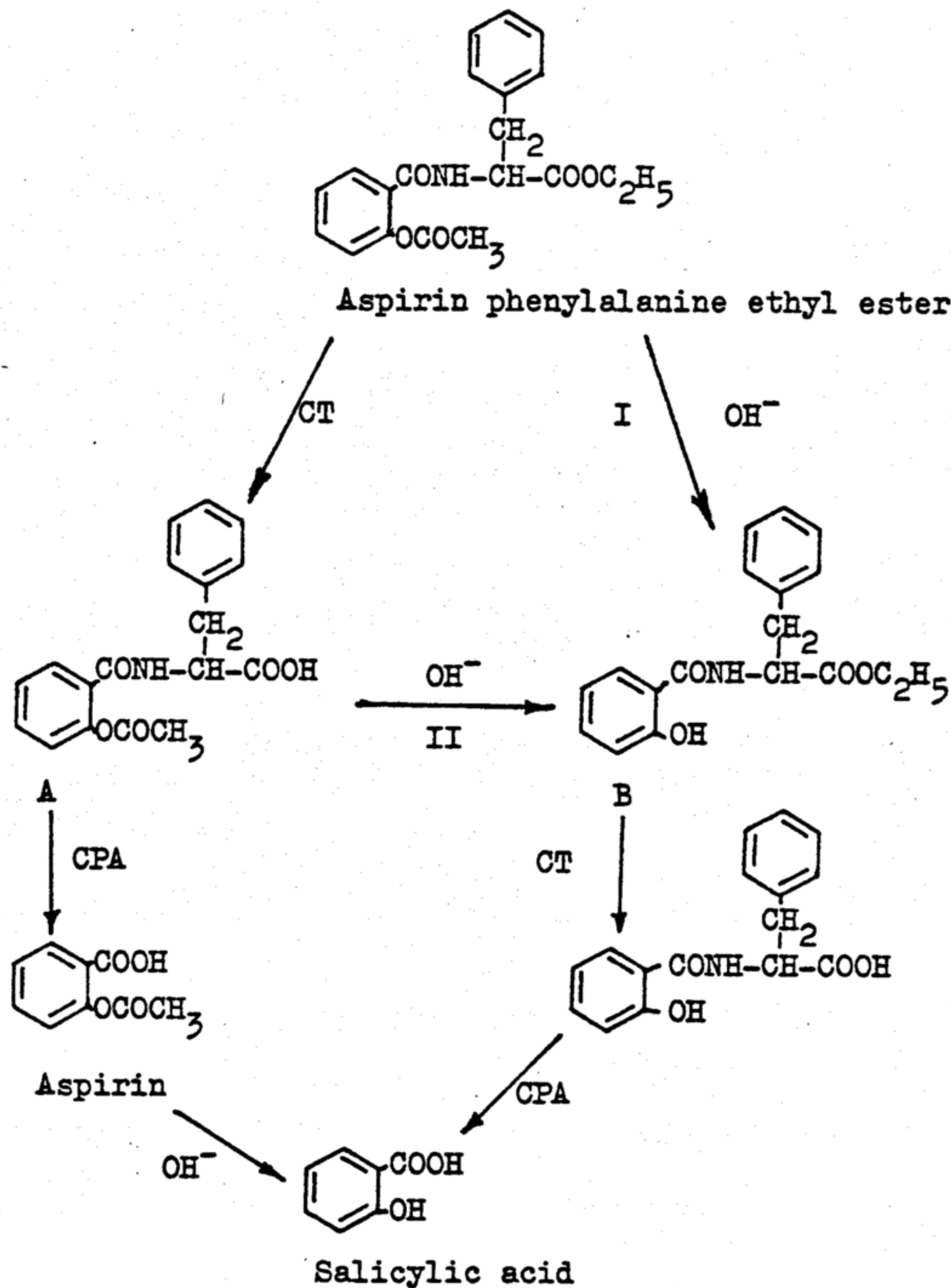
Solvent front
CH2
CONH-CH-COOH
OH
CH2
CONH-CH-COOH
OCOCH3
Asp SA Blank 1 2 3 4 5 8 9 12 13 14 15

A.



B.





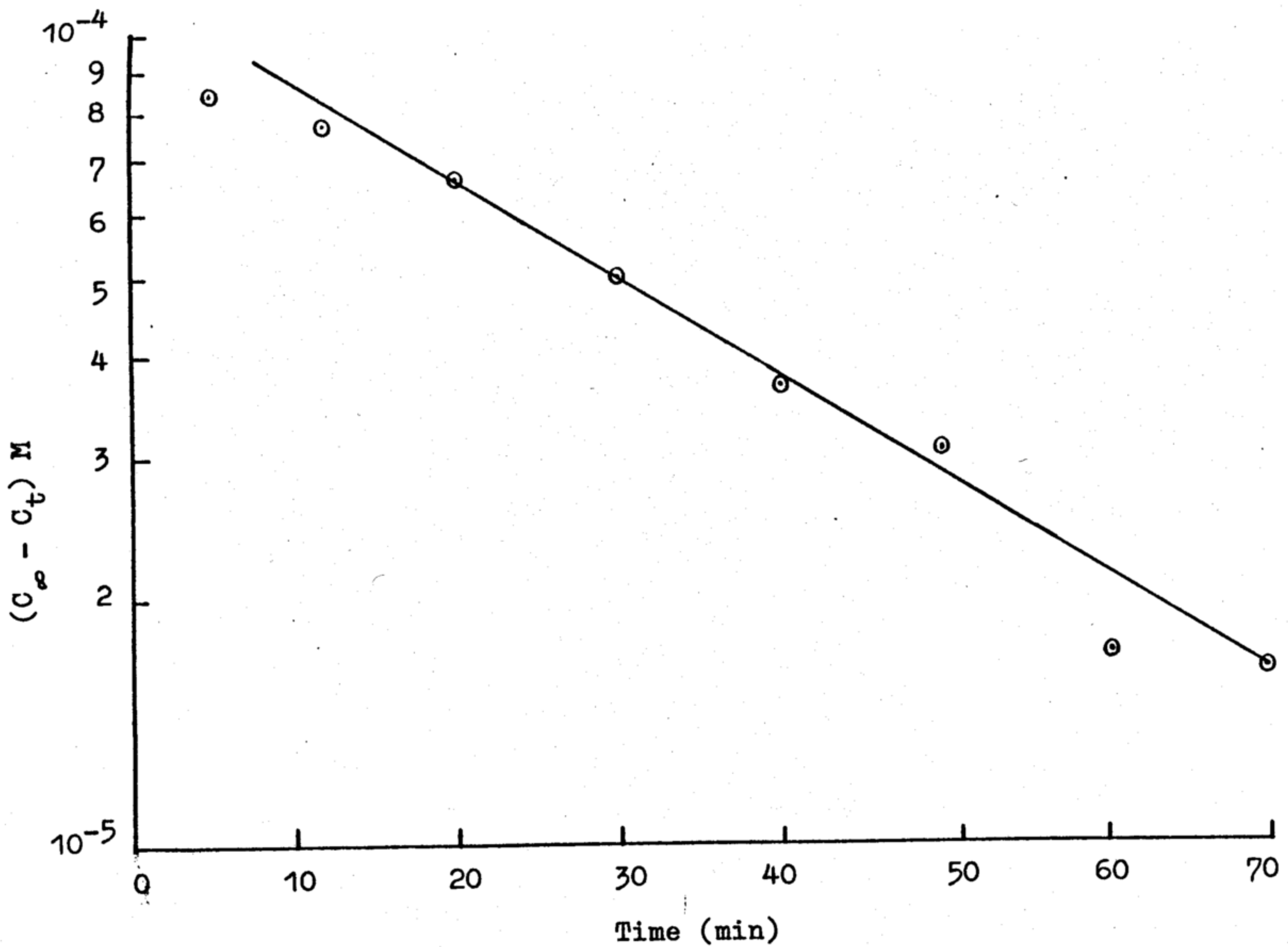
Scheme V. Possible hydrolytic pathways of aspirin phenylalanine in presence of mixture of chymotrypsin and carboxypeptidase A.

phenylalanine and salicylic acid-phenylalanine. From pure samples of aspirin-phenylalanine and salicylic acid-phenylalanine the λ_{max} were found to be 275 nm and 303 nm respectively, and the molar absorptivities at λ_{max} were 1350 $mole^{-1}cm^{-1}$ and 3900 $mole^{-1}cm^{-1}$ respectively. The absorbances of the kinetic samples were measured at 275 nm and 303 nm. From these absorbance measurements the concentrations of the two species present were calculated using the formula

$$C_{Asp\text{-}Phe} = \frac{A_{275}(\varepsilon^{275}_{SPhe}/\varepsilon^{303}_{SPhe})A_{303}}{\varepsilon^{275}_{Asp\text{-}Phe}}$$

$$C_{SPhe} = \frac{A^{303}_{SPhe}}{\varepsilon^{275}_{SPhe}}$$

where, Asp-Phe = aspirin-phenylalanine and SPhe = salicylic acid-phenylalanine. A plot of log $(C_{\infty} - C_t)$ of salicylic acid-phenylalanine is shown in Figure 25. A straight line fit indicates the conformity to a first order degradation. The observed first order rate constant, k_{obs}, was calculated to be 4.66×10^{-4} sec^{-1} corresponding to a half-life of 0.41 hrs. This represents the half-life for the hydrolysis of the O-acetyl group in aspirin-phenylalanine. The presence of large amount of protein (1.0 g/100 ml) in the simulated intestinal fluid may be the reason for the



10^{-4}
9
8
7
6
5
4
3
2
10^{-5}
$(C_\infty - C_t)$ M
0
10
20
30
40
50
60
70
Time (min)

very rapid rate of hydrolysis of the O-acetyl group, compared to the half-life of approximately 52 hours for the hydrolysis of the O-acetyl group of aspirin at pH 7.5 in water.

The half-life of conversion of compound A to B was found to be 0.41 hours and the half-life for carboxypeptidase A catalyzed reaction was calculated to be 5.9 sec (for 10^{-4} M enzyme) and 10 min (for 10^{-6} M enzyme). Demonstration of the formation of aspirin from compound A in presence of a mixture of α-chymotrypsin and carboxypeptidase A, coupled with the faster rate of conversion of the carboxypeptidase A catalyzed step compared to step II indicates that *in vivo* the carboxypeptidase A catalyzed step may be expected to take precedence over step II and lead to the formation of aspirin.

C. Shelf Life Studies of Aspirin-Phenylalanine Ethyl Ester in Aqueous Solutions

Logarithms of concentrations of aspirin-phenylalanine ethyl ester remaining in the aqueous solutions at various pH's, were plotted against time. Reactions were followed up to 10% degradation. From the slopes of these plots the pseudo-first order rate constants, k_{obs}, was determined and at pH 5.6 the corresponding shelf life was 55.2 hours. The pseudo-first order rate constants at different pH's and the

TABLE 19. Kinetic rate constants and shelf lives of aspirin-phenylalanine ethyl ester in aqueous solutions.

pH	$k_{obs} \times 10^3$ (hrs^{-1})	t^{90} (hrs)
7.5	9.84	10.8
6.6	2.77	38.3
5.6	1.92	55.2

corresponding shelf lives are given in Table 19.

In case of a suspension, the rate of degradation is given by

$$\frac{dc'}{dt} = k_1 s$$

where c' is the concentration of the drug in solution (mass/volume), k_1 is the first order rate constant and s is the solubility of the drug expressed in the same unit as c'. For v ml of suspension,

$$v\frac{dc'}{dt} = \frac{dc}{dt}(\text{mass/time})$$

$$= -k_1 vs = -k_0$$

where k_0 is the zero order rate constant for the drug in suspension.

The solubility of aspirin-phenylalanine ethyl ester was found to be 4.7×10^{-4} M (i.e., 0.167 mg/ml) compared to that of 1.85×10^{-2} M (i.e., 3.33 mg/ml) of aspirin. The calculated shelf life of a 600 mg/5 ml of suspension of aspirin-phenylalanine ethyl ester (i.e., an equivalent of 300 mg/5 ml of aspirin) was found to be 4.27 years at pH 5.6 compared to 38 days for 300 mg/5 ml of aspirin suspension at its optimum pH (2.25) for stability. This result indicates

to the possibility of making a stable suspension of aspirin by using amino acid derivatized prodrugs.

D. Absorption Studies *In Situ* from Rat Intestine

To facilitate the understanding of the processes involved in prodrug reconversion and absorption, theoretical models have been developed (83) for the analysis of the *in situ* perfusion experiments described in the experimental section of this report. The objective was to develop a relation between experimentally observed final prodrug/drug concentration and quantities such as intestinal length, flow rate, diffusivity, initial concentration, etc.

In developing the model the following assumptions were made:

i) The intestine can be considered a rigid, straight, smooth walled cylinder of radius γ_w.

ii) Axial diffusion is negligible compared with convection.

iii) The intestinal wall can be considered as a series of anatomically and physiologically distinct barriers, arranged as concentric annuli. The innermost layer is a diffusion layer of thickness of about 100 μ, the middle layer is the zone of combined reaction and diffusion of about 0.1 μ thickness, the outermost layer is a lipid layer

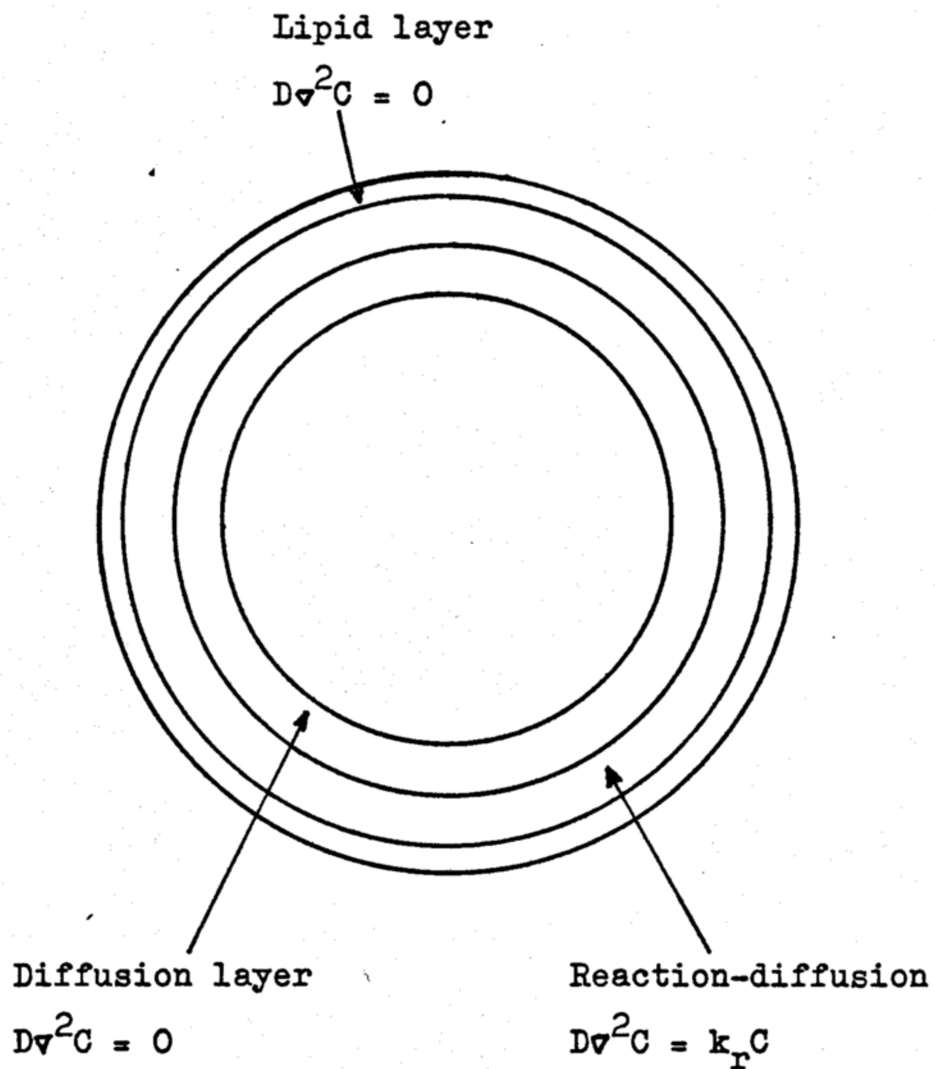


Figure 26. Concentric annuli model for intestine.

with 100 Å as the lower limit of its thickness. These are shown in Figure 26, along with the appropriate differential equations applicable to each layer.

Theoretical expressions have been developed (83) for the ratio c_m/c_o, where c_m = final (cup mixing) concentration of solute and c_o = initial concentration of conditions. For complete radial mixing

$$\frac{c_m}{c_o} = \exp(\frac{-4A}{\Omega}) \quad . \; . \; . \qquad (14)$$

For laminar and plug the expressions assume the same form

$$c_m/c_o = \sum_{i}^{roots}(\frac{4A^2}{bi^4 + [Abi)^2})(\exp(\frac{-2bi^2}{\Omega})) \quad . \; . \; . \qquad (15)$$

where, bi is a root of

$$bJ_1(b) - AJ_0(b) = 0 \quad . \; . \; . \qquad (16)$$

bi is different for laminar and plug flow. Here,

J_1 = first order Bessel function

J_0 = zero order Bessel function (83)

Ω = $\frac{Q}{30\pi DL}$ = $Re \cdot Sc \cdot \gamma_w / L$

Q = flow rate, ml/min

D = diffusivity, cm^2/sec

A = effective permeability = $\frac{K_w \gamma_w}{D}$

L = length of intestine

Re = Reynolds number = $\frac{\langle v \rangle \cdot 2\gamma_w}{\nu}$

Sc = Schmidt number = $\frac{\nu}{D}$

$\langle v \rangle$ = average velocity

K_w = wall permeability

ν = Stokes viscosity, cm^2/sec

γ_w = radius of the intestine

Results of the perfusion experiments with aspirin-phenylalanine ethyl ester are summarized in Table 20. Computer program was written (83) to calculate the effective permeability, A, from known values of c_m/c_o and the corresponding Ω, for the different flow conditions. Table 21 shows the computed A values for different c_m/c_o and Ω values. c_m/c_o vs. Ω values obtained experimentally were fitted by nonlinear regression analysis against the theoretical expressions equations 14-16. The results of the regression analysis is shown graphically in Figure 27. All three theoretical models could fit the data adequately with laminar flow model giving marginally better fit. The A values which gave the best fit are given in Table 22. These results underscore the validity of the assumptions given in the beginning of this section.

From the results of this experiment it can be said that

TABLE 20. Absorption of aspirin and aspirin-phenylalanine ethyl ester from rat intestine *in situ* (c_o for prodrug = 1.408×10^{-4} M, c_o for aspirin = 1.281×10^{-4} M).

Rat no.	Length of intestine (cm)	Flow rate (ml/min)	Omega (Ω)	c_m/c_o for aspirin	c_m/c_o for prodrug
1	8	0.0494	14	---	0.63
		0.123	35	---	0.78
		0.247	70	---	0.86
		0.494	139	0.995	0.88
2	8	0.123	35	---	0.75
		0.247	70	---	0.84
		1.23	347	---	0.96
3	8	0.0494	14	---	0.60
		0.247	70	---	0.87
		1.23	347	---	0.94
4	9	0.0494	12	---	0.60
		0.123	31	---	0.75
		0.494	123	1.01	0.90
		1.23	308	---	0.97
5	8.5	0.494	130	0.98	0.89
6	8.5	0.494	130	0.987	0.92

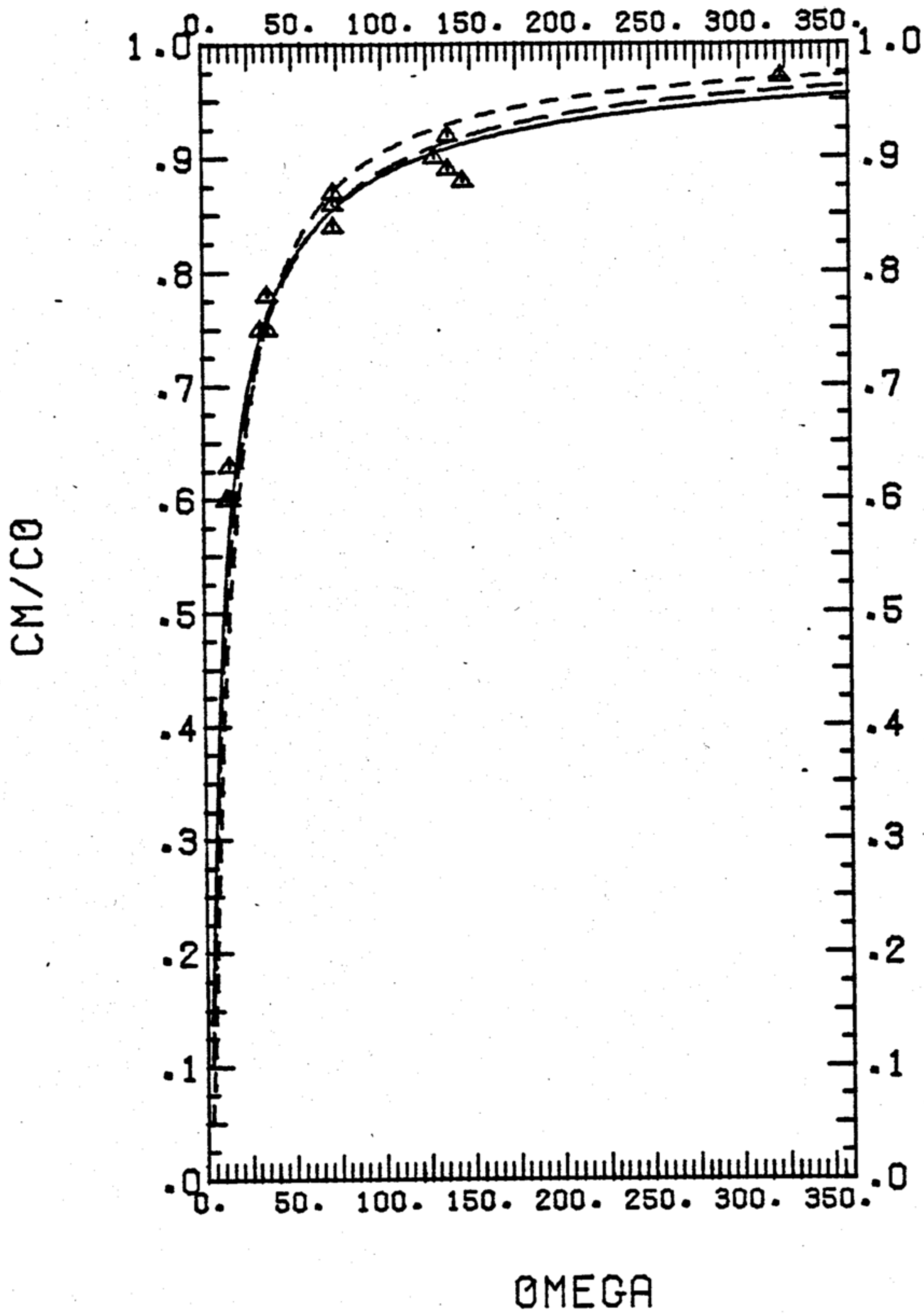
TABLE 21. Effective permeabilities under different flow conditions.

Omega (Ω)	c_m/c_o	A (CRM)	A (plug)	A (Lam)
13.98	0.610	1.728	2.656	5.049
34.82	0.760	2.389	3.707	8.483
72.80	0.856	2.383	4.129	8.748
130.5	0.897	3.724	5.414	13.63
334	0.957	3.826	4.812	7.908

CRM = complete radial mixing.

plug = plug flow.

Lam = laminar flow.



0. 50. 100. 150. 200. 250. 300. 350.
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
.0
CM/C0
OMEGA

TABLE 22. Effective permeabilities obtained from nonlinear regression analysis.

Type of flow	A ± s.e.	Correlation coefficient (R^2)
CRM	1.99 ± 0.25	0.9975
Plug	3.23 ± 0.48	0.9988
Lam	6.87 ± 1.52	0.9993

CRM = complete radial mixing.

Plug = plug flow.

Lam = laminar flow.

the prodrug is satisfactorily absorbed from the rat intestine as measured by the loss of the prodrug from the intestine. The effective permeability value, A, depends on the membrane/water partition coefficient of the prodrug. The high A value for the laminar flow model (the model which best fits the data) may be the effect of high partition coefficient (524 in octanol/water estimated by the methods in ref. 84) of the prodrug. High A value would also result from high rates of simultaneous enzymatic reaction and transport occurring in the membrane. The actual situation is likely to be intermediate between these two extreme cases. Aspirin being completely ionized at pH 7.5 was not absorbed from the intestine of rats (Table 20). This experiment does not provide us with any information as to whether the prodrug is absorbed intact (i.e., unmetabolized) into the blood or is simultaneously reconverted to aspirin by the brush border enzymes and absorbed. Separate experiments need to be designed to determine the mechanism of absorption from the intestine.

E. Dissolution of Aspirin-Phenylalanine Ethyl Ester in Presence of Enzyme

The dissolution rate of aspirin-phenylalanine ethyl ester in the rotating disc apparatus can be predicted from the Levich equation (52),

$$J = 0.62D^{2/3}\nu^{-1/6}\omega^{1/2}c_o \quad . . . \qquad (17)$$

where D is the diffusion coefficient of the solute, ν is the kinematic viscosity of the dissolution medium, ω is the angular velocity of rotation and c_o is the solubility of the solute. The flux, J, is defined as the amount of substance passing per unit time perpendicularly through a unit surface area. Denoting the dissolved mass m, the volume of the dissolution medium v and the surface area of the disc s,

$$J = \frac{v}{s}\frac{dc}{dt} = \frac{1}{s}\frac{dm}{dt}$$

$\therefore$ The dissolution rate can be written as

$$v \cdot \frac{dc}{dt} = \frac{dm}{dt} = 0.62D^{2/3}\nu^{-1/6}\omega^{1/2}Sc_o \quad . . . \qquad (18)$$

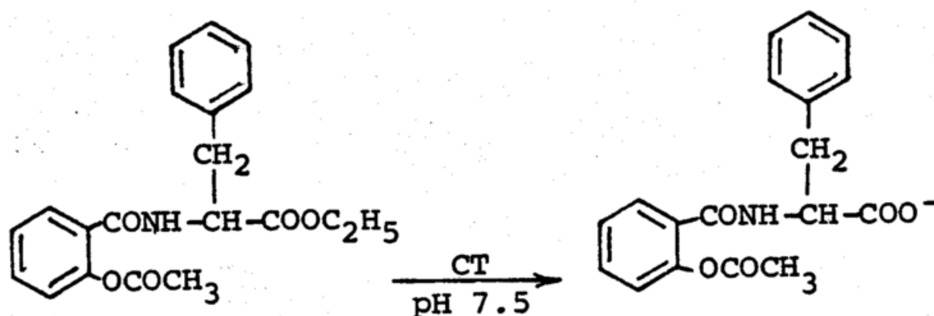
From this, the dissolution rate constant k can be written as

$$k = 0.62D^{2/3}\nu^{-1/6}\omega^{1/2} \quad . . . \qquad (19)$$

Based on Krevelen's (77) treatment of dissolution of carbon dioxide in an alkaline medium, theoretical expressions were derived (78) for the dissolution rate with simultaneous irreversible hydrolytic reaction utilizing the film model. These expressions were shown to give reasonable

estimates of the dissolution rates of 7-acetyltheophylline (78) and 7,7'-succinylditheophylline (79) with simultaneous OH^- ion catalyzed hydrolysis.

In our case the dissolution medium contained α-chymotrypsin. Aspirin-phenylalanine ethyl ester after being dissolved in the dissolution medium reacts with α-chymotrypsin in a way which may be considered irreversible for all practical purposes.



Let C be the concentration of aspirin-phenylalanine ethyl ester at a distance x from the solid surface, t the time, k_{obs} the first order observed rate constant for the enzymatic reaction, D the diffusion coefficient of the prodrug in the medium. At steady state dc/dt = o and

$$D \frac{d^2c}{dx^2} = k_{obs}C \quad . \; . \; . \qquad (20)$$

A solution of this equation assuming constant enzyme concentration throughout and with the boundary conditions:

$C = c_o$ at x = o and

C = 0 at x = h where h is the diffusion layer thickness,

gives

$$C = \frac{c_o \sinh [\alpha(h - x)]}{\sinh (\alpha h)} \quad . . . \qquad (21)$$

where $\alpha = \sqrt{\frac{k_{obs}}{D}}$

$\therefore$ The flux into the medium is given by

$$J = -D(\frac{dc}{dx})_{x=0}$$

$$= \frac{D\alpha[c_o \cosh (\alpha h)]}{\sinh (\alpha h)}$$

i.e., $J = D\alpha c_o \coth (\alpha h)$ . . . (22)

Let $X = \alpha h$

$\therefore J = \frac{D}{h} c_o \frac{X}{\tanh X}$ . . . (23)

When the reaction rate approaches zero, X is nearly equal to tan hX and equation 23 reduces

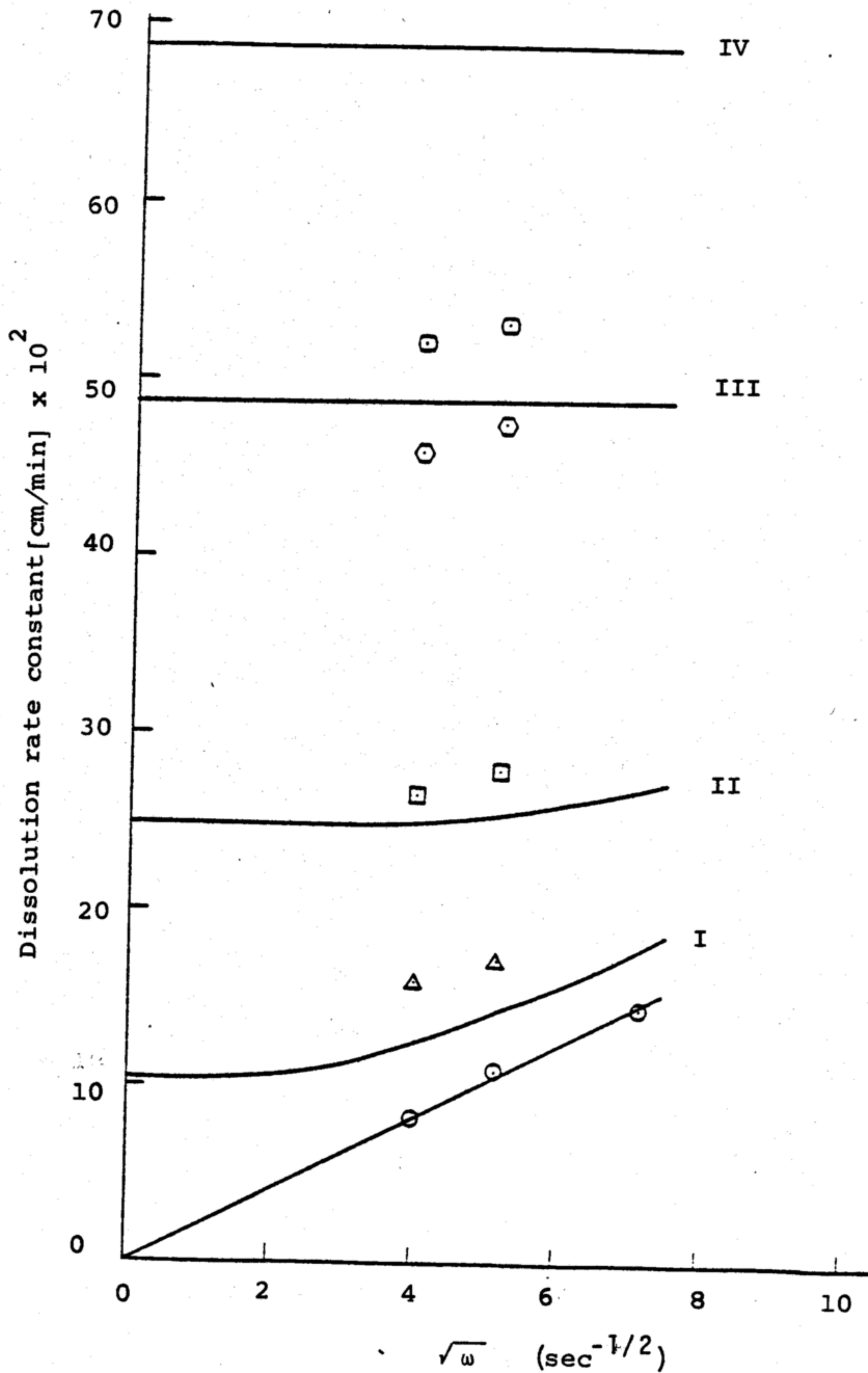
$$J = \frac{D}{h} c_o = 0.62D^{2/3}\nu^{-1/6}\omega^{1/2}c_o \quad . . . \qquad (24)$$

where $$h = 1.61\ D^{1/3}\nu^{1/6}\omega^{-1/2} \quad . . . \qquad (25)$$

Equation 24 is Levich equation (52). Therefore, equation 22 satisfies the limiting conditions and has the appropriate hydrodynamic terms in it entering through substitution of h given by equation 25.

The dissolution rate constants determined at 150, 300 and 500 RPM for dissolution in pH 7.5 phosphate buffer containing no enzyme, and at 150 and 300 RPM for dissolution in pH 7.5 phosphate buffer containing different concentrations of α-chymotrypsin are plotted in Figure 28. Tables 23, 24 and Figures 29, 30 summarize the experimentally observed dissolution data at 150 and 300 RPM respectively. At low enzyme concentration (9.6×10^{-7} M) the observed rate enhancement was more at 150 RPM than at 300 RPM. At higher enzyme concentrations the dissolution rates were comparable at both rotations. It can be noticed from Figure 31 that the observed dissolution rate constants are leveling off at higher enzyme concentrations. Dissolution rate constant increased linearly with $\sqrt{\omega}$ for dissolution in the absence of enzyme in the medium, as is expected from equation 19. In presence of enzymes the relative increase in the dissolution rate constants was lower at higher rotational speed. This is evident from Figure 28.

Dissolution rate constants were estimated using equation 22. These are collected in Table 26 along with the experimentally measured constants and the theoretically



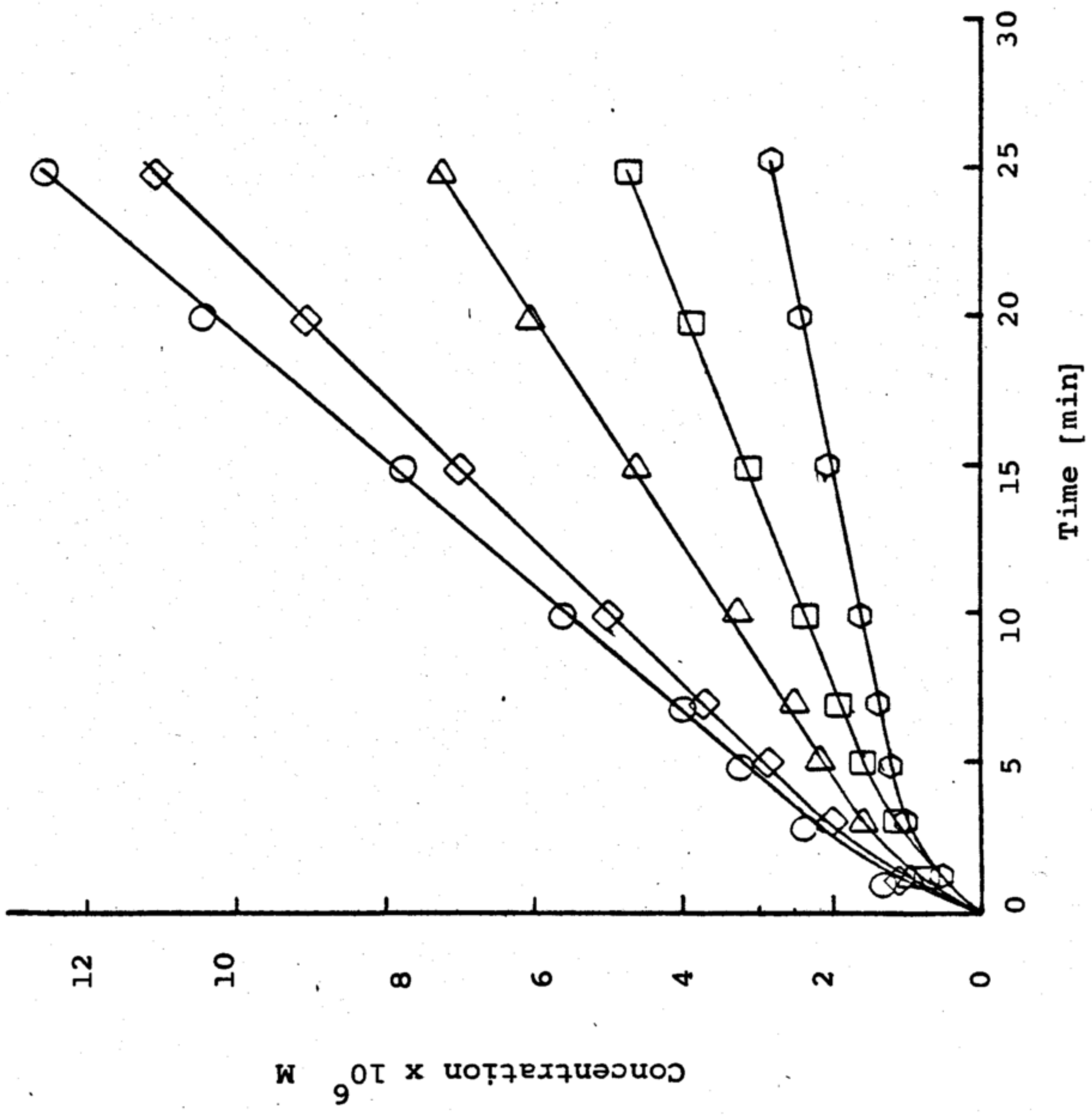
Dissolution rate constant [cm/min] x 10^2
70
60
50
40
30
20
10
0
IV
III
II
I
0
2
4
6
8
10
√ω (sec^-1/2)

TABLE 23. Experimentally observed dissolution data at various concentrations of α-chymotrypsin at 150 RPM.

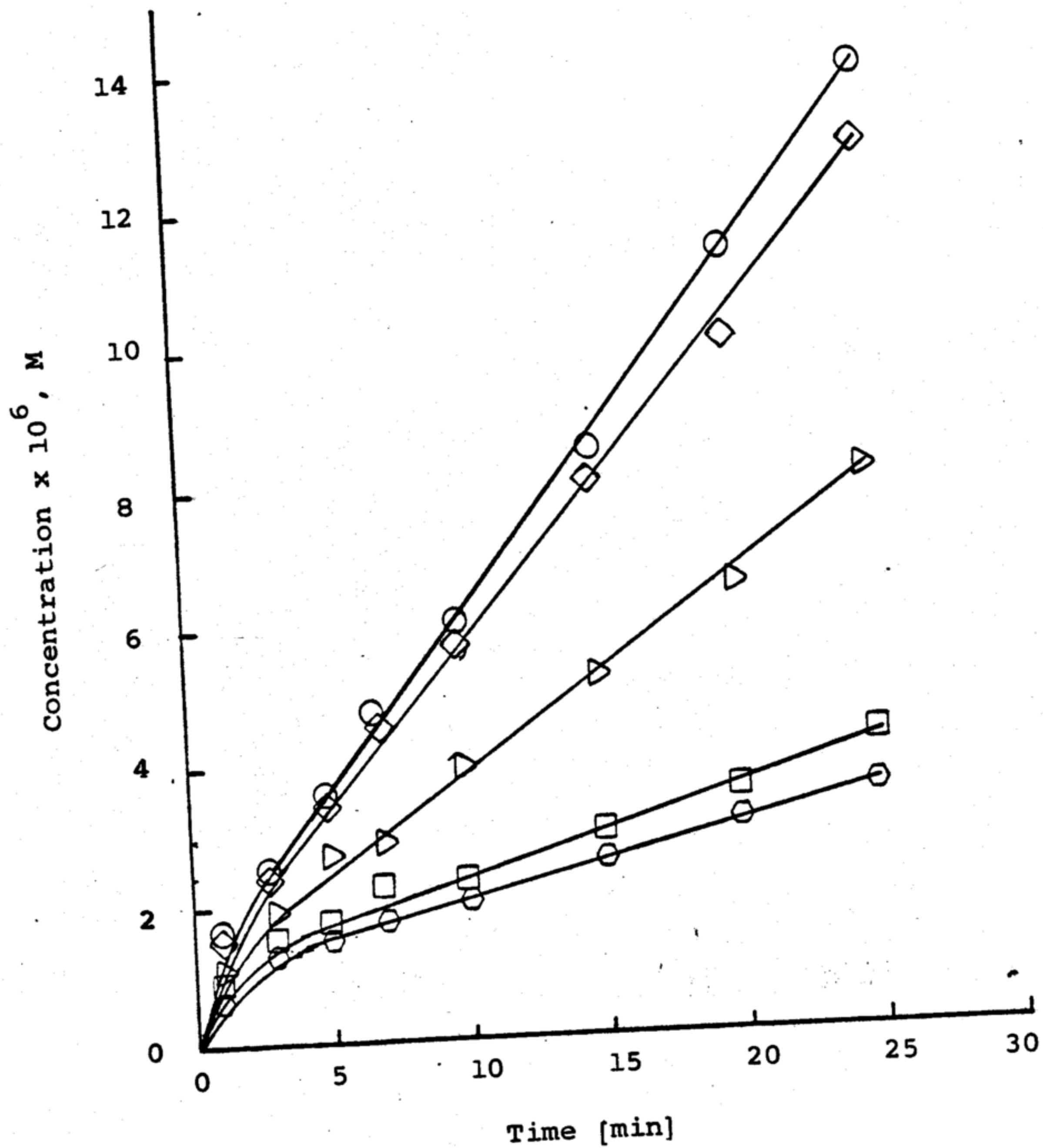
	Concentrations of aspirin phenylalanine-ethyl ester x 10^6 (moles/liter)				
Time (min)	$E_o = 0$	$E_o = 9.6 \times 10^{-7}$ M	$E_o = 5.3 \times 10^{-6}$ M	$E_o = 2 \times 10^{-5}$ M	$E_o = 4 \times 10^{-5}$ M
1	0.8	0.96	1.10	1.24	1.40
3	1.1	1.35	1.55	2.00	2.37
5	1.27	1.60	2.20	2.86	3.32
7	1.43	1.92	2.48	3.70	4.00
10	1.70	2.35	3.31	4.97	5.63
15	2.1	3.13	4.63	7.05	7.76
20	2.5	3.70	6.20	8.86	10.53
$\frac{v}{s}(\frac{dc}{dt})_{t=0}$ ($mole \cdot cm^{-2} sec^{-1}$)	7.32×10^{-7}	1.428×10^{-6}	2.374×10^{-6}	4.107×10^{-6}	4.643×10^{-6}

TABLE 24. Experimentally observed dissolution data at various concentrations of α-chymotrypsin at 300 RPM.

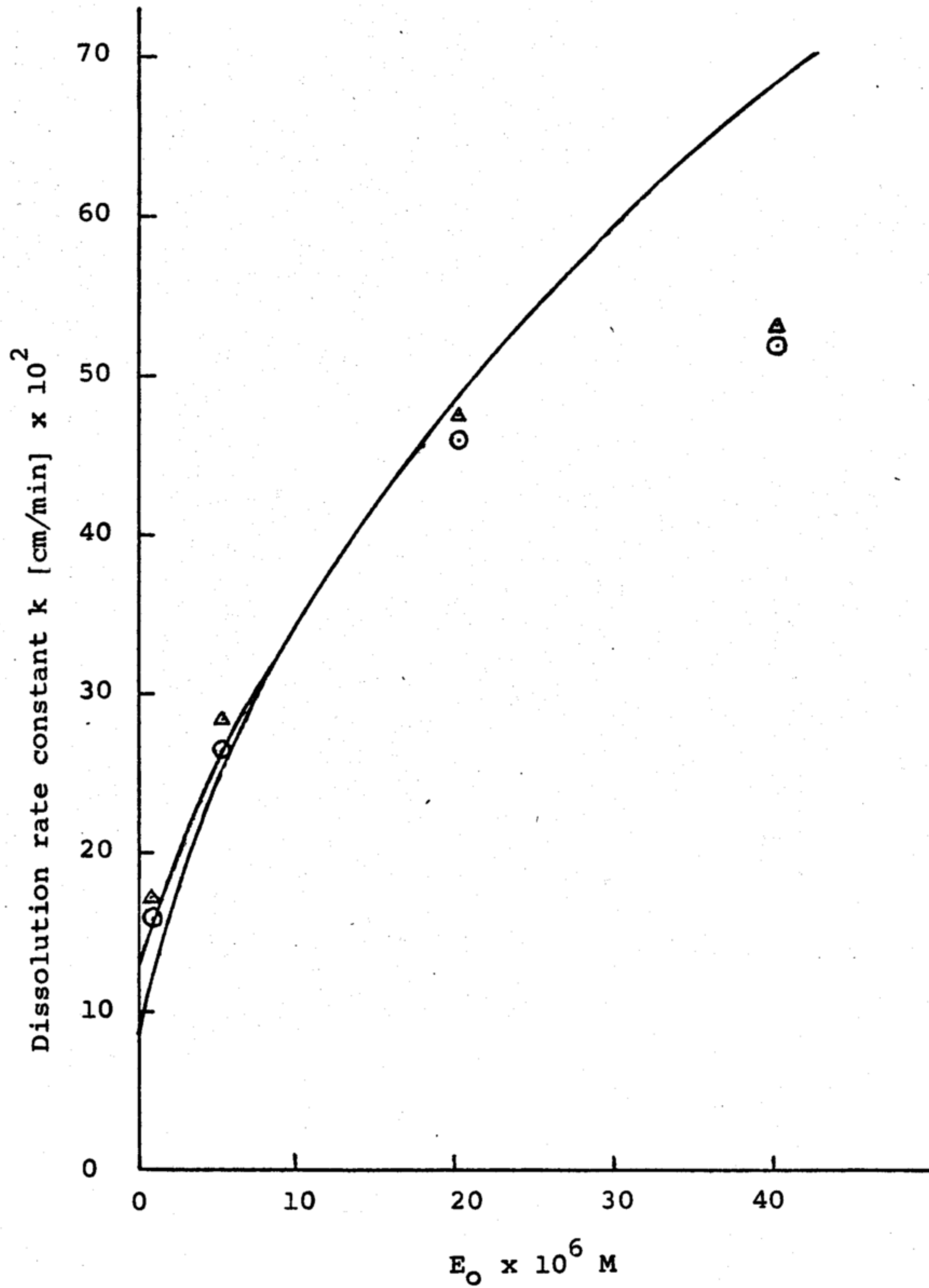
Time (min)	Concentrations of aspirin-phenylalanine ethyl ester x 10^6 (moles/liter)				
	$E_o = 0$	$E_o = 9.6 \times 10^{-7}$ M	$E_o = 5.3 \times 10^{-6}$ M	$E_o = 2 \times 10^{-5}$ M	$E_o = 4 \times 10^{-5}$ M
1	0.75	0.93	1.53	1.63	1.70
3	1.34	1.65	1.94	2.40	2.56
5	1.52	1.80	2.72	3.45	3.62
7	1.78	2.14	2.90	4.60	4.80
10	2.00	2.40	3.94	5.72	6.03
15	2.57	3.03	5.27	8.00	8.47
20	3.16	3.52	6.48	9.97	11.50
$\frac{v}{s}(\frac{dc}{dt})_{t=0}$ $(mole \cdot cm^{-2} sec^{-1})$	1.0×10^{-6}	1.536×10^{-6}	2.500×10^{-6}	4.250×10^{-6}	4.75×10^{-6}



Concentration x 10^6 M
0
2
4
6
8
10
12
Time [min]
0
5
10
15
20
25
30



14
12
10
8
6
4
2
0
Concentration x 10^6, M
0
5
10
15
20
25
30
Time [min]


Dissolution rate constant k [cm/min] x 10^2
0
10
20
30
40
50
60
70
0
10
20
30
40
E_o x 10^6 M

generated curves at both RPM are shown in Figure 31. The first order rate constants, k_{obs}, used in equation 22 were obtained from the *in vitro* enzyme kinetic data from pH-stat experiments by plotting the logarithm of concentrations of the substrate remaining against time. The pseudo-first order rate constant k_1 obtained from the slope gave a second order rate constant, k_2, on division by E_o. k_{obs} for the dissolution experiment was obtained by multiplying k_2 by E_o. The diffusion coefficient of aspirin-phenylalanine ethyl ester was estimated from Hayduk-Laudie correlation (81):

$$D^{\circ}_{A\omega} = 13.26 \times 10^{-5} \eta_W^{-1.4} V_A^{-0.586} \quad . \; . \; . \qquad (26)$$

where η_W = viscosity of water, cp

V_A = solute molal volume at normal boiling point, $cm^3/g\text{-}mol$.

$D^{\circ}_{A\omega}$ = binary diffusion coefficient at infinite dilution, cm^2/sec.

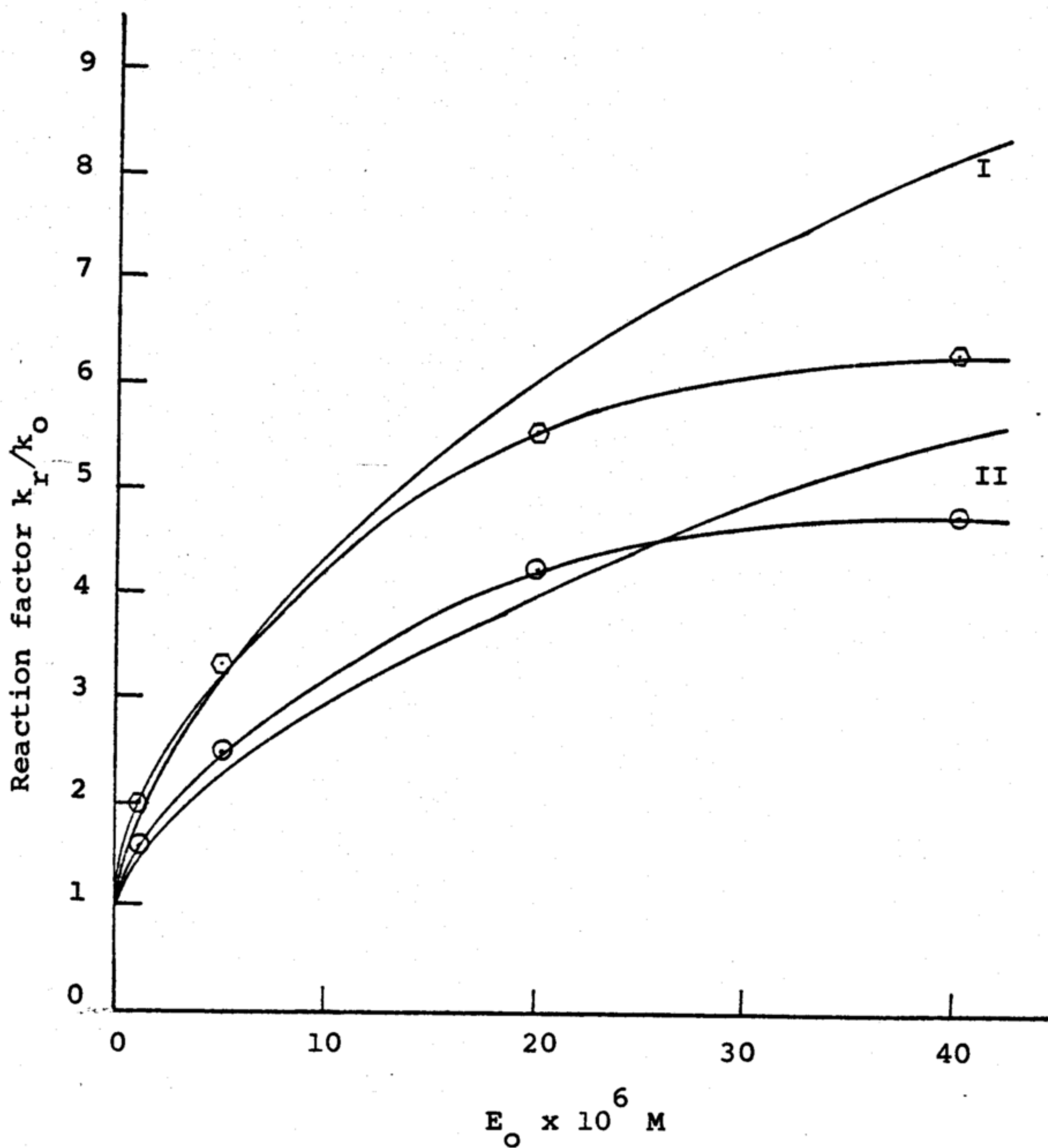
V_A was estimated by the additive-volume procedure of Le-Bas (82). A first order rate constant could also be obtained from Michaelis-Menten equation as $k_1 = k_{cat}E_o/K_m$. This represents the rate constant when no product has been formed and also has the assumption that $S_o >> E_o$. Under experimental situation we were measuring the rate in presence of products of reaction and at high enzyme concentration S_o is

TABLE 26. Comparison of experimentally observed dissolution rate constants with those estimated theoretically.

	RPM 150		RPM 300	
$E_o \times 10^6$ M	Experimental $k \times 10^2$ (cm/min)	Calculated from equation 22 $k \times 10^2$ (cm/min)	Experimental $k \times 10^2$ (cm/min)	Calculated from equation 22 $k \times 10^2$ (cm/min)
0	8.2	8.2	11.2	12.2
0.96	16.0	12.8	17.2	15.4
5.3	26.6	25.4	28.0	26.5
20	46.0	48.7	47.6	48.7
40	52.1	68.6	53.2	68.6

only about eight times higher than E_o. Therefore, use of k_{obs} represents a better approximation to the real situation than using k_1 from Michaelis-Menten equation.

A reaction factor $\phi_R = k_r/k_o$ can be defined (86), where k_r is the dissolution rate constant in presence of enzyme and k_o is that without enzyme. Table 25 summarizes these calculations and Figure 32 shows plots of k_r/k_o against enzyme concentrations for experimental data as well as for those derived from equation 22 at both RPM. It can be noticed from both Figures 31 and 32 that experimentally observed dissolution rate constants were comparable to those estimated from equation 22 at low enzyme concentrations. Whereas the observed values at higher enzyme concentrations were significantly lower than those estimated. Several factors may account for these differences. This model was derived by assuming the diffusion layer to be a stagnant film. It is known (52) that within the diffusion boundary layer the radial and tangential velocity components are not zero. The magnitude of the axial component of velocity decreases and that of the radial component increases as the surface of the disc is approached. When the reaction rates become very fast at high enzyme concentrations, then because of the relatively slow turnover rate ($k_{cat} \simeq 3\ sec^{-1}$) of α-chymotrypsin at pH 7.5 the actual concentration of the free enzyme in the diffusion layer is likely to be lower than



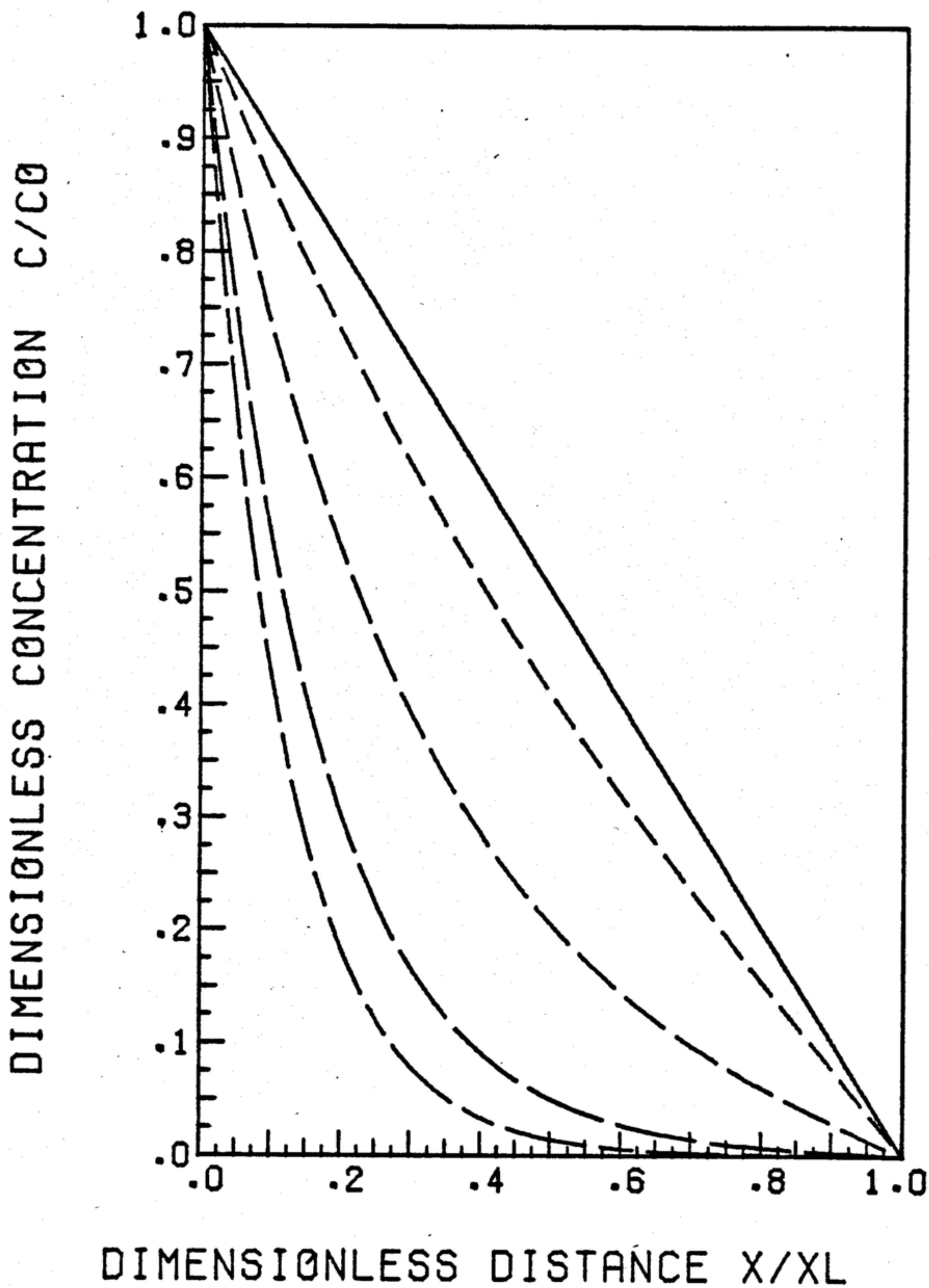
Reaction factor k_r/k_o
0
1
2
3
4
5
6
7
8
9
I
II
0
10
20
30
40
$E_o \times 10^6$ M

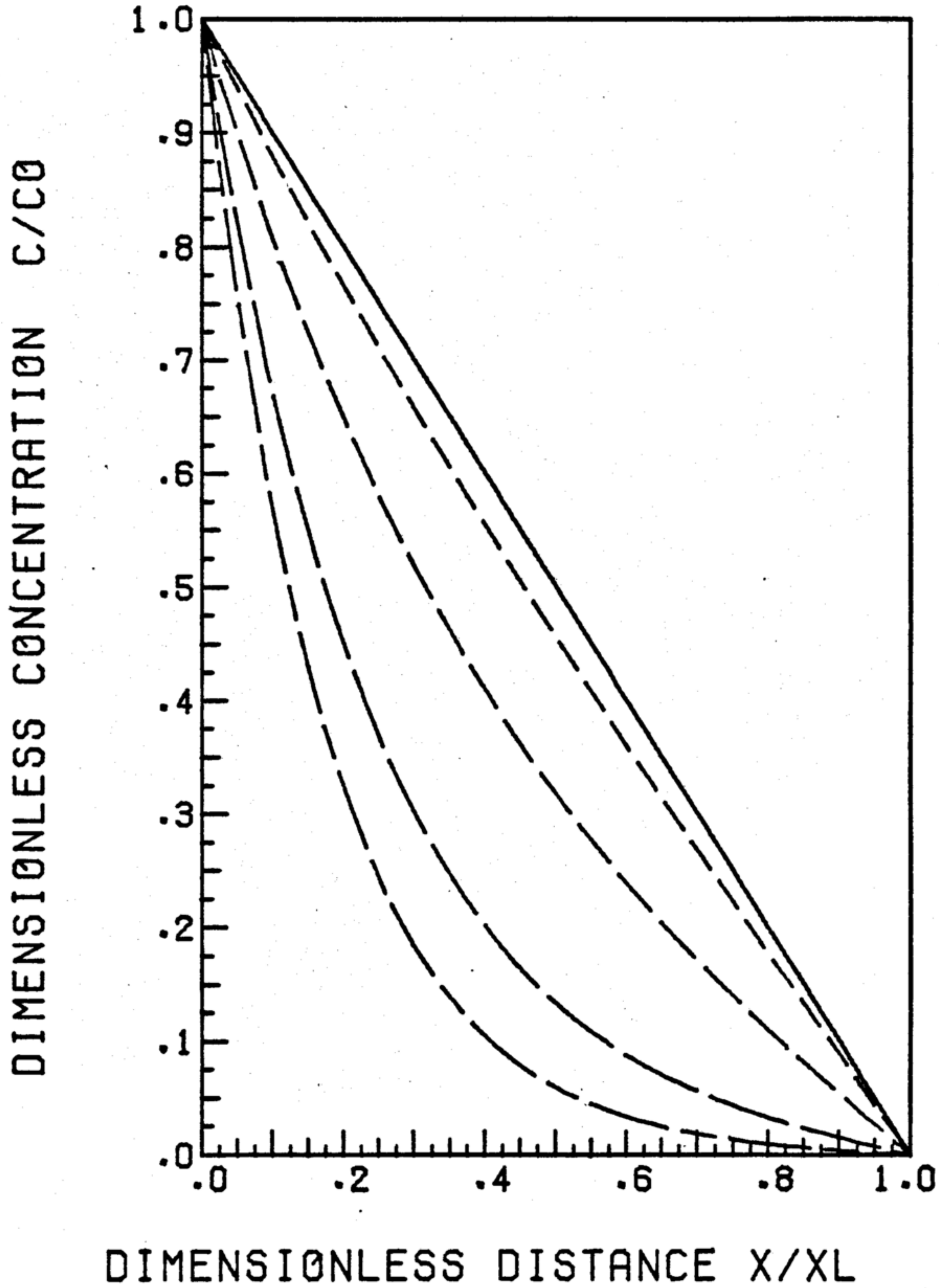
TABLE 25. Reactions factors k_r/k_o at 150 and 300 RPM.

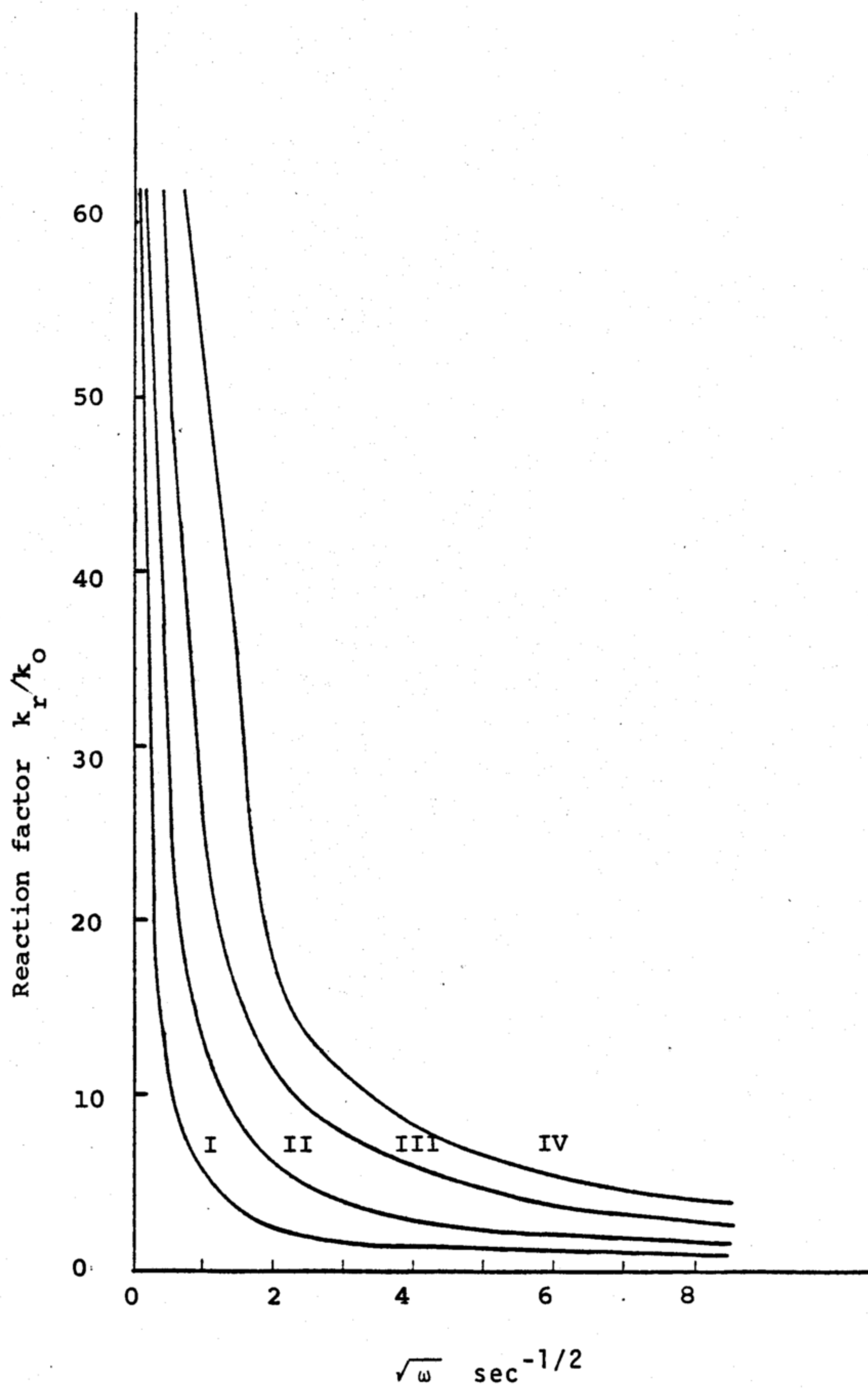
$E_o \times 10^6$ M	150 RPM		300 RPM	
	Observed k_r/k_o	k_r/k_o Calculated using equation 22	Observed k_r/k_o	k_r/k_o Calculated from equation 22
0	1	1	1	1
0.96	1.95	1.56	1.54	1.26
5.3	3.24	3.1	2.5	2.2
20	5.61	5.94	4.25	4.0
40	6.35	8.36	4.75	5.62

that in the bulk. In other words an enzyme concentration gradient may come into the picture, which is not accounted for in this model. Also, at high reaction rate the solute concentration at the disc surface may be lower than the saturation concentration C_0, i.e., the system may become interfacial transfer controlled rather than diffusion controlled. Both of these factors have the obvious effect of slowing down the dissolution rate, thus explaining the lower experimental values than those estimated from equation 22 at higher enzyme concentrations.

Figures 33 and 34 show the plots of dimensionless concentration C/C_0 as a function of dimensionless distance x/x_L (x_L = diffusion layer thickness) generated using equation 22. As can be seen from these plots that the relative change in dissolution rate (given by the slopes of the curves evaluated at $x/x_L = 0$) is less at higher RPM than at lower RPM. This fact becomes more evident from the theoretical plots of dissolution rate constants against $\sqrt{\omega}$ at different enzyme concentrations, in Figure 28. Figure 35 shows plots of reaction factor k_r/k_0 vs. $\sqrt{\omega}$ at different enzyme concentrations as predicted by equation 22. These plots demonstrate that the dissolution rate enhancement effect of enzymatic reactions are greatest under more quiescent conditions. Figures 33 and 34 also show that at high enzyme concentrations the effective diffusion layer thickness is lower than


1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
.0
.0
.2
.4
.6
.8
1.0
DIMENSIONLESS CONCENTRATION C/C0
DIMENSIONLESS DISTANCE X/XL


1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
.0
.0
.2
.4
.6
.8
1.0
DIMENSIONLESS CONCENTRATION C/C0
DIMENSIONLESS DISTANCE X/XL


Reaction factor k_r/k_o
60
50
40
30
20
10
0
0
2
4
6
8
I
II
III
IV
$\sqrt{\omega}$ sec$^{-1/2}$

that predicted by Levich equation.

In this model an experimental first order rate constant, k_{obs}, was used to account for the enzymatic reactions in the diffusion layer. Based on Michaelis-Menten equation the enzymatic reaction near the disc surface is expected to be zero order (since $C_o >> K_m$). Using a zero order rate constant, $K = k_{cat}E_o$, in the stagnant film model one obtains

$$D\frac{d^2c}{dx^2} = K$$

Solution of this equation with the boundary conditions previously used gives

$$C = \frac{K}{D}\frac{x^2}{2} - (\frac{C_o}{h} + \frac{Kh}{2D})x + C_o \quad . . . \qquad (29)$$

and

$$J = \frac{DC_o}{h} + \frac{Kh}{2} \quad . . . \qquad (30)$$

Starting from principles of convective diffusion coupled with zero order reaction one can obtain (85)

$$V_y \frac{dc}{dy} = D\frac{d^2c}{dy^2} - K \quad . . . \qquad (31)$$

where y indicates the axial direction. Solution of this equation using the boundary conditions $C = C_o$ at $y = 0$ and $C = 0$ at $y = h$, gives

$$J = 0.62D^{2/3}\nu^{-1/6}\omega^{1/2}c_o + \alpha \cdot K \qquad (32)$$

$$\alpha = \frac{\int_0^{\delta_0} \int_0^{t} \exp[-\frac{0.51}{3D} \sqrt{\frac{\omega^3}{\nu}} (t^3 - s^3)] ds dt}{1.61 D^{1/3} \nu^{1/6} \omega^{-1/2}} \quad . . . \quad (33)$$

Both equations 30 and 32 predict a linear dependence of the dissolution rate on enzyme concentration which is not experimentally observed. Dissolution rates predicted by equation 30 were found to be much lower than those observed experimentally at all enzyme concentrations. A solution was also obtained for a first order reaction coupled with convective diffusion. Starting from

$$V_y \frac{dc}{dy} = D\frac{d^2c}{dy^2} - k_1 c \quad . . . \quad (34)$$

and using appropriate boundary conditions one can obtain (85)

$$J = \frac{0.62 D^{2/3} \nu^{-1/6} \omega^{1/2} C_0}{1 - 0.43 k_1 D^{-1/3} \nu^{1/3} \omega^{-1} + . . .} \quad (35)$$

Equation 35 was obtained by retaining the first term in the series solution of equation 34 by the Forbenius method. Although the dissolution rates predicted by equation 35 are comparable to those obtained experimentally at lower enzyme concentrations, at enzyme concentrations greater than 5 x 10^{-6} M (with $k_1 = k_{obs}$ defined earlier) it predicts negative fluxes. The truncation error in the series approximation may be responsible for this behavior.

Analysis of the four models derived shows that the models with zero order reaction rate constant fails to predict the observed dissolution rate constants at all the enzyme concentrations studied. Both the models with first order reaction rate constants give reasonable prediction of the experimental results at low enzyme concentrations. This creates a physical inconsistency since a zero order reaction is to be expected at the saturation concentration of the prodrug. A more appropriate model would incorporate a zero order rate constant, the turnover rate of the enzyme and the free enzyme concentration in the diffusion layer in relation to the hydrodynamic conditions.

A final point to be noted is that the rate of aspirin at 430 RPM in 0.1 N HCl was reported (64) to be 1.62×10^{-7} $mole \cdot cm^{-2} sec^{-1}$. It can be seen from Table 26 that even at 150 RPM in presence of 4×10^{-5} M α-chymotrypsin the dissolution rate is about 25 times higher than that of aspirin in 0.1 N HCl. These results show that even though the prodrug is less soluble in water than aspirin itself, the dissolution rate of the prodrug at pH 7.5 and in presence of enzymes may actually be several times higher than that of aspirin in 0.1 N HCl.

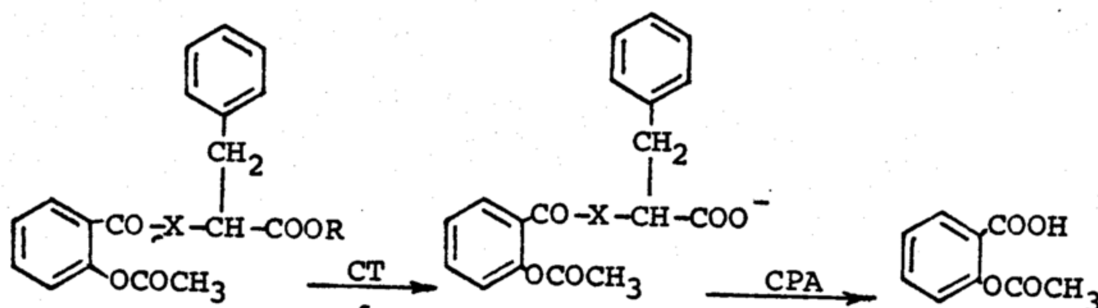
IV. SUMMARY AND CONCLUSIONS

The objective of this research project was to develop and test a rationale for prodrug design utilizing the digestive enzymes of the gastrointestinal tract as the reconversion sites. Central to the prodrug design is the requirement that the prodrug be reconverted *in vivo* rapidly or at a controlled rate to the parent compound. Almost all of the prodrugs designed and tested so far depended on the hydroxyl ion, proton and water molecule catalyzed hydrolytic reconversion to the parent molecule. With the structural requirements of the digestive enzymes known, it is now possible to consider these enzymes as the target reconversion sites. It is expected that this approach would ensure *in vitro* stability and *in vivo* lability of the prodrug.

In order to test the validity of this approach several prodrugs of aspirin were made. Chymotrypsin is known to specifically cleave the ester linkage of aromatic amino acid residues. Substrate specificity of carboxypeptidase A is such that it requires a free terminal carboxyl group and it hydrolyzes amide or ester bonds formed through the α-amino or α-hydroxy groups of aromatic amino acids or their analogs. The free carboxyl group of aspirin is known to aid in the hydrolysis of the O-acetyl group, thereby reducing the half life of aspirin. The unionized carboxyl group has also been

indicated to be responsible for gastric irritation and bleeding. Classical esterification of the carboxyl group has been shown to lead to nonirritating, more stable compounds, but they did not revert to aspirin. If esters or amides of aspirin could be designed to become substrates of the intestinal enzymes, reconversion to aspirin could be assured and would lead to a stable, nonirritating dosage form of aspirin.

The prodrugs synthesized were aspirin-phenylalanine ethyl ester, aspirin-phenylalanine amide and aspirin-phenyllactic ethyl ester. They were so designed that after the initial cleavage of the terminal ethyl ester or amide group by chymotrypsin, carboxypeptidase A will hydrolyze the amide linkage to form aspirin



where X = -O-, or -NH, and R = $-OC_2H_5$, or $-NH_2$.

From the <u>in vitro</u> enzyme kinetic studies with pure chymotrypsin and carboxypeptidase A it was found that for chymotrypsin catalyzed hydrolysis the rate of reaction was fastest for aspirin-phenylalanine ethyl ester. For carboxypeptidase A catalyzed hydrolysis aspirin-phenyllactic acid

was found to be most rapidly reconverted to aspirin. Although aspirin-phenyllactic acid showed competitive product inhibition, these findings were exactly what was expected from the knowledge of the substrate specificities of these enzymes. Also, the rates of reconversion obtained were comparable to those reported in the literature for different -N- and -O- acyl derivatives of phenylalanine and phenyllactic acid. Thus, one of the ideal requirements of the prodrug design utilizing the digestive enzymes as the reconversion sites that the rate of reconversion be independent of the exact structure of the drug molecule was closely met and the reconversion rates obtained were within the range of expectation based on prior knowledge about the catalytic properties of the digestive enzymes.

That aspirin-phenylalanine ethyl ester is actually reconverted to aspirin in presence of a mixture of α-chymotrypsin and carboxypeptidase A was shown by TLC studies. Shelf life of a 600 mg/5 ml suspension (equivalent of 300 mg/5 ml aspirin) of aspirin-phenylalanine ethyl ester at pH 5.6 was calculated to be approximately four years, whereas that of 300 mg/5 ml of aspirin suspension at its optimum pH of 2.5 is about 41 days. Aspirin-phenylalanine ethyl ester was found to be adequately absorbed from rat intestine at pH 7.5 as evidenced by the loss of the prodrug on perfusion through the intestine. The absorption was shown to be dependent on

flow rate and a laminar flow model best fitted the experimental data. The dissolution rate of aspirin-phenylalanine ethyl ester was significantly enhanced by the presence of α-chymotrypsin in the dissolution medium. In presence of 4 x 10^{-5} M α-chymotrypsin at pH 7.5 the dissolution rate of the prodrug was found to be about 25 times faster than that of aspirin in 0.1 N HCl. A simplistic film model was found to best fit the experimental results at low enzyme concentrations, but showed significant deviations at higher enzyme concentrations. The differences could be explained by the development of an enzyme concentration gradient and/or by the change of the rate controlling step from diffusion to interfacial mass transfer resistance.

These results show the viability of this approach of making amino acid derivatized prodrugs. The pharmaceutical properties of drugs with free functional groups can be potentially altered in almost any direction by using this approach since the prodrugs so made depends on the hydrolytic enzymes for _in vivo_ reconversion, stable dosage forms can be formulated while being assured of the reconversion at a predictable rate based on the known biochemical properties of the enzymes to whom the prodrugs are designed to be directed. This represents a significant advantage over the conventional prodrugs which because of the dependence on solution hydrolysis for reconversion are likely to be less

stable in the dosage form. The amino acids being indigenous to the body are not likely to impart any new toxicity to the parent molecule. Appropriate selection of amino acid residues and the enzymes to be targeted as reconversion sites would enable the pharmaceutical scientists to obtain better biopharmaceutical properties of drugs with undesirable properties. Thus this approach could usher in a new era in the science of designing dosage forms with improved biopharmaceutical properties.

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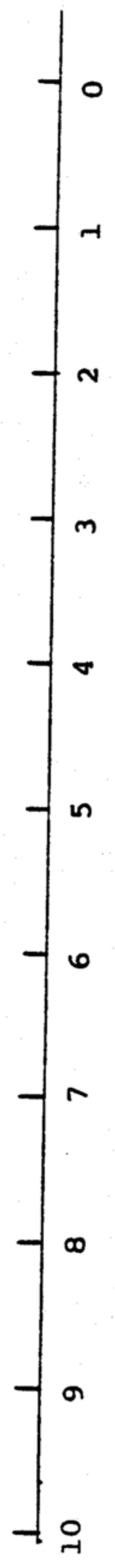
APPENDIX A

Nuclear Magnetic Resonance Spectra of the Prodrugs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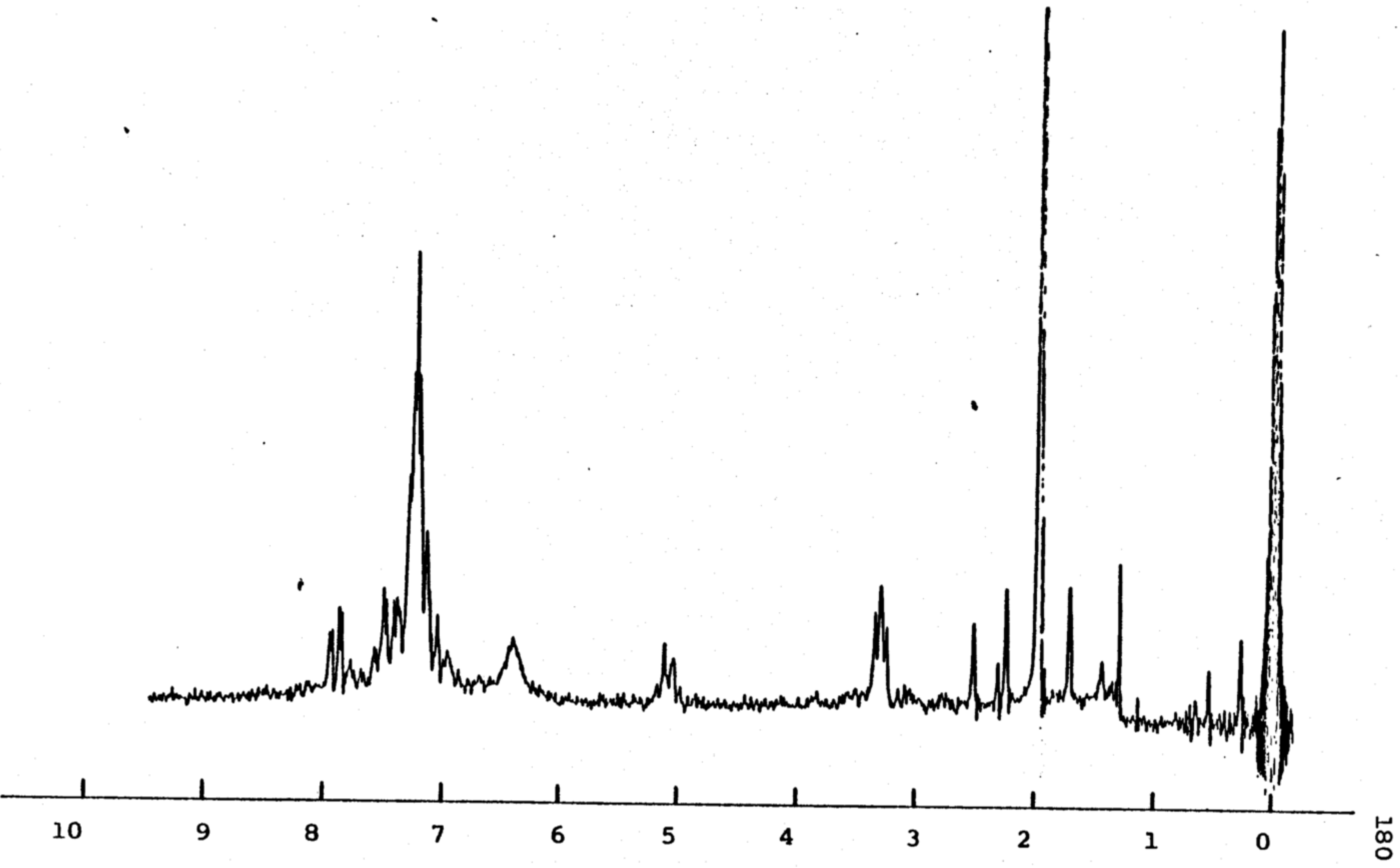
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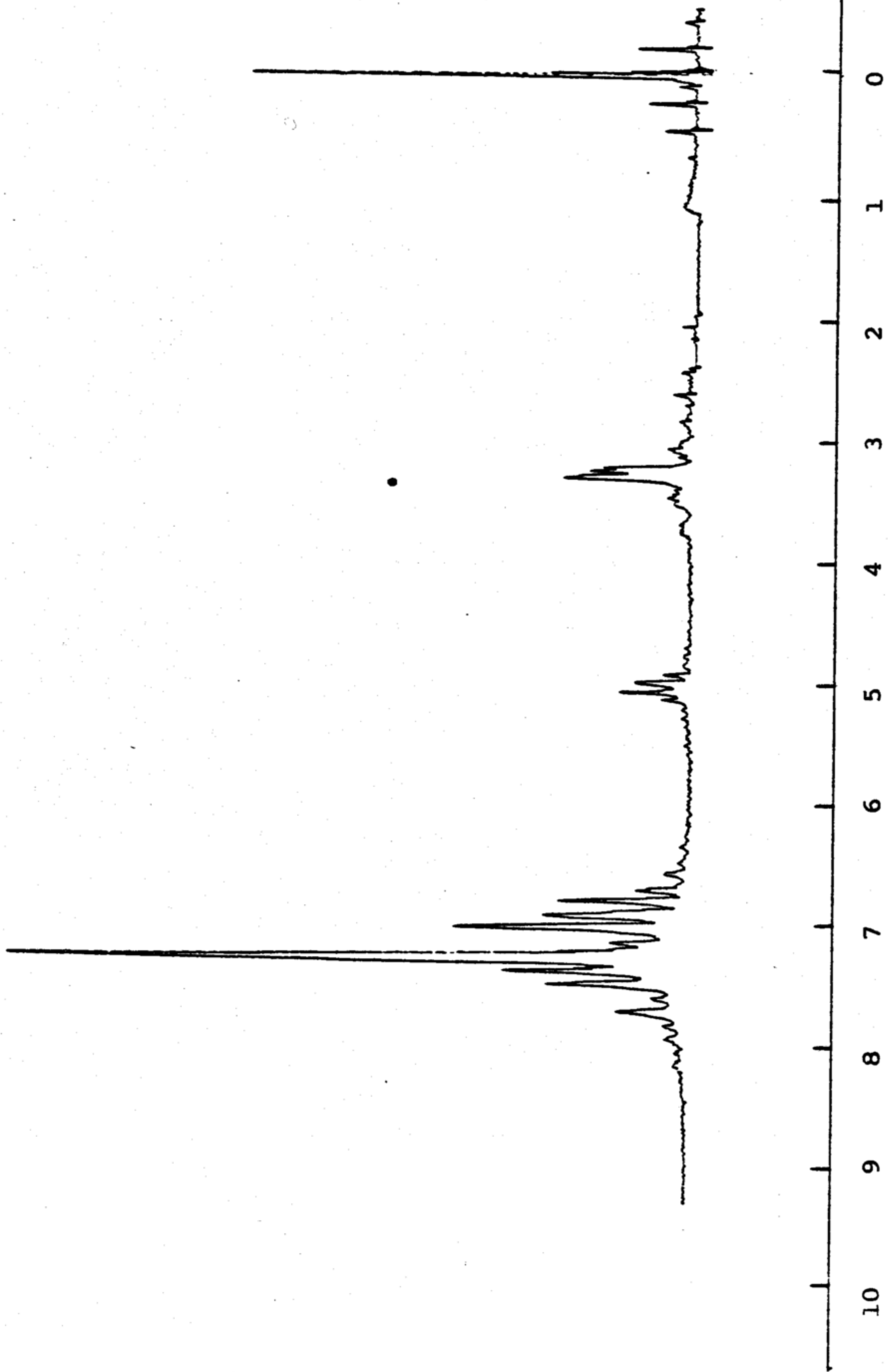
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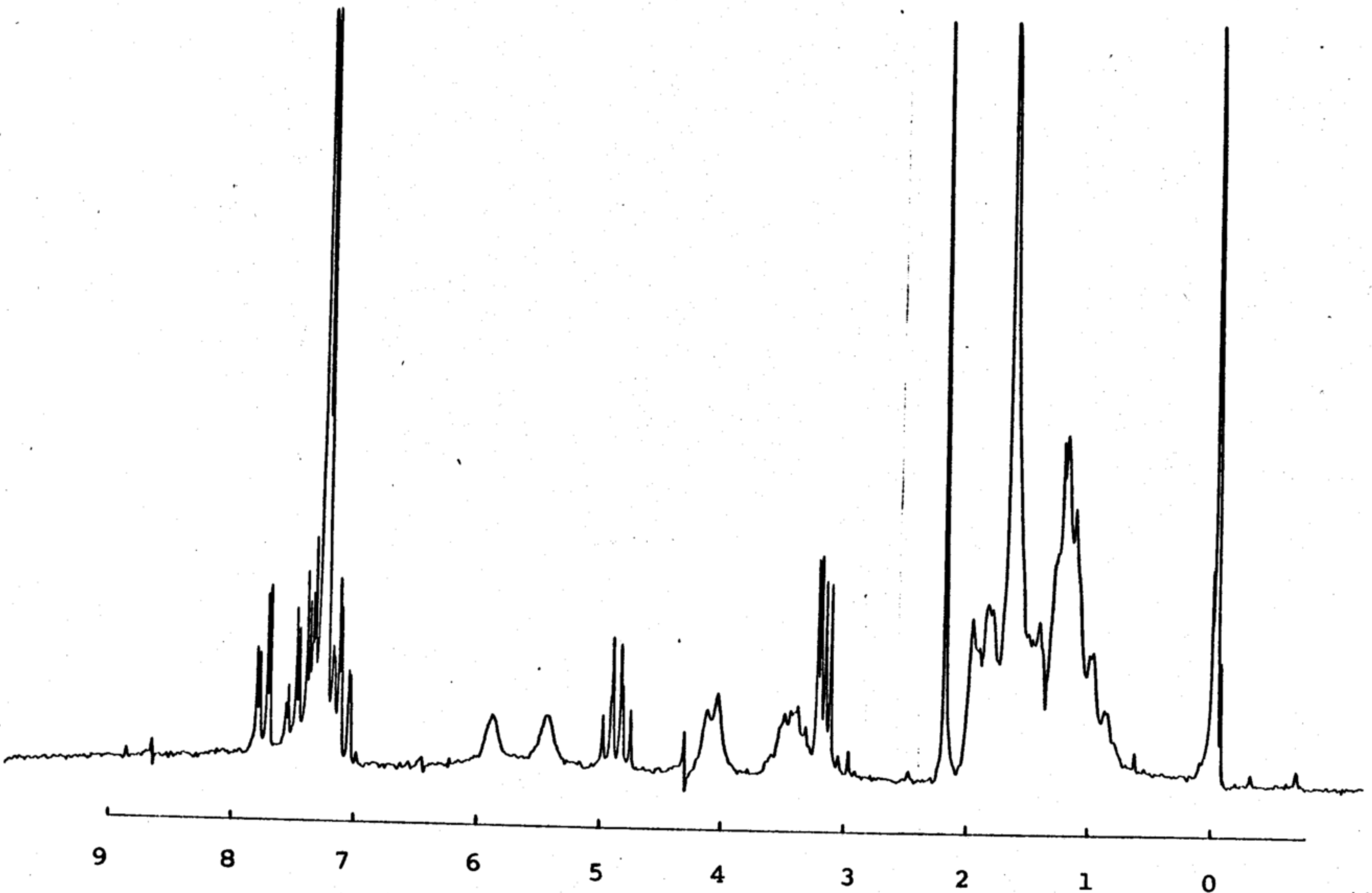
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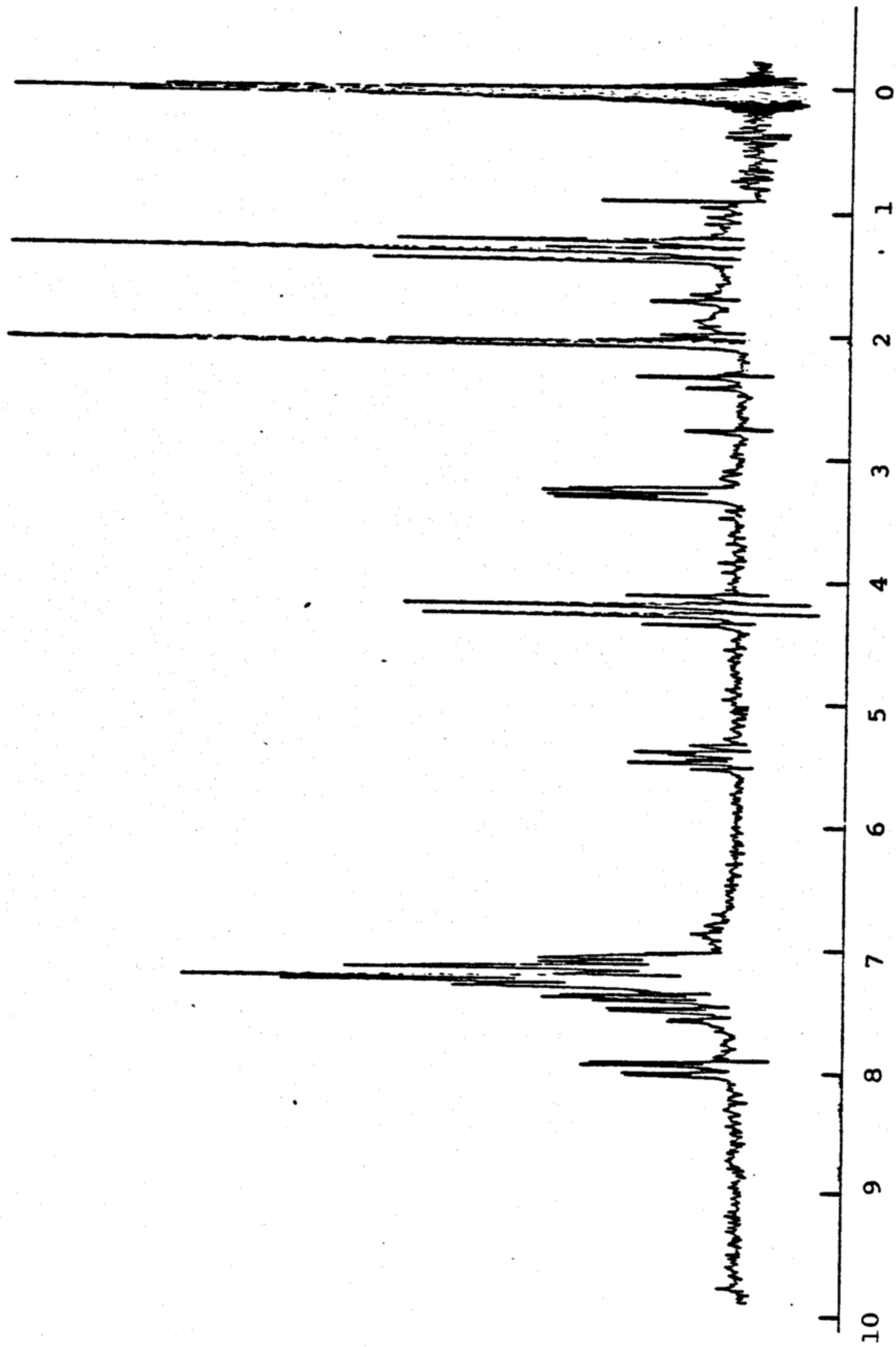
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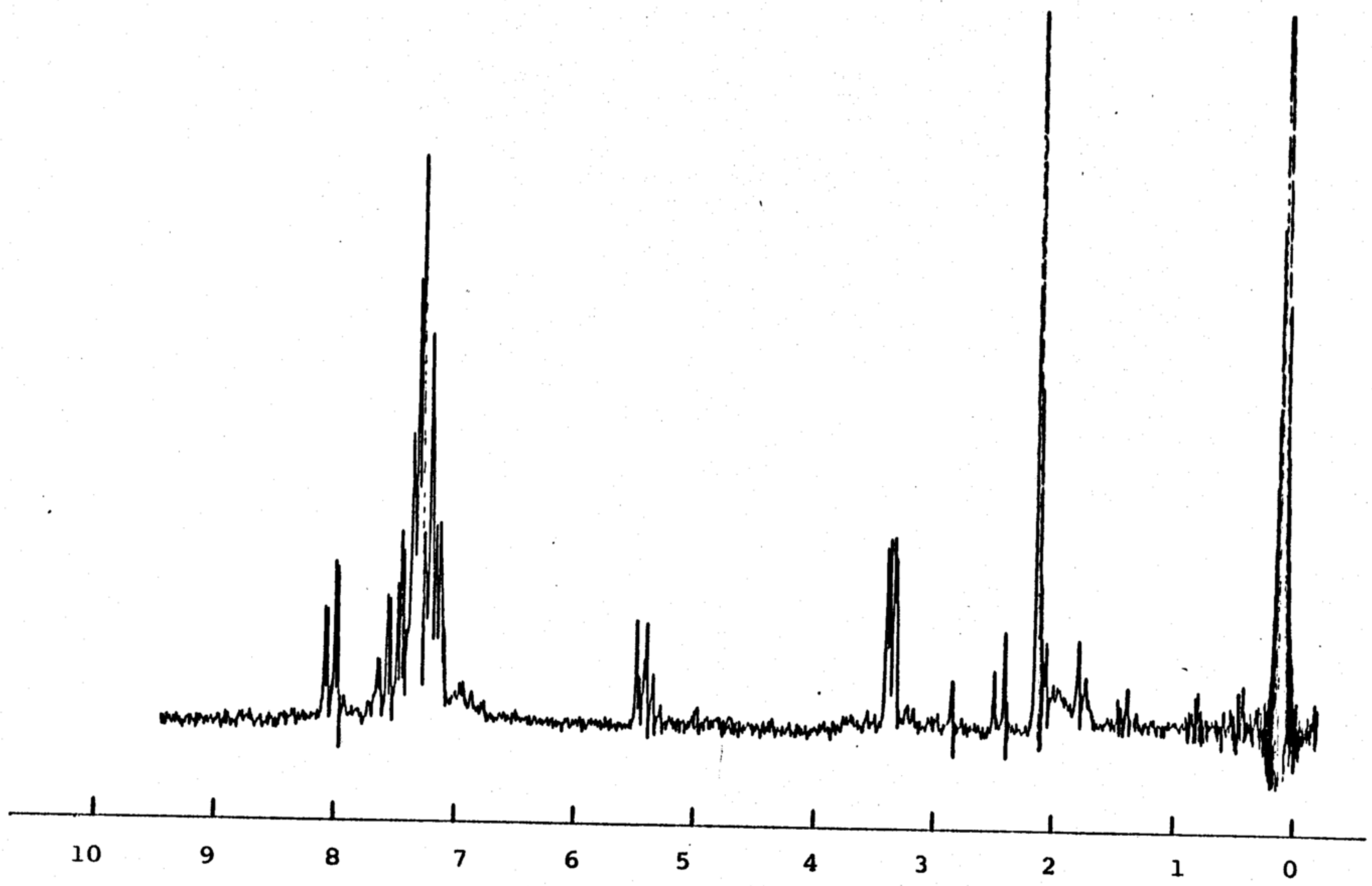

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