Title
The mechanisms of action of sodium salicylate on glucosamine biosynthesis

Author
Chan, James,

Publication Date
1974

Peer reviewed|Thesis/dissertation

THE MECHANISMS OF ACTION OF SODIUM SALICYLATE ON GLUCOSAMINE BIOSYNTHESIS

by

James Chan
Pharm.D., University of California, San Francisco, 1968

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

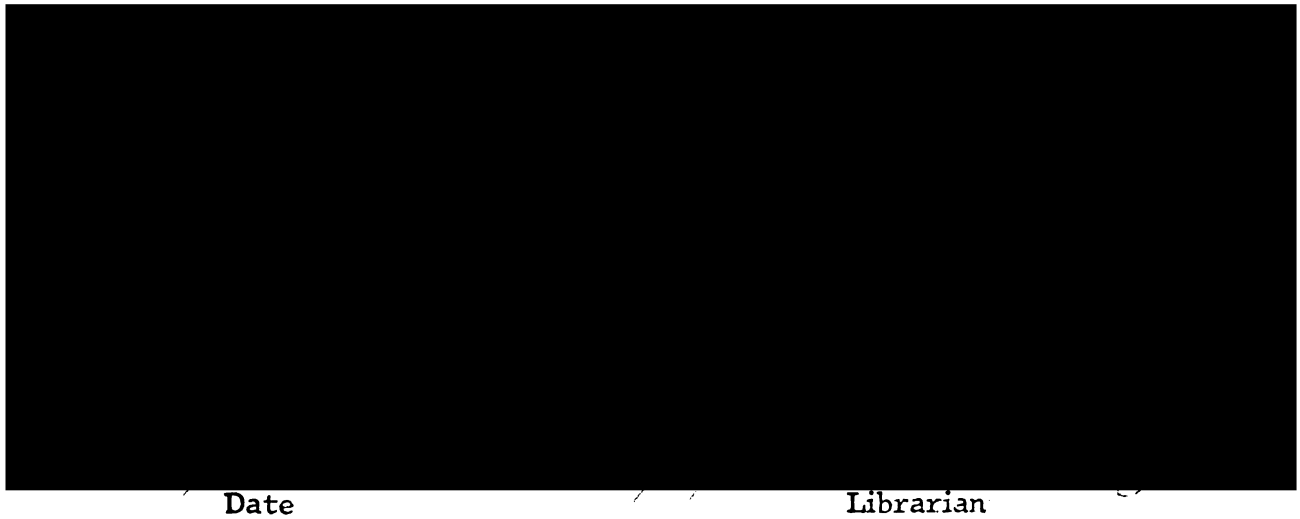
in the

GRADUATE DIVISION

(San Francisco)

of the

UNIVERSITY OF CALIFORNIA



Date Librarian

01 JAN 1974

Degree Conferred: . . . . . .

ABSTRACT

Mechanisms of inhibitory action of sodium salicylate on L-glutamine:D-fructose-6-PO_4 aminotransferase, prepared from rat gastric mucosa, have been studied. Sodium salicylate, at lower concentrations (10-20 mM), inhibited reversibly aminotransferase activity by competing with fructose-6-PO_4. At higher concentrations, sodium salicylate inactivated the enzyme irreversibly with an inactivation rate following first order kinetics. UDP-N-acetylglucosamine is an endogenous negative feedback inhibitor. It inhibited aminotransferase catalyzed reaction also by competing with fructose-6-PO_4 but with an inhibiting activity 1,000 times that of sodium salicylate. UDP-N-acetylglucosamine reduced the salicylate inhibition of the enzymic reaction and protected the enzyme from salicylate induced irreversible inactivation.

Estimation of the <u>in vivo</u> concentration of UDP-N-acetylglucosamine was made.

Administration of sodium salicylate (600 mg./Kg.) to rats induced gastric lesions both orally and intraperitoneally (3 and 6 hours after administration). However, only salicylate administered orally caused an observable decrease in L-glutamine:D-fructose-6-PO_4 aminotransferase activity (5 hours after administration). The decrease in activity resulted from an increase in K_m and a decrease in V_{max}. No concomitant changes in the hexosamine content were observed.

The inhibition of a multienzyme system similar to that

involved in aminosugar metabolism was studied using enzyme kinetic models and linear system models.

The disposition of salicylate after oral and intraperitoneal administration was studied. The pharmacokinetics of salicylate disposition were treated using compartment analysis.

ACKNOWLEDGEMENTS

I am particularly grateful to my research advisor, Dr. K. H. Lee for his guidance, encouragement, and helpful advice throughout all phases of my graduate studies and research.

I wish to thank the other members of my thesis committee, Dr. C. T. Peng and Dr. Richard Fineberg, for their many helpful suggestions in preparing this dissertation.

I am grateful for the financial support received during my graduate studies as a NSF Trainee and especially grateful to Dr. K. H. Lee for providing a research assistantship during my last year of graduate studies and research.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

1-1 Work Done By Others

Nonsteroidal anti-inflammatory drugs in general and salicylate in particular are probably one of the most widely studied group of drugs. Voluminous amount of literature on salicylates appears as books (1,2,3) and review articles (4,5). Other nonsteroid anti-inflammatory agents as well as salicylates have been extensively reviewed by Whitehouse (6,7,8,9,10). The sites of action of these agents at the biochemical and molecular level for the observed clinical effects are not completely known at this time. There has been a great deal of work done by Whitehouse (6,7,8,9), Bostrom (10,11), and Bollet (12) and more recently by Kent and Allen (13) and Lukie and Forstner (14) who have attempted to elucidate the biochemical pharmacology of these agents.

A persistent side reaction of anti-inflammatory drugs at a dose level required for their desired clinical effect is the gastrointestinal irritation. Hemorrhage and ulceration have been produced in many species of experimental animals by administration of these agents (15,16,17,18,19,103,103). Although the mechanism(s) of the adverse effect is poorly understood, many workers believed that it may involve inhibition of gastrointestinal glycoprotein synthesis. Apparently the mucus or more accurately the glycoprotein exerts a protective effect on mucosal cells. It has been suggested by Anderson (33) that the presence of sulfated polysaccarides and mucoprotein decreases

the diffusion of secreted pepsin and hydrochloric acid through the mucoprotein back to the mucosa.

Aspirin administration has been shown to reduce glycoproteins at the canine gastric surface (20) and decreases mucus secretions in isolated canine gastric pouches (21). Phenylbutazone diminishes the overall quantity of radiosulfate eliminated in the gastric secretions (22). Indomethacin decreases the mucus secretion in rat stomach and lowers the concentration of mucopolysaccarides containing sialic acid and L-fucose (23). Many other workers have also proposed that the inhibition of gastrointestinal mucus formation or of secretions or both may play a vital role in the gastro-intestinal pathology (24,25,26,27).

Sodium salicylate inhibits the activities of many of the enzymes involved in mucopolysaccaride synthesis, such as L-glutamine:D-fructose-6-PO_4 aminotransferase (glucosamine synthetase) (12,28,29,40), acetyl-CoA synthetase (13,14), UDP-N-acetylglucosamine UDP-N-acetylgalactosamine epimerase (30), UDPG-dehydrogenase (31), and UDP-glucuronyl transferase (32).

There seems to be little doubt that anti-inflammatory agents affect the mucopolysaccaride synthesis and that mucopolysaccarides or glycoproteins exert some protective action on the gastric mucosa. A convincing piece of work by Ezer and Spzporny (27) appeared in 1970 showed that the compound, -p-chlorocarbobenzoxy-L-lysin-OMe-HCl can stimulate the synthesis of mucopolysaccarides as evidenced by an increased

incorporation of radioactive sulfate into the gastric mucosa of rats. The compound prevents the decrease, at a statistically significant level, in radiosulfate incorporation caused by phenylbutazone and sodium salicylate administration. These investigators also showed that the compound reduces Shay ulcers induced by pylorus ligation, but it is not known whether it can also reduce the ulcers induced by phenylbutazone or sodium salicylate. It may be noted that ligation can more consistently induce ulcerations than drugs.

Figure 1-1 shows that the enzyme, L-glutamine:D-fructose-6-PO_4 aminotransferase, which synthesizes glucosamine-6-PO_4, is located at a branch point in carbohydrate metabolism and will compete for fructose-6-PO_4 with other pathways utilizing hexose monophosphate namely glycolysis, glycogenesis, hexose monophosphate shunt, etc. In addition, Kornfeld (38), Winterburn and Phelps (52), and Bates and Handschumaker (37,38) have reported that hexosamine biosynthesis was subjected to feedback regulation by UDP-N-acetylglucosamine. This inhibition is on the initial enzyme of the pathway, catalyzing the apparent irreversible formation of glucosamine. The generally accepted pathway of mucopolysaccaride biosynthesis is given in Figure 1-2.

Perrey (28) reported that the activity of L-glutamine: D-fructose-6-PO_4 aminotransferase from the rat mucosa was reduced by sodium salicylate <u>in vitro</u> as well as <u>in vivo</u> and suggested possible correlations between the inhibition of this enzyme and occurrance of gastric lesions.

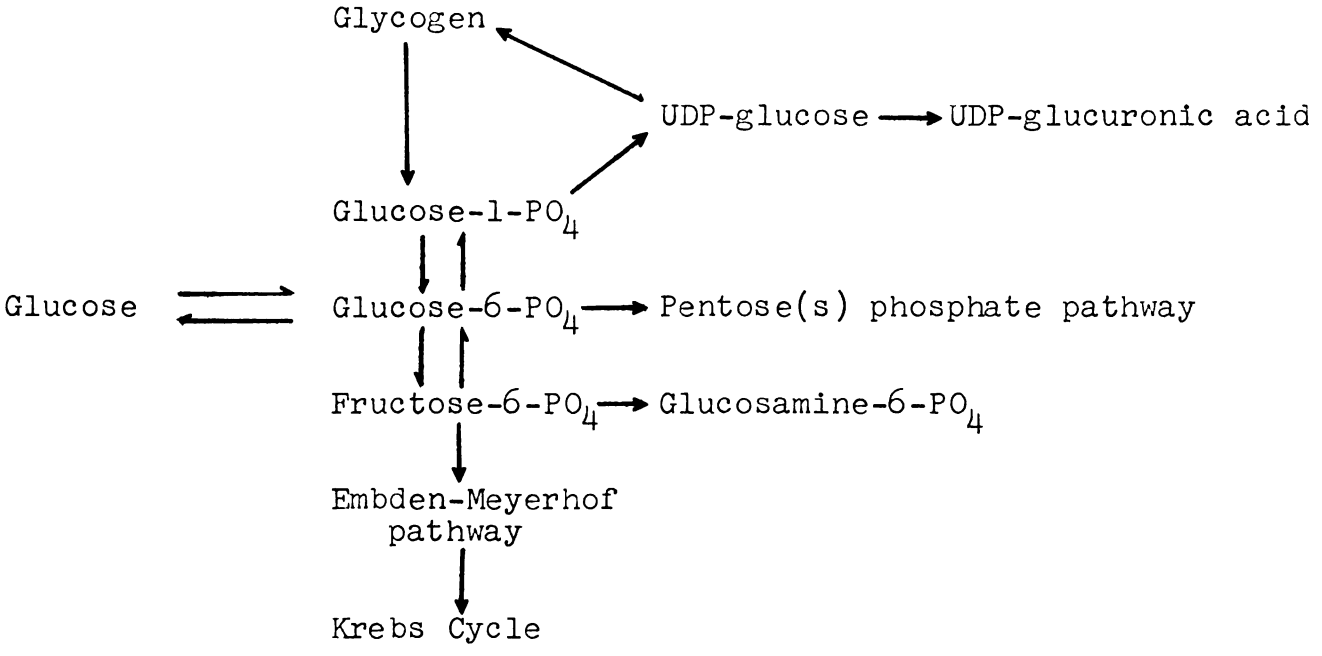


Figure 1-1. Metabolic Pathways of Hexose Monophosphate(s)

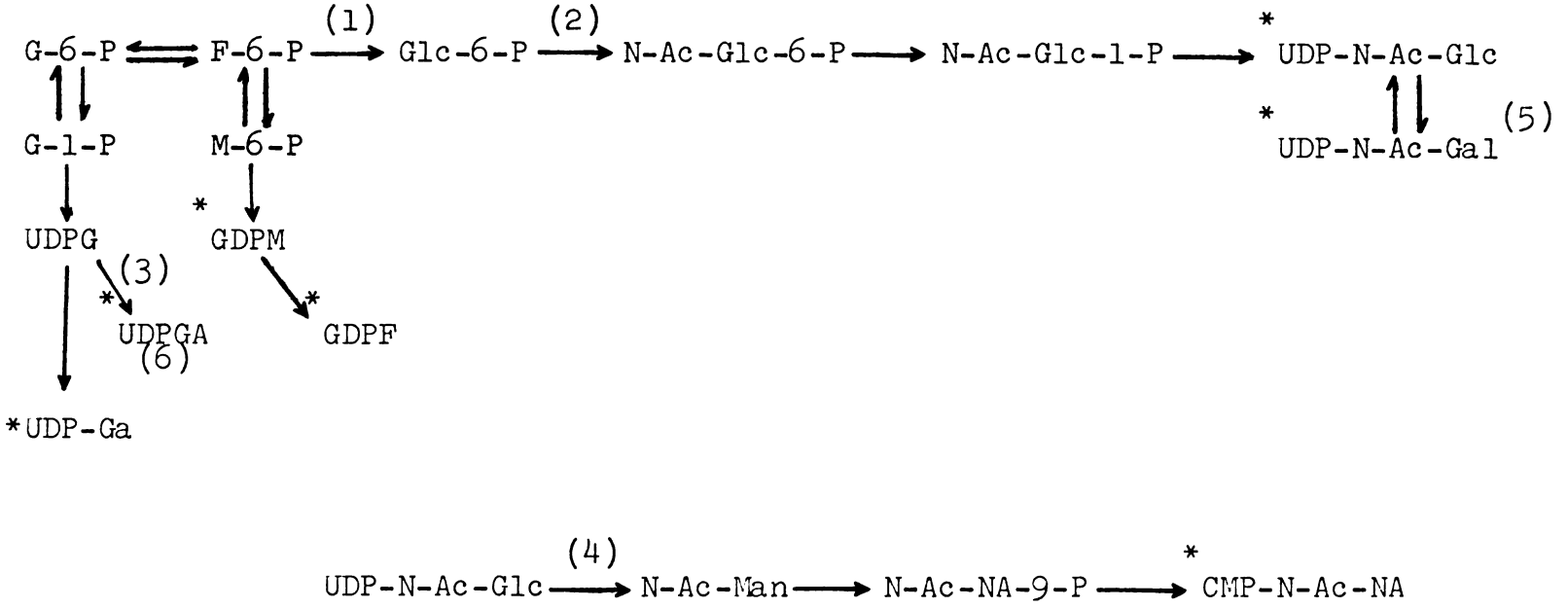
Figure 1-2. Biosythesis of Mucopolysaccarides Indicating Sites of Inhibition by Sodium Salicylate

Sites of Inhibition by Salicylate (references)

(1) 12,28,29,40
(2) 13,14
(3) 31
(4) 13
(5) 30
(6) 32

Abbreviations

G-6-P	Glucose-6-PO_4
F-6-P	Fructose-6-PO_4
Glc-6-P	Glucosamine-6-PO_4
N-Ac-Glc-6-P	N-Acetylglucosamine-6-PO_4
N-Ac-Glc-1-P	N-Acetylglucosamine-1-PO_4
UDP-N-Ac-Glc	UDP-N-Acetylglucosamine
UDP-N-Ac-Gal	UDP-N-Acetylgalactosamine
G-1-P	Glucose-1-PO_4
M-6-P	Mannose-6-PO_4
UDPG	UDP-Glucose
GDPM	GDP-Mannose
UDPGA	UDP-Glucuronic acid
GDPF	GDP-fucose
UDP-Ga	UDP-Galactose
UDP-N-Ac-Glc	UDP-N-Acetylglucosamine
N-Ac-Man	N-Acetylmannosamine
N-Ac-NA-9-P	N-Acetylneuraminic acid-9-PO_4
CMP-N-Ac-NA	CMP-N-Acetylneuraminic acid
UDP	Uridine diphosphate
GDP	Guanosine diphosphate
CMP	Cytidine Monophosphate



*Donors of the respective carbohydrate moieties in the biosynthesis of mucopolysaccarides

Figure 1-2. Biosythesis of Mucopolysaccarides Indicating Sites of Inhibition By Sodium Salicylate

1-2 Problems of *In Vitro* to *In Vivo* Correlations

Application of a model based on *in vitro* data to an *in vivo* system is a difficult task. The fact that a drug inhibits the enzyme activity *in vitro* is not a sufficient guarantee that the *in vivo* biological effects attributed to that drug are due to the same inhibition(s). Many reasons may be responsible for complicating these correlations. The composition of the *in vitro* incubation mixture may beam little resemblance to physiological requirements. The relative proportion of enzyme, substrate, cofactors, pH, and ionic strength of the incubation media may not reflect the normal biochemical system. Extreme and artificial conditions are frequently used to facilitate the quantitive measurement of reaction velocity and enzyme activity. *In vivo*, the concentration of the drug may vary with both tissue and time in the body whereas *in vitro* it remains effectively constant.

Some of the more important considerations when correlating *in vitro* enzyme inhibition with *in vivo* biological observable effects are summarized as follows:

I. Pharmacokinetics of the inhibitor
   1. Time course of the inhibitor concentration in the tissue(s) and/or blood.
   2. Plasma and tissue binding of the inhibitor.
   3. Metabolism of the inhibitor and activity of metabolites.
   4. Mode of administration of the inhibitor.

II. Nature of the inhibition
    1. Mechanism of action: competitive, noncompetitive, uncompetitive, etc., with substrate, coenzyme, activator, cofactor, etc.
    2. Reversibility of inhibition.

III. Nature of the enzyme system
    1. Organization of the enzyme system: monolinear, branched, cyclic, etc.
    2. Existence of feedback and nature of feedback.
    3. Compartmentation of enzyme and substrates, products, etc., intracellular enzyme reserve.

IV. The role the enzyme or enzyme system plays in maintaining the homeostasis of the biological event under observation.

If an interaction between a drug and an enzyme is to be relevant to an <u>in vivo</u> effect, then it must be elicited at drug concentration equal to those occuring at the target site. The time course of the drug at the site of action generally determines the time of onset, duration, and the rate of dissipation of the effect as well as the onset, duration, and magnitude of the maximal effect. Since many drugs are bound to plasma proteins and only a fraction is available to enter the body cells and available to interact with enzymes, it is the free concentration of the inhibitor in the body fluids which is important and not the total concentration. The bound inhibitor is generally not available for interaction with enzymes. If the activity of the drug resides in a metabolite or if metabolite(s) are also active,

then the time course of the metabolite(s) and plasma and tissue binding of the metabolite(s) would take on importance. The route of administration is important especially in cases where the drug is rapidly metabolized by the liver. An oral dose will enter the systemic circulation by way of the portal vein and the liver. Therefore, the entire administered dose, assuming complete absorption, would be subjected to metabolism before reaching the site of action. After an intravenous dose, only a fraction of the dose is subjected to metabolism in its first pass through the liver. Therefore, a higher fraction of the administered dose would be able to reach the site of action. The route of administration would produce drastic differences in tissue concentrations if the drug was administered directly into the site of action. For example, if the site of action was the gastric mucosa and the drug was given orally compared to a parenteral route, the mucosa would receive a much higher fraction of the administered dose via the oral route.

The mechanism of inhibition is another important consideration in extrapolating *in vitro* data to *in vivo* situations. If the inhibitor competes reversibly with a substrate, coenzyme, activator, or cofactor, then the *in vivo* concentration of the inhibitor as well as the substrate coenzyme, activator, or cofactor will determine the degree of inhibition. For example, if the in *vitro* incubation with 1 mM of substrate and 1 mM of

inhibitor showed competitive inhibition[1] and we achieved a concentration of 1 mM of the inhibitor *in vivo* but the *in vivo* concentration of substrate was 10 mM, we would not expect to see any correlation between *in vivo* and *in vitro* results. If the mechanism of inhibition was noncompetitive[2], only the *in vivo* concentrations of the inhibitor and enzyme are important. Another important consideration is that of reversibility of inhibition. An important characteristic of reversible inhibitors is that a definite degree and duration of inhibition depends on the concentration of the inhibitor and the degree of inhibition will dissipate in a rate directly related to the rate of elimination of the inhibitor from the site of action. Irreversible inhibition, in contrast to the reversible type, will cause a gradual loss of the enzyme activity, the extent of which depends on the concentration of the inhibitor attained at the site of action and its rate of elimination from the site.

---

[1] The usual meaning attached to competitive inhibition is that the inhibitor reacts reversibly with a site on the enzyme and thereby prevents combination of that component of the enzyme reaction that normally forms a complex at that site; thus an inhibitor may be competitive with substrate, coenzyme, or activator. The degree of inhibition is depended on the concentration of the substrate (coenzyme, cofactor, or activator) and the inhibitor and their respective dissociation constants.

[2] Noncompetitive inhibition is assumed to involve reaction of the inhibitor with a region other than the active center so that combination of the substrate with the substrate site is unaffected, but the breakdown of the enzyme substrate complex is prevented. In this case, the degree of inhibition will not depend on the substrate concentration or the Michalis Menten constant but only on the inhibitor concentration and the inhibitor dissociation constant.

The enzyme activity will return to normal at a rate depending on the rate of biosynthesis of the new enzyme providing that the elimination of the inhibitor from the site is sufficiently rapid not to inhibit newly synthesized enzyme.

A major difference between the *in vitro* and *in vivo* studies on enzyme inhibition is that in the living cell an enzyme normally functions as one step in a chain. The role of the enzyme in the metabolic sequence determines the vulnerability of the enzyme to inhibition. If the enzyme in question is part of a monolinear chain, a branched chain, a cyclic system, or feedback system, its role in controlling the formation of the product of the multienzyme system may vary. Some enzymes normally function at only a fraction of their maximum capacity and their potential activity is far in excess of the flux of their substrates. On the other hand, there are enzymes that normally function at near their maximum capacities and inhibition of the latter would be much more significant *in vivo*. In the former case, the intracellular reserve of the enzyme will allow for the replacement of the inhibited enzymes and restore the preinhibition substrate flux. If the enzyme in question was localized in certain compartments or subcellular structures, the *in vivo* effect of the inhibitor must therefore depend on the ability of the inhibitor to penetrate the barrier. An inhibitor which is a potent inhibitor in vitro may be totally inactive *in vivo* because of its inability to reach the enzyme *in vivo*. However, a drug may exert an effect on the barrier between enzyme and substrate and thus change metabolism without direct effect on the enzyme involved.

In order to induce a pathology as gastric lesions, we must cause a disturbance in homeostasis of factor(s) preventing gastric lesions. There are no doubt many possible target sites and the vulnerability as well as resistance of each to disturbance may differ. Therefore, if we attempt to correlate the inhibition of a highly vulnerable and poorly resistant site to an observed pathology, we may be expected to be more successful than if we chose a highly resistant and invulnerable site. Also, if the drug acted on more than one target site, correlation between biological event and inhibition at one of the sites would be difficult.

1-3 Aims of This Research

In this project, we will study the correlation between the action of sodium salicylate on glucosamine biosynthesis and gastric lesions. We will explore the feasibility of achieving sufficient concentration in the gastric mucosa to inhibit glucosamine synthesis via oral and parenteral administration of the drug. This is of particular importance as Brodie and Chase (41) reported similar dose response curves for oral and intraperitoneal administration of aspirin in inducing gastric lesions in male Holtzmann rats. We will consider the inhibition of single and multienzyme system(s) involved in synthesizing mucopolysaccarides from a mechanistic point of view and treat pharmacokinetically the gastric mucosa receiving the drug from oral and intraperitoneal dosing by compartment models. These models will be useful in interpreting results of others as well

as the experimental results obtained in this study.

The mechanism(s) of action of sodium salicylate on the enzyme L-glutamine:D-fructose-6-PO_4 aminotransferase will be studied. We will also study the effects of UDP-N-acetylglucosamine, the endogenous feedback inhibitor of the enzyme, on the mechanism of action of sodium salicylate. The dose level of sodium salicylate that would induce gastric lesions both by oral and IP routes with minimal of toxicities will be established to guide the in vivo studies. The time course of the drug in the plasma and gastric mucosa after oral and IP administration will be monitored. We will also ascertain the activity of L-glutamine:D-fructose-6-PO_4 aminotransferase and the hexosamine content after giving the drug. Finally, we will attempt to correlate, based on the data obtained, the action of sodium salicylate on glucosamine synthesis and gastric ulceration.

II. THEORETICAL CONSIDERATIONS

2-1 Inhibition of Single Enzyme Systems

The basic principles of enzyme kinetics and Michaelis-Menten theory are presented in all basic tests on biochemistry and in elaborate details by Webb (46) and Dixon and Webb (45). For this reason it will not be discussed here. We will simply assume that the rate of the enzymatic reactions we are studying are adequately described by the Michaelis-Menten equation.

$$v = \frac{V_{max}(S)}{K_m + (S)} \qquad (2\text{-}1)$$

v is the rate of the enzyme catalyzed reaction

(S) is the substrate concentration

V_{max} is the maximum rate

K_m the Michaelis constant which is equal to the substrate concentration that produces half-maximal rate.

We are basically interested in inhibitory mechanisms which could be obtained from kinetic data. The basic approach to determination of the inhibitor constant and characterizing the type of inhibition was made by Lineweaver and Burk (47) but other procedures have been developed which may be more accurate and applicable in specific areas (46).

The two basic types of inhibition can be categorized on the basis of reversibility. Irreversibility may be characterized by the state in which restoration of enzyme activity is not significant over an interval commensurate with the periods involved in the kinetic experiments (53). Kinetics of

irreversible inhibition are thus relatively simple since the inhibition increases with time as the active enzyme can be written as:

$$\frac{d(E)}{dt} = k(E)(I) \quad \text{or} \quad (E) = (E_o)\, e^{-k(I)t} \qquad (2\text{-}2a,b)$$

(E) is the enzyme concentration

(E_o) enzyme concentration at time zero

k the reaction rate constant

(I) the inhibitor concentration

e the case of natural logarithm

The usual formulation of inhibition kinetics apply only to reversible inhibitions. Under this classification are included competitive, noncompetitive, and uncompetitive[3] inhibition. Naturally under each category complete or partial inhibition is possible as well as mixture of more than one type of inhibition. Also, the inhibition may involve substrates, co-factor, coenzymes, and other modifiers of a particular enzyme's activity.

In our case, we are concerned with a two substrate reaction forming two products. We will restrict our discussion to situations applicable to the reaction catalyzed by L-glutamine:D-fructose-6-PO_4 aminotransferase.

---

[3] In this type of inhibition, the inhibitor increases the affinity of the enzyme for the substrate but the enzyme-inhibitor-substrate complex does not break down to products (46).

If we assume a simple model for a two substrate reaction with one enzyme

$$a + b + E \longleftrightarrow abE \longrightarrow \text{products} + E \qquad (2\text{-}3)$$

the rate of reaction will be:

$$v = \frac{V_{max}}{1 + K_3/(b) + K_4/(a) + K_1K_3/(a)(b)} \qquad (2\text{-}4)$$

$$K_1 = \frac{(a)(E)}{(aE)} \qquad K_3 = \frac{(aE)(b)}{(abE)} \qquad (2\text{-}5a,b)$$

$$K_2 = \frac{(b)(E)}{(bE)} \qquad K_4 = \frac{(bE)(a)}{(abE)} \qquad (2\text{-}5c,d)$$

If we assume that the binding of each substrate is independent of the other, we can further simplify by letting $K_1=K_3=K_a$ and $K_2=K_4=K_b$. Therefore, if (b) or (a) is held constant, the reaction velocities will be:

$$v = \frac{V_{max}\,(b)/(K_a + (b))}{1 + K_a/(a)} \qquad (2\text{-}6a)$$

$$v = \frac{V_{max}\,(a)/(K_a + (a))}{1 + K_b/(b)} \qquad (2\text{-}6b)$$

If (a) or (b) was in excess or much larger than K_a or K_b in magnitude respectively, then:

$$v = V_{max}/(1 + K_a/(a)) \qquad (2\text{-}7)$$

$$v = V_{max}/(1 + K_b/(b)) \qquad (2\text{-}8)$$

If inhibitor I should compete with the substrate a for the substrate binding site, then K_a will be increased by a factor of $(1 + (I)/K_i)$ (46). In our simple model K_i is the dissociation constant for the enzyme inhibitor complex. The velocity of the inhibited reaction will be:

$$v = \frac{V_{max}\,(b)/(K_b + (b))}{1 + K_a\,(1 + (I)/K_i/(a)} \qquad (2\text{-}9)$$

If I is a noncompetitive inhibitor of a, meaning that the inhibitor does not affect the combination of the substrate with the enzyme but affects only V_{max} by a factor of $1/(1 + (I)/K_i)$ (46). The inhibited velocity would then be:

$$v = \frac{V_{max}\,(a)/(1 + (I)/K_i)((a) + K_a)}{1 + K_b/(b)} \qquad (2\text{-}10)$$

An interesting situation arises as we look at the two substrate reaction; if we had a competitive inhibition of the substrate held constant, we will get a rate equation indicating a noncompetitive inhibition:

$$v = \frac{V_{max}\,(a)/((a) + K_a(1 + (I)/K_i)}{1 + K_b/(b)} \qquad (2\text{-}11)$$

If (a) was very large, we would not observe any inhibition, as (a) decreased a greater degree of noncompetitive inhibition would be observed.

There are many graphic methods to determine the mechanism of inhibition and to determine constants that quantitatively characterize the inhibition. The most common method is the

plotting of 1/v versus 1/(S) suggested by Lineweaver and Burk (47). This equation comes from taking the reciprocal of the Michaelis-Menten equation:

$$1/v = 1/V_{max} + K_m/V_{max}(S) \qquad (2\text{-}12)$$

Examples of competitive and noncompetitive inhibitions are shown in Figures 2-1 and 2-2.

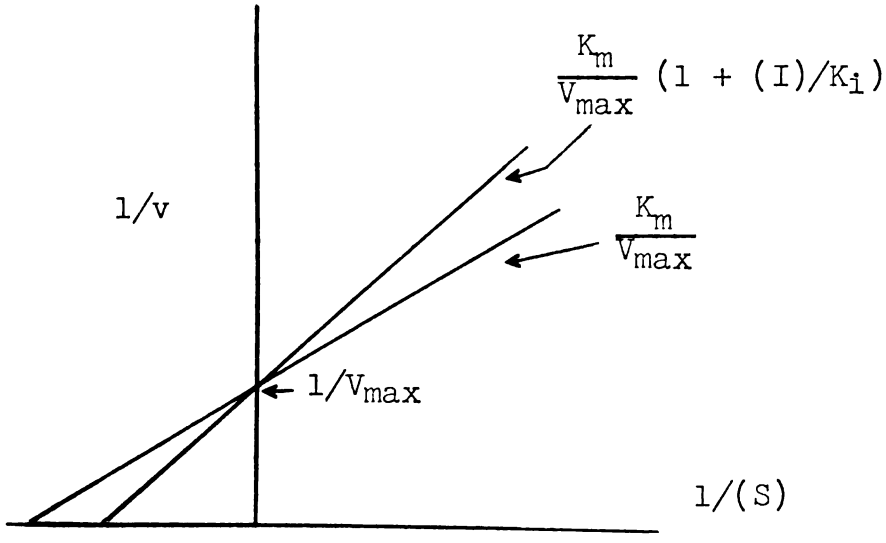


Figure 2-1. Competitive Inhibition, Double Reciprocal Plot

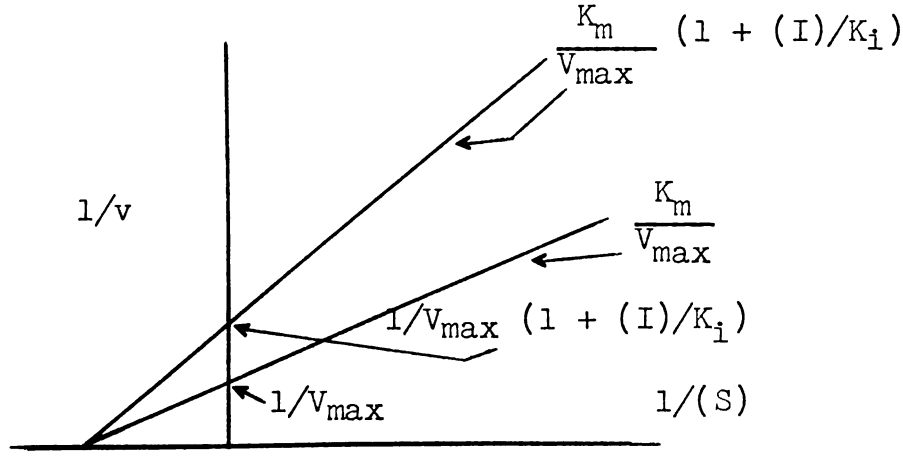
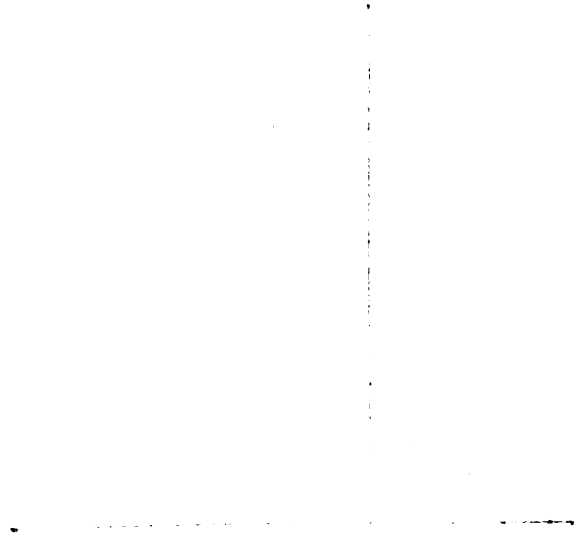


Figure 2-2. Noncompetitive Inhibition, Double Reciprocal Plot



Another procedure suggested by Lineweaver and Burk provides parallel straight lines of constant slope. This method has the advantage that by plotting (S) rather than 1/(S) we are able to spread the data out along the x axis; plotting 1/(S) tends to bunch the data towards the origin. Therefore, in using the double reciprocal plotting, the slope of the lines will be determined more heavily by the larger 1/(S) values. Figures 2-3 and 2-4 show the single reciprocal method of plotting.

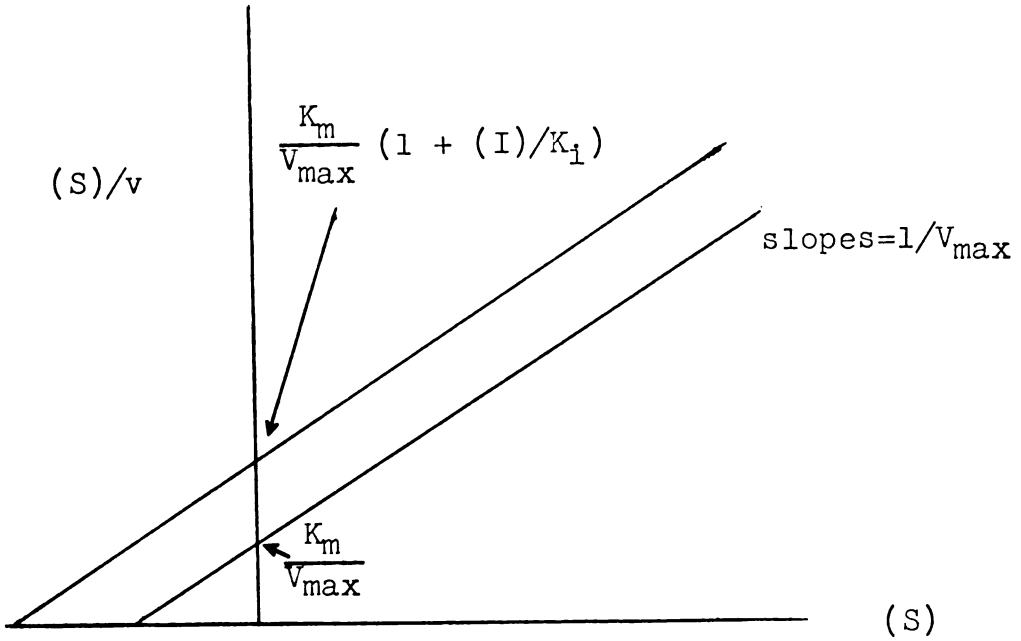


Figure 2-3. Competitive Inhibition, Single Reciprocal Plot

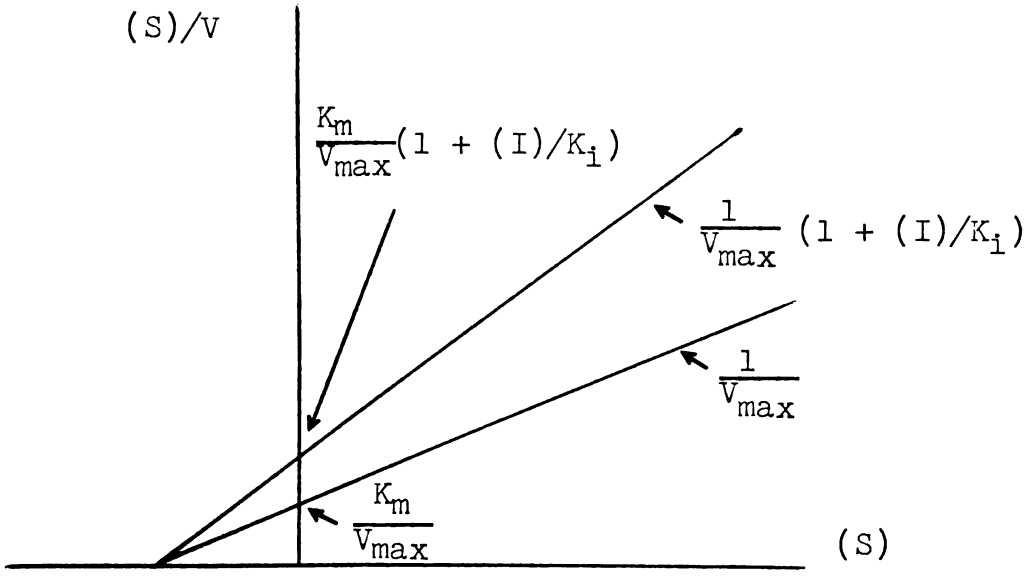


Figure 2-4. Noncompetitive Inhibition, Single Reciprocal Plot

One of the most useful methods was developed by Dixon (48) which allows for the direct determination of K_i. In this method one plots 1/v versus (I) as shown in Figures 2-5 and 2-6.

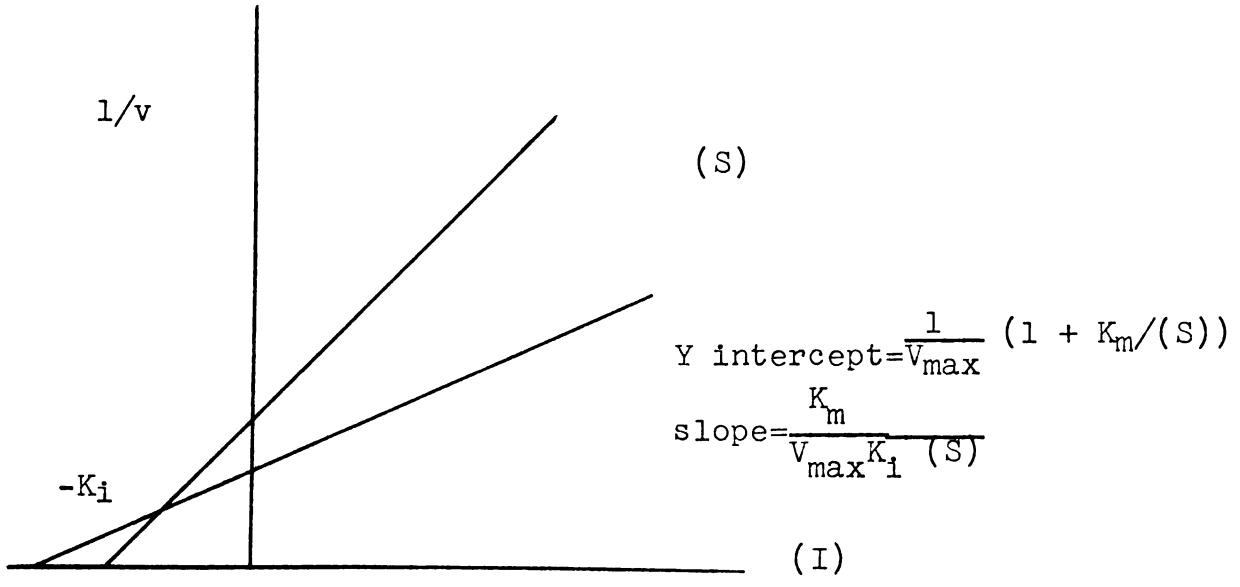


Figure 2-5. Competitive Inhibition, Dixon Plot

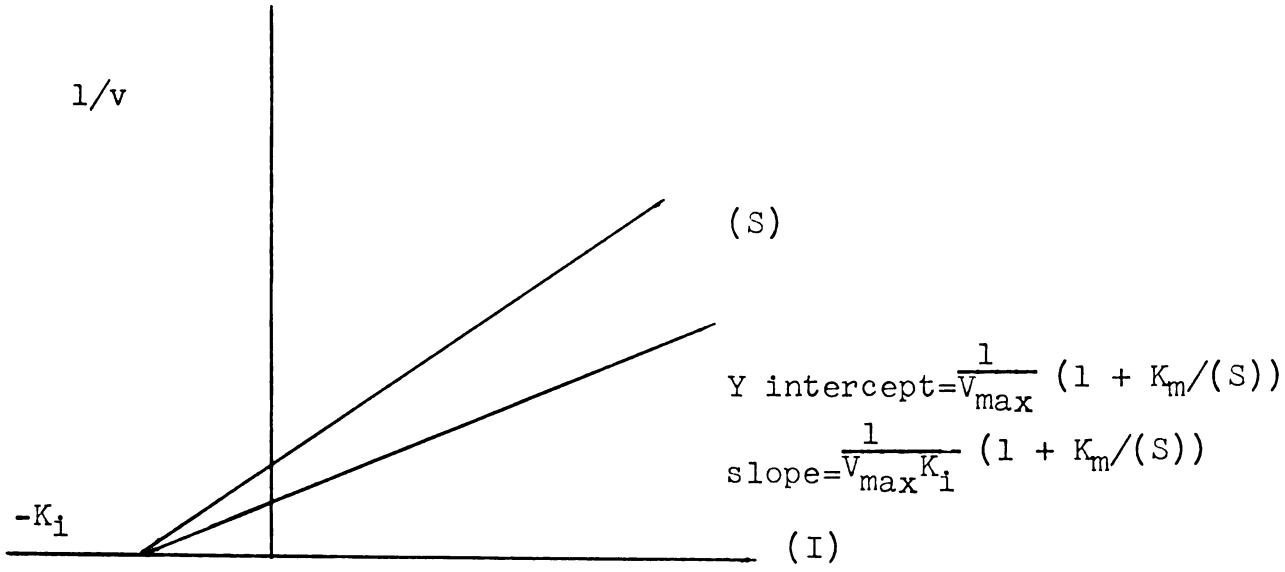


Figure 2-6. Noncompetitive Inhibition, Dixon Plot

There are other types of inhibition mechanisms as partially competitive, partially noncompetitive, uncompetitive, and mixtures or combinations of more than one of the above. These will not be discussed because preliminary studies showed that they were not applicable. Also, since we are using an enzyme extract rather than a pure enzyme, some of the more complex details of inhibition would escape detection.

2-2 Enzyme Inhibition In Vivo

A review article by Smith and Dawkins (4) discussed the inhibition of a number of cellular enzymes by salicylates. These included many dehydrogenases, aminotransferases, decarboxylases, and others. If we wish to extrapolate the action of salicylate from an *in vitro* enzyme inhibition to an *in vivo* setting, an important factor is the reversibility of the inhibition.

If the inhibitor is a reversible competitor of the substrate

for the enzyme, then the degree of inhibition achieved in any tissue *in vivo* would vary not only with the salicylate concentration but also with the substrate concentration provided that the substrate concentration is essential in determining the reaction velocity. In a case where the inhibitor acts as a reversible noncompetitive inhibitor of the enzyme reaction rate, the degree and duration of inhibition *in vivo* will depend on the concentration time course of the inhibitor interacting with the enzyme and the enzyme concentration. In both cases the inhibition time profile should be reflected by the concentration of the inhibitor at the site of action.

Irreversible inhibition, compared to the reversible type tends to become progressive with time. A single dose of an inhibitor can cause a gradual loss of enzyme activity and the return of this activity to preinhibition levels will depend on its rate of biosynthesis of new enzyme. Inhibition can therefore persist long after the inhibitor has been eliminated from the site of action.

Salicylates have been reported to inhibit a wide variety of enzymes with a variety of mechanisms (4). The most interesting may be the ability of sodium salicylate to denature protein. This was first reported by Anson and Hirsky (49) in 1934. Their work showed that sodium salicylate denatured bovine methmoglobin in a reversible manner. Grisolia et al (50) reported that salicylate inactivated muscle triosephosphate dehydrogenase. Gould and Smith (51) showed irreversible

inhibition of rat brain glutamate decarboxylase and Kent and Allen (13) showed irreversible inactivation of L-glutamine: D-fructose-6-PO_4 from sheep intestinal mucosal scrapings.

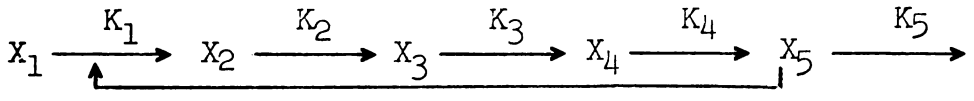
Another very important consideration is that <u>in vivo</u> an enzyme function as one step in a chain. Manifestation of inhibition of a multienzyme system resulting from inhibition of one enzyme depends, on large part, the spatial organization of the multienzyme system and its inherent ability to resist inhibition.

In Figure 2-7 depicts the generally accepted metabolic pathway involved in amino sugar metabolism. It can be subdivided into a monolinear chain with a feedback loop followed by a divergant branched linear chain.

a) Inhibition of a Monolinear Chain with Feedback Inhibition

In order to establish a mathematical model for the monolinear chain with the feedback loop, two conditions must be satisfied. First, the system must be at a steady state. Since steady state systems are highly characteristic of biological processes (109,110,111), the concentrations of various intermediates in the metabolic sequence should remain relatively constant to allow constant flux of substrates and products. Second, we assume that (S) is much smaller than K_m which is not unreasonable for most <u>in vivo</u> metabolic sequences (54).

Looking first at the monolinear chain with the feedback loop:



(2-13)

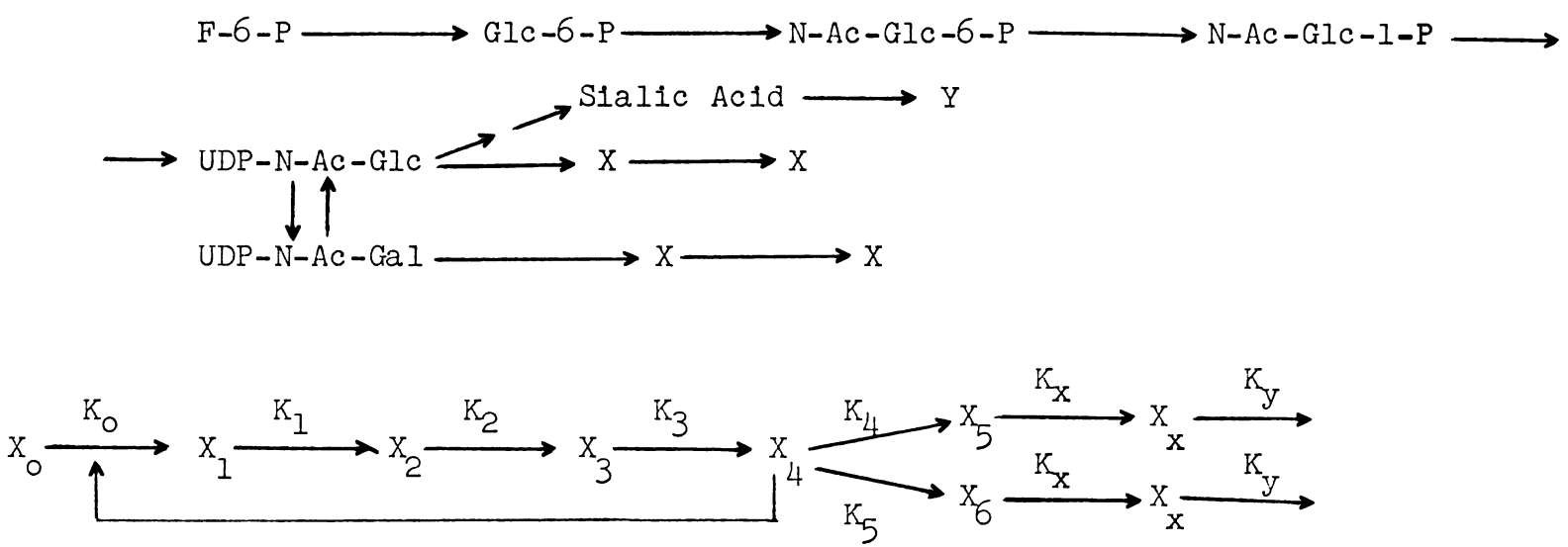


Figure 2-7. Pathways of Hexosamine Metabolism and Its Biochemical Sequence Represented By Linear Analysis

(Abbreviations are the same as in Figure 1-2; X and Y are unknown intermediates; K is a first order rate constant.)

If we let X_i equal the concentrations of the various intermediates and K equal V_{max}/K_m and the feedback inhibitor X_5 is a competitive inhibitor of X_1 for the enzyme, we get the following differential equations:

$$\frac{dX_2}{dt} = \frac{V_{max} X_1}{K_m (1 + X_5/K_i)} - K_2 X_2 \qquad (2\text{-}14)$$

$$\frac{dX_3}{dt} = K_2 X_2 - K_3 X_3 \qquad (2\text{-}15)$$

$$\frac{dX_4}{dt} = K_3 X_3 - K_4 X_4 \qquad (2\text{-}16)$$

$$\frac{dX_5}{dt} = K_4 X_4 - K_5 X_5 \qquad (2\text{-}17)$$

At steady state:

$$X_2^\circ = \frac{V_{max} X_1^\circ}{K_m (1 + X_5^\circ/K_i) K_2} \qquad (2\text{-}18a,b)$$

X° is the steady state concentration

$$X_i^\circ = \frac{K_j}{K_i} X_j^\circ$$

$i = 3,4,5$
$j = i - 1$

In order to look at the stability of the system at steady state, we must transform the singular point to the new origin in the rectangular coordinate system. The singular point will be the chemical concentrations at which there is steady state flux for the chemical reactants and an obvious solution of the differential equations. If we let $u = X - X^{\circ}$, where u is the new variable and X° the concentration at steady state, the singularity will now occur at the origin, $u = 0$. We can now allow for positive as well as negative deviation from steady state value.

$$\frac{du_2}{dt} = \frac{V_{max} X_1}{K_m \left(1 + \frac{X_5^{\circ} + u_5}{K_1}\right)} - K_2 X_2^{\circ} - K_2 u_2 \qquad (2\text{-}19)$$

$$\frac{du_3}{dt} = K_2 u_2 - K_3 u_3 \qquad (2\text{-}20)$$

$$\frac{du_4}{dt} = K_3 u_3 - K_4 u_4 \qquad (2\text{-}21)$$

$$\frac{du_5}{dt} = K_4 u_4 - K_5 u_5 \qquad (2\text{-}22)$$

If we simplify equation 2-19 to $\frac{du_2}{dt} = F(u_5) - K_2 u_2$

and assuming X_1 remains constant, we will obtain the following simultaneous fourth order differential equation:

$$\frac{d^4u_5}{dt^4} + (K_2 + K_3 + K_4 + K_5)\frac{d^3u_5}{dt^3} +$$

$$(K_2K_3 + K_2K_4 + K_2K_5 + K_4K_5 + K_3K_5 + K_4K_3)\frac{d^2u_5}{dt^2} +$$

$$(K_2K_3K_4 + K_2K_3K_5 + K_2K_4K_5 + K_3K_4K_5)\frac{du_5}{dt} +$$

$$K_2K_3K_4K_5u_5 = K_4K_3K_2\ (F(u_5)) \qquad (2\text{-}23)$$

This system is a stable system based on the Hurwitz criterion for stability (54). This means that any disturbance which tends to displace it from steady state will be met with resistance inherent in the system. Therefore, inhibition of individual enzymes may not necessarily result in reduction of the steady state output of the product. If we assume small changes in X_5 around the steady state value X_5, then we can approximate the change in steady state rate of flux in a manner proportional to the change in X_5 from X_5, we can write the system in a classical control system diagram.

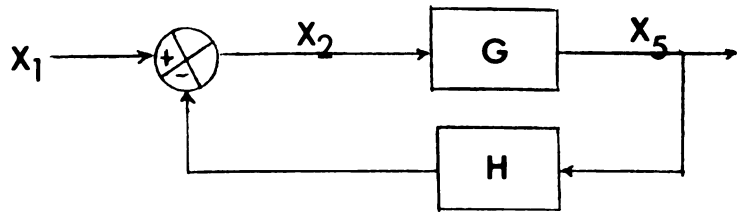


Figure 2-8. Classical Control System Diagram for Equation 2-13. G represents the controlling system. H is the proportionality constant $\Delta v = H(X_5 - X_5)$, where Δv is the change from steady state velocity. H would be a function of K_i, X_5, X_1, V_{max}, and K_{m_1}.

G represents the controlled system which has the following differential equation:

$$\frac{d^3u_5}{dt^3} + (K_3 + K_4 + K_5)\frac{d^2u_5}{dt^2} + (K_4K_5 + K_5K_3 + K_4K_3)\frac{du_5}{dt} +$$

$$K_5K_4K_3u_5 = K_4K_3K_2u_2 \qquad (2\text{-}24)$$

and the following transfer function can be obtained by taking the Laplace transform:

$$\frac{K_2K_3K_4}{s^3 + (K_3 + K_4 + K_5)s^2 + (K_4K_5 + K_5K_3 + K_4K_3)s + K_3K_4K_5} \qquad (2\text{-}25)$$

at steady state we can apply the Final Value Theorem, lim f(t)= lim sF(s), and using a step function as input for X_2 we obtained:

$$\lim s\left(\frac{K_2K_3K_4}{s^3 + as^2 + bs + K_3K_4K_5}\right)\frac{1}{s} = \frac{K_2}{K_5} = G \qquad (2\text{-}26)$$

where a and b are coefficients as shown in Equation 2-25.

The change in steady state output of a control system described above is given by Cushman (55):

$$\Delta ss = \frac{1}{1 + GH_{ss}} \Delta GH \qquad (2\text{-}27)$$

Thus the larger the GH the greater the ability of the control system to resist change. If the system remains first order, then enzymes one, two, and five are the key enzymes controlling the steady state rate.

Taking a more empirical approach into this system without the linear approximation. We will first look at the controlled system as a monolinear chain:

$$A \xrightarrow{v_1} B \xrightarrow{v_2} C \xrightarrow{v_3} D \longrightarrow \qquad (2\text{-}28)$$

at steady state $v_1 = v_2 = v_3$. The intermediate concentrations will then be as follows:

$$(B) = \frac{V_1 K_2 (A)}{(A)(V_2 - V_1) + V_2 K_1} \qquad (2\text{-}29a)$$

$$(C) = \frac{V_2 K_3 (A)}{(A)(V_3 - V_1) + V_3 K_1} \qquad (2\text{-}29b)$$

or in a general form:

$$(J) = \frac{V_1 K_j}{(A)(V_j - V_1) + V_j K_1} \qquad (2\text{-}30)$$

where $V = V_{max}$ and $K = K_m$.

One sees that the ratio of V_1 to V_j is important in maintaining steady state. If V_j is close in value to V_1, then the system can be taken out of steady state with greater ease. The maximal inhibition allowable for a system to remain in steady state is given by Webb (46):

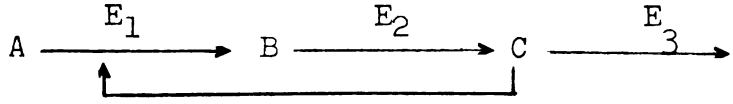
$$i_{max} = 1 - \frac{V_1}{V_j}\left(\frac{(A)}{(A) + K_1}\right) \qquad (2\text{-}31)$$

where V_j is the lowest in the monolinear chain. If the first enzyme was inhibited, we would expect the steady state rate to decrease similar to an isolated enzyme. Naturally, if we deal only with small changes in intermediate concentrations and if the substrate concentrations are smaller than K_m, then the relative values of the intermediate concentrations are proportional to their respective V_{max}'s and K_m's.

$$(J) = \frac{(V_{max}/K_m)_i (I)}{(V_{max}/K_m)_j} \qquad (2\text{-}32)$$

From this we can see that inhibition of one or more of the enzymes after the first will not lead to reduction of the steady state rate as long as the substrate concentrations are smaller than K_m's. Also, in the linear region, the nature of the inhibition whether competitive or noncompetitive will not make any difference.

For a feedback system, we would expect a greater resistance to inhibition. For example, if we look at the simple case:



(2-33)

and at steady state where $v_1 = v_2 = v_3$

and:

$$v_1 = \frac{V_1(A)}{(A) + K_1(1 + (C)/K_c} \qquad (2\text{-}34)$$

$$v_3 = \frac{V_3(C)}{K_3 + (C)} \qquad (2\text{-}35)$$

or:

$$C^2 + K_c(1+(A)/K_1(1 - V_1/V_3))\ C - \frac{V_1 K_c K_3 (A)}{V_3 K_1} \qquad (2\text{-}36)$$

One can see that inhibition of the first enzyme is resisted which was not the case in the monolinear chain. To further illustrate this we will use values reported for glucosamine synthetase and UDP-glucosamine pyrophosphorylase and simulate a velocity-substrate plot, figures 2-9a and 2-9b. Glucosamine formation is in fact a bimolecular reaction but since glutamine concentration is believed to be much greater than fructose-6-PO_4 (56) we will assume that the latter is rate limiting. K_1 is then K_m for fructose-6-PO_4.

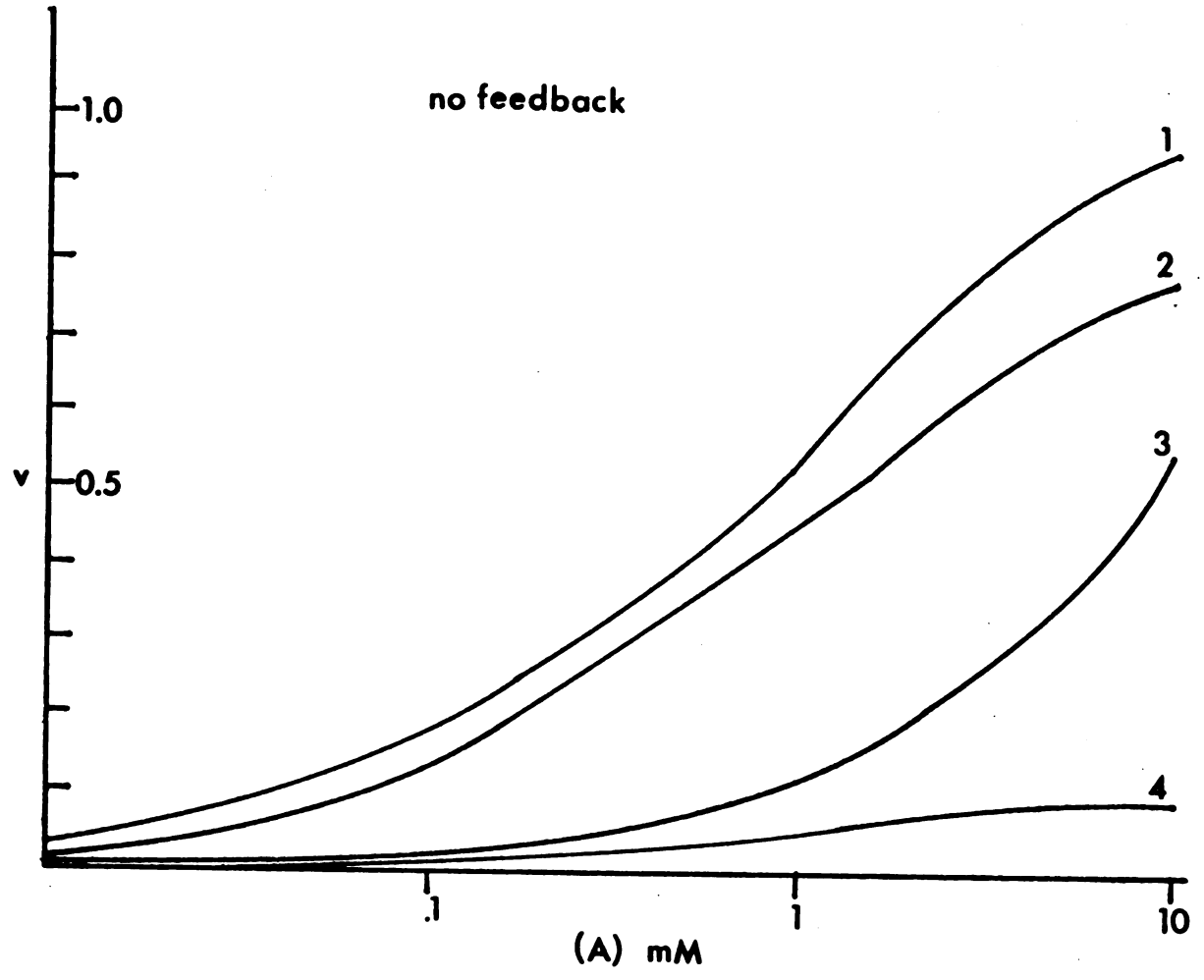
Figures 2-9a and 2-9b. Effect of inhibition on E_1 on the variation of the relative rate with substrate concentration in a system with and without feedback. $V_1 = V_3 = 1$, $K_1 = 4.5 \times 10^{-4}$M, $K_3 = 3 \times 10^{-4}$M, and $K_c = 5 \times 10^{-6}$M.

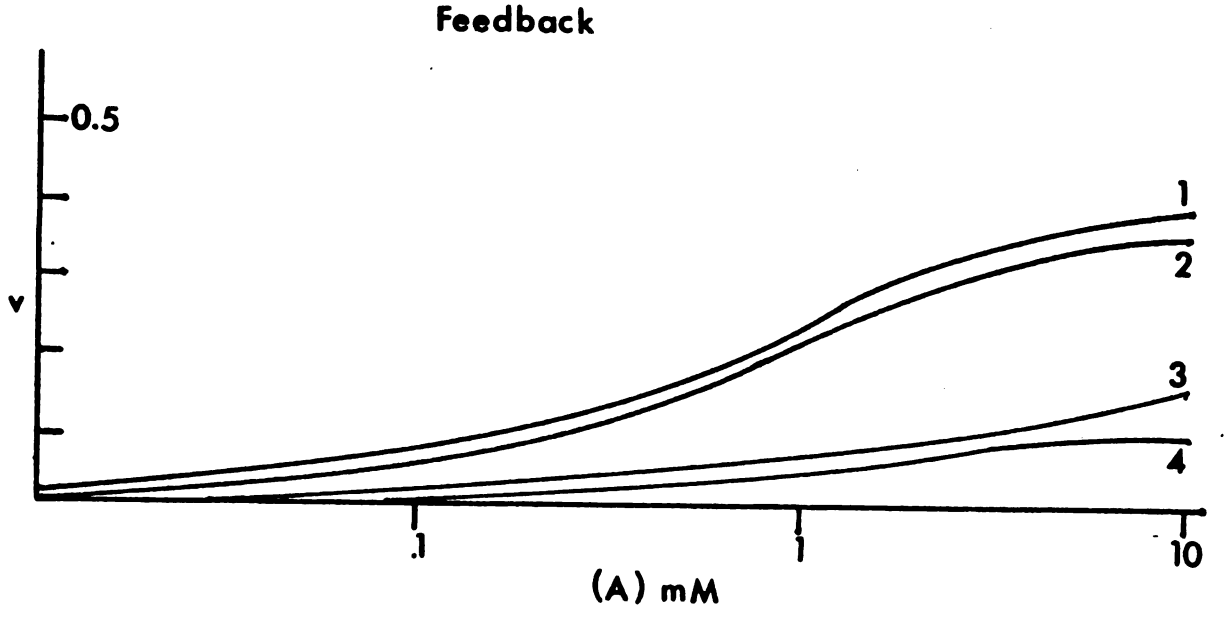
Curve 1: Uninhibited steady state rate v.

Curve 2: V_1 reduced by 20 per cent.

Curve 3: K_1 increased by a factor of 10.

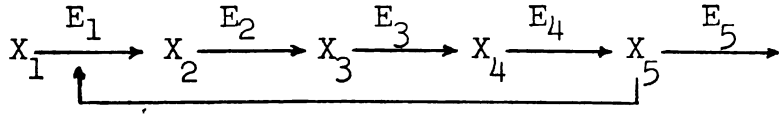
Curve 4: V_1 reduced by 90 per cent.


no feedback
1.0
0.5
v
1
2
3
4
.1
1
10
(A) mM


Feedback
0.5
v
1
2
3
4
.1
1
10
(A) mM

Figures 2-9a and 2-9b show the differences between a system with and without feedback. Below 10 mM or physiological range the system without feedback shows a steeper slope while the system with feedback tends to maintain a more or less "buffer" to changes in (A). At the lower substrate concentrations especially the feedback system has a much greater resistance to inhibition to E_1. For example, when V_1 is reduced by 20 per cent and (A) equals to 0.1 mM, the feedback system showed 8 per cent inhibition in product formation while the monolinear chain showed 20 per cent reduction. The system without feedback can overcome competitive inhibition at high (A) while the feedback loop reduces this effect. Both systems gave essentially the same profile for noncompetitive inhibition.

Contrary to the monolinear chain, the feedback system is sensitive to inhibition at the enzyme after the feedback at E_3. Inhibition at E_3 will lead to increase in C thus reducing the steady state rate. If we look at our five enzyme system:



(2-37)

and assume that a linear approximation after E_1 would be valid we would have:

$$v = \frac{V_1 X_1}{X_1 + K_1(1 + X_5/K_I)} \qquad (2\text{-}38)$$

$$X_i = \frac{(K_i/V_i)}{(K_5/V_5)} X_5 \qquad (2\text{-}39)$$

$$v_i = \frac{V_i X_i}{K_i} \qquad (2\text{-}40)$$

where K_I feedback inhibitor constant

$K = K_m$

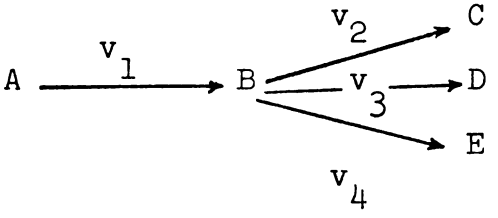
$V = V_{max}$

X_i concentration of any intermediate except X_1

At steady state v_1 is equal to v_i and we would arrive at the following equation indicating that enzymes one and five are important not only in determining the steady state rate but also the concentration of each intermediate.

$$X_i^2 (K_1 K_i V_5 / K_I K_5) + X_i (V_i X_1 + V_i K_1) = V_1 K_i X_1 \qquad (2\text{-}41)$$

b) Inhibition of the Divergent Branch Chain



(2-42)

implies that $v_1 = v_2 + v_3 + v_4$ and reduction in v_1 due to inhibition of E_1 will reduce the sum of $v_2 + v_3 + v_4$. Each velocity need not be reduced equally and would depend on the

state of the system before inhibition. For example, if one or more of the steps after B are close to saturation, inhibition of v_1 will not lead to drastic reduction in rates of the steps close to saturation. If, however, all steps are operating in the linear region, then each v after B would be reduced proportionally to v_1. If one or two of the velocities after B are inhibited and one of the steps are saturated, then the uninhibited step will increase due to an increase in B and thus maintain the steady state velocity ($v_1 = v_2 + v_3 + v_4$). One can then actually get stimulation of one pathway resulting from inhibition of others.

A close examination into the enzyme system which synthesizes mucopolysaccarides reveals two vulnerable sites for inhibition. The first enzyme apparently regulates the steady state rate of the whole sequence as it is located at a branch point of carbohydrate synthesis and is regulated by the feedback inhibitor. The second site is immediately after the feedback loop which also contains the divergent branch chain. Inhibition at this site can lead to build up of the feedback inhibitor and stimulation of certain branches of the divergent chain.

An experiment done by Kent and Allen (13) will illustrate to a certain extent what we have discussed. These authors incubated sheep colonic mucosal scrapings with radioactive glucose and determined the incorporation into various intermediates under the influence of sodium salicylate.

Table 2-1. Incorporation of 2-^{14}C Glucose into the Monosaccharide Constituents of a Glycoprotein Fraction From Sheep Colonic Scrapings.*

Monosaccharide	Control	3.75 mM SA	Per Cent Inhibition	Per Cent Inhibition Calculated
salic acid	8.26	2.27	63	-41
glucosamine	3.54	1.65	53	-53
galactosamine	4.11	2.50	39	+30
glycoprotein	11950	7050	41	

* Kent, P.W. and A. Allen, Biochem. J. 106, 645 (1968).

Activity for glycoprotein in counts/min/mg and other given as 10^{-3}x counts/min/umole.

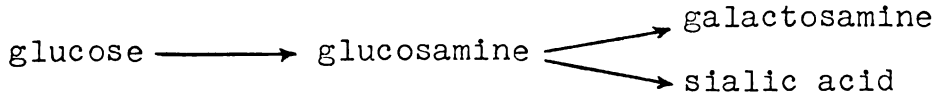
Calculation of Per Cent Inhibition

glucose ⟶ glucosamine $\quad 1 - \frac{(1.65/3.54)}{1} \times 100 = -53.4\%$

glucosamine ⟶ sialic acid $\quad 1 - \frac{(2.27/8.26)}{(1.65/3.54)} \times 100 = -41\%$

glucosamine ⟶ galactosamine $\quad 1 - \frac{(2.50/4.11)}{(1.65/3.54)} \times 100 = +30\%$

Simplified Reaction Sequence



The inhibition of incorporation given by Kent and Allen do not reflect the true inhibition. Since inhibition of an earlier step will naturally depress the later steps, compensations should be made for a more realistic approach. Therefore, we should recalculate the inhibition by using the value of the precursor for the control. Kent and Allen's data along with the recalculated inhibition percentages are given in Table 2-1.

There are two major reasons why there is a reduction in activity at some time t:

(1) The steady state flux is reduced. In this case, the rate constant of the rate limiting step is reduced and the pool size is relatively stable.

(2) The steady state flux is constant meaning that the rate limiting step is not inhibited but a reduction in a non rate limiting step(s); this results in a decrease in k (s) causing a compensation by an increase in pool size, thus diluting the isotope and decreasing the activity.

If we consider the reaction:

$$\text{glucose} \xrightarrow{k_1} \text{glucosamine} \xrightarrow{k_2} \qquad (2\text{-}43)$$

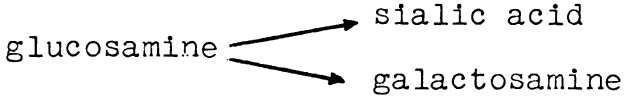
and k_1 is reduced, glucose pool can increase to compensate. However, since glucosamine pathway is expected to be a small fraction of glucose metabolized, pool size adjustment would be minimal.

If the metabolism of glucosamine anywhere along the pathway

is reduced, then the pool size will increase leading to reduction in activity. Since "glucosamine" is included in all the following--glucosamine-6-PO_4, N-acetylglucosamine-6-PO_4, N-acetylglucosamine-1-PO_4, and UDP-N-acetylglucosamine--reduction in total activity of "glucosamine" can result from inhibition of any step. Logically, the earlier the inhibition occurs the greater the inhibition as subsequent steps will cumulate the inhibition.

The inhibition of a glycoprotein fraction of 41% may indicate a reduction in the metabolic flux. The composition of this fraction is N-acyl neuraminic acid 13.7%, galactose 13.8%, N-acetylglucosamine 27.4%, and N-acetylgalactosamine 13.7%. Since N-acetylglucosamine comprised the largest component, its synthesis may be rate limiting.

In the case of the divergent branch chain:



(2-44)

the steady state flux in the formation of glucosamine is equal to that used up by conversion to sialic acid and galactosamine and any other side reaction. As discussed earlier, if one branch is inhibited, then the other pathways would have to increase to maintain the steady state flux. In the data by Kent and Allen, recalculation showed a decrease in incorporation of tracer into sialic acid of 41% and an increase in the galactosamine pathway by 30%. One may consider the possibility that this decrease

may be caused by decrease in utilization of sialic acid and resultant increase in pool size; but this does not explain the increase in galactosamine synthesis. This data is consistent with the hypothesis that sodium salicylate inhibits the synthesis of sialic acid and results in the increase in galactosamine levels.

Lukie and Forstner (14) attempted to locate the site of action of sodium salicylate in the rat intestine using labelled glucosamine and determining the counts in various intermediates as N-acetylglucosamine, glucosamine-PO_4, and UDP-N-acetyl-glucosamine. They found that there was a reduction in counts of all intermediates after glucosamine compared to control values. There is one problem, however, as a reduction in steady state flux can also lead to the same results as the activity time curves will be displaced in time. For example, the peak activity for a_2 in the sequence $a_1 \xrightarrow{k_1} a_2 \xrightarrow{k_2} a_3 \rightarrow$ will occur at time T where:

$$T = \frac{1}{k_2 - k_1} \ln \frac{k_2}{k_1} \qquad (2\text{-}45)$$

If k_1 is decreased, then T will increase and the curve will have a peak at a later time. Subsequent activity curves will also be displaced in time in accordance with Zilversmits rule stating that if i is a precursor of j, then the specific activities a_i and a_j must cross at the time of maximum of a_j. At any time, therefore, the total radioactivity distributed among the various intermediates will be less than the control. One might get the

impression that glucosamine to N-acetylglucosamine is being inhibited by the drug as at time t the incorporation of tracer into N-acetylglucosamine is reduced compared to the control. This can be nothing more than displacement of the activity-time curve along the time axis due to the reduction of the steady state synthesis rate of glucosamine.

From Kent and Allen's work, we conclude that there is some inhibition by sodium salicylate occurring somewhere between glucose to UDP-N-acetylglucosamine perhaps at synthesis of glucosamine-6-PO_4 from glucose-6-PO_4 thus reducing the steady state synthesis rate. Also, salicylate seems to inhibit the synthesis of sialic acid and a concomitant stimulation of galactosamine synthesis. We cannot rule out inhibition at other sites but these are more sensitive based on the organization of the enzyme system. More elaborate tracer studies must be done to learn more about the incorporation of various monosaccarides as well as the synthesis of various types of glycoproteins with varying composition.

c) Effects of Inhibitions on Hexosamine Content

Examining the metabolic scheme of hexosamine metabolism, Figure 2-7, the key enzyme in determining the steady state rate of hexosamine synthesis is apparently glucosamine synthetase. Therefore, if we monitored the hexosamine content, we could get some estimate of the steady state rate. To obtain a mathematical expression of how the hexosamine content would change as a result of inhibition, we can use a linear analysis

of the biochemical system. This means that a linear differential equation of the type $dX_i/dt = K_j X_j - K_i X_i$ can adequately describe the kinetics of each intermediate. At steady state, we will have $dX_i/dt = 0$ or $X_j = (K_i/K_j)(X_i)$. Since the nature of the various intermediates beyond UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine leading to glycoproteins are not known, we will designate them as X_x. Glucosamine and galactosamine will be designated as X_4. Under these conditions, we can equate, at steady state:

$$K_o X_o = K_1 X_1 = K_2 X_2 = K_3 X_3 = (K_4 + K_5)X_4 = K_x(X_5 + X_6) = K_y X_x \qquad (2\text{-}46)$$

From this the total hexosamine will be as follows:

$$T = X_1(1 + K_1/K_2 + K_1/K_3 + K_1/(K_4 + K_5) + K_1/K_x + K_1/K_y) \qquad (2\text{-}47)$$

Based on the premise that steady state will be maintained and decreases in K_i will be compensated by an increase in X_i to maintain the steady state rate. Keeping in mind that K is equal to V_{max}/K_m we can foster some interpretations regarding changes in T.

T will decrease if the formation of X_i is decreased namely the formation of glucosamine or if the decrease in K_1 is greater than the decrease in $K_i (i \neq 1)$, K_x, or K_y.

T will increase if $K_i (i \neq 1)$ in any combination and/or K_x, K_y are decreased as the result of decrease in V_{max} or increase in K_m. Also, if the decrease in $K_i (i \neq 1)$, K_x and/or

K_y are greater than the decrease in K_1.

There will be no change in T if none of the K's are altered or the combination of effects cancel each other out leaving a net no effect.

If for any reason the system is taken out of steady state by the inhibition(s), this model will not be applicable in interpreting the results.

2-3 Kinetics and Compartment Model for Salicylate Disposition

A necessary condition to achieve some kind of correlation between the drug and its action is that the drug must be available at sufficient concentration for a sufficient period of time at the site of action. In our case the drug must have an adequate concentration time profile to significantly inhibit the synthesis of glucosamine. The route of administration is of prime importance in determining the time-concentration profile of the drug at the target tissue, the gastric mucosa. The tissue concentration time profile will in turn determine the tissue variation of the intensities of the drug response.

Perrey suggested correlation between inhibition of glucosamine synthetase activity and gastric ulceration after oral administration of salicylate (28). Brodie and Chase (41), using aspirin, reported that the dose response curves in inducing ulcers in rats were similar for oral and IP routes. Djahanguiri (43), using indomethacin, produced very similar dose response curves with oral and intraperitoneal dosing in rats. This has also been confirmed by Lee (44).

The pharmacokinetics of salicylates are published in many sources and is highlighted by the work of Levy (1,3,61) and more recently by Rowland et al (64). The drug in solution is well absorbed from the gastrointestinal tract. Salicylic acid has a pK_a of 3.0; therefore, the low pH of the stomach should favor the absorption of the drug. The greater surface area of the small intestine, however, makes it the optimum site for rapid absorption. The rate of absorption is believed to obey Fick's law of diffusion and the rate of flux into the gut is governed primarily by the concentration gradient. Smith and McArthur (62) reported that the drug is bound to plasma proteins and that the amount of protein binding varies with the total concentration of the drug in the plasma. At concentrations of 200mcg/ml. to 600mcg/ml., 45% to 60% of the total drug in the plasma is bound to plasma proteins. The same authors also reported that only the liver and kidney seem to have a capacity to serve as significant tissue binding sites for the salicylate molecule.

The accepted metabolic pathways both in man and laboratory animals are qualitatively similar (Figure 2-10).

The metabolites listed above in addition to free salicylate accounts for over 95% of the administered dose. The amount of unchanged salicylate excreted in man varies from 5% to 20% (61); therefore, the majority of the administered dose is eliminated as metabolites. All the major metabolites and free salicylate are rapidly eliminated by renal excretion. Levy (61) reported that in man, even at doses that saturate the metabolic enzymes,

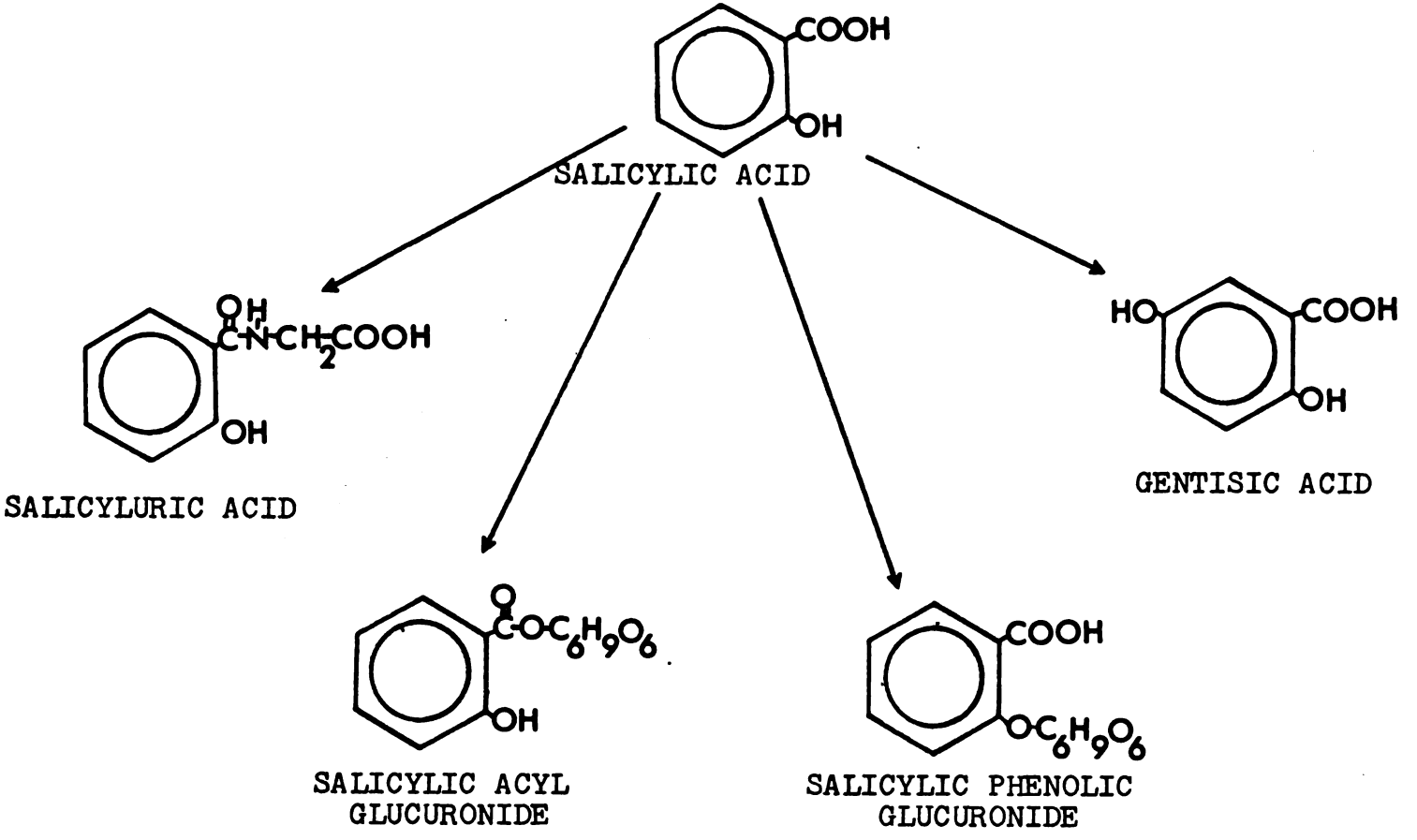


Figure 2-10. Metabolic Pathways of Salicylic Acid

the excretion of metabolites are so rapid that accumulation is negligible. Mandel (63) suggested that only 7% of the total salicylate in the blood is in the form of derivatives and less than 1% may be in the form of glucuronides.

Despite all the work done on the pharmacokinetics of salicylates, no one has followed the concentration time course of the drug in the gastric mucosa after oral or parenteral dosing.

We will preface the experimental work by taking a theoretical look at the concentration time course of the drug in the plasma and the gastric mucosa using a simple compartmental analysis. Compartmental analysis is widely used in simplifying the overwhelming complexities that would be involved in an attempt to measure the rates of transfer through each region of a system. Here we are concerned with two compartments, the plasma and the gastric mucosa. We will assume that the drug can be metabolized from the plasma only, meaning that the liver and kidney are the major metabolizing organs. Hanninen (57) reported that the rat gastric mucosa is capable of forming glucuronides with nitrophenol but the amount of salicylate metabolites in various tissues are generally reported to be low. Wolf and Austen (58) found only trace amounts of salicylic glucuronide in the rat tissues with the major metabolic organs, the liver and kidney, containing only 2% to 6%. Since more than 80% of salicylic acid is metabolized, this would indicate that metabolites of salicylate are very rapidly excreted from the body.

We can treat the general kinetics of the drug by using a physiologically consistent model where the flux of the drug, in and out of tissues, is limited by blood flow (59,60). When the drug is given by an intraperitoneal route, the drug input is essentially into the plasma compartment. The gastric mucosa compartment acquires the drug via the circulation. The accumulation of the drug in the plasma and mucosa compartments can be described by the following equations based on conservation of mass:

$$dA_p/dt = (A_{in} - A_{out})_p \qquad (2\text{-}48)$$

$$dA_m/dt = (A_{in} - A_{out})_m \qquad (2\text{-}49)$$

where p is the plasma compartment, m is the mucosa compartment, A_{in} and A_{out} represent the flux of material into and out of each compartment.

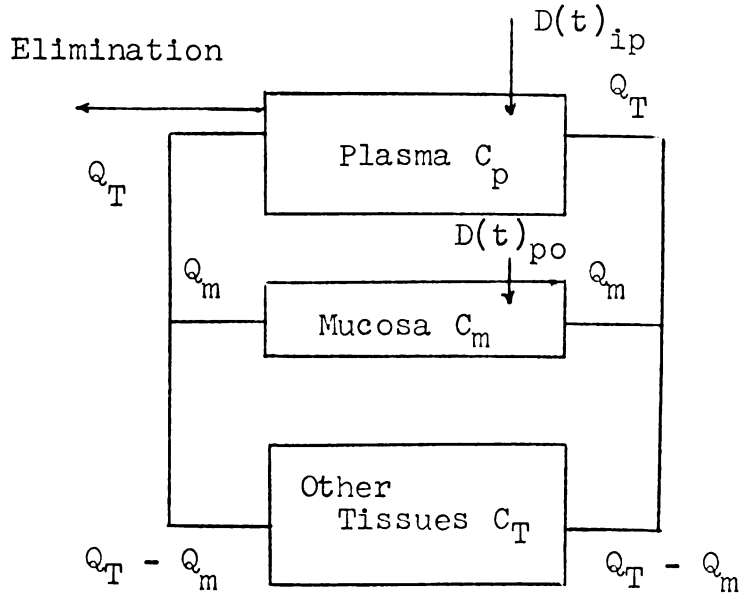


Figure 2-11. Compartment Model For Salicylate Disposition

For the IP route the input of drug into the plasma compartment is made up of a time dependent drug input D(t) and material flux into the plasma via the circulation, $Q_T C_T$ where Q_T is the rate of plasma flow to and from the tissues and C_T the tissue concentration of the drug. Mass flux out of the plasma compartment is represented by plasma flow to the tissues, $Q_T C_p$, and metabolism represented by an enzyme substrate type of equation $V_{max} C_p/(K_m + C_p)$, where V_{max} and K_m have the usual meaning and C_p is the plasma concentration. An excretion term $K_e C_p$ is also included for the unchanged drug, where K_e is a first order excretion rate constant of the unchanged drug. If we assume that the drug in the plasma is in a hypothetical volume V_p, we will have the following:

$$V_p \frac{dC_p}{dt} = D(t)_{ip} - Q_T C_P + Q_T C_T - \frac{V_{max} C_p}{K_m + C_p} - K_e C_p \qquad (2\text{-}50)$$

If we treat the mucosa the same way, and assuming no significant metabolism by the mucosa, we will have:

$$V_m \frac{dC_m}{dt} = Q_m C_p - Q_m C_m \qquad (2\text{-}51)$$

where V_m is the apparent volume of the mucosal compartment and Q_m is the plasma flow to and from the mucosa.

If we look into the case of oral dosing, the plasma equation will be the same but the mucosal compartment will have an additional input, $D(t)_{po}$.

$$V_m \frac{dC_m}{dt} = D(t)_{po} + Q_m C_p - Q_m C_m \qquad (2\text{-}52)$$

If we integrate the equations from t = 0 to t = T,

for the oral dose:

$$V_m \int_0^T \frac{dC_m}{dt} dt = V_m C_m = \int_0^T D(t)_{po} dt + Q_m \int_0^T C_p dt - Q_m \int_0^T C_m dt \qquad (2\text{-}53)$$

for the IP dose:

$$V_m C_m = Q_m \int_0^T C_p dt - Q_m \int_0^T C_m dt \qquad (2\text{-}54)$$

$\int C_p dt$ and $\int C_m dt$ represent the areas under the plasma and tissue concentration time curves respectively. $V_m C_m$ is the amount in the mucosa at time T and $\int_0^T D(t)_{po} dt$ is the amount absorbed into the mucosa from time zero to time T. Since we can get the areas by monitoring the plasma and mucosal concentrations, we should be able to estimate the net amount absorbed into the gastric mucosa by using the following relationships obtained from the above equations 2-53 and 2-54 by eliminating Q_m and rearranging,

$$\int_0^T D(t)_{po} dt = V_m C_{m(po)} - V_m C_{m(ip)} \frac{\int_0^T C_{p(po)} dt - \int_0^T C_{m(po)} dt}{\int_0^T C_{p(ip)} dt - \int_0^T C_{m(ip)} dt} \qquad (2\text{-}55)$$

where po and ip represent the data obtained from oral and IP administration. Since only the free drug is able to diffuse

into the tissue, the equation should be corrected to account for this. If we let α equal the fraction of free drug and assuming negligible mucosal tissue binding, we will have the following corrected equation:

$$\int_0^T D(t)_{po}\,dt = V_m C_{m(po)} - V_m C_{m(ip)} \frac{\alpha\int_0^T C_{p(po)}dt - \int_0^T C_{m(po)}dt}{\alpha\int_0^T C_{p(ip)}dt - \int_0^T C_{m(ip)}dt} \qquad (2\text{-}56)$$

III. MATERIALS AND METHODS

3-1 Materials and Chemicals

D-glucosamine HCl, D-glucose-6-PO_4, D-fructose-6-PO_4, UDP-glucuronic acid, L-glutamine HCl, UDP-N-acetylglucosamine, and beta glucuronidase were obtained from Sigma Biochemicals. Sodium salicylate and benzoic acid were from J. T. Baker. p-Hydroxybenzoic acid, nicotinic acid, meta hydroxybenzoic acid, 2-amino benzoic acid, 6-methoxy benzoic acid, and (ethylene dinitrilo) tetraacetic acid disodium salt were from Eastman Kodak. Boric acid, potassium hydroxide, potassium phosphate (monobasic and dibasic), and ether were from Mallinckrodt. Glacial acetic acid, concentrated hydrochloric acid, and acetic anhydride were from Allied Chemicals. Mercaptoethanol was from Matheson, Coleman, and Bell.

Sephadex G-25 was from Pharmacia, Uppsala, Sweden. Sprague Dawley rats were purchased from Simenson Laboratory. They were fed a standard commercial laboratory diet and fasted 24 hours before use.

3-2 <u>In Vitro</u> Studies

a) L-Glutamine:D-Fructose-6-PO_4 Aminotransferase Assay in Crude Liver and Mucosal Extracts

Assay was carried out similar to the method described by Bates (39). Sprague Dawley male rats (120-150 Gms.) were etherized and decapitated to drain the blood. Liver or stomach was then excised and placed into an ice cold extraction medium

of pH 7.4 containing 10 mM glucose-6-PO_4 or fructose-6-PO_4, 20 mM glutamine, 0.085 M potassium phosphate buffer (pH 7.4), 0.1% mercaptoethanol, and 0.1 mM (ethylene dinitrilo) tetraacetic acid disodium salt. The liver was cut into small pieces before homogenization and the stomach was cut along its greater curvature, spread on a watch glass placed over ice, and the mucosal surface was scraped with a small spatular. Liver or mucosal scraping was homogenized in a hand homogenizer with equal volume to weight of the extraction medium. The homogenate was centrifuged in a refrigerated Spinco ultra-centrifuge at 35,000 x g for 60 minutes. One milliliter of the extract was incubated with three milliliters of incubation medium (pH 7.4) containing 0.085 M potassium phosphate buffer, 0.1% mercaptoethanol, 1 mM (ethylene dinitrilo) tetraacetic acid disodium salt, and graded concentration of glucose-6-PO_4 or fructose-6-PO_4, and glutamine. Sodium salicylate and/or UDP-N-acetylglucosamine were added at various concentrations in the inhibition studies. The incubation was carried out in a 25 ml. volumetric flask on a Dubnoff shaker incubator at 37 degrees Centigrade for a period of 30 minutes for liver extracts and 45 minutes for mucosal extracts. The mixture was then heated in boiling water for 2 minutes to terminate enzyme activity. After cooling to room temperature, the mixture was centrifuged at 35,000 x g for 15 minutes and the supernatant removed for hexosamine assay.

Hexosamine was analyzed by the method described by Benson

and Friedman (65). In a 15 ml. centrifuge tube with a ground glass stopper was added 0.8 ml. of the supernatant containing hexosamine, 0.5 ml. of borate buffer (pH 9.0), prepared by adding equal parts of 1.12 M of boric acid and 0.56 M potassium hydroxide, and 0.1 ml. of 5% acetic anhydride in ice cold water. This reaction mixture was kept at room temperature for 10 minutes and heated in boiling water for 10 minutes. After cooling, 6 ml. of dimethylaminobenzaldehyde (Eastman Kodak) solution (1 Gm. in 1 ml. concentrated HCl to 100 ml. of glacial acetic acid) was added and heated at 37 degrees Centigrade for 20 minutes. The resultant solution was read at 585 nm in a Beckman-B spectrophotometer using glucosamine HCl as the standard. No correction was applied for the fact that glucosamine-6-PO_4 on a molar basis gave 85% of the color produced by glucosamine (66). Reaction mixtures tested either before or after incubation in the absence of enzyme produced negligible amount of color for glucosamine. It has been assumed throughout this work that the isomerization of glucose-6-PO_4 to fructose-6-PO_4 was not rate limiting due to the presence of isomerases in the enzyme extracts (39). In fact, Bates (39) and Pogell (34,67) found that glucose-6-PO_4 is a better substrate for the crude enzyme. The change in absorbance was linear over the incubation times used. Incubation with sodium salicylate and UDP-N-acetylglucosamine did not alter the linear absorbance time relations. Studies showed that the mechanism of inhibition of sodium salicylate on the enzyme from liver or mucosa were essentially identical. Due to the fact that the liver extracts

possessed higher activity, the inactivation studies and inhibitions with higher inhibitor concentrations were done with the liver extract.

b) Enzyme Inactivation Studies

Inactivation studies were done by preincubating sodium salicylate with the enzyme and the reaction mixture in the absence of fructose-6-PO_4 for a designated time. In these studies, glucose or fructose were also excluded from the extraction medium. Preincubation of the enzyme with salicylate in the absence of glutamine did not produce any greater loss of enzyme activity compared to the control.

The study involving the reversibility of inhibition of sodium salicylate was carried out by passing the preincubation mixture through a sephadex G-25 column (20 x 1.25 cm.) (39), which has been equilibrated with the extraction medium. All separations were carried out between 0 to 4 degrees Centigrade to reduce loss of enzyme activity. Two milliliters of the preincubation mixture was passed through the column. Three milliliters of the enzyme (protein) fraction was collected and one milliliter was incubated with two milliliters of the incubation medium for 30 minutes. Activity was compared to the control treated under identical conditions but not including sodium salicylate.

Structural activity relationships of the inactivation involves preincubating with compounds similar in structure to salicylate, passing through sephadex, and the activity

determined as described above.

c) Determination of Glucuronyl Transferase Activity in the Gastric Mucosa

The microsomal fraction containing the glucuronyl transferase activity was separated by the method described by Hanninen (57). Mucosal scraping was homogenized with three times the tissue weight to volume of 0.15 M KCl at 0 degrees Centigrade. This homogenate was then centrifuged at 2,000 x g for 10 minutes and 18,000 x g for 30 minutes. It was then resuspended in its original volume of KCl solution. Two mililiters of the enzyme extract was added to 2 ml. of phosphate buffer (pH 7.5) containing 10 mM of disodium EDTA, 2 mM UDP-glucuronic acid, and 1 mM of sodium salicylate and incubated for 2 hours at 37 degrees Centigrade in a Dubnoff shaker. The reaction was stopped by heating the mixture in boiling water for 2 minutes, then centrifuging at 30,000 x g for 10 minutes. The supernatant was removed and assayed for free salicylate and glucuronide.

The supernatant was divided into two groups. One was analyzed for sodium salicylate without further treatment. The other was treated with 1,000 units/ml. of beta glucuronidase in pH 4.7 acetate buffer overnight before extracting with ether for analysis of salicylate. The difference between the two groups is the amount of glucuronide formed.

3-3 *In Vivo* Studies

a) Gastric Lesions Induced by Sodium Salicylate

Sprague Dawley male rats (100-120 Gms.) were fasted for 24 hours before the test. Sodium salicylate was dissolved in 0.1 M phosphate buffer (pH 7.4) for both oral and parenteral use. Oral dosing was accomplished by extending a polyethylene tubing into the oral cavity to the stomach and a volume of one milliliter was delivered with a syringe. The same volume was used for intraperitoneal dosing. Gastric lesions were examined immediately after dosing: 1 hour, 3 hours, and 6 hours later. The rats were killed by placing them in an ether saturated chamber, and the stomachs were removed. The stomachs were opened along the greater curvature and examined for the presence of hemorrhagic spots, petichiae, edema, and erosions. Any hemorrhagic area 2 mm. or greater in its largest dimension was considered as positive. Severity of ulceration was evaluated by the method of Lin (68). An ulcer score was used which included the per cent of the population in which ulcers were found. The size and number of lesions and general severity of the ulcers were considered. The ulcer index is made up of a total of three individual parameters.

$$\text{ulcer index} = UI = \frac{\text{ulcer rats}}{\text{total rats}} \times 10 + \frac{\text{total ulcer score}}{\text{total \# of rats}} + \frac{\text{total general severity}}{\text{total \# of rats}}$$

The ulcer score is graded according to size

mm.	3	2	1	.1
points	3	2	1	.2

General severity is based on observation of severity

hemorrhagic spots	0.5
petichiae	0.2
edema	1.0
erosion	0.5 - 1.1

A maximum ulcer index of 24 is possible.

Preliminary studies showed that a dose of 600 mg./Kg. of sodium salicylate induces gastric lesions both by oral and the intraperitoneal route while causing a minimal or other side effects.

b) Analysis of Sodium Salicylate in the Plasma and Gastric Mucosa

Sodium salicylate was administered either orally or intraperitoneally at a dose of 600 mg./Kg. in pH 7.5 phosphate buffer. Sprague Dawley male rats were fasted 24 hours before administration of the drug. Two rats were used for each experimental time point from zero to six hours per experiment. The later time points in the oral dosing experiment represent the pooling of three rats. Blood was obtained from each animal after ether anesthesia by cardiac puncture using a heparinized syringe. The plasma fraction was collected after centrifuging for 15 minutes at 30,000 x g in a refrigerated Spinco centrifuge.

Mucosal scrapings from two animals were homogenized with four milliliters of normal saline and centrifuged at 2,000 x g for 5 minutes. The supernatant was obtained by centrifugation at 35,000 x g for 30 minutes in a Spinco refrigerated ultra-centrifuge.

Analysis of sodium salicylate was carried out by the procedure described by Riegleman and Harris (69) with some modifications. The plasma samples were diluted by a factor of 1 to 100 with normal saline. The mucosal supernatant from oral and IP administration were diluted 1 to 10 and 1 to 3 respectively. To one milliliter of the plasma and the mucosal supernatant was added one milliliter of normal saline and 5 ml. of ether (containing 8 mg./ml. of oxalic acid). This was shaken in a 15 ml. centrifuge tube with a ground glass stopper on a mechanical shaker. Three milliliters was then transferred to another 15 ml. centrifuge tube containing 3 ml. of phosphate buffer (pH 7.5) and shaken again. The ether layer was aspirated and the concentrations of salicylate were determined flurometrically at an uncorrected wavelength of activation of 315 nm and fluorescence at 420 nm. Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer. Quenching correction was applied by the method described by Peng (70). Results were compared to a standard curve of sodium salicylate. Recoveries of the salicylate molecule from extraction was essentially complete in the concentration range under study.

Mucosal concentrations were expressed in mcg./mg. of the mucosal supernatant protein. Protein concentrations were

determined by the Biuret method (71) using bovine serum albumin as the standard.

c) Determination of Glucuronide Content in the Mucosa

Rats were given 600 mg./kg. of sodium salicylate orally and four rats were examined at one and three hours after drug administration. The supernatant of the gastric mucosal scraping was removed and assayed for glucuronide as described in Section 3-2c on the determination of glucuronyl transferase activity in the gastric mucosa.

d) Determination of L-Glutamine:D-Fructose-6-Phosphate Aminotransferase Activity After Drug Administration

Sprague Dawley male rats were fasted for 24 hours and given sodium salicylate at a dose of 600 mg./kg. both orally and intraperitoneally. Cycloheximide was administered intraperitoneally at a dose of 2.5 mg./kg. Cycloheximide was dissolved in normal saline and 1 ml. of the drug was administered. At designated times, rats were sacrificed and mucosal scrapings removed for assay of L-glutamine:D-fructose-6-PO_4 aminotransferase activity. Protein concentration was determined by the Biuret reaction (71).

e) Determination of Hexosamine Content After Drug Administration

Hexosamine content was determined from treated and untreated rats by analyzing the mucosal homogenate supernatant (35,000 x g) and the pellet formed after centrifugation. One milliliter of the supernatant was placed in a culture tube with a screw top

and 1 ml. of 6 N HCl was added and heated at 100 degrees Centigrade in an oil bath for 6 hours. The hydrolysate was neutralized with NaOH and the tube rinsed with water to make a final volume of 10 ml. Aliquots were removed for hexosamine analysis. The pellet was heated to dryness and constant weight in an oven at 60 degrees Centigrade. Aliquots of 50 mg. were hydrolyzed with 2 ml. of 3 N HCl at 100 degrees Centigrade for 12 hours in culture tubes with screw tops. The hydrolysate was then analyzed in the same manner as the supernatant fraction. Preliminary studies indicated that hydrolysis time of 10 to 14 hours yielded maximum hexosamine content for the pellet and 5 to 7 hours for the supernatant. The analysis of hexosamine was carried out by the method of Cessi (72).

IV. EXPERIMENTAL RESULTS

4-1 Inhibition of L-Glutamine:D-Fructose-6-PO_4 Aminotransferase By Sodium Salicylate In Vitro

a) Inhibition of the Enzyme Catalyzed Reaction

The inhibition of sodium salicylate on glucosamine synthetase activity was studied by using the enzymes prepared from both the rat liver and gastric mucosa. Figures 4-1 and 4-2 show the rate of the enzyme catalyzed reaction as a function of glucose-6-PO_4. From the double reciprocal plot (Figure 4-1), sodium salicylate acted as a competitive inhibitor with respect to glucose-6-PO_4. This means that the maximum rate was not affected but the K_m for glucose-6-PO_4 was increased. The model of this type of inhibition is depicted in Figure 2-1.

In the case of the liver enzyme, it required a higher concentration of salicylate to achieve inhibition. The effect of salicylate on the enzyme activity as a function of the concentration of glucose-6-PO_4 is shown in Figure 4-2. At a lower substrate concentration (3 mM of glucose-6-PO_4) the data deviated from the straight line which represents competitive inhibition. The data was replotted using the Dixon method (48) and eliminating the lowest concentration glucose-6-PO_4. The resultant plot (Figure 4-3) depicts competitive inhibition similar to that described in Figure 2-5.

The plots of the rate of the enzymatic reaction as a function of the glutamine concentration (Figures 4-4 and 4-5) showed that salicylate acted as a noncompetitive inhibitor with

Figure 4-1. Effect of salicylate on L-glutamine:D-fructose-6-PO_4 aminotransferase activity in the supernatant preparation of rat gastric mucosa tissue as a function of the concentration of glucose-6-PO_4. V is expressed as micromoles of glucosamine formed per minute. Reaction mixture contained in 4 ml.: 85 mM phosphate buffer, pH 7.4, 1 mM EDTA, 0.1% mercaptoethanol, 20 mM glutamine, 0.45 mg./ml. of supernatant protein, glucose-6-PO_4 as shown, and sodium salicylate: ● , none; ◆, 1 x 10^{-2}M; ▲, 1.5 x 10^{-2}M.

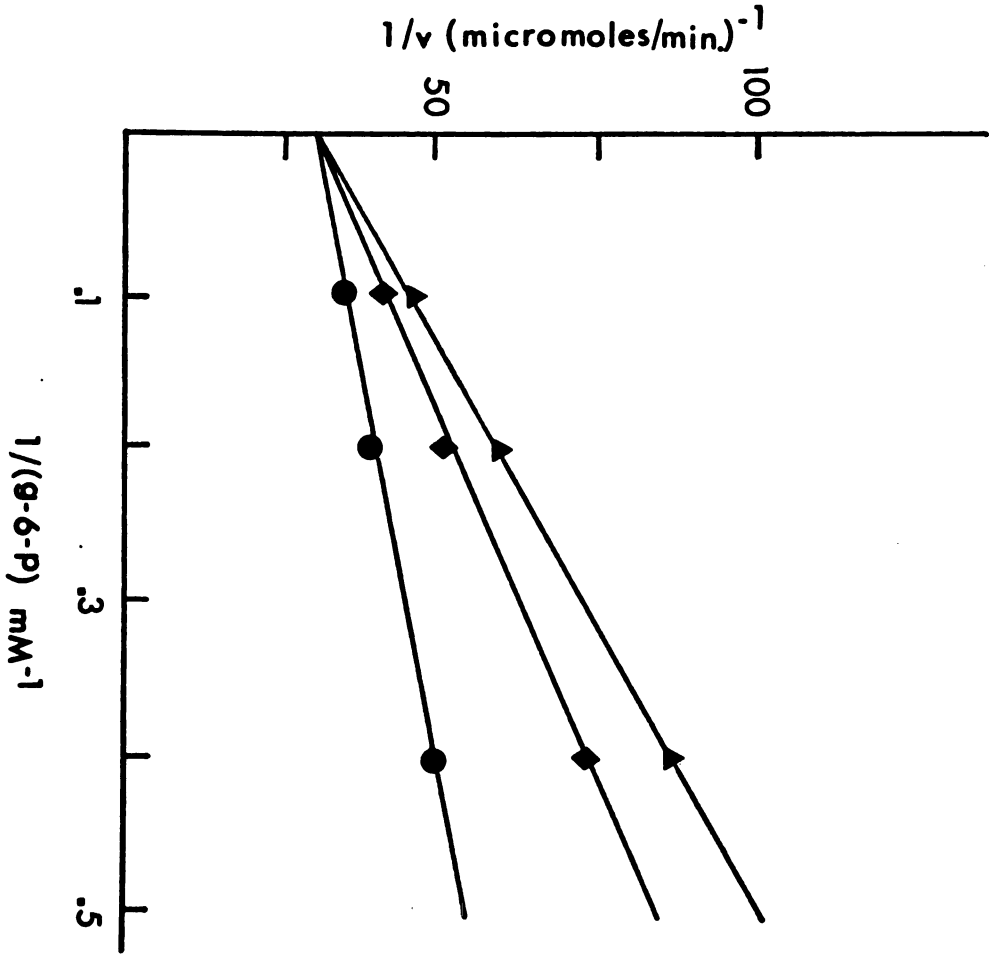

1/v (micromoles/min)$^{-1}$
50
100
.1
.3
.5
1/(S-S-P) mM^{-1}

Figure 4-2. Effect of salicylate on L-glutamine:D-fructose-6-PO_4 aminotransferase activity in the supernatant preparation of rat liver tissue as a function of the concentration of glucose-6-PO_4. V is expressed as micromoles of glucosamine formed per minute. The reaction mixture was as detailed for Figure 4-1 except that the protein concentration was 1.6 mg./ml. ● , control; ■ , 3 x 10^{-2} M sodium salicylate; ◆ , 4 x 10^{-2} M sodium salicylate.

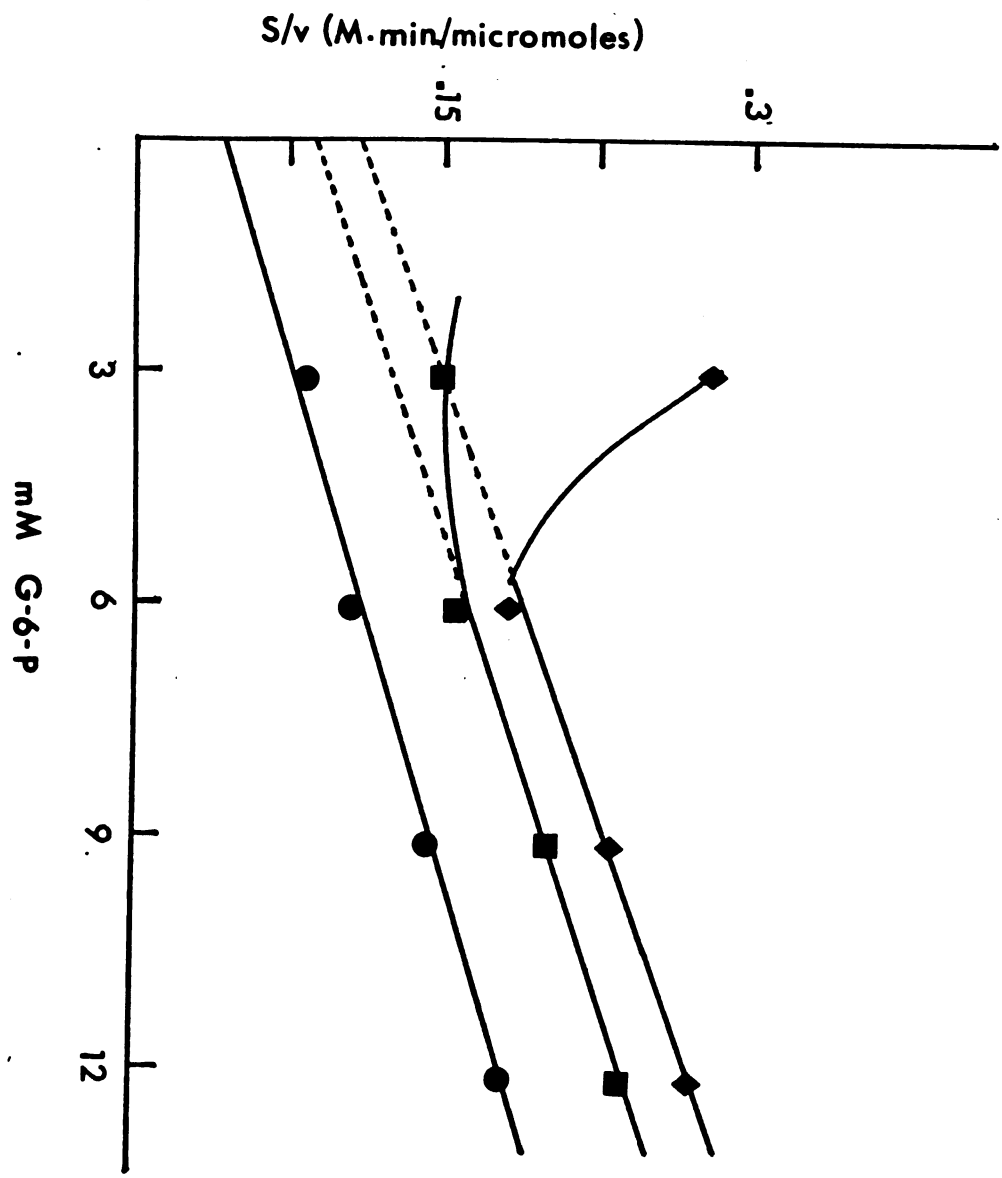

S/v (M·min/micromoles)
·15
·3
3
6
9
12
mM G-6-P

Figure 4-3. Dixon plot showing the effect of salicylate on aminotransferase activity in the supernatant preparation of rat liver tissue as a function of the concentration of glucose-6-PO_4. Reaction mixture contained in 4 ml.: 85 mM phosphate buffer, pH 7.4, 1 mM EDTA, 0.1% mercaptoethanol, 20 mM glutamine, 1.6 mg./ml. of supernatant protein, sodium salicylate as shown, and glucose-6-PO_4: ● , 12 mM; ▲ , 9 mM; ◆ , 6 mM.

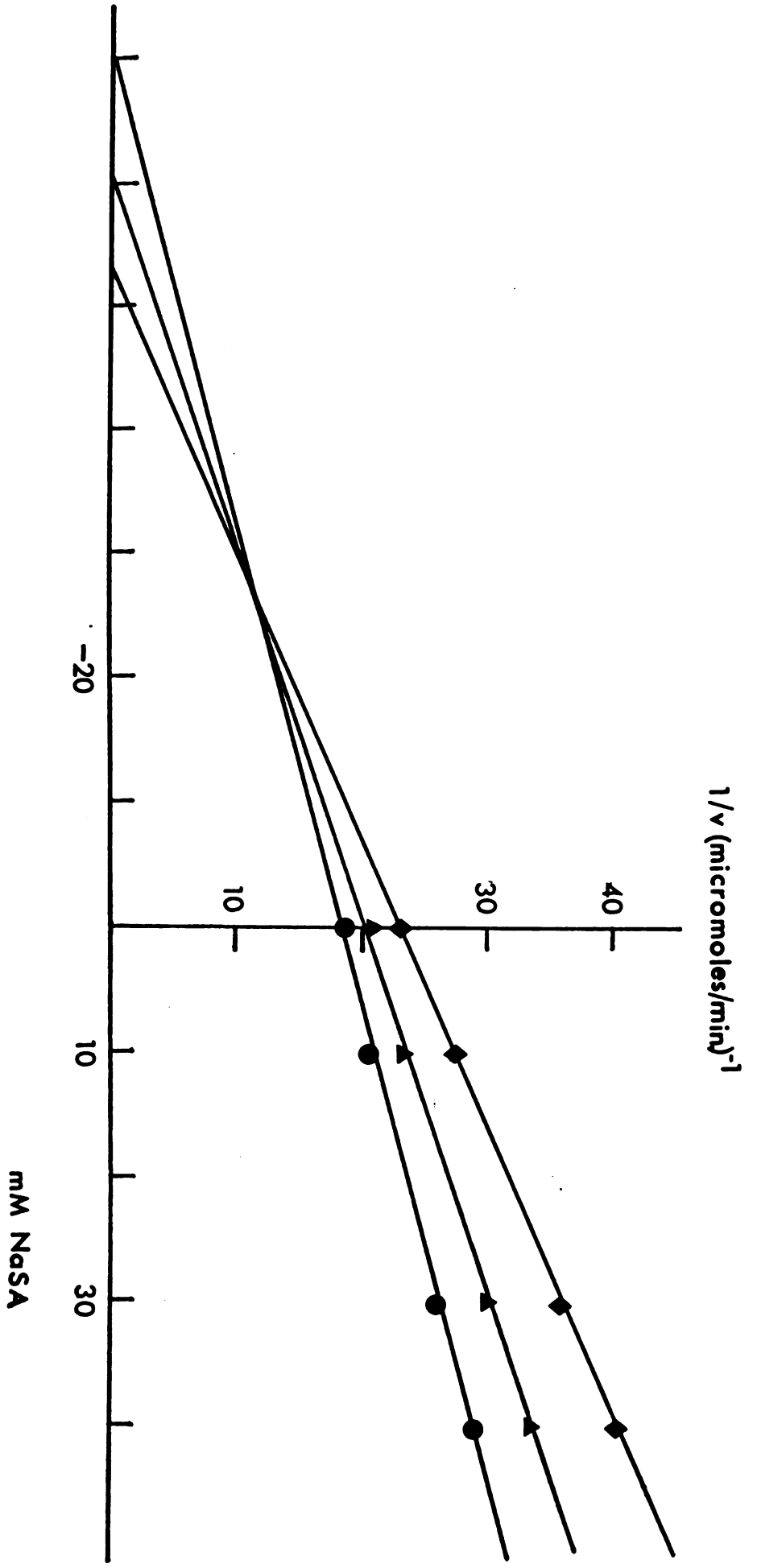

1/v (micromoles/min)$^{-1}$
10
30
40
−20
10
30
mM NaSA

Figure 4-4. Effect of salicylate on aminotransferase activity in the supernatant preparation of rat gastric mucosa tissue as a function of the concentration of glutamine. V is expressed as micromoles of glucosamine formed per minute. Reaction mixture was a detailed in Figure 4-1 except that glucose-6-PO_4 was held constant at 10 mM, glutamine as shown, and sodium salicylate: ●, none; ▲, 1 x 10^{-2} M; ◆, 1.5 x 10^{-2} M.

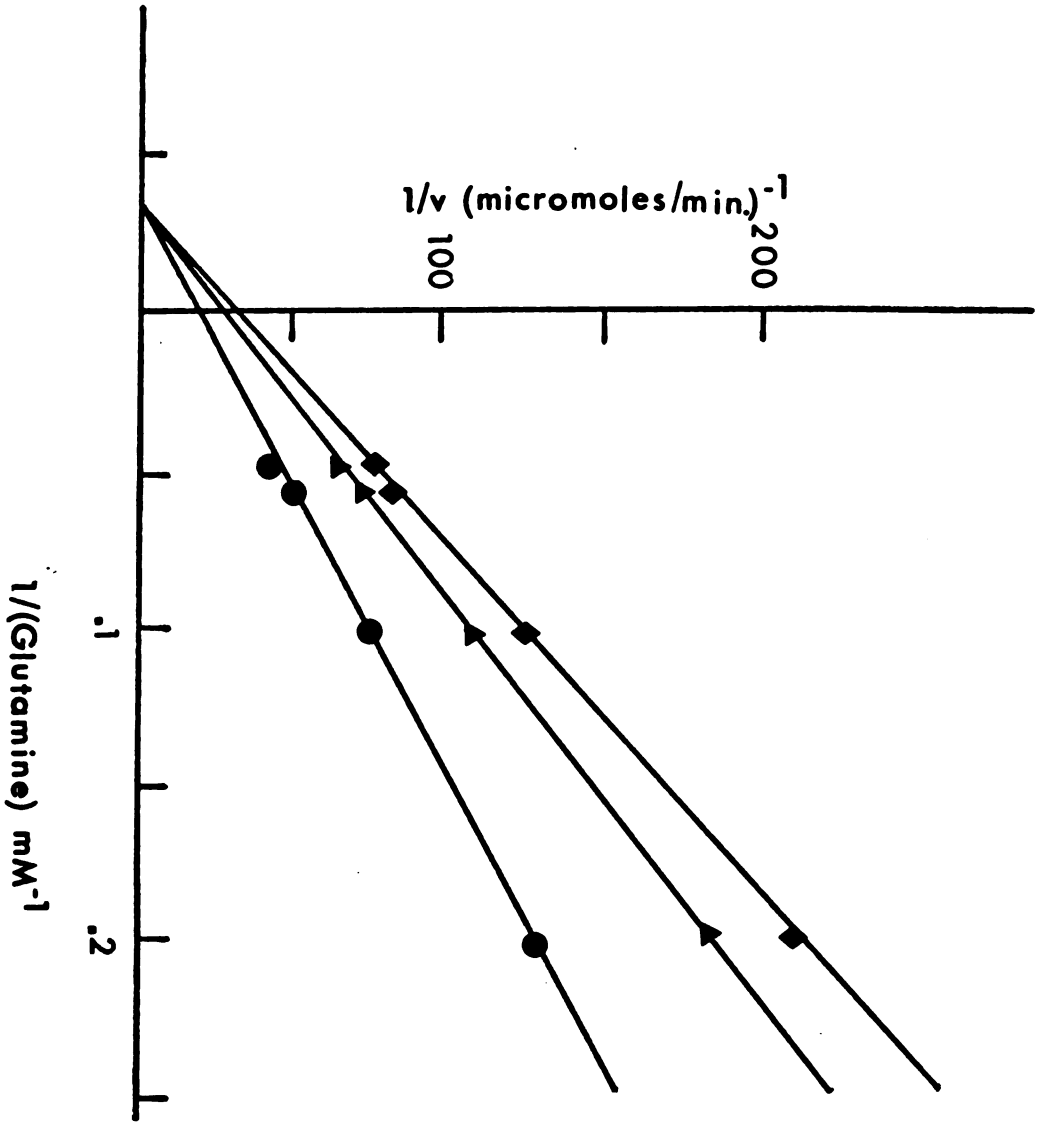

1/v (micromoles/min.)$^{-1}$
100
200
1/(Glutamine) mM^{-1}
.1
.2

Figure 4-5. Effect of salicylate on aminotransferase activity in the supernatant preparation of rat liver tissue as a function of the concentration of glutamine. Reaction mixture was a detailed in Figure 4-1 except that the protein concentration was 1.4 mg./ml. and the glucose-6-PO_4 concentration was held constant at 10 mM, glutamine as shown, and sodium salicylate: ● , none; ◆ , 3×10^{-2}M; ○ , 4×10^{-2}M.

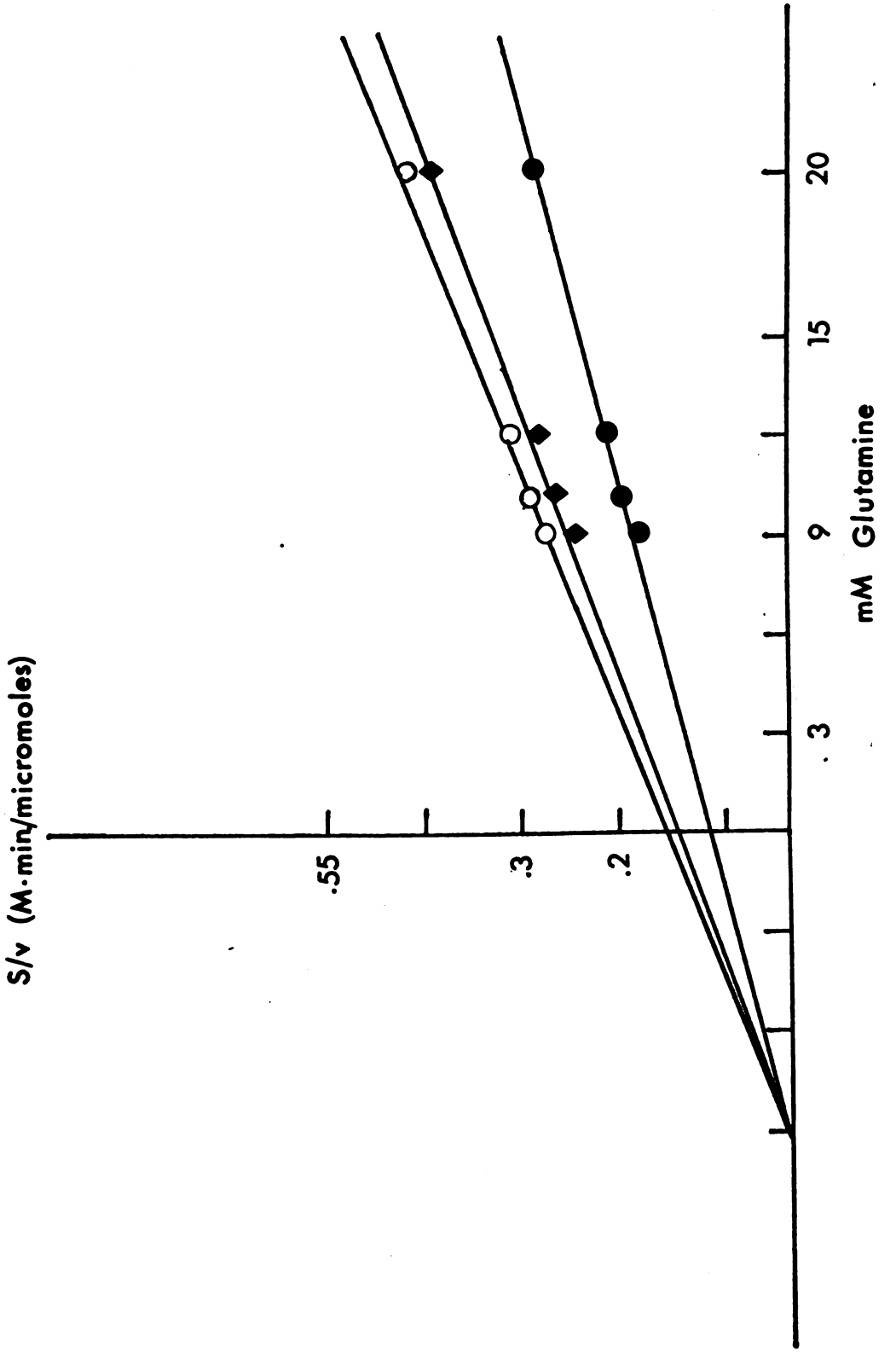

S/v (M·min/micromoles)
.55
.3
.2
.3
9
15
20
mM Glutamine

Figure 4-6. Calibration curve for glucosamine HCl.

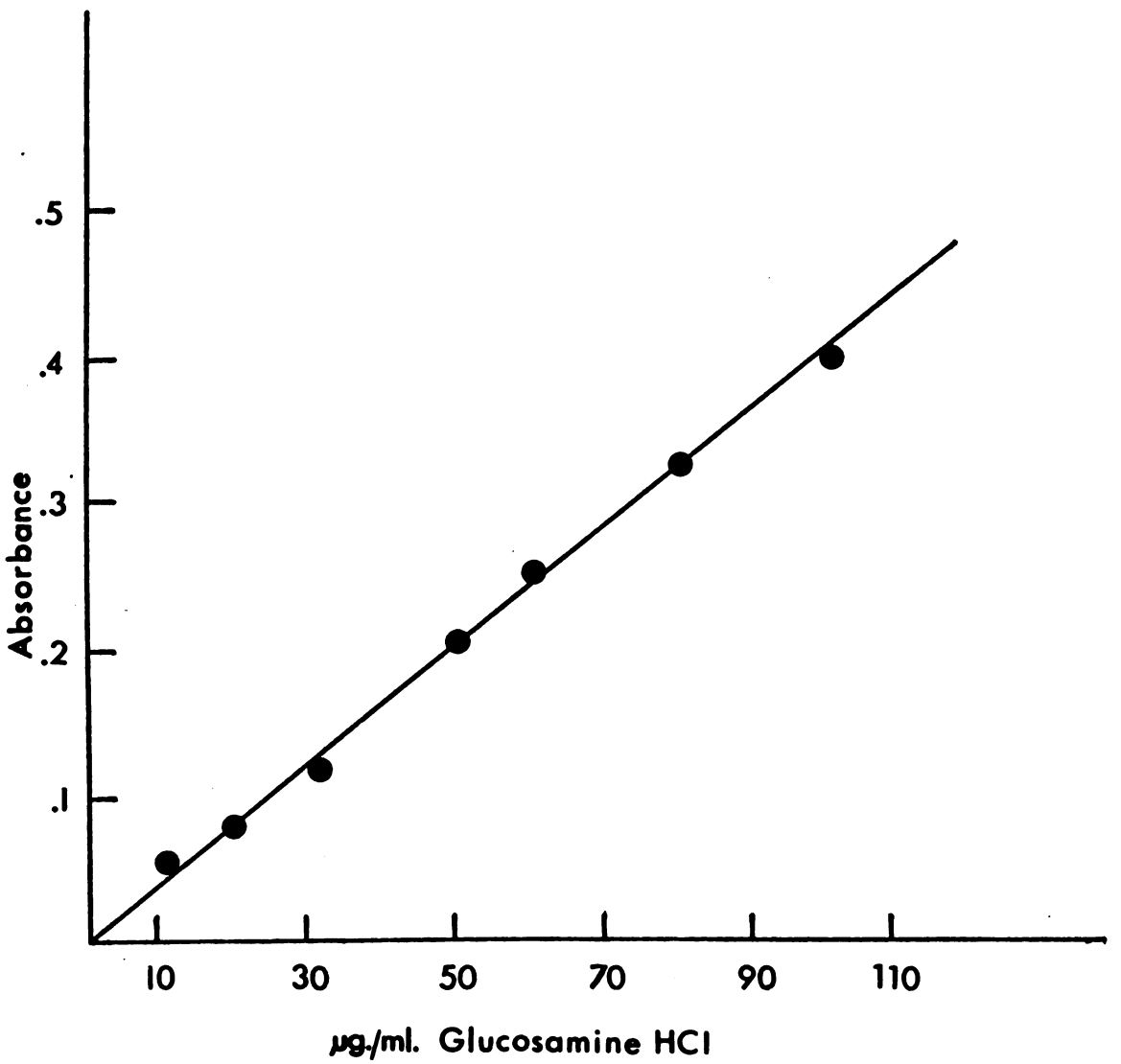

.5
.4
.3
.2
.1
Absorbance
10
30
50
70
90
110
µg./ml. Glucosamine HCl

Table 4-1. Effects of the Concentrations of Glucose-6-PO_4 and Glutamine on the Inhibition of Aminotransferase Activity by Sodium Salicylate.

	12 mM G-6-P		20 mM G-6-P	
Glutamine	0 mM SA	30 mM SA	0 mM SA	30 mM SA
10 mM	1.63	1.22	1.90	1.83
12 mM	1.78	1.32	2.03	2.08
20 mM	2.19	1.56	2.41	2.34

Experimental conditions were as those described in Figure 4-5 with the concentrations of glucose-6-PO_4, glutamine, and salicylate as listed. Enzyme activity given as micromoles of glucosamine formed per 30 minutes of incubation.

Table 4-2. Comparative K_m and K_i Values From the Rat Liver and Gastric Mucosa Enzyme Preparations.

Parameters	Liver	Mucosa
K_m		
G-6-P	5.2×10^{-3}M	1.7×10^{-3}M
Glutamine	8.0×10^{-3}M	3.0×10^{-2}M
K_i (competitive)	2.6×10^{-2}M	7.5×10^{-2}M
K_i (calculated)	2.7×10^{-2}M	6.8×10^{-2}M
K_i/K_m	5.2*	4.4*

*Variation in enzyme assay 8 to 12%.

Data determined from Figures 4-1, 4-2, 4-3, 4-4, and 4-5.

respect to glutamine. From the discussion in Section 2-1, equations 2-10 and 2-11 may be used to express this type of inhibition. The first case represented noncompetitive inhibition according to the true sense of the definition while in the second case, the apparent noncompetitive inhibition was caused by competition with the second substrate which was maintained at a constant concentration.

In the latter case we should be able to overcome the inhibition by increasing the concentration of the constant substrate. As shown in Table 4-1, at a concentration of 20 mM of glucose-6-PO_4, the inhibition became negligible. The K_i calculated from equation 2-11 also agreed with that determined from Figures 4-1 and 4-2. The results indicate that the observed noncompetitive inhibition is a result of competitive inhibition of the enzyme with respect to glucose-6-PO_4. The close agreement of the ratios of K_i/K_m for the liver and mucosal enzyme extracts suggests that sodium salicylate inhibits both enzymes in an identical manner.

b) Enzyme Inactivation Studies

Studies were carried out to determine if sodium salicylate could inactivate glucosamine synthetase. Liver enzyme extracts were used in this study. Figure 4-7 showed the activity of the enzyme incubated in the absence of fructose-6-PO_4. To determine the reversibility of the inactivation, samples were passed through sephadex G-25, after a designated preincubation period, in order to remove sodium salicylate. Table 4-3 showed that

the inactivation of L-glutamine:D-fructose-6-PO_4 aminotransferase by sodium salicylate was irreversible.

The inactivation kinetics were also studied and the results are shown in Figure 4-9. In the range of 15 mM to 35 mM of sodium salicylate, the inactivation followed first order kinetics. Since the inhibitor was present in excess, a pseudofirst order kinetics would probably be more correct. The data seems to fit a single exponential function, $i = 1 - e^{-kt}$, where i is the fraction inactivated, t is the time of preincubation, and k the inactivation rate constant which is a function of the inhibitor concentration and other factors.

Various analogs of salicylate were tested to study the structure-activity relationship. The results of the study are given in Table 4-4. The results indicate that the inactivation is specific to the salicylate molecule. Slight activity shown by 2-amino benzoic acid seems to indicate that the inactivation may involve metal chelation.

The two substrates, fructose-6-PO_4 and glutamine, as well as the endogenous negative feedback inhibitor, UDP-N-acetylglucosamine, were studied as compounds which may possibly afford protection to the enzyme from inactivation by sodium salicylate. Results shown in Figure 4-10 indicate that fructose-6-PO_4 at 10 mM and UDP-N-acetylglucosamine at 3.5×10^{-6} M can protect the enzyme against inactivation by 40 mM of sodium salicylate. Glutamine, up to 20 mM, showed no protection for the enzyme.

Figure 4-7. Effect of salicylate on aminotransferase activity in the rat liver tissue supernatant preparation as a function of the preincubation time. Preincubation mixture contained in 3 ml.: 85 mM phosphate buffer, pH 7.4, 1 mM EDTA, 0.1% mercaptoethanol, 20 mM glutamine, 2.5 mg./ml. of supernatant protein, and sodium salicylate: ■ , none; ● , 4×10^{-2} M. After the preincubation period, fructose-6-PO_4 was added to give a final concentration of 10 mM and a final volume of 4 ml. The resultant reaction mixture was incubated for 30 minutes to determine the enzyme activity.

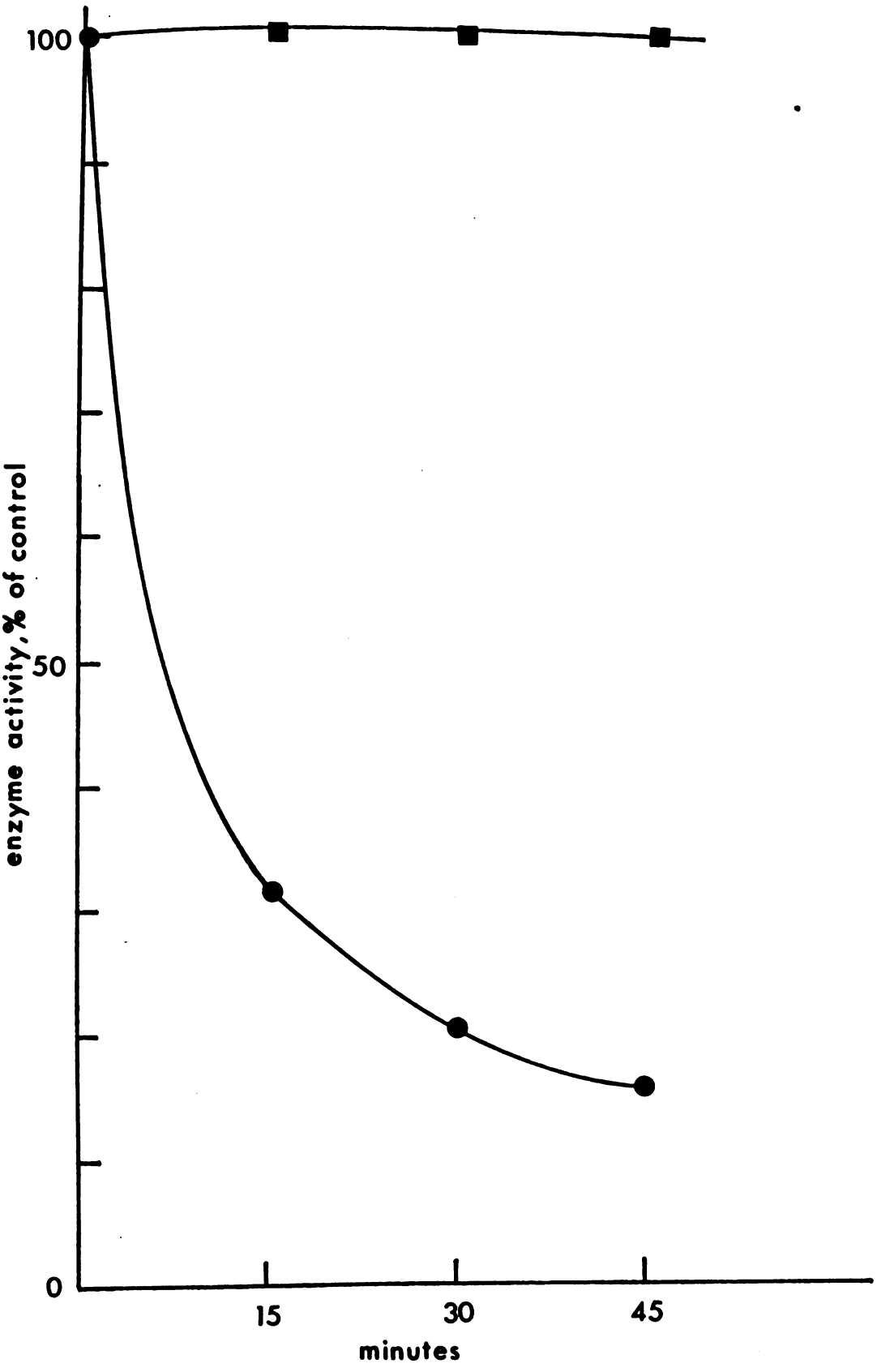

100
50
0
enzyme activity, % of control
15
30
45
minutes

Figure 4-8. Separation of sodium salicylate and the protein fraction of the rat liver supernatant preparation by sephadex G-25.

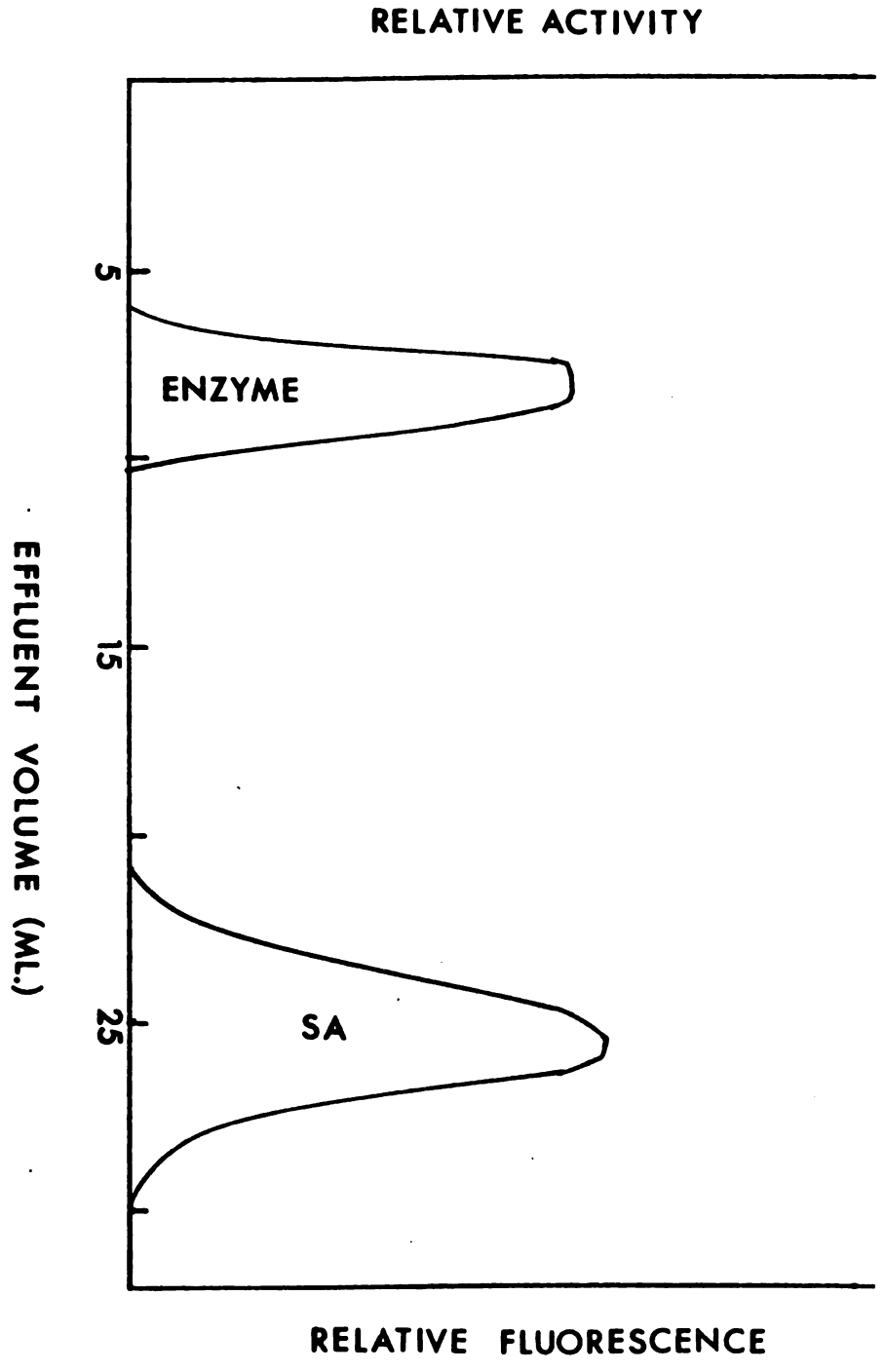

RELATIVE ACTIVITY
5
ENZYME
15
25
SA
EFFLUENT VOLUME (ML.)
RELATIVE FLUORESCENCE

Table 4-3. Irreversible Inactivation of Glucosamine Synthetase By Sodium Salicylate.

Salicylate (mM)	Per Cent Activity Remaining
0	100
15	96
30	70
40	41

Experimental conditions were as detailed in Figure 4-9 with a preincubation period of 30 minutes.

Table 4-4. Structural Activity Relationship of Enzyme Inactivation.

Compounds	Per Cent Activity Remaining
Control	100.0
Benzoic Acid	99.0
p-OH Benzoic Acid	98.7
o-Methoxy Benzoic Acid	96.7
Nicotinic Acid	92.6
m-OH Benzoic Acid	90.6
2-Amino Benzoic Acid	86.3
Sodium Salicylate	55.3
Salicylate Acid	56.4

All compounds were tested for their ability to inactivate the enzyme at a concentration of 30 mM. Experimental conditions were as described in Figure 4-9 except a protein concentration of 2.5 mg./ml. was used.

Figure 4-9. Effect of varying concentrations of salicylate on the aminotransferase activity in the rat liver tissue supernatant preparation as a function of preincubation time. Preincubation mixture is as detailed in Figure 4-7 except that the 2.1 mg./ml. of supernatant protein was used. After the preincubation period, salicylate was removed by passage through sephadex G-25. Fructose-6-PO_4 was added to determine the enzyme activity as described on Page 53.

Curve 1, 15 mM; curve 2, 25 mM; curve 3, 30 mM; curve 4, 35 mM.

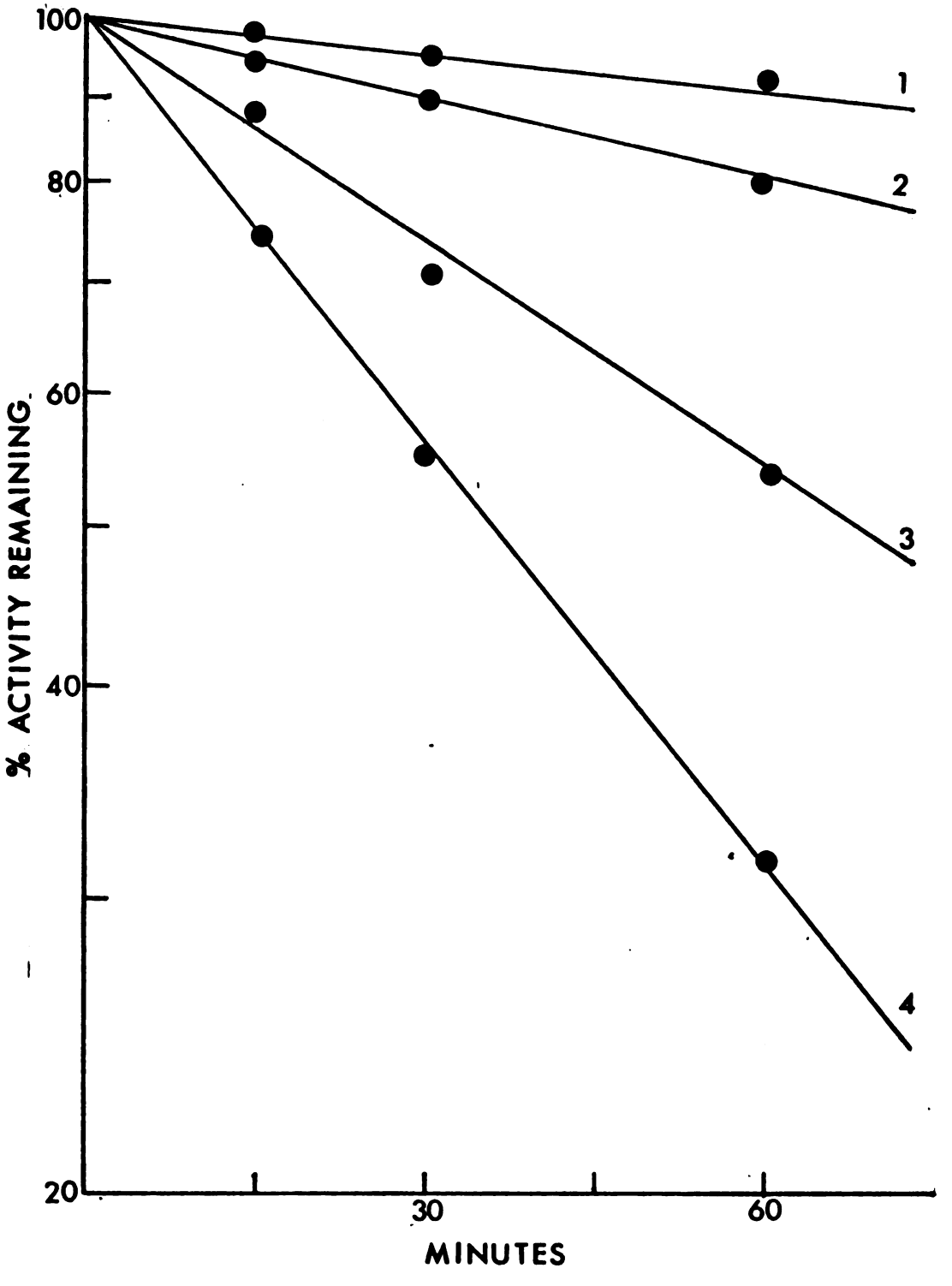
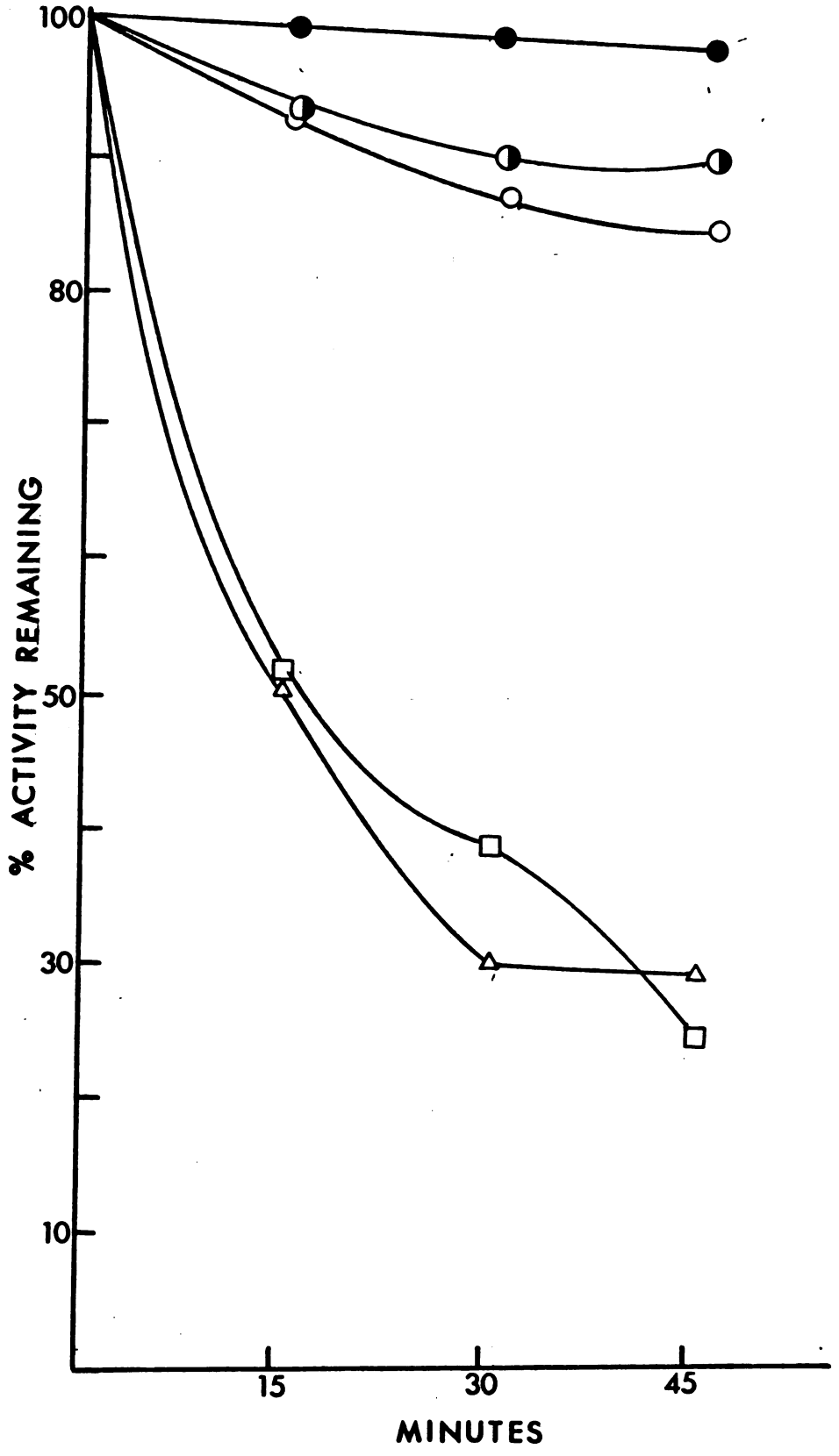

100
80
60
40
20
% ACTIVITY REMAINING
1
2
3
4
30
60
MINUTES

Figure 4-10. Protective effect of D-fructose-6-PO_4, L-glutamine, and UDP-N-acetylglucosamine on salicylate induced enzyme inactivation. Enzyme activity is expressed as per cent of the contro and the time of preincubation given in minutes. Preincubation reaction mixture contained in 3 m 1 ml. of liver supernatant fraction, 85 mM phos buffer (pH 7.4), 1 mM EDTA, and 0.1% mercaptoet and the following additions: ● , none; ◑ , 40 sodium salicylate and 3.5×10^{-6} M UDP-N-acetyl-glucosamine; ○ , 40 mM sodium salicylate and 1 fructose-6-PO_4; ◘ , 40 mM of sodium salicylate and 20 mM of glutamine; ▲ , 40 mM of sodium salicylate. After preincubation, the samples were passed through sephadex G-25 and the enzym activity determined as described in Section 3-2


100
80
50
30
10
% ACTIVITY REMAINING
15
30
45
MINUTES

c) Effect of UDP-N-acetylglucosamine on the Inhibition by Sodium Salicylate on L-Glutamine:D-Fructose-6-PO_4 Aminotransferase

Kornfeld (38) reported that UDP-N-acetylglucosamine was a competitive inhibitor of the enzyme by increasing the K_m for fructose-6-PO_4 and not affecting V_{max}. Her studies were carried out by using partially purified preparation from the rat liver. We repeated the study by using an enzyme extract from the rat gastric mucosa. Results shown in Figures 4-11 and 4-12 indicate that UDP-N-acetylglucosamine inhibited the mucosal enzyme in the identical manner as sodium salicylate, competitively for fructose-6-PO_4 and noncompetitively for glutamine. From the competitive plot, a value of 1×10^{-6} M was obtained for K_i as compared to that of 5×10^{-6} M obtained by Kornfeld (38).

Examining the inhibition of the enzyme over a concentration range of UDP-N-acetylglucosamine from 0 to 28×10^{-6} M, the resultant curve (Figure 4-13) was hyperbolic in nature and the inhibition seemed to level off at 85 to 90%.

The addition of UDP-N-acetylglucosamine to a system inhibited by sodium salicylate modified the action of the salicylate inhibition. Figure 4-10 showed that UDP-N-acetylglucosamine at a concentration of 3.5×10^{-6} M can protect the enzyme against inactivation by 40 mM of sodium salicylate. A further study involving the per cent inhibition of the enzyme activity as a function of salicylate concentration at various concentrations of UDP-N-acetylglucosamine (Figure 4-14) showed

Figure 4-11. Inhibition of mucosa supernatant aminotransferase activity as a function of the concentration of UDP-N-acetylglucosamine. Reaction mixture contained in 4 ml.: 1 mg./ml. of mucosal supernatant protein, 85 mM of phosphate buffer (pH 7.4), 1 mM EDTA, 0.1% mercaptoethanol, 20 mM L-glutamine, 10 mM glucose-6-PO_4, and UDP-N-acetylglucosamine as given.

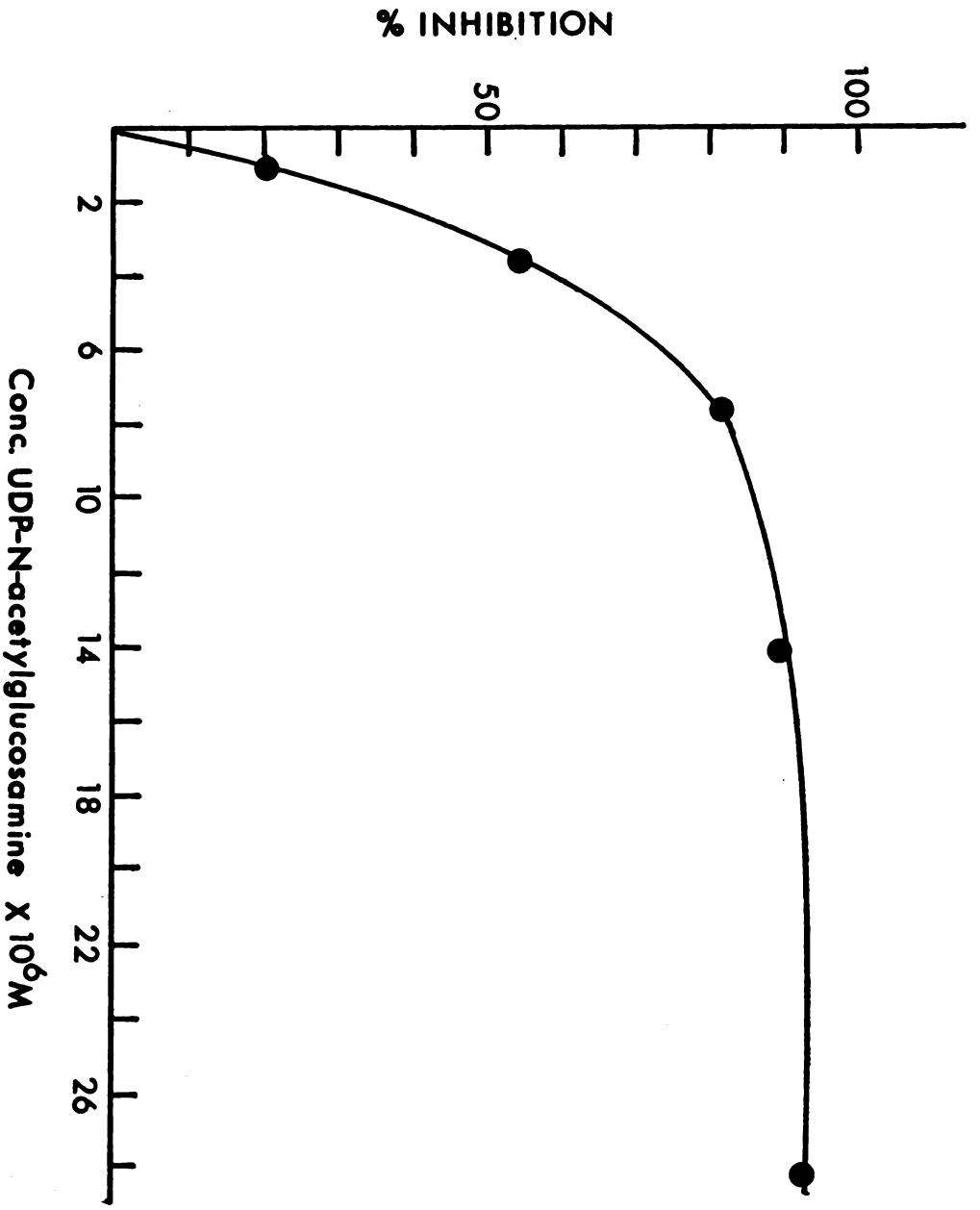

% INHIBITION
50
100
2
6
10
14
18
22
26
Conc. UDP-N-acetylglucosamine X 10⁶M

Figure 4-12. Effect of UDP-N-acetylglucosamine on aminotransferase activity in the supernatant preparation of rat gastric mucosa tissue as a function of the concentration of glutamine. V is expressed as micromoles of glucosamine formed per minute. Reaction mixture was as detailed in Figure 4-11 except the protein was 0.51 mg./ml., glutamine as shown, and UDP-N-acetylglucosamine: ● , none; ◆ , 4×10^{-6}M; ▲ , 1×10^{-5}M.

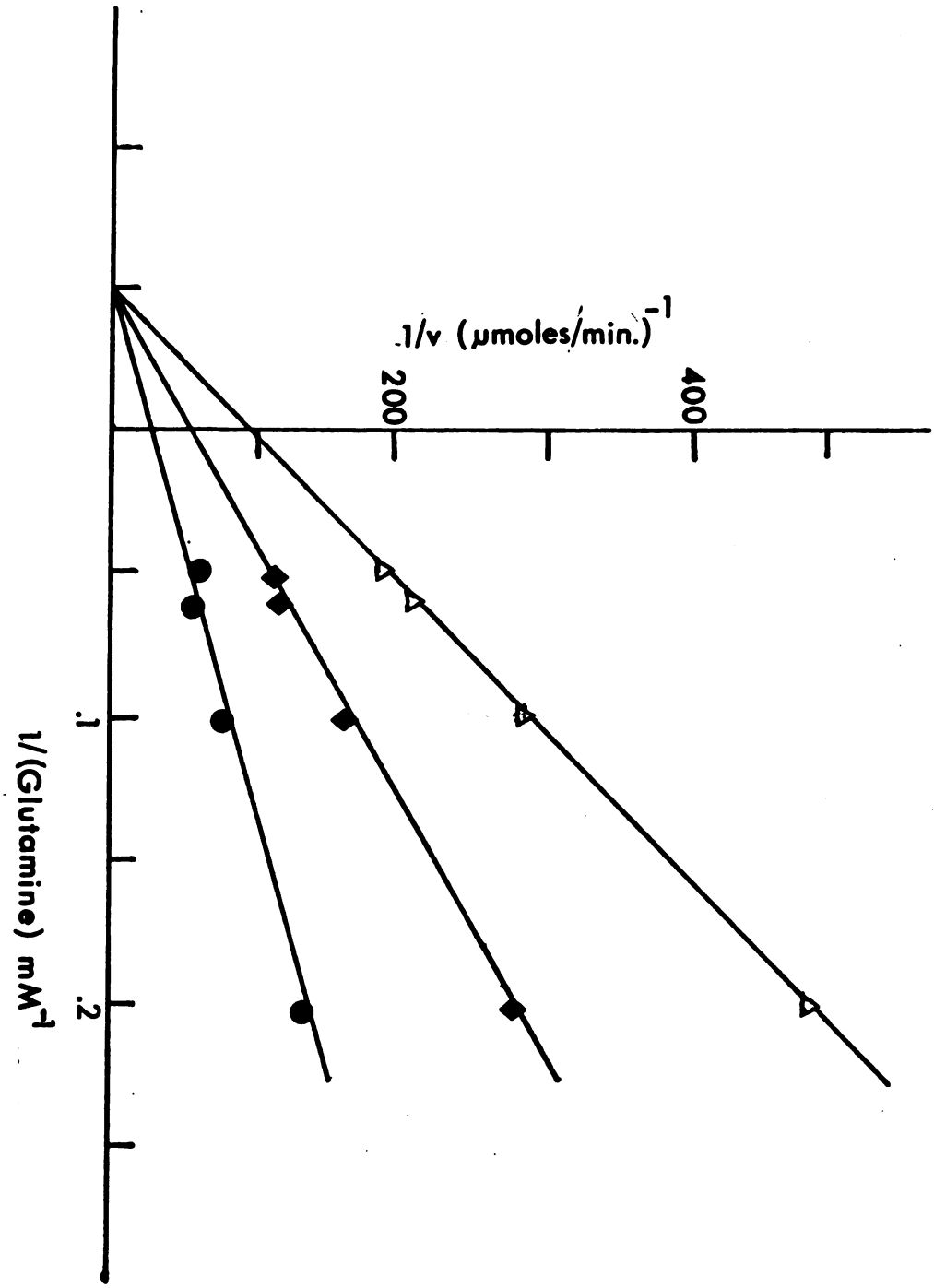

1/v (μmoles/min.)$^{-1}$
200
400
.1
.2
1/(Glutamine) mM^{-1}

Figure 4-13. Effect of UDP-N-acetylglucosamine on aminotransferase activity in the supernatant preparation of rat gastric mucosa tissue as a function of the concentration of glucose-6-PO_4. Reaction mixture was as detailed in Figure 4-11 except glucose-6-PO_4 is as shown and UDP-N-acetylglucosamine: ● , none; ◇ , 1×10^{-5}M; ○ , 5×10^{-5}M.

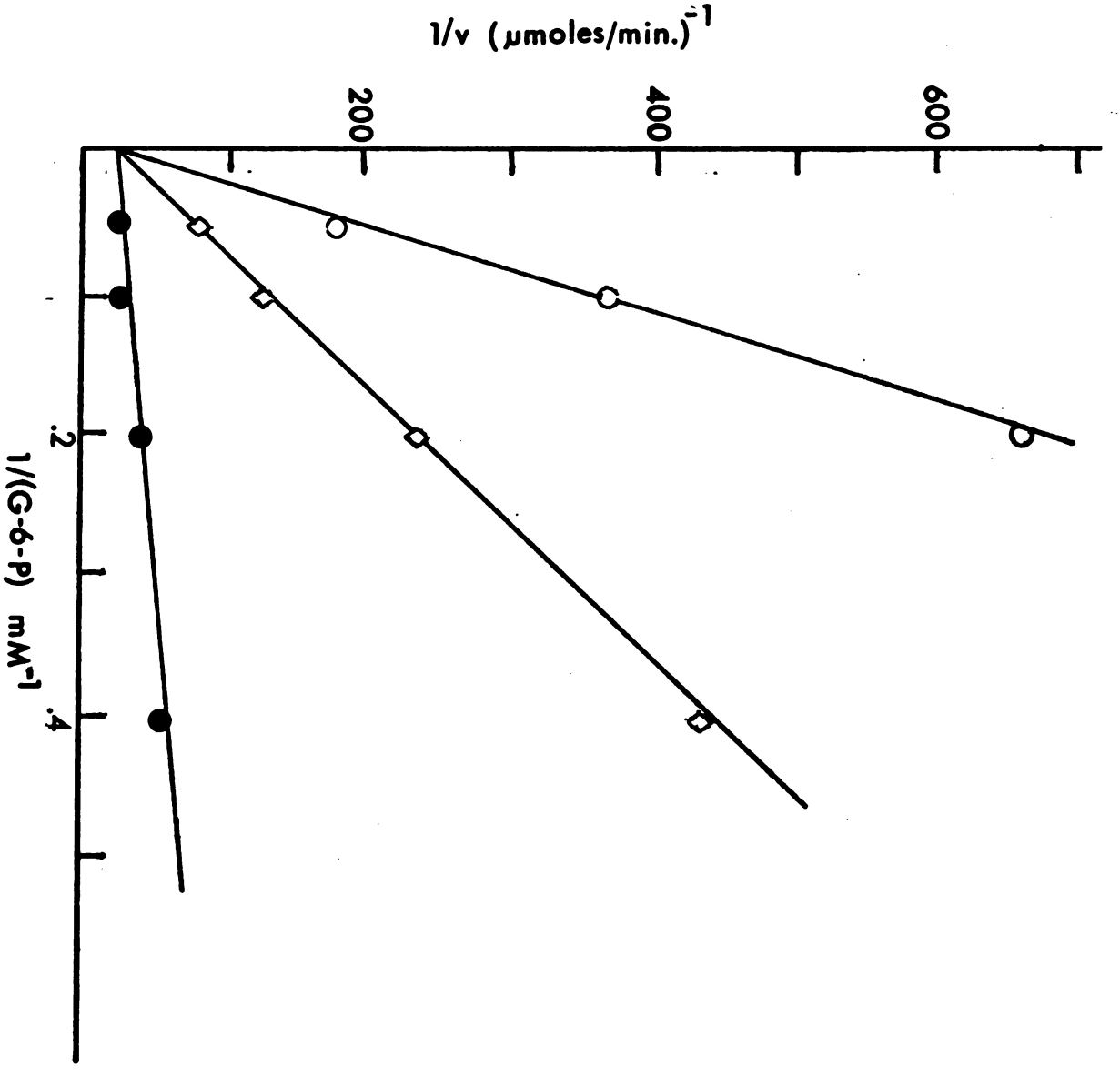
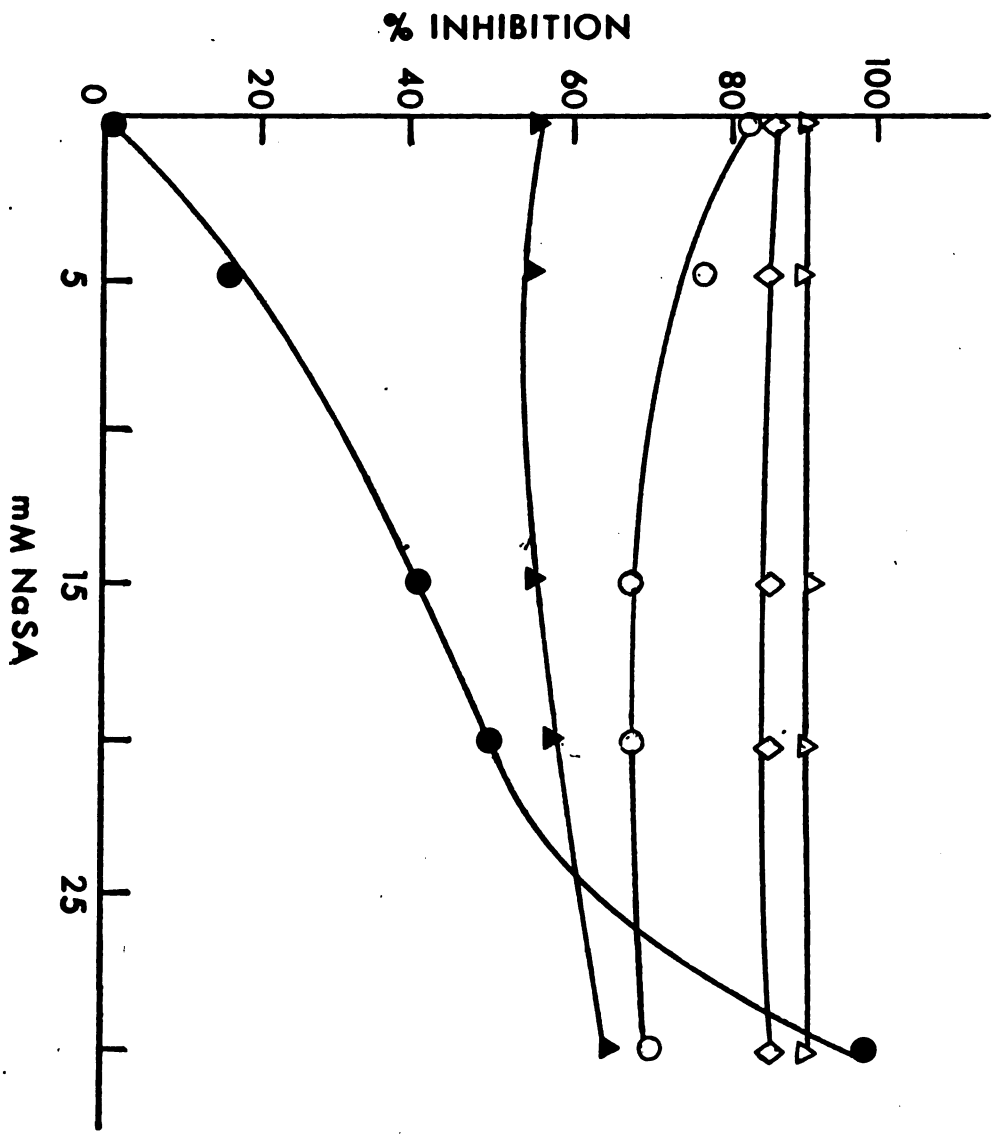

1/v (μmoles/min.)$^{-1}$
200
400
600
1/(G-6-P) mM^{-1}
.2
.4

Figure 4-14. Effect of sodium salicylate on the activity of glucosamine synthetase prepared from the rat gastric mucosa supernatant in the absence and presence of UDP-N-acetylglucosamine. Reaction mixture was as detailed in Figure 4-11 except the protein concentration was 0.76 mg./ml., salicylate concentrations are as shown, and UDP-N-acetyl-glucosamine concentrations were: ● , none; ▲ , 3.5×10^{-6}M; o , 7×10^{-6}M; ◇ , 1.4×10^{-5}M; △ , 2.8×10^{-5}M.


% INHIBITION
0
20
40
60
80
100
5
15
25
mM NaSA

this interaction. With no added UDP-N-acetylglucosamine, we observed that up to 20 mM of salicylate, inhibition of glucosamine formation appears to result from inhibition of the enzymatic reaction. At 30 mM of salicylate, the loss of activity can be explained by inactivation of the enzyme as well as inhibition of the reaction. At 3.5×10^{-6} M of UDP-N-acetylglucosamine, essentially no effect was observed until at 30 mM of salicylate where there was some loss of activity. At 7×10^{-6} M, there was about 80% inhibition of the enzyme. Sodium salicylate appears to be able to displace some of the inhibitor from the binding site on the enzyme leading to an increase in activity at 15 to 20 mM of salicylate. At 30 mM of salicylate, we again observe a decrease in activity apparently due to enzyme inactivation. At higher concentrations of UDP-N-acetylglucosamine, more than 80% of the enzymes were inhibited; sodium salicylate at concentrations up to 30 mM cannot further inhibit the enzyme nor displace the feedback inhibitor giving rise to increases in enzyme activity.

In constructing Lineweaver Burk plots at various concentrations of sodium salicylate, we observed an increase in activity of the enzyme extract by addition of 2 mM of salicylate (Figure 4-15). In light of the apparent competitive binding of salicylate and UDP-N-acetylglucosamine for the same site, we can, by adding a known concentration of UDP-N-acetylglucosamine determine graphically the slope from the double reciprocal plots and arrive at an approximate concentration of UDP-N-acetylglucosamine <u>in vivo</u>. A value of 10^{-5} M was calculated.

Figure 4-15. Estimation of the <u>in vivo</u> concentration of UDP-N-acetylglucosamine in the rat gastric mucosa supernatant. Reaction mixture was as detailed in Figure 4-11 except the protein concentration was 0.5 mg./ml., and glucose-6-PO_4 as shown, and ● , 2 mM sodium salicylate; ○ , 1 x 10^{-6}M UDP-N-acetylglucosamine; ◑ , no added salicylate nor UDP-N-acetylglucosamine.

Calculation of the <u>in vivo</u> concentration of UDP-N-acetyl-glucosamine:

Slopes from Figure 4-15

Sample + 2 mM SA = 70

Sample only = 110

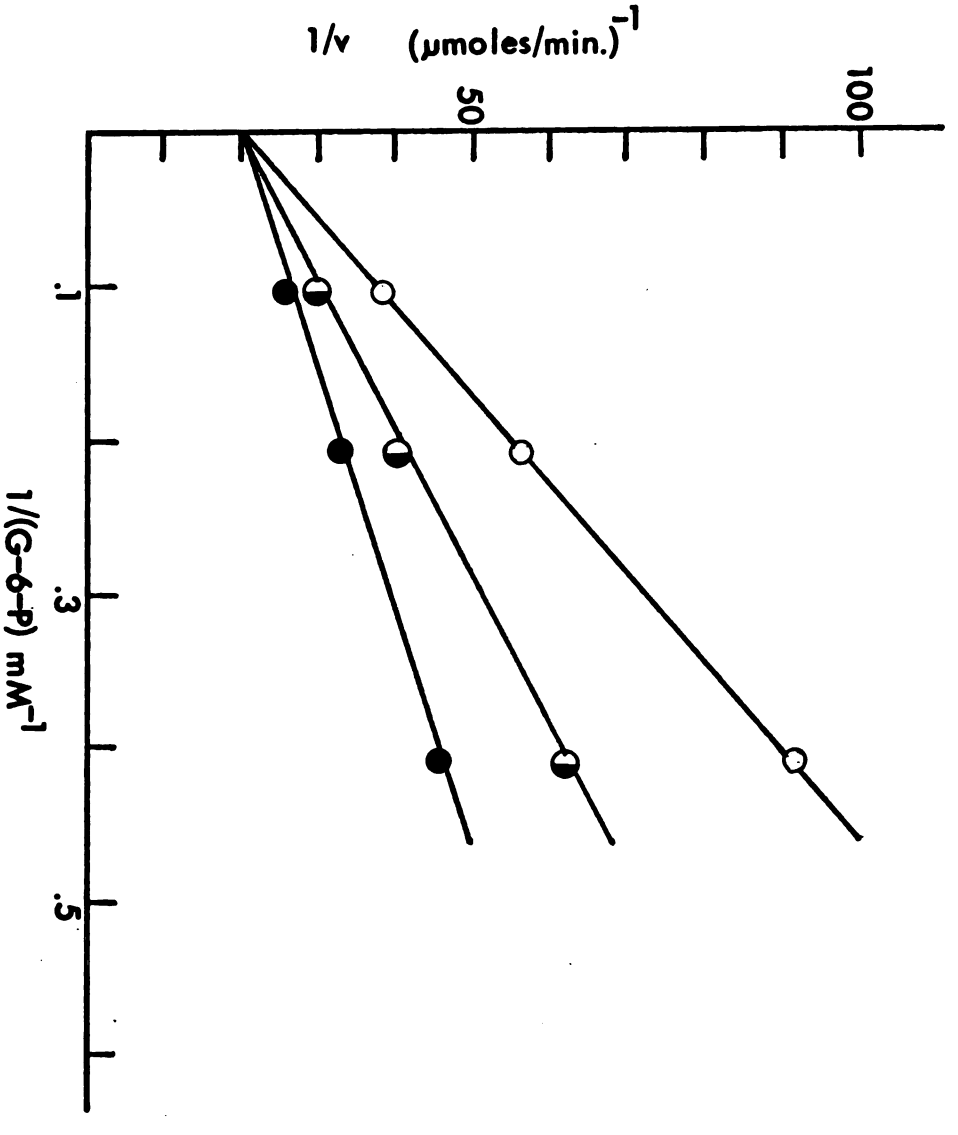
Sample + 1 x 10^{-6}M UDP-N-acetylglucosamine = 180

If we then let the endogenous UDP-N-acetylglucosamine be i the slopes of lines 2 and 3 would be:

$$70\ (1 + i\ /K_i) = 110$$

$$70\ (1 + (1 \times 10^{-6} + i\ )/K_i) = 180$$

By equating K_i's, we arrived at a value of 5.7 x 10^{-7}M for i . The average weight of a scraping from one rat stomach is about 250 mg. If we assume 60% water or approximately 0.15 ml., this is diluted by a factor of 40 in preparing the incubation mixture. The estimated <u>in vivo</u> concentration of UDP-N-acetylglucosamine in the gastric mucosa is about 10^{-5}M.


1/v (μmoles/min.)$^{-1}$
50
100
.1
.3
.5
1/(G-6-P) mM^{-1}

4-2 *In Vivo* Action of Sodium Salicylate

Since a dosage of 600 mg./kg. was capable of inducing gastric lesion both by oral and intraperitoneal administration with a minimum of other side effects, we, therefore, used this dose throughout the *in vivo* studies.

a) Gastric Lesions Induced by Sodium Salicylate

Table 4-5 shows the effect of the route of administration on the salicylate induced gastric lesions. Statistical analysis by the Fisher Exact Probability Method (73) of the data indicated that oral and intraperitoneal dosing of sodium salicylate were not significantly different at the .05 level with the number of rats used. In observing the severity of the gastric lesions, orally administered sodium salicylate produced more severe lesions than that given intraperitoneally.

b) Pharmacokinetics of Sodium Salicylate

The plasma and gastric mucosal concentrations of sodium salicylate after oral and IP dosing are given in Table 4-6. If we were to estimate the *in vivo* mucosal concentration of sodium salicylate, we would have a better idea of the concentration range we are working in when comparing inhibition data. The average mucosal weight for two mucosal scrapings in about 500 mg. If we assume 60% water in these preparations, we will get 0.3 ml. of water for the two scrapings. The tissue extract from two mucosal scrapings is 5 ml. with an average protein concentration of 2.5 mg./ml. We, therefore, can arrive at an estimate of the *in vivo* concentrations (Table 4-7). Based on

Table 4-5. Effect of the Route of Administration on Salicylate Induced Gastric Lesions.

Time (Hours)	Route	No. of Rats With Ulcer	No Ulcer	Ulcer Index
0	Oral	0	10	0
0	IP	0	10	0
1	Oral	1	9	1.6
1	IP	0	10	0
3	Oral	5	5	5.6
3	IP	1	9	1.3
6	Oral	9	1	13.2
6	IP	5	5	6.8

the *in vitro* results, a concentration range of 10 mM to 16 mM (30 minutes to 2 hours after oral dosing) of salicylate is sufficient to inhibit L-glutamine:D-fructose-6-PO_4 amino-transferase *in vivo* by competitive inhibition with respect to fructose-6-PO_4 and perhaps even by irreversible inactivation.

If we examine the areas under the concentration versus time curves between 0 to 6 hours (Figures 4-16 and 4-17) and determine the area by the trapezoidal rule, we see that the area under the plasma concentration-time curve was greater by 1.18 times that of the IP plasma-time curve. The area under the mucosal concentration-time curve, however, was greater by 6.9 times after oral dosing compared to IP dosing (Table 4-7).

The area of a concentration time profile is a good indication of the availability of the drug, namely how much drug is there and for how long. As one would expect, oral administration provided a much higher concentration and for a longer duration of the drug in the gastric mucosa than intraperitoneal administration.

McArthur et al (74) reported that up to 5 mM, the per cent of free salicylate in the plasma was about 60%. They also reported that only two tissues, the liver and kidney, seem to be able to bind the drug to any significant extent therefore justifying the assumption of neglible mucosal tissue binding. Using Equation 2-56 and a value of 0.6 for α, the value calculated for $\int_0^6 D(t)_{po}dt$ was 2.6 mg. This seems to indicate that less than 5% of the administered dose was absorbed into the gastric mucosa.

Table 4-6. Plasma and Gastric Mucosal Concentrations of Sodium Salicylate After Oral and IP Administration Of A Dose Of 600 mg./kg.

Time (Hours)	Oral		IP	
	Plasma	Mucosa	Plasma	Mucosa
0.5	350 ± 36	66.4 ± 4.5	650 ± 41	5.97 ± 0.84
1	625 ± 26	64.3 ± 5.1	850 ± 36	8.33 ± 1.3
2	775 ± 30	41.7 ± 4.3	850 ± 51	6.92 ± 0.82
3	725 ± 50	33.8 ± 3.0	800 ± 30	6.56 ± 0.45
4	650 ± 53	34.4 ± 2.3	800 ± 60	6.56 ± 0.67
6	650 ± 43	35.2 ± 2.5	600 ± 43	5.00 ± 0.52
8	605	19.2		
15	300	9.62		

Plasma concentration expressed as mcg./ml. ± standard deviation and mucosal concentrations expressed as mcg./mg. of protein ± standard deviation. Data is graphically depicted in Figures 4-16, 4-17, and 4-18.

Table 4-7. Estimated In Vivo Concentrations of Salicylate In the Gastric Mucosa Supernatant After Salicylate Administration.

Time (Hours)	Oral (mM)	IP (mM)
0.5	16.5	1.5
1	16.0	2.08
2	10.5	1.72
3	8.5	1.64
8	4.75	

Table 4-8. Areas Under the Curve After Oral And IP Administration of Sodium Salicylate.

Route	Plasma (mcg./ml. x hr.)	Mucosa (mcg./mg. x hr.)
Oral	3848	251.5
IP	4565	36.4

Areas were determined from Figures 4-16 and 4-17 by using the trapezoidal method.

Figure 4-16. Plasma salicylate concentration as a function of time after oral and intraperitoneal administration of the drug to rats at a dose of 600 mg./Kg.

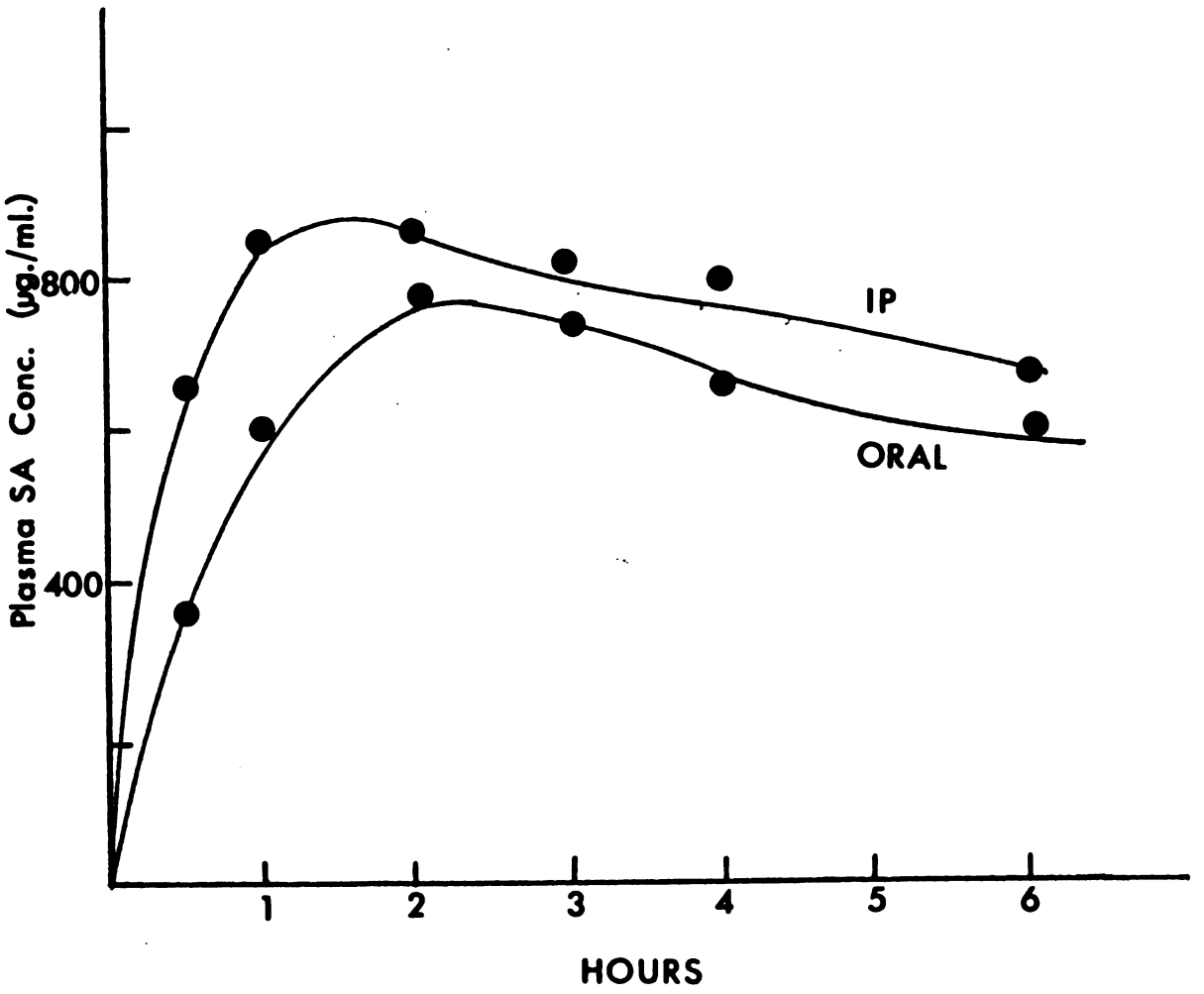

Plasma SA Conc. (µg./ml.)
800
400
IP
ORAL
1
2
3
4
5
6
HOURS

Figure 4-17. Mucosa concentration of salicylate as a function of time. Mucosa concentration is expressed as mcg. of salicylate per mg. of gastric mucosal supernatant protein. Conditions of drug administration were as detailed in Figure 4-16.

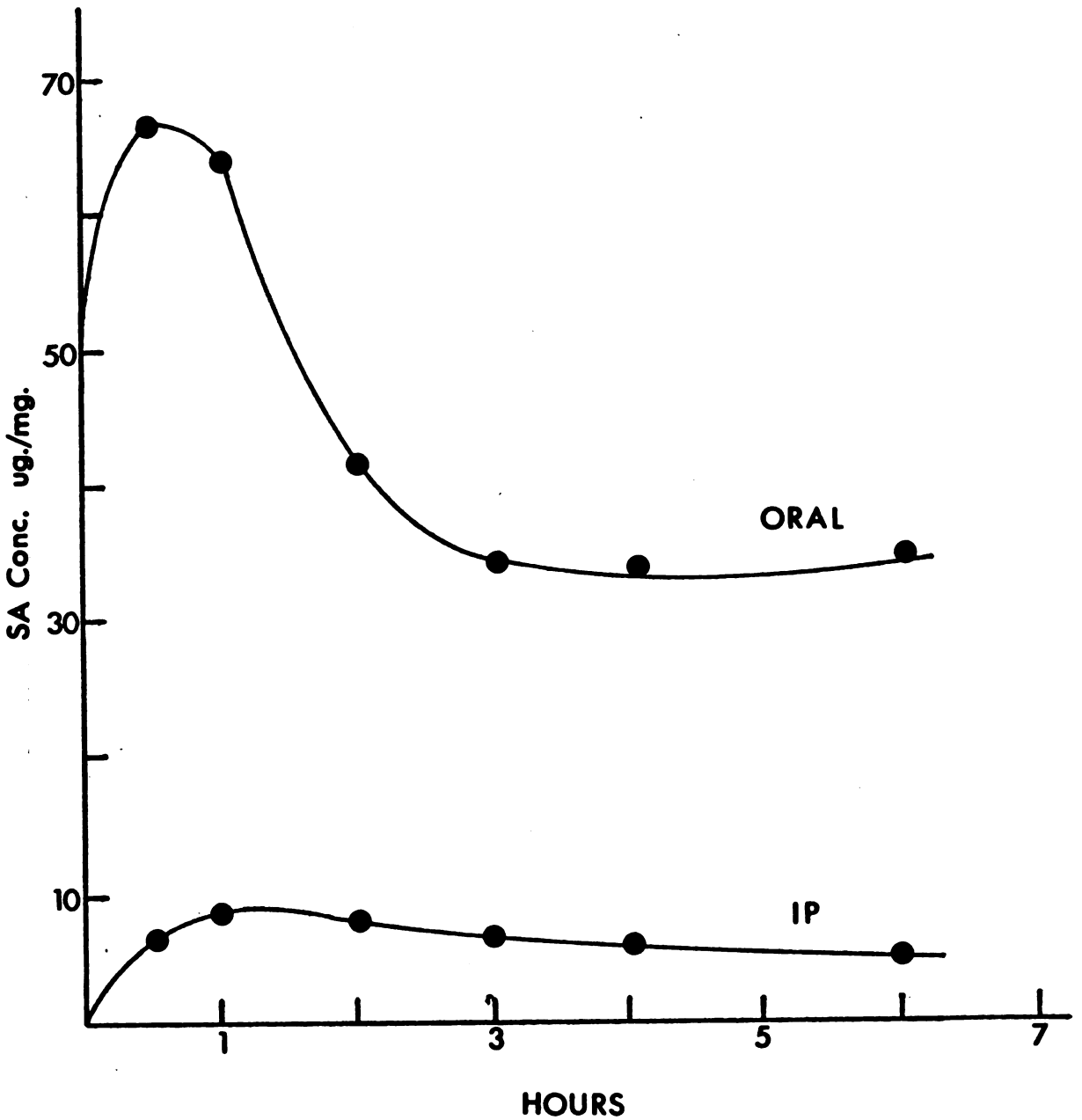
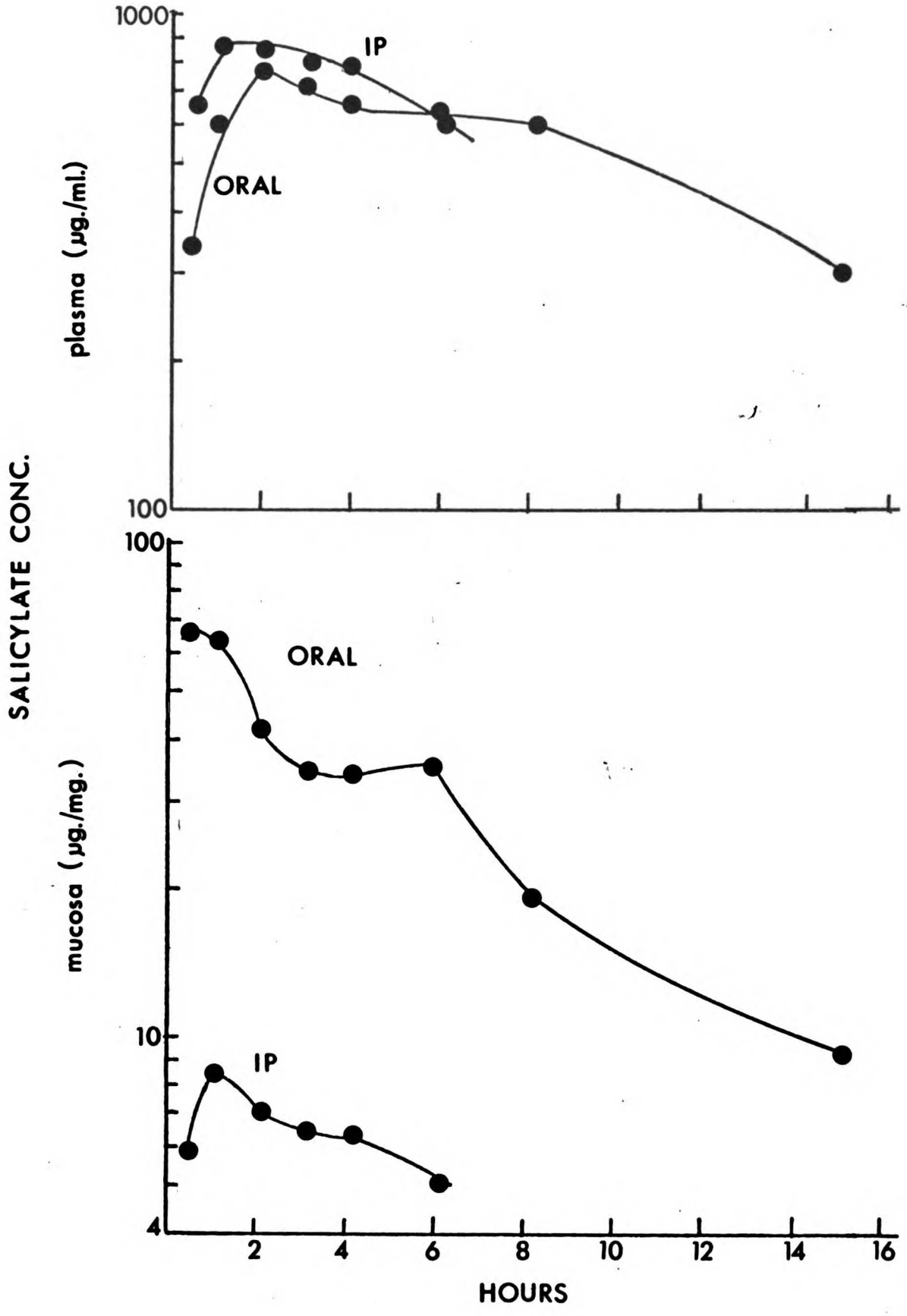

70
50
30
10
SA Conc. ug./mg.
ORAL
IP
1
3
5
7
HOURS

Figure 4-18. Semilog plots of the data presented in Figures 4-16 and 4-17.


SALICYLATE CONC.
plasma (µg./ml.)
1000
100
IP
ORAL
mucosa (µg./mg.)
100
10
4
ORAL
IP
2
4
6
8
10
12
14
16
HOURS

A semilog plot of the concentration versus time profile indicated that the drug, both in the plasma and mucosal supernatant, declined in a nonlinear manner (Figure 4-18) indicative of saturation of metabolic pathways occuring at high doses of salicylate (61). Since only the excretion of unchanged salicylate follows first order kinetics and that represents only a small fraction of the administered dose, saturation of the metabolic pathway will give rise to elimination kinetics approaching zero order in the early stages and first order at later time points.

The mucosal concentration-time plots of salicylate displayed an apparent steady state at 3 to 6 hours. The IP dose yields a level of salicylate in the mucosa much lower than the oral dose while the plasma concentrations are relatively similar. Using the blood flow limited model derived earlier (Equations 2-51 and 2-52), at steady state we have:

$$V_m \frac{dC_m}{dt} = 0 = D(t)_{po} + Q_m C_{p(po)} - Q_m C_{m(po)}$$

$$V_m \frac{dC_m}{dt} = 0 = Q_m C_{p(ip)} - Q_m C_{m(ip)}$$

where C_m and C_p are the concentrations in the mucosa and plasma respectively. $D(t)_{po}$ is the drug input into the mucosa from an oral dose. Q_m is the blood flow to and from the mucosa compart-

ment. If we assume that $C_{p(po)}$ is equal to $C_{p(ip)}$ and no changes in Q_m, we will arrive at the following:

$$D(t)_{po} = Q_m (C_{m(po)} - C_{m(ip)})$$

If this model is correct, then we are seeing a saturation of the ability of the circulation to remove salicylate from the mucosa. The amount of drug removed would be $Q_m(C_{m(po)} - C_{m(ip)})$ and this is replaced by the input $D(t)_{po}$ accounting for the observed steady state. The small amount of drug removed from the mucosal compartment is not reflected in a significant change in plasma level as the fraction of the cardiac output to the stomach is small. Caster (76) reported that the volume of blood in the rat stomach is 41.1 microliters per Gm. but the total blood volume is reported to be 62 ml./Kg. (75). The average weight of the rat stomach from a 120 Gm. rat is about 1 Gm. meaning that the blood of the stomach represents about 0.55% of the total blood volume.

c) Metabolism of Sodium Salicylate By the Gastric Mucosa

Hanninen (57) reported glucuronyl transferase activity in the rat stomach mucosa using nitrophenol as an acceptor of glucuronic acid. We were unable to find any glucuronides 1 and 3 hours after an oral dose of sodium salicylate of 600 mg./kg. This could mean that there was very little metabolism or the metabolite was eliminated very rapidly.

Incubation of the microsomal fraction of the rat's gastric

mucosal scrapings with 0.5 mM of sodium salicylate and 1 mM of UDP-glucuronic acid showed that less than 10% of the sodium salicylate was converted to glucuronide in 2 hours. This suggests that the rat gastric mucosa has a low capacity for glucuronide formation.

d) Inhibition of L-Glutamine:D-Fructose-6-PO_4 Aminotransferase Activity In Vivo

A plot of the activity of the enzyme and the amount of protein in the enzyme extract used in the incubation mixture showed a curvilinear behavior (Figure 4-19). The data showed that at protein concentrations greater than 3 mg./ml., the activity deviated from linearity. Based on the above observations, all enzyme extracts from the rat gastric mucosa scrapings were diluted to the region of 1 to 3 mg./ml. to obtain a more accurate comparison of enzyme activities.

Following oral administration of sodium salicylate at the dose of 600 mg./kg. to the rat, the animal was sacrificed at time 0, 3 , and 5 hours and the aminotransferase activity in the mucosal scrapings was determined. The results are presented in Table 4-8.

Intraperitoneal administration of sodium salicylate at 600 mg./kg. to the rat did not produce any significant changes in enzyme activity over a six hour period. An average value of $3.36 \pm 0.28 \times 10^{-3}$ µmole/min./mg. of protein was obtained from two experiments with three rats per time point when samples were taken at 0, 3, and 5 hours after salicylate administration.

Figure 4-19. Enzyme activity as a function of the protein concentration of the mucosal supernatant extract. Reaction mixture contained in 4 ml.: 85 mM phosphate buffer (pH 7.4), 1 mM EDTA, 0.1% mercaptoethanol, 20 mM glutamine, 10 mM glucose-6-PO_4, and 1 ml. of the supernatant extract with the protein concentration as shown.

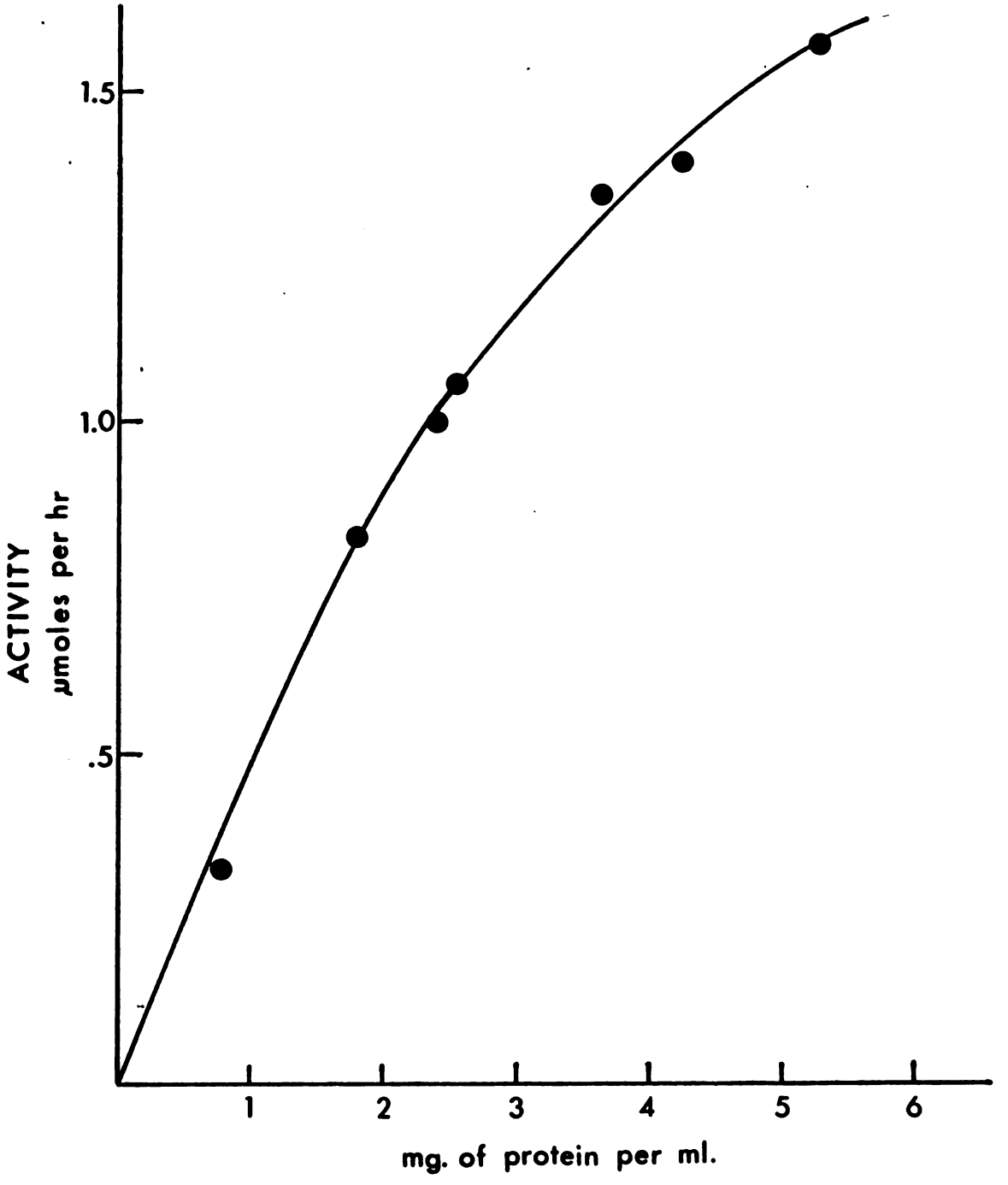

ACTIVITY
μmoles per hr
1.5
1.0
.5
1
2
3
4
5
6
mg. of protein per ml.

Table 4-9. Effect of Oral Administration of Sodium Salicylate on L-Glutamine:D-Fructose-6-PO_4 Aminotransferase Activity From the Rat Gastric Mucosal Supernatant.

Time (Hours)	Aminotransferase Activity
0	$3.02 \pm 0.25 \times 10^{-3}$
3	$2.94 \pm 0.31 \times 10^{-3}$
5	$2.36 \pm 0.22 \times 10^{-3}$

Enzyme activity expressed as micromoles of glucosamine formed per minute per milligram of protein ± standard deviation.

The data represented three experiments of three rats per time point. Protein concentration varied from 1.5 to 2 mg./ml. Activity at the 5-hour time point was significantly different from the zero time point at the 0.05 level of significance using the Student t Test.

Table 4-10. Effect of the Administration of Cycloheximide on L-Glutamine:D-Fructose-6-PO_4 Aminotransferase Activity From the Gastric Mucosal Supernatant.

Time (Hours)	Aminotransferase Activity
0	3.50×10^{-3}
2	3.24
4	3.75
6	3.33
24	$2.90 \pm 0.35 \times 10^{-3}$

Enzyme activity expressed as micromoles of glucosamine formed per minute per milligram of protein ± standard deviation.

The 24-hour time point represents two groups of three rats each; other points are one group of three rats. The average of the first 6 hours was $3.45 \pm 0.23 \times 10^{-3}$ micromoles/min./mg. protein.

Since the reduction of enzyme activity after oral administration of sodium salicylate did not reveal the mechanism of the inhibition, a different approach was used. A Lineweaver Burk plot was constructed using the data of enzyme activities determined at various concentrations of the substrate glucose-6-PO_4. The resulting double reciprocal plot is shown in Figure 4-20. The results indicate that the decrease in enzyme activity 5 hours after salicylate administration resulted from an increase in K_m and a decrease in V_{max}.

The possibility exists that sodium salicylate may inhibit the synthesis of the aminotransferase enzyme resulting in a decrease in total enzyme. This inhibition of enzyme synthesis would only manifest itself over a six hour period if the turnover of the enzyme was rapid. To test this possibility, cycloheximide, a potent inhibitor of protein synthesis in mammalian cells at the ribosomal level, was used. Jondorf (78) reported that this compound can cause a substantial inhibition of protein synthesis for 24 hours when administered at a dose of 2.5 mg./kg. to rats. Cycloheximide administration at a dose of 2.5 mg./kg. intraperitoneally did not show a significant change in aminotransferase activity over the first six hours after administration, and only a small reduction in activity in 24 hours (Table 4-10).

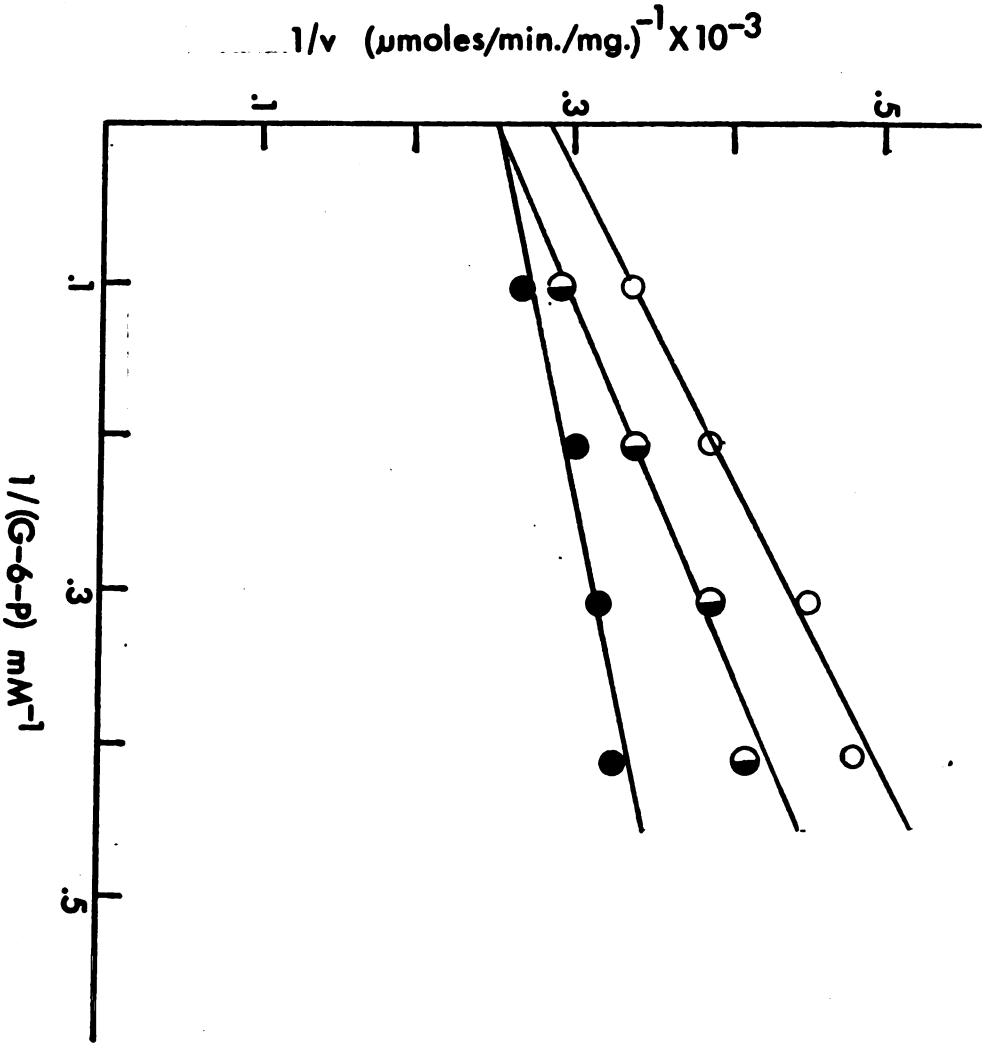
e) Effects of Sodium Salicylate on Hexosamine Content

After oral administration of sodium salicylate at a dose of 600 mg./kg. to the rat, the hexosamine content of the soluble

Figure 4-20. Enzyme activity of the mucosal extract as a function of the glucose-6-PO_4 concentration after oral administration of sodium salicylate at a dose of 600 mg./Kg. Enzyme activity is expressed as micromoles of glucosamine formed per minute per mg. of supernatant protein. Time after salicylate administration: ● , 0 hours; ◑ , 3 hours; ○ , 5 hours. Kinetic Parameters, K_m and V_{max}.

Time (Hours)	K_m (mM)	V_{max} (micromoles/min./mg.)
0	0.8 ± 0.7	4.00 ± 0.75 x 10^{-3}
3	1.8 ± 1.1	4.08 ± 1.1
5	1.83 ± 0.37	3.5 ± 0.28

Standard deviation of K_m and V_{max} are determined by the method of propagation of error(77).


1/v (μmoles/min./mg.)$^{-1}$ X 10^{-3}
1
3
5
.1
.3
.5
1/(G-6-P) mM^{-1}

fraction (35,000 x g) and the pellet fraction of the rat gastric mucosal scrapings were determined. The results, shown in Table 4-11, indicate no significant changes in hexosamine content in either the soluble fraction or the pellet fraction after salicylate administration.

Table 4-11. Effect of Oral Administration of Sodium Salicylate on the Hexosamine Content of the Soluble Fraction and the Pellet Fraction of the Rat Gastric Mucosal Scrapings.

Time (Hours)	Soluble Fraction	Pellet Fraction
0	15.4 ± 1.8	2.66 ± 0.32
1	13.2 ± 2.3	2.42 ± 0.13
3	12.8 ± 1.3	2.31 ± 0.41
6	12.5 ± 0.8	2.21 ± 0.45

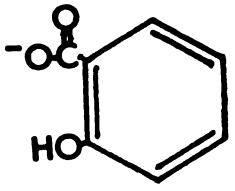
Hexosamine content of the soluble fraction is expressed as micrograms per milligram of supernatant protein. Hexosamine content of the pellet fraction is expressed as micrograms per milligram of the dry weight of the pellet. Mean values and their standard deviations are given.

The results represent three experiments with three rats per experimental time point. Untreated animals gave 14.7 ± 1.7 mcg./mg. and 2.17 ± 0.23 mcg./mg. for the soluble and pellet fraction respectively.

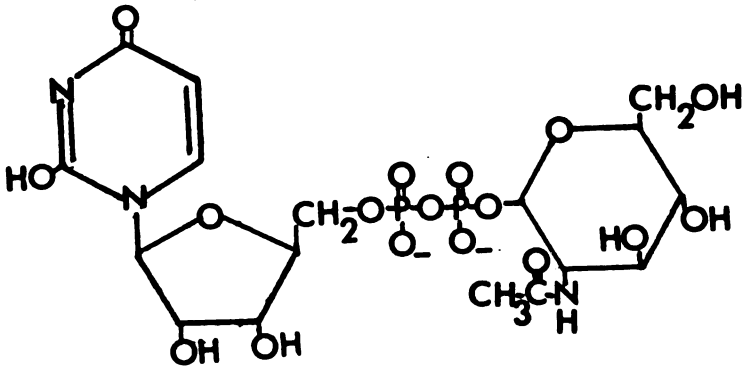
V. DISCUSSION

The *in vitro* results indicated that sodium salicylate can either inhibit the reaction catalyzed by the enzyme L-glutamine:D-fructose-6-PO_4 aminotransferase by a reversible competition with fructose-6-PO_4 or cause inactivation of the enzyme in an irreversible manner.

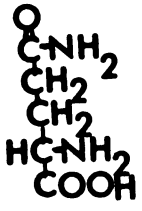
The results indicated that both salicylate and UDP-N-acetylglucosamine apparently compete for the same binding site. Based on a consideration of the molecular structures (Figure 5-1), one would not expect all three molecules to bind to the same site. The only similarity one can conceive is the similarity of the benzene ring of salicylate to the pyrimidine ring of UDP. Salicylate can then bind to the enzyme at the site normally occupied by the pyrimidine ring of UDP-N-acetyl-glucosamine. Kornfeld (38) was able to partially desensitize the enzyme to the inhibition by UDP-N-acetylglucosamine suggesting that the inhibitor site was distinct from the active site. Winterburn and Phelps (53) using a highly purified liver enzyme, demonstrated that UDP-N-acetylglucosamine was actually a noncompetitive (decrease in V_{max}) inhibitor with respect to fructose-6-PO_4 and an uncompetitive (decrease in V_{max} and K_m) inhibitor with respect to glutamine. In the presence of glucose-6-PO_4, the inhibition became competitive with fructose-6-PO_4 along with a decrease in the inhibitor constant K_i. In our crude enzyme extracts, we used glucose-6-PO_4 to stabilize our enzyme as well as for the substrate.



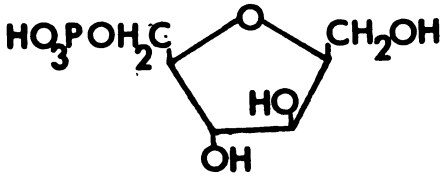
SALICYLATE



UDP-N-ACETYLGLUCOSAMINE



GLUTAMINE



FRUCTOSE-6-PO_4

Figure 5-1. Chemical Structures of Salicylate, UDP-N-Acetyl-glucosamine, Fructose-6-PO_4, and Glutamine

Bates and Handschumaker (37) suggested that L-glutamine: D-fructose-6-PO_4 aminotransferase can exist in an inhibited as well as an uninhibited state. The active state is favored by the binding of fructose-6-PO_4 to the enzyme and the inhibited state is induced by the binding of UDP-N-acetylglucosamine. The structural requirements are very selective, as UDP-N-acetyl-glucosamine can protect the enzyme against inactivation by 6-diazo-5-oxo norleucine, a glutamine analog capable of under-going alkylation reactions. Compounds of similar structure as UTP, UDP, and UDP-glucuronate afforded no protection while N-acetylglucosamine-1-PO_4 showed slight protection (37). Kornfeld et al (79) reported that other UDP-sugars showed some inhibitory action on the enzyme, as with both substrates (fructose-6-PO_4 and glutamine), saturating the concentrations of UDP-N-acetylglucosamine, UDP-glucose, UDP-galactose, UDP-xylose, and UDP-glucuronate at 0.025 mM, 1.5 mM, 2.8 mM, 2.6 mM, and 3.4 mM respectively gave the same inhibition. UDP-N-acetyl-galactosamine gave 1/5th the activity of the glucosamine counterpart (38). Our results indicated that it would require 30 mM of salicylate to give the inhibition equivalent to 0.025 mM of UDP-N-acetylglucosamine or approximately 1/1000th the activity.

Our <u>in vitro</u> results also showed that UDP-N-acetylglucosamine protected the enzyme against inactivation by salicylate. This may involve competitive binding of the salicylate molecule for the UDP-N-acetylglucosamine binding site or at some other site made less vulnerable by conformational changes induced by

UDP-N-acetylglucosamine. The fact that fructose-6-PO_4 also protected the enzyme against salicylate inactivation suggests that the uninhibited state favored by fructose-6-PO_4 is less vulnerable to the action of salicylate. This would mean that fructose-6-PO_4 protects against inactivation by causing a conformational change in the enzyme making it less favorable for the binding of salicylate. UDP-N-acetylglucosamine protects by actually competing with salicylate for the same binding site. This also suggests that salicylate does not compete with fructose-6-PO_4 at the same binding site but actually increases the K_m of fructose-6-PO_4 by binding to a site normally occupied by UDP-N-acetylglucosamine. This analysis is not completely in accordance with the claims made by Winterburn and Phelps (52) who reported that glucosamine synthetase does not behave as other classical allosteric enzymes and with the possible exception of the behavior towards fructose-6-PO_4, any homotropic interactions that occur in this enzyme are of a minor nature.

The <u>in vivo</u> data indicated a decrease in enzyme activity after salicylate administration. This decrease was reflected by a decrease in V_{max} and an increase in K_m. The decrease in V_{max} may be caused by enzyme inactivation as observed <u>in vitro</u> or an inhibition of enzyme synthesis. Salicylate has been reported by Dawkins and McArthur (81) to inhibit the incorporation of radioactive leucine and histidine into the protein of a mitochondrial supernatant fraction from mouse liver. Lukie and Forstner (14) and Kent and Allen (13) showed that incorpora-

tion of amino acids into the protein portion of glycoprotein can be inhibited by 15 mM of salicylate. Burleigh and Smith (82,83) reported that salicylate inhibited aminoacyl-t-RNA formation. The data obtained from the cycloheximide experiment indicated that the enzyme turnover was relatively slow as the enzyme levels did not change significantly over a six hour period. This is further supported by Perrey (28) who administered 1,000 mg./Kg. of salicylate and observed a very rapid loss of enzyme activity (half life about 5 hours). The activity was 20% at 12 hours and 18% of the original activity at 24 hours. The slow recovery of the enzyme to the basal value would tend to support the fact that the turnover was slow enough that the inhibition of enzyme synthesis would not manifest any decrease in enzyme levels over a six hour period. The decrease in V_{max} can then be attributed to enzyme inactivation.

The use of inhibitors of protein synthesis do not always lead to unambiguous results, these drugs will block hormonal induction of the enzyme but they do not always affect the basal enzyme levels (84). Kenney (85) found that cycloheximide blocks the degradation as well as the synthesis of tyrosine aminotransferase leading to erroneous determination of the turnover rate of this enzyme. Therefore, we still need a more accurate determination of the turnover rate of L-glutamine: D-fructose-6-PO_4 aminotransferase to be absolutely certain about the effects of salicylate on the synthesis of the enzyme.

The increase in K_m is a perplexing problem. The possibility that the increase may be caused by the salicylate in the incubation mixture was precluded as the concentration of salicylate in the final incubation mixture were in the region of 0.1 mM and much below the range shown to cause inhibition. Inhibition by a metabolite or metabolites of sodium salicylate is possible but not likely as significant amounts of glucuronide were not found in the mucosa and the glycine conjugate (salicyluric acid) has been reported in the kidney and liver only and rats excrete only small quantities of this metabolite (86). Our experimental results further indicated that all the salicylate detected in the gastric mucosal supernatant after drug administration occurred as free salicylate.

Another possible explanation for the increase in K_m involves changes in the in vivo concentration of UDP-N-acetylglucosamine. Kent and Allen (13) showed that sodium salicylate inhibited the conversion of UDP-N-acetylglucosamine to sialic acid while stimulating the conversion to UDP-N-acetylgalactosamine. Since the glucosamine and galactosamine nucleosides are at equilibrium at the ratio of 2 to 1 (38), an increase in galactosamine formation must mean a concomitant increase in UDP-N-acetylglucosamine. Therefore, by increasing I, we increase the K_m of fructose-6-PO_4 by the factor of $(1 + (I)/K_i)$. A decrease in K_i can also result in an increase in K_m. Winterburn and Phelps (80) reported that AMP potentiated the inhibition of glucosamine synthetase by UDP-N-acetylglucosamine. An increase

in glucose-6-PO_4 concentrations also can decrease the value of K_i (52). Since salicylate produced hyperglycemic response in experimental animals (1) as well as to increase ADP and AMP levels (87), these mechanisms could conceivably be operative in vivo. One would not expect the effects to occur in vitro after the dilution procedure used to determine the enzyme activity although the equilibrium constants for AMP, ADP, glucose-6-PO_4, and the enzyme are not known. An increase in the in vivo concentration of UDP-N-acetylglucosamine resulting in the increase in the observed K_m appears to be the more feasible explanation. Examining Figure 4-13, one sees that inhibition in the asymtotic portion of the hyperbolic curve would change slightly over a wide range of inhibitor concentration of UDP-N-acetylglucosamine. Upon dilution, we enter a region of the graph with a steeper slope where small differences in concentration could be amplified into large differences in inhibition. This explanation would be consistent if majority of the enzyme in vivo was inhibited by UDP-N-acetylglucosamine. We estimated that the in vivo concentration in the gastric mucosa to be approximately 10^{-5}M meaning we would expect 85 to 90% inhibition of the enzymes. Winterburn and Phelps (52) estimated that in the liver, the glucosamine synthetase enzyme was inhibited in excess of 90%. If the intracellular reserve of the enzyme is high and most of the enzyme is bound by the feed-back inhibitor, salicylate would not be expected to have any effect on glucosamine synthesis. The feedback inhibitor not

only protects the enzyme against inactivation by salicylate but also prevents the binding of salicylate to the enzyme. If salicylate should inactivate some of the enzyme or reduce glucosamine synthesis by competitive inhibition, this would lead to a decrease in UDP-N-acetylglucosamine levels, the feedback control system would release from inhibition more enzymes, and therefore retain the initial steady state synthetic rate of glucosamine. The fact that the hexosamine content in both the supernatant and insoluble fractions did not show any significant decrease tends to support this idea of a large intracellular reserve of the enzyme. This enzyme seemed to fit into the catagory of equilibrium enzymes described by Krebs (106). This class of enzymes function at only a fraction of their maximum capacity and their potential activity is far in excess of the flux rate of their substrates. This excess enables these enzymes to establish near-equilibrium between starting materials and end products, not only when the flux rate of the material varied widely, but also when a substantial proportions of the enzyme has been inhibited. Therefore, if only 10% of the enzyme is operating to maintain the flux rate, theoretically 90% inhibition of the total enzyme potential would not alter the rate of flux. For example, a 90% inhibition of fumarase is not likely to affect the flux rate of metabolic intermediates through the tricarboxylic acid cycle (4). Since glucosamine synthetase is also subjected to regulation by the negative feedback inhibitor, the rate of glucosamine synthesis rate

appeared to be stabilized by two mechanisms, intracellular reserve of enzymes and feedback regulation.

Examining the role of mucopolysaccaride synthesis or more accurately hexosamine synthesis in terms of a larger system of carbohydrate metabolism, there are other factors which may result in the reduction of the rate of synthesis of hexosamine. The enzymes which synthesize the various intermediates involved in mucopolysaccaride synthesis at least up to UDP-N-acetyl-glucosamine in the rat liver and neonatal rat skin occur in the cytosol (88). Hardingham and Phelps (88) reported that there was no compartmentation of intermediates in hexosamine synthetic pathway. L-glutamine:D-fructose-6-PO_4 aminotransferase is situated at a branch in the main stream of carbohydrate metabolism. The amount of fructose-6-PO_4 commited to hexosamine synthesis will be subjected to various controls depending on the state of metabolism. The fraction of fructose-6-PO_4 eventually converted to hexosamine varies from tissue to tissue. In the mouse liver a value of conversion of 0.5 to 2% was quoted by Reich (89), but Hardingham and Phelps (88) studying rat skin quoted a value of 15 to 20%. Draper and Kent (90) using sheep colonic mucosal scrapings reported that 10% of the total administered radioactive glucose converted to hexosamine.

The key enzymes believed to regulate carbohydrate metabolism are phosphofructokinase, phosphorylase, pyruvate dehydrogenase system, and glucosamine synthetase. A common

denominator of regulation seems to be the adenine nucleotides AMP, ADP, and ATP. Since carbohydrate is one of the major sources of the energy for the high energy bonds, one is not surprised to see ATP/ADP ratio being an important regulatory signal. ATP decreases the activities of phosphofructokinase and pyruvate dehydrogenase complex (91,92), while ADP and AMP increase the activity of phosphofructokinase. AMP on the other hand decreases the activity of glucosamine synthetase.

The metabolic effects of salicylates are many and the literature is voluminous. The uncoupling of oxidative phosphorylation by salicylates have been reported in simple mitochondrial preparations as well as isolated rat diaphragms. The direct demonstration of an uncoupling action of salicylate in the whole animal has not been accomplished. Smith and Jeffrey (87) showed that salicylate as low as 0.1 mM caused a decrease in the levels of creatine phosphate and ATP in isolated rat diaphragm. One would certainly expect that a decrease in ATP would result in a concomitant increase in ADP and AMP levels leading to an increase in activities of phosphofructokinase and pyruvate dehydrogenase complex and a decrease in the activity of glucosamine synthetase. This would result in a greater flux of glucose into the Embden Meyerhof pathway and eventually through the Krebs cycle to correct the disturbance in the ATP/ADP ratio. The fact that we did not observe a measurable change in hexosamine content may suggest that only a small fraction of the fructose is converted to hexosamine and

the rerouting of fructose from the hexosamine pathway to the mainstream of carbohydrate metabolism is not an important adjustment. Also, the existence of the feedback loop would tend to resist this change in flux of substrate. As the level of substrate is reduced, the associated decrease in the level of the feedback inhibitor will release more enzymes to compete for the substrate.

There has been much controversy regarding the effect of the route of administration of salicylate and its ability to cause gastric lesions. Davenport (93), Martin (94), and Levy (95) support the premise that salicylate acted locally on the gastric mucosa to induce lesions. They further believe that the high concentrations required at the mucosal surface can only be achieved by oral administration. On the contrary, Grossman (96), Djahanguiri (101), and Brodie and Chase (41) reported that parenteral administration of salicylate can also produce gastric lesions. Brodie and Chase in fact showed a practically superimposable dose response curve (per cent incidence of gastric lesions as a function of the dose) for oral and intraperitoneal administration. Other anti-inflammatory drugs including phenylbutazone and indomethacin have been reported to induce gastric lesions both by oral and parenteral administration (43,44,98,99,107).

Our data showed that both oral and intraperitoneal administration of salicylate (600 mg./Kg.) produced gastric lesions. The severity of the lesions and the incidence of

occurrance was slightly greater for the oral route. Oral administration also provided sufficient salicylate levels to decrease the activity of L-glutamine:D-fructose-6-PO_4 aminotransferase at 3 and 5 hours post administration. The concentrations of salicylate achieved via IP administration were not sufficient to cause an observable decrease in enzyme activity. Based on *in vitro* data, we do not expect the concentration achieved by IP administration to have any effect on the enzyme *in vivo*. We, therefore, were unable to establish a cause and effect relationship or correlation between salicylate concentration, inhibition of hexosamine formation, and incidence and severity of gastric lesions.

Two explanations seem feasible at this time. First, there may be another site in the biosynthetic pathway of glycoproteins which may be more sensitive to the effect of salicylate. Our mathematical model of the biochemical pathway of aminosugar metabolism indicated that the aminotransferase enzyme and the enzyme(s) immediately after the feedback loop (UDP-N-acetylglucosamine branching to sialic acid and UDP-N-acetylgalactosamine) are important in maintaining the steady state rate of aminosugar metabolism. Since our data indicated that the inhibition of hexosamine formation by inhibition of the aminotransferase enzyme was not correlated to incidence and severity of gastric lesions, the possibility exists that sialic acid formation may be a more sensitive parameter with which to seek correlation.

The second explanation is an idea proposed by Skoryna (100) who suggested that certain combinations of causative factors may contribute to the induction of gastric lesions. One may consider the success of salicylate in inducing gastric lesions as the sum of the effects of salicylate on the various causative factors.

The following have been listed as possible sites of action of salicylates leading to drug induced ulcers (100) and these can be categorized into three sites:

Systemic

hypothalamic-pituitary-adrenal system

gastric secretions (CNS, vagus)

hypoprothrombinemia

Mucosal Level

glycoprotein synthesis

glycoprotein secretions

cellular energetics (uncoupling of oxidative phosphorylation)

HCl secretion

Local Effects

mucus denaturation

mucosal necrosis

local pH effects

The toxic response can be simplified to the action of the drug at the three sites, local, mucosal, and systemic. We would expect factors as time of onset of drug action, duration

of maximal response, and rate of dissipation of the effect to be a function of the concentration of the drug at the site of action. The concentration time relationship at each site will then be a function of the input of the drug and its disposition. At each site, the intensity of effect would be described by the following expression (108): $E = m \log C + e$ where E is the intensity of the pharmacologic effect, C is the concentration at the site, m is the slope of the line which is obtained when E is plotted against log C, and e is the intercept of the line on the E axis. The intensity of the effect related to the incidence and severity of ulceration may be the sum of the effects at each site with the weight of each contribution described by a weighting factor w:

$$E(t) = w_1 E(t)_1 + w_2 E(t)_2 + w_3 E(t)_3$$

where E(t) represents the intensity of the effect as a function of time. Further research would be required to confirm or reject these possibilities.

VI. CONCLUSION

The *in vitro* studies indicated that sodium salicylate can inhibit the activity of L-glutamine:D-fructose-6-PO_4 aminotransferase from the rat liver and gastric mucosal scrapings. Salicylate can inhibit the enzyme catalyzed reaction by acting as a reversible competitive inhibitor of fructose-6-PO_4 or by an irreversible inactivation of the enzyme.

In vivo studies showed that salicylate decreased the activity of the enzyme from the rat gastric mucosal scraping after an orally administered dose of 600 mg./Kg. but did not show a reduction in the hexosamine content. L-glutamine: D-fructose-6-PO_4 aminotransferase is part of a feedback multienzyme system with the feedback inhibitor, UDP-N-acetyl-glucosamine, inhibiting the step catalyzed by the aminotransferase enzyme. Two explanations can be proposed to explain the lack of effect on the hexosamine content. (1) Salicylate binds to the site occupied by the feedback inhibitor with the latter having 1,000 times the affinity for the enzyme as the former. (2) A large fraction (in excess of 80%) of the enzyme appears to be in an inactive form bound to the feedback inhibitor thus providing an intracellular reserve of the enzyme against inactivation induced by sodium salicylate.

From this study, we do not see any correlation between inhibition L-glutamine:D-fructose-6-PO_4 aminotransferase activity in the rat gastric mucosa and gastric lesions induced by sodium salicylate.

REFERENCES

1. Smith, M.J.H. and P.K. Smith, The Salicylates, A Critical Bibliographic Review, Interscience Publishers, New York, 1966.

2. Gross, M. and L.A. Greenberg, The Salicylates, Hillhouse Press, New Haven, 1948.

3. Dixon, A. St. J., B.K. Martin, M.J.H. Smith, and P.H.N. Wood, eds, Salicylates, An International Symposium, Little Brown, Boston, 1963.

4. Smith, M.J.H. and P.D. Dawkins, J. Pharm. Pharmacol. 23, 729 (1971).

5. Collier, H.O.J., Advan. Pharmacol. Chemotherap. 7, 337 (1969).

6. Whitehouse, M.W. and H. Bostrom, Biochem. Pharmacol. 7, 135 (1964).

7. Whitehouse, M.W. and H. Bostrom, Biochem. Pharmacol. 11, 1175 (1962).

8. Bostrom, H., K. Berntsen, and M.W. Whitehouse, Biochem. Pharmacol. 13, 413 (1964).

9. Whitehouse, M.W., Biochem. Pharmacol. 13, 319 (1964).

10. Bostrom, H., A. Moretti, and M.W. Whitehouse, Biochem. Biophys. Acta 74, 213 (1963).

11. Bostrom, H. and B. Maansson, J. Pharm. Pharmacol. 7, 185 (1955).

12. Bollet, A.J., Arthritis Rheumat. 4, 624 (1961).

13. Kent, P.W. and A. Allen, Biochem. J. 106, 645 (1968).

14. Lukie, B.E. and G.G. Forstner, Biochem. Biophys. Acta 273, 380 (1972).

15. Anderson, K.W., Arch. Int. Pharmacodyn. Ther. 152, 379 (1964).

16. Brodie, D.A. and B.J. Chase, Gastroenterol. 53, 604 (1967).

17. Croft, D.N., J. Pharm. Pharmacol. 18, 354 (1966).

18. Kirsner, J.B. and H. Ford, Gastroenterol. 29, 18 (1955).

19. Levrat, M. and R. Lambert, Gastroenteralogia (Basel) 94, 273 (1960).

20. Hakkinen, I.P., T. Johansson, and M. Pantio, Gut 9, 712 (1968).

21. Menguy, R. and Y.F. Masters, Surg. Gynecol. Obstet. 120, 92 (1965).

22. Lambert, R., C. Andre, J.P. Leonard, and F. Martin, Acta Gastroentero Belgica 30, 787 (1967).

23. Menguy, R. and L. Desbaillet, Am. J. Dig. Dis. 12, 862 (1967).

24. Lindquist, B., Nord. Med. 85, 3 (1971).

25. Denko, C.W., J. Lab. Clin. Med. 63, 953 (1964).

26. Emmanuel, J.H. and R.D. Montgomery, Postgrad. Med. J. 47, 227 (1971).

27. Ezer, E. and L. Szporny, J. Pharm. Pharmacol. 22, 143 (1970).

28. Perrey, K.H., Arch. Int. Pharmacodyn. 176, 2 (1968).

29. Schonhofer, U.P., Med. Pharmacol. Exp. 15, 491 (1966).

30. Nakagawa, H. and P. Bentley, J. Pharm. Pharmacol. 23, 399 (1970).

31. Lee, K.H. and M.R. Spencer, J. Pharm. Sci. 58, 1152 (1970).

32. Lee, K.H. and M.R. Spencer, J. Pharm. Sci. 58, 464 (1969).

33. Anderson, W., J. Pharm. Pharmacol. 13, 2 (1961).

34. Pogell, B. and R. Gryder, J. Biol. Chem. 228, 701 (1957).

35. Ghosh, S., H.J. Blumenthal, E. Davidson, and S. Roseman, J. Biol. Chem. 235, 5 (1960).

36. Winterburn, P.J. and C.F. Phelps, Biochem. J. 121, 701 (1971).

37. Bates, C.J., W.R. Adams, and R.E. Handschumaker, J. Biol. Chem. 241, 1705 (1966).

38. Kornfeld, R.H., J. Biol. Chem. 242, 3135 (1967).

39. Bates, C.J. and R.E. Handschumaker, Advan. in Enzyme Regulation, F. Weber, ed., 7, 183, Academic Press, New York, 1968.

40. Bostrom, H. and B. Jacobson, Biochem. Biophys. Acta 83, 152 (1964).

41. Brodie, D.A. and B.J. Chase, Gastroenterol. 53, 604 (1967).

42. Anderson, K.W., Arch. Int. Pharmacodyn. 152, 379 (1964).

43. Djahanguiri, B., Scand. J. Gastroenterol. 4, 265 (1969).

44. Lee, K.H., unpublished data.

45. Dixon, M. and E.C. Webb, Enzymes, Chap. 4, Academic Press, New York, 1964.

46. Webb, J.F., Enzymes and Metabolic Inhibitors, Vol. 1, Chaps. 5 and 7, Academic Press, New York, 1963.

47. Lineweaver, H. and D. Burk, J. Am. Chem. Soc. 56, 658 (1934).

48. Dixon, M., Biochem. J. 35, 170 (1953).

49. Anson, M.L. and A.E. Mirsky, J. Gen. Physiol. 17, 399 (1934).

50. Grisolia, S., I. Santos, and J. Mendelson, Nature 219, (5160) 1252 (1968).

51. Gould, B.J. and M.J.H. Smith, J. Pharm. Pharmacol. 12, 15 (1965).

52. Winterburn, P.J. and C.F. Phelps, Biochem. J. 121, 711 (1971).

53. Dixon, M. and E.C. Webb, Enzymes, Pg. 52, Academic Press, New York, 1964.

54. Walter, C.F., in Biochemical Regulatory Mechanisms in Eukaryotic Cells, E. Kun and S. Grisolia Eds., Wiley-Interscience, New York, 1972.

55. Cushman, P.G., in Control Engineer's Handbook, J. G. Truxal ed., McGraw Hill, New York, Chap. 2, 1958.

56. Brosnan, J.T., H.A. Krebs, and P.H. Williamson, Biochem. J. 117, 91 (1970).

57. Hanninen, O., Annal. Acad. Scient. Fenn. Series A 123 (1966).

58. Wolff, J. and F.K. Austen, J. Clin. Invest. 37, 1144 (1958).

59. Bischoff, K.G., R.L. Dedrick, D.S. Zaharko, and J.A. Longstreth, J. Pharm. Sci. 60, 1128 (1971).

60. Bischoff, K.B., R.L. Dedrick, and D.S. Zaharko, J. Pharm. Sci. 59, 149 (1970).

61. Levy, G., T. Tsuchiya, and A.P. Lewis, Clin. Pharm. Therap. 13, 258 (1972).

62. McArthur, J.N., P.D. Dawkins, and M.J.H. Smith, J. Pharm. Pharmacol. 22, 801 (1970).

63. Mandel, H.G., in Fundamentals of Drug Metabolism and Drug Disposition, edited by B.N. LaDu, H.G. Mandel, and E.L. Way, Williams and Wilkins C., Baltimore, 1971.

64. Rowland, M., L.A. Benet, and S. Riegleman, J. Pharm. Sci. 59, 365 (1970).

65. Benson, R. and S. Friedman, J. Biol. Chem. 245, 2219 (1970).

66. Ghosh, S., H. J. Blumenthal, E. Davidson, and S. Roseman, 235, 1265 (1960).

67. Pogell, B.M. and R.M.Gryder, J. Biol. Chem. 235, 558 (1960).

68. Lin, T.M., Lilly Research Laboratories, personal communications.

69. Harris, P.A. and S. Riegleman, J. Pharm. Sci. 56, 6 (1967).

70. Peng, C.T., Anal. Chem. 32, 1292 (1960).

71. Colowick, S. and N.O. Kaplan eds., Methods in Enzymology, Vol. 3, Pg. 447, Academic Press, New York, 1957.

72. Cessi, C. and F. Piliego, Biochem. J. 77, 508 (1960).

73. Siegel, S., Nonparametric Statistics for the Behavorial Sciences, Pg. 96, McGraw Hill, New York, 1956.

74. McArthur, J.N., P.D. Dawkins, and M.J.H. Smith, J. Pharm. Pharmacol. 22, 801 (1970).

75. Bonnycastle, P.D., B.A. Cleghorn, Am. J. Physiol. 137, 380 (1942).

76. Caster, W.O., A.B. Simon, and W.D. Armstrong, Am. J. Physiol. 183, 317 (1955).

77. Bevington, P., Data Reduction and Error Analysis for the Physical Science, Chap. 4, McGraw Hill Co., New York, 1969.

78. Jondorf, W.R., Biochem. Pharmacol. 17, 839 (1969).

79. Kornfeld, S., R. Kornfeld, E.F. Neufeld, and P.J. O'Brien, Proc. Natn. Acad. Sci. 53, 371 (1964).

80. Winterburn, P.J. and C.F. Phelps, Biochem. J. 121, 721 (1971).

81. Dawkins, P.D., J.N. McArthur, and M.J.H. Smith, Biochem. Pharmacol. 20, 1303 (1970)

82. Burleigh, M. and M.J.H. Smith, J. Pharm. Pharmacol. 23, 590 (1971).

83. Burleigh, M. and M.J.H. Smith, J. Pharm. Pharmacol. 23, 519 (1971).

84. Greengard, O., M.A. Smith, and G. Acs, J. Biol. Chem. 238, 1548 (1963).

85. Kenney, F.T., Science 156, 525 (1967).

86. Quilley, E. and M.J.H. Smith, J. Pharm. Pharmacol. 4, 624 (1952).

87. Smith, M.J.H. and S.W. Jeffrey, Biochem. J. 64, 589 (1956).

88. Hardingham, T.E. and C.F. Phelps, Biochem. J., 108, 9 (1968).

89. Reich, J.G., U. Till, J. Gunther, D. Zahn, M. Tschisgale, and H. Frunder, Biochem. J. 6, 384 (1968).

90. Draper, P. and P.W. Kent, Biochem. J. 86, 248 (1963).

91. Lorenson, M.Y. and T.E. Marson, J. Biol. Chem. 244, 6420 (1969).

92. Linn, T.C., F.H. Pettit, F. Hucho, and L.J. Reed, Proc. Natl. Acad. Sci. 64, 227 (1969).

93. Davenport, H.W., Gastroenterol. 46, 245 (1964).

94. Dixon, A. St. J., B.K. Martin, M.J.H. Smith, and P.H.N. Wood, eds., Salicylates , An International Symposium, Pgs. 210-211, Little Brown, Boston, 1963.

95. Dixon, A. St. J., B.K. Martin, M.J.H. Smith, and P.H.N. Wood, eds., Salicylates, An International Symposium, Pgs. 209-210, Little Brown, Boston, 1963.

96. Grossman, M.E., K.K. Matsumoto, and R.J. Lichter, Gastroenterol. 40, 383 (1961).

97. Hucker, H.B., A.G. Zacchei, S.V. Cox, D.A. Brodie, and N.H.R. Cantwell, J. Pharmacol. Exp. Thera. 153, 237 (1966).

98. Watt, J. and C.W.M. Wilson, Gastroenterol. 37, 96 (1959).

99. Freedman, J.T., M.A. Geiss, and R. Hafkesbring, Am. J. Med. Sci. 237, 67 (1959).

100. Skoryna, S.C., in Pathophysiology of Peptic Ulcers, S.C. Skoryna ed., McGill Univ. Press, Montreal, 1963.

101. Djahanguiri, B., Chem. Biol. Act. 251, 5508 (1969).

102. Hitchens, J.T., S. Goldstein, A. Sambuca, and I. Shemano, Pharmacologist 9, 242 (1967).

103. Kent, T.H., R.M. Cardelli, and F.W. Stamler, Amer. J. Pathol. 54, 237 (1969).

104. Sun, D.C., Ann. N.Y. Acad. Sci. 140, 747 (1967).

105. Cammarata, D.S., R.G. Bianchi, and F.J. Fago, Gastroenterol. 61, 850 (1971).

106. Krebs, H.A., in Current Topics in Cellular Regulation, Vol. 1, Pgs. 45-56, Ed. B.L. Horecker and E.R. Stadtman, Academic Press, New York, 1969.

107. Varro, V., L. Csernay, and T. Javor, Gastroenterol. 37, 463 (1959).

108. Levy, G., Clin. Pharmacol. Therap. 7, 364 (1966).

109. Resigno, A. and G. Segre, Drug and Tracer Kinetics, Chap. 3, Blaidell Publ. Co., London, 1966.

110. Davison, A.N. and J. Dobbing, Nature 191, 844 (1961).

111. Schoenheimer, R., The Dynamic State of Body Constituents, Cambridge, Mass.,1946.