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FACTORS AFFECTING THE ABSORPTION OF DRUGS ACROSS THE IN VIVO RAT INTESTINE

by

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FACTORS AFFECTING THE ABSORPTION OF DRUGS ACROSS THE IN VIVO RAT INTESTINE

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Several factors that are considered to have a possible influence on the intestinal absorption rates of drugs were investigated in an attempt to further delineate the mechanisms by which drugs penetrate the intestinal barrier, in vivo. The absorption rates of two model drugs, an ionized acidic drug, salicylate, and a non-ionized basic drug, antipyrine, were studied using an absorption model in which the small intestines of rats were cannulated and perfused in situ under a variety of experimental conditions.

Various periods of fasting were utilized to study surface area effects since fasting has been shown to reduce absorptive surface area by reducing the mucosal epithelial cell population. Absorption rates of salicylate and antipyrine were significantly reduced under the influence of fasting, with salicylate being more affected than antipyrine. At the same time, fasting caused a reduction in the intestinal weight of the test animals that was shown to be linear with length of time of the fast. Mucosal cell separation studies showed that weight losses for both the mucosal and the underlying musculature fractions of the total intestinal mass occurred at about the same rate under the influence of fasting. It was surmised that mucosal cell surface area is not by itself the most important factor influencing drug absorption rates.

To further investigate mucosal epithelial surface effects, drug absorption rates from the intestines of rats exhibiting experimental diabetes were studied. In this disease state, mucosal cell number is significantly increased over normal values and it was predicted that the increased surface area for absorption would result in increased drug absorption rates. The absorption rate for antipyrine was unchanged while the rate for salicylate

was significantly decreased compared to the rates from non-diabetic control rats. This decrease was noted in spite of a substantial increase in the mucosal fraction of the total intestinal weight. While the mucosal cell mass was increased, the underlying musculature mass was severely decreased. These results infer that possible increases in absorption rates with increased mucosal cell surface area are more than offset by reduced musculature mass. Reduced blood perfusion of the mesenteric vascular bed is suggested as the major cause of the observed reduction in absorption rates. Mucosal cell hypoxia following reduced blood perfusion would also suggest decreased mucosal cell metabolic function. These results led to a consideration of factors other than mucosal cell surface area that could be important in their effects on intestinal drug absorption rates.

The ionic and nutritional milieu of the mucosal cells was deemed to be important for optimal absorption of compounds like salicylate. The effects of mucosal cell viability and cellular metabolic processes on drug absorption rates and the possibility of co-transport of drug molecules were investigated. Glucose, fructose and 3-O-methylglucose were chosen as metabolic and transport stimulants, while acetazolamide, probenecid, ouabain, phlorizin, and dinitrophenol were selected as metabolic and transport inhibitors. Results showed that the absorption rates of salicylate were stimulated in the presence of the sugars but that these rates were not depressed in the presence of the metabolic inhibitors used. The results are interpreted as inferring that salicylate absorption is not directly linked to an active transport process but is readily influenced by the level of metabolic activity or the nutritional state of the intestinal mucosal cells.

A second disease state model, the spontaneously hypertensive rat, was employed to investigate the possible effects of hypertension and vascular disease on the intestinal absorption rates of drugs. Ancillary effects

associated with hypertension involve changes in the total peripheral vascular resistance and probable changes in the volume of blood perfusing the mesenteric vascular bed. Salicylate and antipyrine absorption rates from hypertensive rats were reduced considerably compared to normotensive rats of the same species and sex. Aterio-venous shunting in the mesenteric vascular bed may be the reason for the depressed rates since intestinal mass per unit length studies show no difference in mass between hyper- and normo-tensive animals.

Conclusions drawn from these studies indicate that salicylate and antipyrine appear to be absorbed from the intestinal tracts of rats by passive processes. Further, the nutritional state of the intestinal epithelial tissue is deemed an important factor influencing intestinal drug absorption and is easily modified by fasting or by nutrient supplementation. It was also concluded that certain disease states may seriously alter intestinal drug absorption rates, especially when vascular changes are involved.

Thus it appears evident that mucosal cell viability, cell number and mesenteric blood flow are three major factors influencing the intestinal absorption rates of drugs.

To Kersten, Kira and Elizabeth

with Love

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San Francisco James M. Orr

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF TABLES (CONTINUED)

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

INTRODUCTION

The oral route of drug administration is the most convenient and most frequently used means of drug input into the vascular system of the body. The intestinal barrier that separates the lumen of the intestine from the mesenteric vasculature is a complex structure composed of lipids, lipoproteins, proteins and polysaccharides. This barrier allows the ready passage of nutrients and electrolytes necessary for normal function but acts to prevent the passage of many biological substances that could prove harmful to the host if absorbed. Thus the intestinal membrane is said to be a differentially permeable membrane.

A. Absorption Processes:

The forces that cause transepithelial movement of molecules and ions out of the intestinal tract can be classified as: cellular metabolic forces, electrical gradients, hydrogen ion gradients, net water flux and concentration or activity gradients. Cellular metabolic forces, electrical, and hydrogen ion gradients are generally linked closely together in systems which are involved in the active transport of substances, and net water flux is a consequence of osmotic differences caused by active transport and activity gradients. Therefore two broad categories termed passive transport and energy requiring transport are often used to describe the transmembrane movement of substances.

1. Active Transfer

Sodium, potassium, and chloride ions, several hexoses and some amino acids have been shown to be actively transported (1-4). However only a few drugs such as the anti-tumor agent 5-fluorouracil (5) and some derivatives of nitrogen mustard (6) have been shown to be actively absorbed.

These compounds are probably actively absorbed via the amino acid transport mechanism.

ii. Passive Transfer

The majority of drugs are assumed to be absorbed by an activity driving force which gives rise to passive diffusion and can be approximated by Fick's first law applied to membrane transport as described by Turner et al. (7):

$$\frac{dQ_b}{dt} = D_m A_m R_{m/s} (C_i - C_b) / \Delta X_m \qquad \text{Eq. 1.}$$

where:

Q_b = amount of drug in the blood at any time, t.

D_m = the effective diffusivity of the drug in the intestinal membrane.

A_m = the area of the membrane available for free diffusion.

$R_{m/s}$ = the drug partition coefficient between membrane and bathing solution.

ΔX_m = the thickness of the intestinal membrane.

C_i = free concentration of drug in the intestinal lumen at time, t.

C_b = free concentration of drug in the blood at any time, t.

The blood usually acts as a sink for the drug molecules appearing on the serosal side of the intestine. Therefore, a large concentration gradient may exist across the barrier and C_i may be considered to be much greater than C_b. Thus Eq. 1 can be simplified to:

$$\frac{dQ_b}{dt} = K C_i \qquad \text{Eq. 2}$$

where: K = is the apparent absorption rate constant for a particular drug and encompasses all drug and membrane parameters in Eq. 1.

Equation 2 is a first order rate equation and can be applied to the absorption from the intestine of most drugs in solution. The absorption of a drug from the intestinal tract is largely dependent upon: the physicochemical state of the molecule, the physiologic and metabolic state of the intestinal mucosal cells, the structure of the intestinal epithelium, the drug transit time in the intestine and the rate of blood circulation in the mesenteric vasculature. The factors that can alter the above states and thereby influence drug absorption, are of great and continuing interest in the area of health science that has been described as biopharmaceutics. Biopharmaceutics has been defined (8) as the "study of the factors influencing the bioavailability of a drug in man and animals and the use of this information to optimize pharmacologic or therapeutic activity of drug products in clinical applications".

B. Factors Involved in Drug Absorption

One of the major factors influencing intestinal drug absorption is the physical nature of the drug entity. The relationship between the physicochemical properties of a drug and its gastrointestinal absorption rate has been the subject of intensive investigation since the early 1900's. From the classical studies on membrane permeability, drug molecular size and lipid solubility carried out by Overton (9), Collander and Bärlund (10) and by Höber and Höber (11) to more recent work relating intestinal absorption rates to intestinal hydrogen ion concentration, drug solubility rates and ionization constants, a generalized theory of passive intestinal drug absorption has emerged. Based on the experimen-

tal results of these early investigators and the Danielli-Davson (12) concept that the intestinal membrane is lipoidal, Shore et al. (13) proposed that only the non-ionized species of a drug molecule passed the intestinal barrier, and that gastrointestinal drug absorption could be explained by a pH-partition hypothesis. This hypothesis stated that the intestinal barrier was essentially a lipoidal sieve; that the non-ionized form of the drug was the only permeant species; that the mechanism of movement of drug molecules was by passive diffusion; and that the rate of absorption of a drug molecule was a function of its oil:water partition coefficient. The pH-partition hypothesis explains a large amount of absorption data. However, although some evidence from their steady state experiments did not fit a model consistent with exclusive absorption of non-ionized species, Hogben et al. (14) chose to ignore the possibility of drug ion absorption. Instead these investigators explained the discrepancies by postulating the existence of a region of pH (calculated as pH 5.3) at the luminal border of the intestinal barrier which was lower than the usual pH (about 6.8) found in the bulk luminal fluid of the intestine. This concept of a pH microclimate at the mucosal border became known as the Virtual pH hypothesis.

The rigid concept of the membrane as a lipoid sieve has led to some rather inflexible views regarding absorption. Gibaldi (15) infers that if the intestine can be conceived of as a lipoid sieve, and if hydrophilic pore penetration is of little consequence in drug absorption, then water soluble molecules are unable to cross the barrier unless they are actively transported. There is now a great deal of evidence supporting a mosaic structure for the lipid bilayer membrane. The structure of the membrane is envisaged by many (16) to contain regions occupied by aqueous proteins

of high polarity through which polar molecules and ions can pass with relative ease. The concept of a calculated virtual pH at the intestinal border has come under criticism from Benet as reviewed by Wagner (17) and from Smolen (18) as being thermodynamically unsound. Further, the microclimate has never been determined experimentally and it's existence remains a controversy not yet resolved.

There is, however, a large body of evidence accumulating in the literature that supports the contention that drug ions are absorbed to an appreciable degree. Levine and Pelikan (19) have shown that quaternary ammonium compounds are absorbed from rat intestine and that the amounts absorbed are proportional to the luminal concentrations of the agents. Pindell et al. (20) have shown that tetracycline, a zwitterion at pH 7, was absorbed by a simple diffusion process over a large concentration range. Several workers (21-24) have demonstrated the absorption of salicylate from pH 6.6-7.4 solutions where the drug is greater than 99% ionized. Lanman and associates (25) have published data showing absorption of weak organic acids such as hippuric acid, sulfanilic acid, para aminohippuric acid and phenol red, compounds that are ionized at the pH of the intestinal solution used (pH 7.4). These authors demonstrated that the compounds were absorbed by simple diffusion, and that the rates could be correlated to their chloroform:water partition coefficients, but not to the degree of drug ionization. Lanman et al. (25) concluded that these organic acids were absorbed in their anionic forms, probably through lipoidal regions in the intestinal barrier. Another interpretation of their data and the salicylate data of Schanker et al. (26) could be that the ionized compounds also diffuse through aqueous channels or pores in the intestinal barrier as suggested by Crone and Keen (27) for

the pyridinium aldoximes.

Wagner and Sedman (28) have recently derived a set of equations based on extraction theory which describe gastrointestinal absorption rates as a function of the pH of the gastrointestinal contents and time. Results of their treatment of literature data suggest that the aqueous diffusion layer may not be rate limiting for monomeric drug molecules. Instead, transfer out of the in vivo membrane may be the rate determining step in the absorption process. These workers base their hypothesis on the premise that the membrane concentration of drug is a function of the luminal drug concentration and the drug partition coefficient between the luminal fluid and the membrane. Their treatment is not limited to a concept where only the non-ionized species is assumed to traverse the barrier and it can be readily applied to data describing drug ion absorption. Wagner and Sedman state that their equations "... quantitate the pH-partition hypothesis and explain most, if not all, related observed data in the literature."

Benet et al. (21) have studied in vitro drug absorption from pH 7.4 buffers in which the sodium ion was replaced by potassium ion. These workers showed that a partial or complete replacement of Na^+ caused a 60—80% decrease in the rate of absorption of ionized salicylate but only a 15-30% reduction in the absorption rate for non-ionized acetanilide. Increased K^+ concentration has been shown to cause swelling of intestinal epithelial tissue via water uptake (29). Mayersohn and Gibaldi (22) found that the tissue swelling produced in their studies by replacement of Na^+ with K^+ inhibited solute uptake and that molecular size was an important factor in the degree of solute transfer inhibition. They found a linear correlation between drug molecular weight and degree of transfer

inhibition. As an explanation for these phenomena, it was proposed by Benet et al. (21) that the swelling caused by the increased K^+ closed off the tight junctions between columnar cells in the mucosal epithelium. Frömter and Diamond (30) have proposed that a wide variety of molecules and ions are transferred across epithelial barriers via aqueous or polar pores. They define the zonula occludens or tight junctions, that have been shown to exist between epithelial cells, as being the major anatomical sites for these postulated pores through the epithelium. Results in other tissues have shown similar effects with potassium ion. Clausen (31) studied sugar transport in muscle tissue under the influence of potassium and osmolar concentration changes. He noted that in K^+ rich media, glucose uptake was inhibited and that these media brought about a rapid increase in cell volume. Clausen also found that swelling due to K^+ could be suppressed by increasing the osmolarity of the medium.

In the early 1960's, Nogami and Matsuzawa (32,33) studied the in vitro intestinal absorption of acidic and basic drugs. They employed salicylic acid and aminopyrine as model drugs. On the basis of their results, these authors concluded that the model drugs penetrated the in vitro rat intestine by a simple diffusion process, and that aminopyrine penetrated the intestinal membrane in the non-ionized form, while salicylic acid penetrated the membrane as both the ionized and non-ionized species. The in vivo results of Siurala et al. (34,35) and others (23, 24) support the cautionary statements of Smyth and Whittam (4) that "... it would be wise to consider individual cases in detail before it is too readily assumed that non-ionic diffusion is the force responsible for movement of a particular substance."

C. Methods Used to Study Intestinal Absorption

A wide variety of in vitro and in vivo methods have been employed to study the extent and rate of absorption of drugs from the intestinal tract as has been extensively reviewed by Parsons (36).

i. In Vitro Models

In vitro methods generally employ either a sac of intestine into which the drug solution is placed, or a loop of intestine through which the drug solution may be perfused. The sacs or loops may be used in a normal orientation or they may be everted. Absorption is determined directly as the appearance of drug in the external or serosal solution. One drawback of these systems is that the drug must traverse the whole of the intestinal wall from mucosal to serosal surface. The rates of absorption obtained from these systems are not physiologic because of this distance that the drug must travel and the absence of a mesenteric blood supply. If measurable rates are obtained for a drug using this system, it could be argued that absorption should be much improved in the in vivo environment. This, however, has not always been the case. Anderson and Bowtle (37) have recently shown that macroanionic sulfated polysaccharides with molecular weights of about 25,000 were absorbed across the in vitro rat and guinea pig intestine. Other studies (38) have shown that inulin, a polysaccharide of molecular weight 5000, can diffuse across the in vitro everted sac preparation at measurable rates. However, inulin was almost totally non-absorbed in an in vivo study in rats reported by Miller and Schedl (39). These workers and others (40) have also demonstrated that polyethylene glycol (molecular weight of 4000) is not absorbed from the rat intestine in vivo.

Thus a major disadvantage of the in vitro intestinal preparation is

its possible exaggerated permeability. The usefulness of these in vitro preparations lies in preliminary drug absorption screening procedures and for rank order correlations of chemical modifications of a given drug entity or of various drug - excipient complexes found in dosage forms.

ii. Chronic In Vivo Models

Several studies that could truly be termed in vivo, involving both human and animal subjects have been reported, in which a multilumen catheter was introduced via the naso-pharynx route (41,42). With this type of system, a portion of the intestinal tract can be isolated by inflation of balloon collars in the catheter. This isolated segment of the tract may then be perfused with a drug solution and an appropriate volume marker. Multi-lumen intestinal catheters have been successfully used in human volunteers by Siurala and co-workers (34,35) to study the absorption of warfarin and acetylsalicylic acid from the stomach and small intestine. They noted that these drugs were absorbed faster from the intestine than the stomach even though the intestine was at a pH where the ionized form of the drug was the predominant species. A similar system was used by Rohde and Chen (43) to study the intestinal permeability to sugars and urea in human patients recovering from asian cholera and in dogs with experimental cholera.

An in vivo experimental model for the study of gastrointestinal drug absorption in conscious, restrained, rhesus monkeys has been reported by Nayak and Benet (44,45). The model design is similar in concept to the multi-lumen catheter arrangement described above with some modifications. Instead of a nasal tube, these workers employ two permanent cannulae, one in the stomach, and one in the small intestine, which allow the introduc-

tion of catheters for isolation of various gastrointestinal segments; for perfusion of drug solutions; and for sampling of the luminal contents. Several disadvantages or criticisms of surgically prepared anesthetized animals as models have been avoided. Surgical placement of intra-vascular catheters and intra-gastrointestinal cannulae are effected well in advance of any experiment. This allows time for wound healing, and recovery from surgical shock. Urine and blood samples are taken for periodic analysis to ensure that the animals remain in good health. The animals are non-anesthetized and sit erect rather than supine during experiments. The model allows repeated absorption studies to be carried out in a single animal providing consistent results (44) and the advantage of using each animal as its own control.

iii. Acute *In Vivo* Models (*In Situ*)

Several methods of measuring the *in vivo* intestinal absorption of drugs in experimental animals have been described (19,23,26). These methods are also indirect in that the disappearance of drug from the lumen of the gut is taken to be a measure of drug absorption. These techniques are less physiologic than the methods described above but are more physiologic than *in vitro* techniques. An intact blood supply is maintained although the animal is anesthetized, surgically prepared and supine during the experiment. As in the *in vitro* systems, the intestine may be divided into sacs containing drug solution, or an *in situ* loop of intestine may be perfused with an appropriate buffer solution containing the drug under study. Stomach emptying, drug dissolution, and the presence of food in the g.i. tract are variables that can be avoided with these preparations. Support for the usefulness of this type of system comes from the recent work of Perrier and Gibaldi (46). They studied a series of antibiotics,

comparing the absorption rates from *in vitro* rat intestine with *in situ* intestinal loops and with human g.i. absorption data. Results from the *in vitro* everted rat intestines did not always agree with data from the *in situ* rat intestinal loops. However, these workers did find a rank order correlation between rates from the *in situ* loops in rats and the rates from the human studies.

Ochsenfahrt and Winne (47) have described an *in vivo* intestinal perfusion system in which both the disappearance of drug from the gut loop and the appearance of the drug in the cannulated mesenteric vein are measured. The difference between these two values is reported to be a measure of the permeability of the epithelial membrane, from the lumen to the interstitial spaces, including the unstirred water layer at the luminal border. The above methods of studying intestinal transfer of drugs should be termed *in situ* (in place) rather than *in vivo* since the animals used are not in their normal environment during experiments.

D. Project Goals

The purpose of this work is to study the effects of various factors on the intestinal absorption of two model drugs in an attempt to further define the mechanisms by which drugs in general cross the intestinal barrier.

i. Model Drugs

Two model drugs will be used in these studies. Salicylic acid is chosen as an acidic drug, greater than 99% ionized at the pH of the blood. Antipyrine will be used as a representative basic, non-ionized drug. Both these drugs have been examined by a number of investigators (13-15,21-24) whose results are the bases for several theories of drug absorption.

ii. Experimental Model

The *in situ*, recirculating intestinal perfusion system described by Schanker *et al*. (26) will be used for all absorption experiments. Male Sprague-Dawley rats will be used throughout the experiments except where otherwise noted. In order to eliminate as many variables as possible in the model, drug absorption will be measured from solutions, buffered at the same pH as plasma (pH 7.4) which should eliminate dissolution rate and pH gradient problems. The perfusion buffer chosen is Krebs-Henseleit pH 7.4 buffer (Table A-1, Appendix). Absorption rates will be measured as the rate of disappearance of drug from the perfusion solution. This method has been used by several researchers (13,14,19, 22-24) to obtain values for the apparent absorption rates of drugs.

iii. Factors to be Examined

a. Absorptive Surface Area

(1.) Fasting: According to Fick's principles (Eq. 1), surface area for absorption has an important influence on the rate of drug absorption. Fasting has been shown to reduce mucosal cell number in the intestines of animals and to reduce the intestinal absorption rates of several drugs (23). The effects of total food withdrawal on total body weight, mucosal cell mass, and on absorption rates of salicylate and antipyrine will be examined in a series of experiments.

(2.) Disease states: Absorptive surface area can be further investigated by the use of rats with experimentally induced diabetes. Evidence in the literature has shown that mucosal cell number and size are significantly increased in the diabetic state. It is predicted that if mucosal cell number and hence surface area is an important factor in drug absorption, the rates of absorption of the two model drugs should be decreased with fasting and increased with diabetes.

b. Cell Viability and Transport Mechanisms

Previous experiments (21,22), in which buffer constituents were altered, showed a marked influence on drug absorption rates in vitro. When Na^+ was replaced by K^+ in the buffer, in vitro drug transfer rates for salicylate and acetanilide were significantly reduced. Preliminary in vivo experiments were carried out in our laboratory (Table A-2, Appendix) which showed similar effects, with the absorption rates of salicylate and acetanilide being substantially reduced when Na^+ was replaced by K^+ in the perfusion buffer. These effects were attributed to cell swelling in the presence of the K^+ rich medium. It would seem pertinent to explore the effects of other factors in the environment of the mucosal cells which may affect the absorption of drugs. Experiments will be carried out to examine the effects on drug absorption of the nutritional and metabolic state of the intestinal tissue as well as the possibility of involvement of active transport processes in drug absorption mechanisms. Metabolized and non-metabolized sugars will be employed as possible stimulants and certain general and specific inhibitors such as dinitrophenol, ouabain, phlorizin, acetazolamide, and probenecid will be used to interfere with metabolic and active transport processes in the intestine during drug perfusion studies.

c. Intestinal Blood Flow

The influence of blood flow on drug absorption rate has been demonstrated in rats by Ochsenfahrt and Winne (24) who used a pump to produce various blood flow rates through the mesenteric vascular system. Preliminary stop-flow studies in our laboratory, involving stopping all circulation, showed a rapid and marked decrease in drug absorption rates (Table A-3, Appendix) dramatically demonstrating the close association of ab-

sorption rate and blood flow for salicylate and antipyrine. Since surgery causes shock and blood flow changes, it is important to find a model of abnormal intestinal blood-flow rates. Spontaneously hypertensive rats (48) may provide such a model. Hypertension is associated with increased peripheral vascular resistance and the possibility of vascular arteriovenous shunting accompanying the observed microangiopathy (48,49). Up to the present, drug absorption rates in patients have not been compared with normal persons in order to discern any effects of the disease on the absorption process (50). For these reasons, experiments will be performed to study the absorption of salicylate and antipyrine in normotensive and spontaneously hypertensive female Wistar rats.

It is hoped that this series of experiments will provide further understanding of drug absorption processes.

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CHAPTER 2

THE EFFECT OF FASTING ON THE RATE OF INTESTINAL DRUG ABSORPTION IN RATS

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Introduction

It has become increasingly evident that the small intestine is the major site of absorption for most orally administered drugs whether these drugs are ionized or non-ionized (1,2). Despite an increased understanding of the structure and function of the various cellular and subcellular units that comprise the intestine as an organ, many details regarding the effects of nutritional lack and starvation on the intestinal absorption process are still unknown. Several studies have been carried out showing that fasting alters the intestinal absorption patterns of compounds such as D-glucose, L-histidine, methionine and proline (3-5). However, few reports have been published regarding the effects of fasting or nutritional lack on the intestinal absorption of drugs. Doluisio and coworkers (6) showed that the absorption rates of certain drugs were adversely affected by fasting for periods of 24 hours or longer. Hopper et al. (7) demonstrated that fasting invoked a continual decrease in the number of cells in the germinal crypts of the intestinal epithelium. They noted that this change was apparent as early as the end of the first day of fasting and by the end of the tenth day, the crypt cell population was one-third that of the control rats. Similar results were obtained by McManus and Isselbacher (8) who employed an overnight fast to compare the weights of fed and fasted rats. They demonstrated a difference in weight of intestine, with the fed rats having heavier intestines than the fasted rats, specifically a greater mucosal cell mass. In as much as mucosal cells have a turnover time of about 2 days in mature rats, these findings

and those of Doluisio would seem to indicate that even short periods of fasting would produce a decrease in absorption rates of drugs if mucosal cell number were an important factor in absorption.

In order to attempt to delineate the effects of fasting on drug absorption from the intestine, experiments were carried out in our laboratory using an *in vivo* perfusion system and employing salicylic acid and antipyrine as model drugs.

Experimental:

i. Materials: All chemicals and buffer constituents used were of reagent grade. The drug solutions used for perfusion consisted of 17 mM (2.3 mg/ml) salicylic acid or 5.3 mM (1.0 mg/ml) antipyrine made up in glucose-free Krebs-Henseleit buffer (K-H buffer) adjusted to pH 7.4 (9). See also Table A-1, Appendix. These concentrations are the same as those used in other studies and were chosen for purposes of comparison. The perfusion solutions were maintained at 37°C by a constant temperature bath (Haake, type R21). Determinations of pH were carried out on a pH meter (Beckman, Research Model).

ii. Surgical Procedures: Male Sprague-Dawley rats (Simonsen) initially weighing 300-350 grams were used in all the fasting studies. The animals were properly housed, two per wire mesh cage (to prevent coprophagy) in rooms with adequate ventilation and light and were maintained on Purina rat chow (Ralston-Purina Co.). Water was always allowed *ad libitum*. For the fasting studies, rats were fasted for various periods of time beginning at the same time of day in each experiment since the time of day of food withdrawal has been shown to have an effect on the actual length of fasting time for nocturnal animals (8,10).

After a predetermined period of fasting, the animals were weighed,

and a 40 mg/Kg dose of sodium pentobarbital (Nembutal Sodium, Abbott) was injected intraperitoneally to produce anesthesia. A laparotomy following the linea alba was performed and the small intestine was cannulated with polyethelene tubing (PE 320, Clay-Adams) at the ligament of Treitz and at a point 10 cm. proximal to the ileocecal junction so as to form an in situ loop of intestine suitable for perfusion. When the cannulae were inserted, care was taken in placing the ligatures between the arcuate blood vessels so as to maintain intact the blood circulation in the intestinal loop. This was ascertained by the observed normal pink color of the intestine and regular arterial pulsations. The segment of intestine was then washed free of chyme using 50 ml of warmed (37°C) K-H buffer. The wash buffer was gently expelled using a bubble of air. The abdominal incision was then closed using clamps and covered with a surgical pad moistened with buffer. The cannulae were attached to an inlet and outlet of a reservoir containing the drug solution.

iii. In Vivo Perfusion: Perfusion of the loop at a rate of 1.5 ml/min was carried out in the direction of normal g.i. flow by means of a peristaltic pump (Harvard Instrument Co.). The recirculation system used was similar to that described by Schanker and coworkers (11) and is schematically depicted in Figure 1. The reservoir contained 50 ml of heated, stirred and gassed (95% O_2, 5% CO_2) K-H buffer (pH 7.4) at the appropriate drug concentration. Body temperature was maintained via a small incandescent table lamp, and was monitored by means of a calibrated thermister probe inserted rectally. Figures A-1 and A-2 in the Appendix show the thermister thermometer schematic and a calibration curve relating resistance to temperature. Samples for assay were removed from the reservoir at fixed time periods during the experiment. Intraluminal pressure was

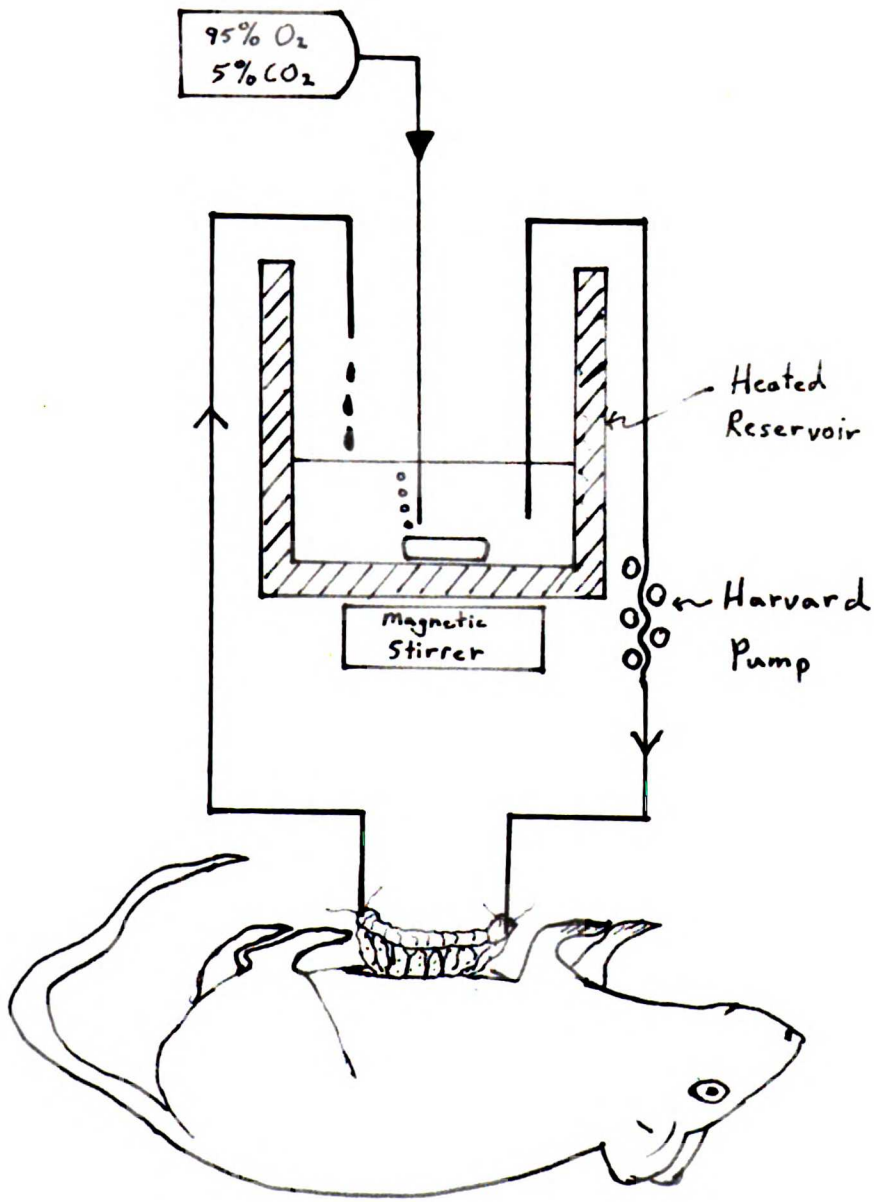

Figure 1. Schematic Diagram of Recirculating In Vivo Intestinal Perfusion System.

monitored using a simple T-tube manometer.

At the end of each experiment, the animal was sacrificed and the intestinal loop was carefully separated from the mesentery. The length of the intestinal loop was measured with the loop suspended vertically and a standard 20 gram weight attached to the lower end. The loop was weighed wet and then dried to a constant dry-weight in an 80°C drying oven so as to obtain wet and dry intestinal weights per unit length.

In experiments where blood samples were required, the left femoral vein was exposed. A polyethylene cannula (PE 50), filled with heparinized normal saline (5 units heparin/ml saline) to prevent clotting, was inserted far enough into the femoral vein to reach the common iliac vein. In some instances, the right external jugular vein was also exposed and a cannula inserted until the tip reached the right precava region.

Following a series of fasting experiments the mucosa was separated from the rest of the intestinal mass. This was accomplished by slitting the loop longitudinally, pinning the flattened intestine on a parafilm strip and removing the mucosal fraction by scraping with a microscope slide as described by Schedl and Wilson (12).

In the initial series of experiments, trace ^{3}H-Inulin (0.034 μCi/ml) was added as a volume marker. Miller and Schedl (13) and Wingate *et al.* (14) have shown that this polysaccharide (molecular weight of 5000) is not absorbed from the g.i. tract. Changes in the concentration of the volume marker can be used to correct the intestinal perfusate drug concentration for net water flux into or out of the intestine using the equation:

$$C_{corr} = C_{obs} \times \frac{C_{mi}}{C_{mt}} \qquad \text{Eq. 1}$$

where:

C_{corr} = corrected reservoir drug concentration at any time, t.

C_{obs} = observed reservoir drug concentration at any time, t.

C_{mi} = initial concentration of volume marker.

C_{mt} = concentration of volume marker at any time, t.

Since our drug-buffer perfusate was in the osmolar range of 310-330 mOsm/L, it was felt that net water flux would be minimal. A typical plot of drug concentration vs time, uncorrected and corrected for water flux, is shown in Figure A-3 of the Appendix. The slopes were not significantly different, therefore net water flux was taken to be negligible in its effect in these experiments and subsequently a volume marker was only employed occasionally to check for water fluxes.

iv. Assays: Salicylic acid was assayed either spectrophotofluorometrically on a Aminco-Bowman spectrophotofluorometer (SPF) (American Instrument Co.) or by liquid scintillation spectrometry.

a. Method I for Salicylate Assay: To an aliquot of drug solution, a 0.5 ml volume of 5% $KHSO_4$ was added to adjust the pH to 2. Exactly 5 ml of cold anhydrous ether was added to each sample in an ice bath. The samples were then mixed on a vibrating mixer for 1 minute and subsequently centrifuged for 10 minutes @ 2000 rpm. Exactly 1.0 ml volumes of the ether phase were transferred to tubes containing 5 ml of pH 7 phosphate buffer. The samples were shaken again, centrifuged at 2000 rpm for 10 min., and the ether layer removed by suction and by bubbling nitrogen through the sample. The aqueous samples were then placed in the SPF and the fluorescence recorded. The excitation and emission wavelengths were

established for each assay. This extraction procedure is reported to exclude glucuronide metabolites of salicylic acid (15). Standards were prepared and treated in the same manner as the samples. An example of a standard curve of salicylate is shown in Figure A-4 in the Appendix.

b. Method II for Salicylic Acid: Salicylic-7-^{14}C acid with a specific activity of 4.888 μCi per mMole was obtained from New England Nuclear (NEN) Corp, Boston, Mass. (Lot #506-076). Purity was established by paper chromatography followed by counting in a windowless strip counter. The appearance of a single peak was taken as proof of chemical purity. Aliquots of the ^{14}C-labelled salicylic acid were added to non-radioactive 17mM salicylate solutions and experiments carried out as before. The samples were then added to 15 ml volumes of Aquasol liquid scintillation cocktail (NEN) and the radioactivity determined in a liquid scintillation spectrometer (Packard Tricarb-Packard Instrument Corp., Model 3375). Automatic external standardization was used to determine counting efficiency (Figure A-5, Appendix). From the literature regarding intestinal microfloral metabolism of substituted benzoic acid acids (16,17) there appears to be no measurable metabolism of salicylic acid by the microflora in the gastrointestinal tract. Although Schachter et al. (18) found some salicyl glucuronide formation in gut tissue, the amount corresponded only to about 2% of a 1 mM luminal salicylate concentration. Kunze et al. (19) detected no metabolism of salicylic acid in the rat intestine during 60 minute perfusion experiments. Several experiments were carried out using both the fluorometric method of assay and the radioactivity assay. The methods were found to yield equivalent results. Thus, when the isotope of salicylic acid became available, all subsequent assays were done by the radioactivity method.

c. Method for Antipyrine: The method of Brodie et al. (20) for the assay of antipyrine is very tedious, therefore antipyrine-N-methyl-^{14}C with a specific activity of 10 μCi/mM was obtained from ICN, Irvine, Ca. (Lot #566262). Spectral purity was ascertained by T.L.C. using chloroform:ether:methanol (85:15:4). A single sharp peak was obtained by radioscan with an r_f value corresponding to pure antipyrine (Merck & Co., Lot #62884). Suitable aliquots of this isotope were added to the 5.3 mM solutions of antipyrine in K-H buffer used as perfusion solutions. Assay was by radioactivity measurement of the ^{14}C label in Aquasol scintillation cocktail (NEN Corp.) as described for ^{14}C-salicylate.

Plasma samples were assayed in a similar manner. As a check for metabolites, the method of Brodie (20) was adopted where duplicate plasma samples were alkalinized, extracted with ethylene dichloride and recounted. No change was noted in the slope of the plasma decay curve and this was taken to indicate that there was no appreciable formation of the major hydroxylate metabolites during the time of the experiment. Apparent absorption rate constants were calculated from the drug concentration versus time data by means of a weighted log-linear least squares method.

Results and Discussion: Typical changes in drug concentration over a 90 minute time period after various fasting times are shown in Figures 2 and 3. These figures depict concentrations from a single experiment at each fasting time, which is indicated as fasting time in hours to the right of each curve. The slopes can be seen to decrease steadily as the length of fast increases.

The effects of fasting on the average apparent absorption rate constants per unit length ($\bar{k}ap$) for salicylate and antipyrine are shown in Table I. There is a continual decrease in the apparent drug absorption

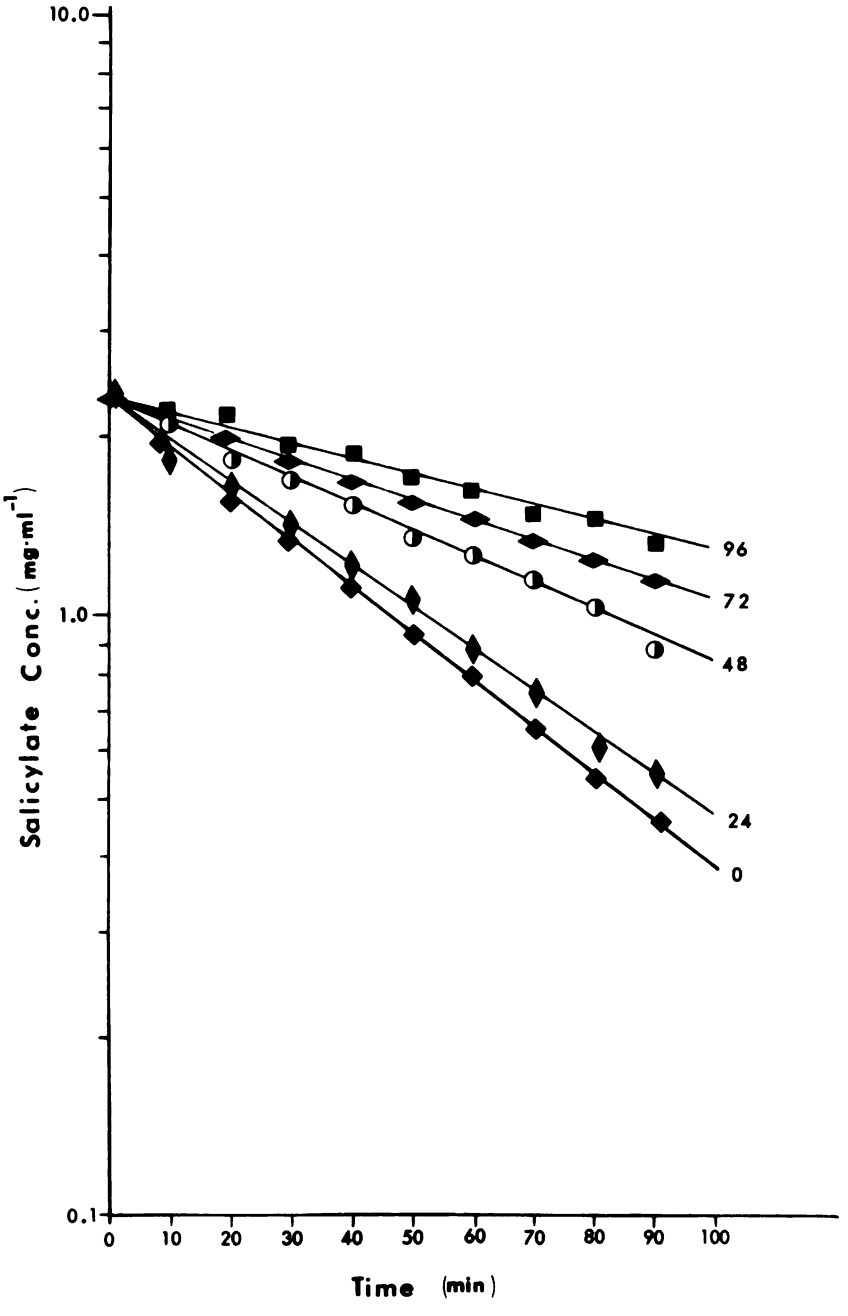

Figure 2. Effect of Length of Fast on the Intestinal Perfusate Concentration of Salicylate.

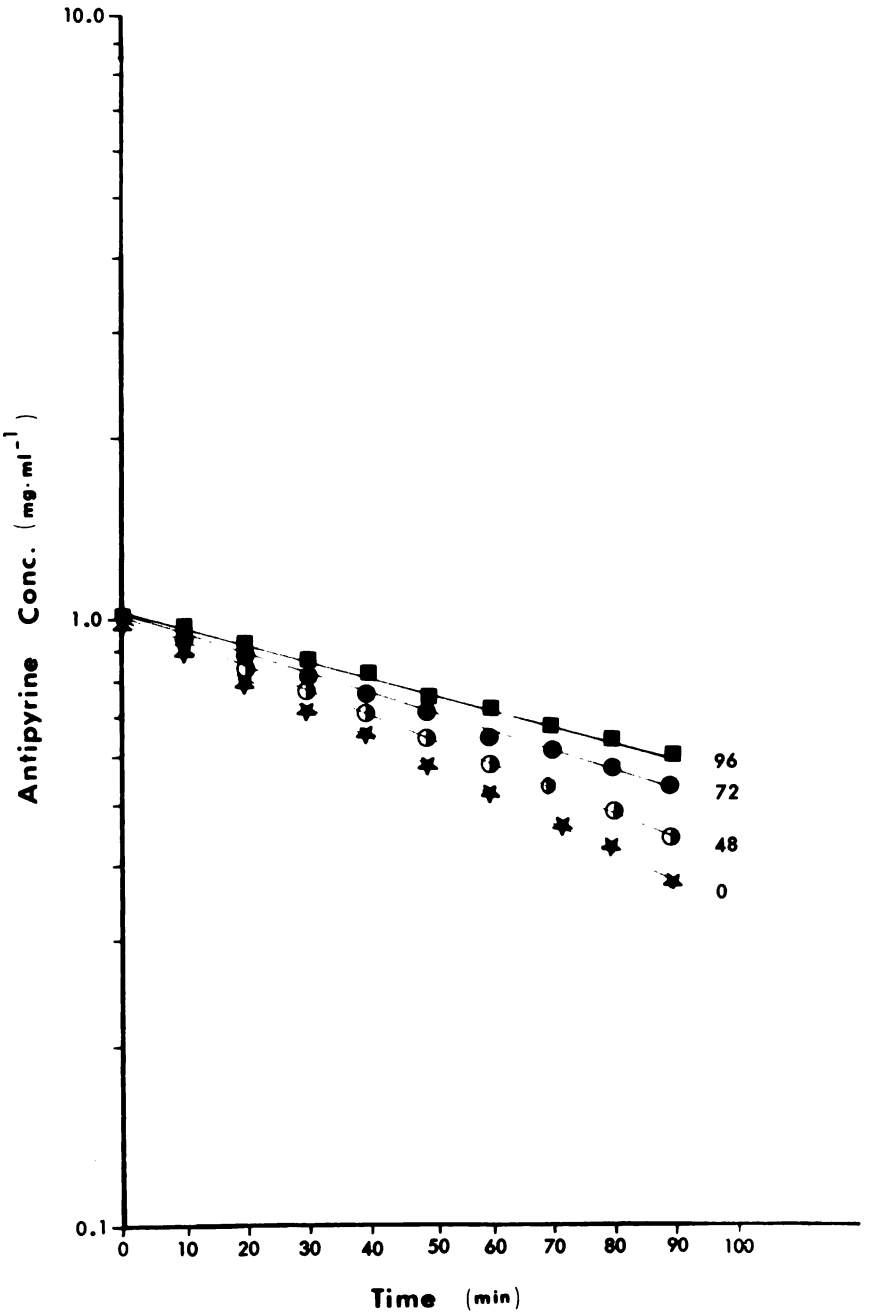

Figure 3. Effect of Length of Fast on the Intestinal Perfusate Concentration of Antipyrine.

Table I

Effect of Fasting on Intestinal Drug Absorption Rate Constants in Rats

Drug Studied: Salicylate (17mM)								
Fasting time (hr)	0	12	24	36	48	60	72	96
Number of studies	7	6	7	5	8	5	4	3
Absorption Rate Constants	2.68	2.11	1.42	1.28	1.29	1.04	0.91	0.88
$\overline{kap}$ ($min^{-1} \cdot cm^{-1} \cdot 10^4$)	(0.68)*	(0.49)	(0.27)	(0.57)	(0.34)	(0.39)	(0.21)	(0.17)
Fraction of Non-Fasted Rate	1.0	.89	.60	.54	.54	.44	.38	.37

Drug Studied: Antipyrine (5.3mM)					
Fasting time (hr)	0	24	48	72	96
Number of studies	7	6	4	3	3
Absorption Rate Constants	1.40	1.32	1.27	1.18	1.16
$\overline{kap}$ ($min^{-1} \cdot cm^{-1} \cdot 10^4$)	(0.10)	(0.22)	(0.30)	(0.02)	(0.04)
Fraction of Non-Fasted Rate	1.0	.94	.91	.84	.83

* Values in parentheses are standard deviations

rates as the fasting interval is increased to 96 hours. The rates for salicylate decreased by 63% over 96 hours of fasting while the rates for antipyrine decreased by only 14% over the same period of fast.

The results observed for salicylate are similar to those reported by Doluisio and coworkers (6). Figures 4 and 5 are plots of the $\bar{k}ap$ values (listed in Table I) vs. fasting time. The bars represent the standard deviations from the averages. Figure 4 shows that the apparent absorption rate for salicylate initially decreases rapidly with fasting and then tends to level off as the fasting time reaches 96 hours. The data depicted in Figure 5 indicate that antipyrine rates are less affected by fasting than are salicylate rates.

The observation that fasting affects the salicylate rates more severely than the antipyrine rates can be demonstrated more clearly by converting the rates to intestinal clearances as depicted in Figure 6. The experimental results show that salicylate clearance was greatly reduced (68%) by 96 hr of fasting while the non-ionized antipyrine clearance was only reduced by 14%. The differences in the effects of fasting on salicylate and antipyrine absorption may be taken as showing that the mucosal cell number or surface area is not as critical for antipyrine absorption as it is for salicylate absorption.

This suggestion is also reflected in previous work (21) which indicated that transfer of antipyrine across the <u>in vitro</u> rat intestine yielded equivalent rates for mucosal to serosal and serosal to mucosal fluxes ($J_{m\to s} = J_{s\to m}$). On the other hand the mucosal to serosal flux for salicylate was about two times the flux in the opposite direction ($J_{m\to s} = 2\ J_{s\to m}$). These workers suggested that the difference in surface area between the mucosal and serosal membranes appeared not to be impor-

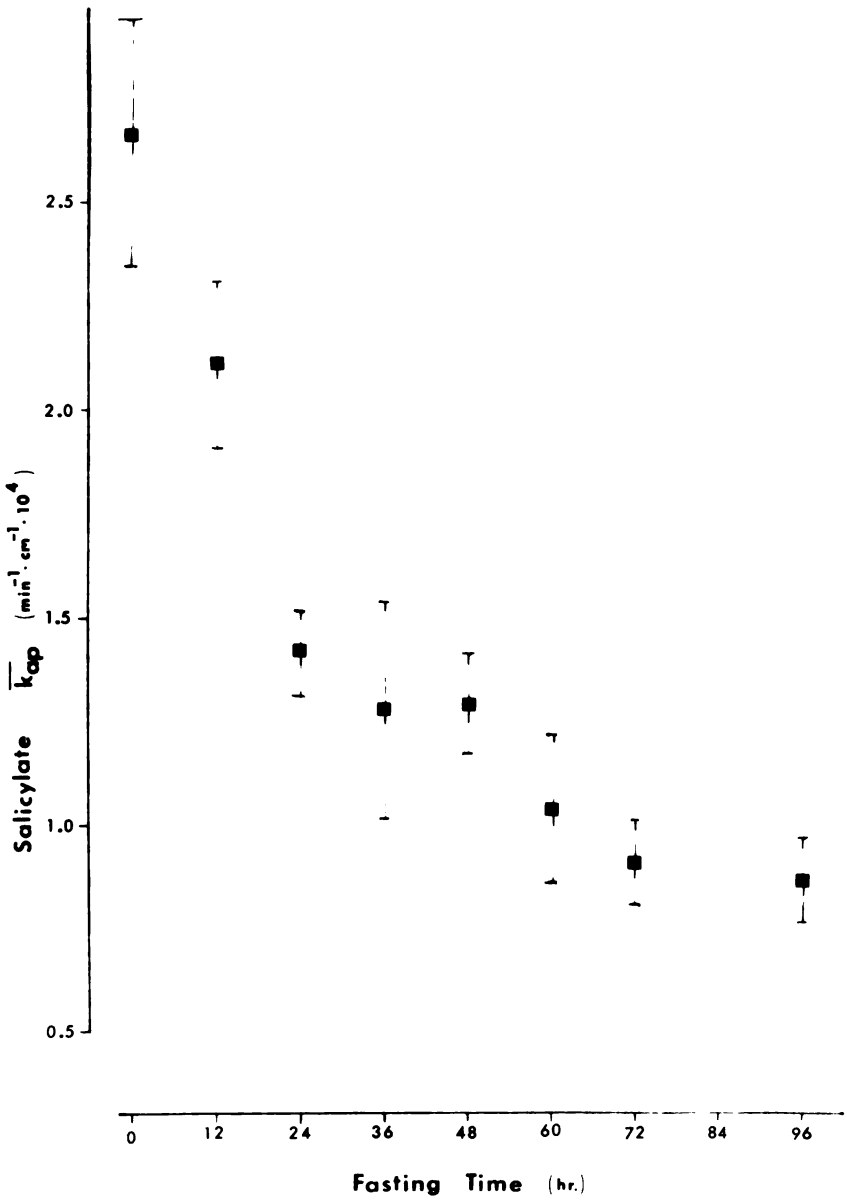

Figure 4. Effect of Fasting on the Average Apparent Intestinal Absorption Rate Constant for Salicylate.

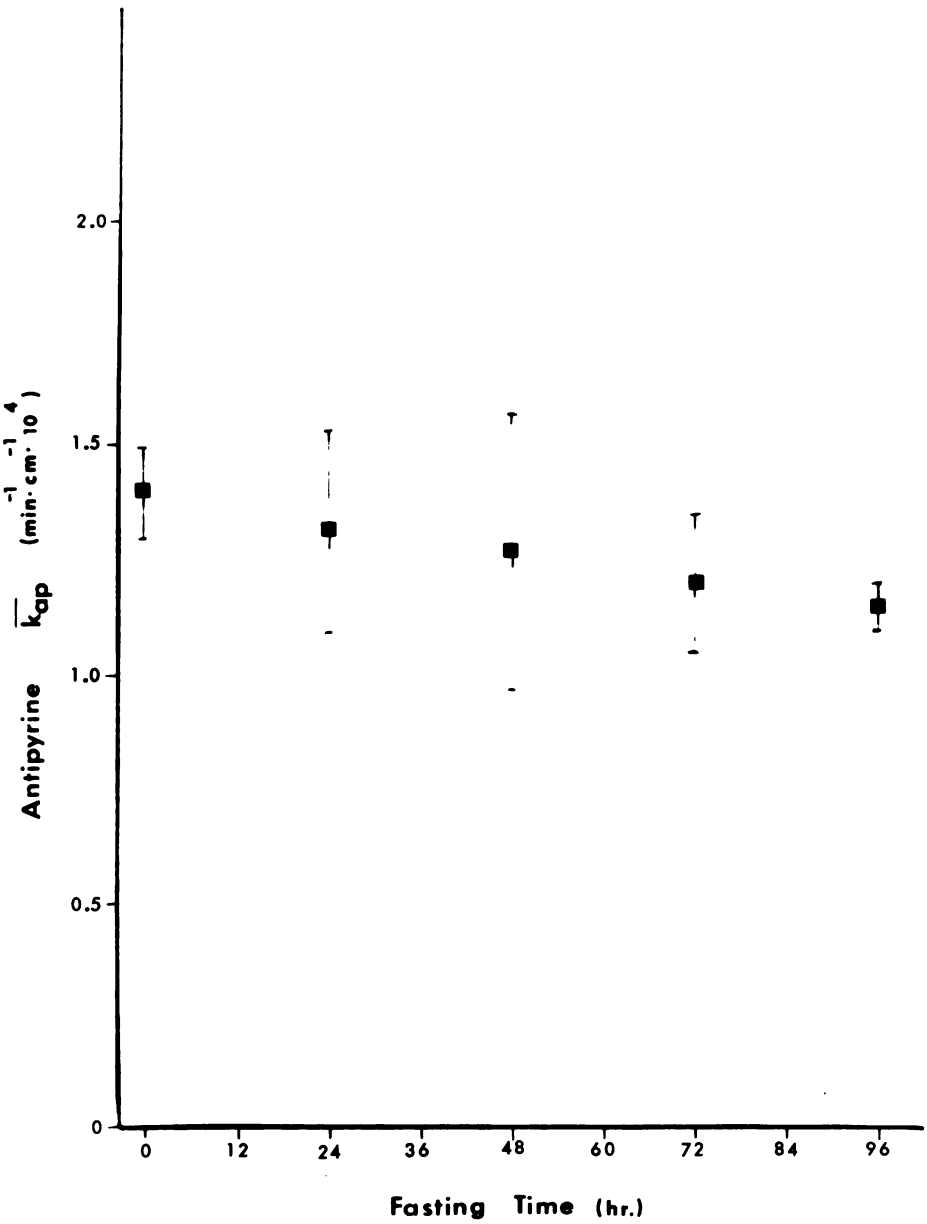

Figure 5. Effect of Fasting on the Average Apparent Intestinal Absorption Rate Constant for Antipyrine.

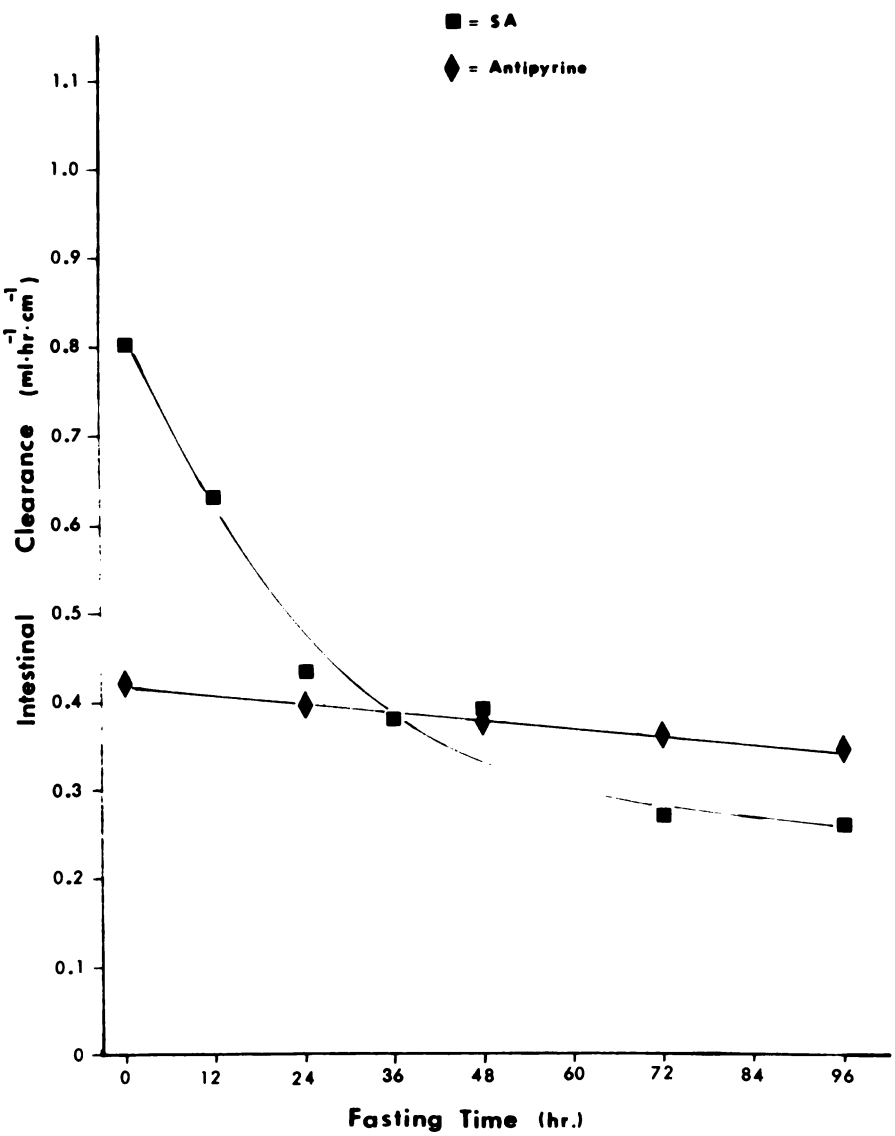

Figure 6. Effect of Fasting on the Apparent Intestinal Clearance of Salicylate and Antipyrine.

tant in the case of antipyrine and thus the rate limiting step in intestinal transfer of non-ionized drugs may be passage through the membrane barrier rather than passage into or out of the membrane.

As depicted in Fig. 6 the clearance values after 24 hours of fasting are 0.43 and 0.39 ml/hr cm for salicylate and antipyrine. These are about 3 to 5 times the in vitro values of 0.08 ml/hr cm and 0.172 ml/hr cm reported by Mayersohn et al (22) for salicylate and antipyrine, respectively. The fact that the in vitro values are lower than the in vivo values is probably due in part to the extra tissue that must be traversed by the drug going through the in vitro preparations, and in part to the loss of all effects of mesenteric blood flow. These suppositions are lent credence by the work of Ochsenfahrt & Winne (23) who demonstrated that a 70% reduction in mesenteric blood flow caused a 45-55% reduction in the in vivo rates of absorption of both salicylate and antipyrine.

The effect of fasting on intestinal mass is shown in Table II and in Figure 7. Intestinal mass is shown to decrease progressively as fasting is prolonged. These data agree with the published reports of Fabry (24) and others (3,8,25) who demonstrated that fasting produces a decrease in intestinal mass.

Newey & associates (3) compared fed and 72 hr fasted rats with respect to their abilities to absorb sugars and amino acids. They found that the transfer rates of glucose and amino acids were decreased in fasted animals. These workers also noted that the average body weight decreased by 20%, and that the intestinal segment average weight decreased by 28% after 72 hours of fasting. The percent decrease in intestinal weight was greater than the percent total body weight loss, which is consistent with the idea that the high rate of proliferation of the germinal

Table II

Intestinal Mass Changes with Fasting

Fasting time (hr)	0 (control)	12	24	36	48	60	72	96
Number of rats	14	6	13	5	12	5	7	6
Average Weight/length (mg/cm)	16.66 (2.05)*	15.45 (1.5)	13.73 (1.26)	13.01 (1.37)	11.47 (1.85)	11.48 (.43)	10.38 (.64)	10.16 (1.88)
% Change from Control	0	7.3	17.6	21.9	31.2	31.1	37.7	39.0

* Values in parentheses are standard deviations

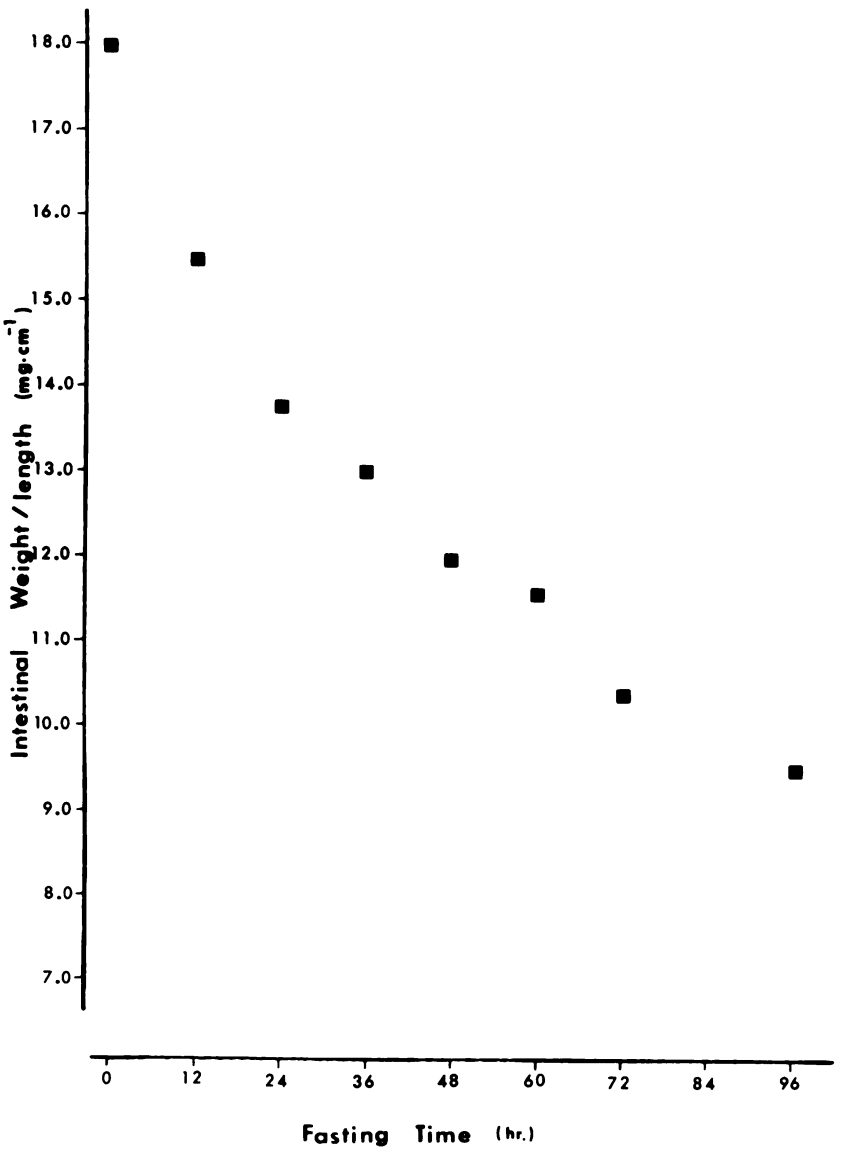

Figure 7. Effect of Fasting on the Dry Intestinal Weight Per Unit Length of Perfused Loop.

cells in the epithelial crypts is rapidly and adversely affected by the nutrient lack caused by fasting. Although McManus and Isselbacher (8) showed no difference in body weight, intestinal length, or lipid content between control and 24 hr fasted rats, they did show a difference in intestinal weight and a difference in DNA content, with lower values noted for fasted rats as compared to control rats. Based on an assumed constancy of cellular DNA, they interpret their findings to indicate that the fasted rats had decreased numbers of mucosal cells. Their results agree with those of Brown (25) and others (7,27), who showed that there was reduced cellular proliferation, shortened villi and a reduction in mucosal cell population with fasting. Hopper et al. (7) noted that with a cell turnover time of 2.1 days for mucosal cells in mature rats (28,29), even short periods of fasting could be considered to have detrimental effects on mucosal cell number.

Since both the intestinal mucosal mass and the apparent absorption rates for salicylate are decreasing as a function of fasting time, it would appear that intestinal mucosal cell mass may be an important factor in the absorption mechanism for drug ions like salicylate. A plot of the averaged apparent absorption rates per unit length for salicylate versus intestinal weight/length at several fasting times is shown in Figure 8. There is a strong positive correlation (0.941) indicating that intestinal mucosal cell number may indeed be an important factor in drug absorption as was suggested by Newey and associates (3) for the absorption of amino acids.

A number of reports have noted that cytotoxic agents such as mechlorethamine (nitrogen mustard) (30), actinomycin D (31), methotrexate (32), as well as antimitotic agents such as colchicine (33,34), have been

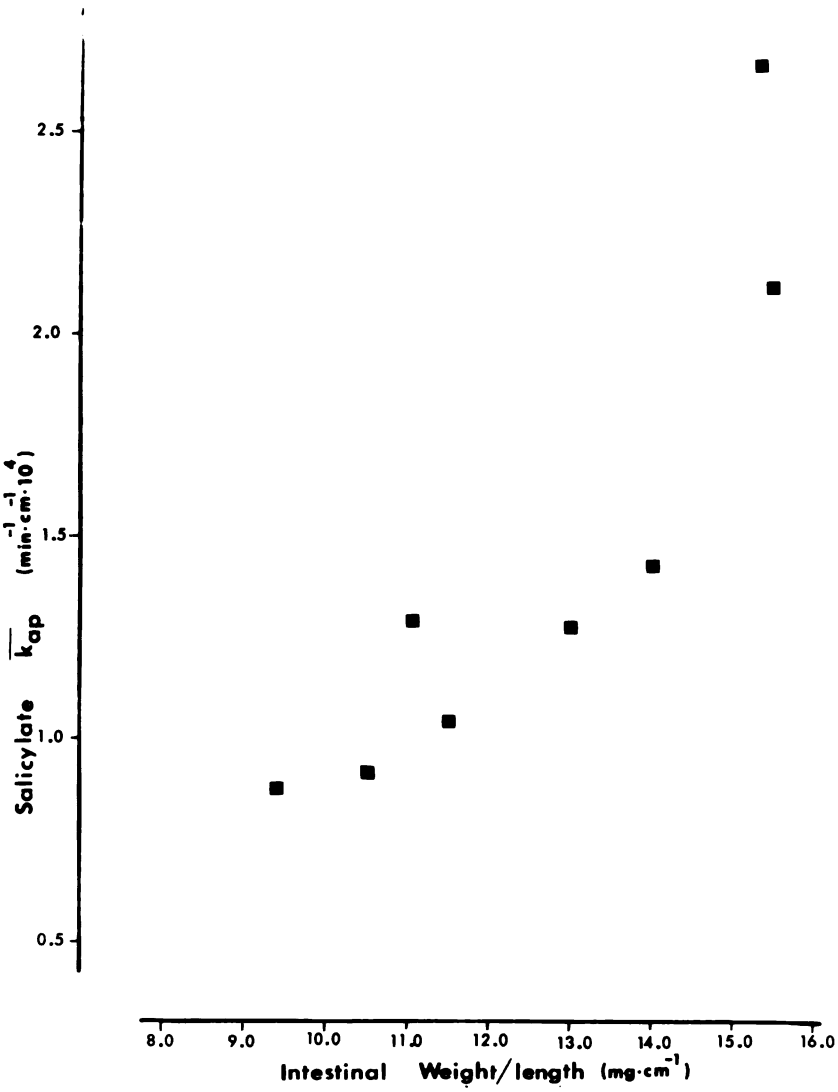

Figure 8. Plot of Average Apparent Absorption Rate Constants for Salicylate Versus Intestinal Weight Per Unit Length at Various Intervals of Fasting.

shown to cause morphological changes in the g.i. tract not unlike those effects seen with long term starvation (7). Some of these morphological changes include: a decrease or abolition of mitosis in the germinative crypts, decreased villi height, and fewer mucosal cells.

Furthermore, these workers (3,30-34) noted that amino acid absorption, glucose transfer, metabolic activity, sodium transport, water absorption, and overall cellular function are all generally inhibited by the action of these treatments on the intestinal mucosa. Hampton (34) noted that the cells remaining after colchicine treatment were depleted of ribosomes and polysomes and were presumed to be functionally inadequate.

The general effect of these agents and presumably of fasting would seem to be two-fold: First, not as many cells are present, therefore absorptive surface is decreased, and second, the capacity of the remaining cells for normal activity is reduced.

To further elucidate the functions of intestinal mass in drug absorption, a series of experiments were carried out in which the intestinal mucosa was separated from the underlying musculature of the rat intestine after various periods of fasting. Table III and Figure 9 show the effect of fasting on the total weight and the separated mucosal and musculature fractions of the intestinal weight. The total intestinal weight decreased with fasting (Table III) in a manner similar to that described in previous experiments (Table I). However, when the mucosa and the musculature are separated it can be seen (Figure 9) that both of these tissue masses lose weight more or less in a parallel fashion as fasting progresses. This indicates that decreased mucosal cell number may not be totally responsible for the decreased absorption rates ob-

Table III

Mucosal, Musculature, and Total Intestinal Mass Changes with Fasting

Fasting time (hr)	0 (control)	24	48	72	96
Intestinal Dry Weights (mg/cm)					
Mucosal weight/length	5.49(1.1)*	4.74(.26)	3.32(.33)	3.07(.25)	2.68(.15)
Musculature weight/length	9.42(1.5)	7.13(1.2)	6.98(1.23)	6.64(1.26)	5.37(.14)
Total weight/length	14.91(2.4)	11.87(1.2)	10.30(1.8)	9.71(1.41)	8.05(.28)
% Change of total weight/ length from control	0	20.4	30.9	34.9	46.0
Mucosa/musculature Ratio	0.590(.10)	0.680(.11)	0.482(.05)	0.473(.09)	0.498(.02)
Mucosal % of total wt.	36.8	40.2	32.5	32.0	33.2

There were 4 animals in each group.

* Values reported are means $\pm$ (SD)

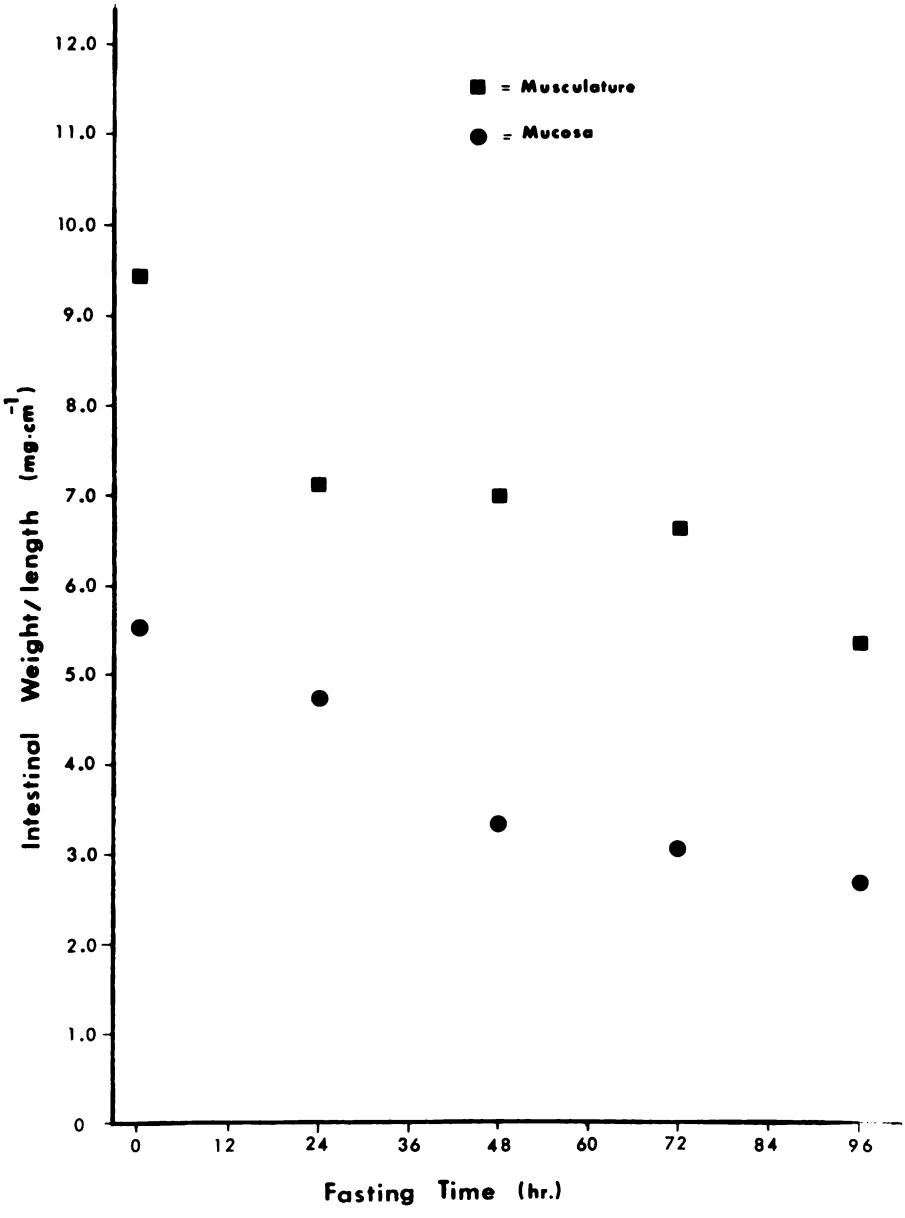

Figure 9. Effect of Fasting on the Separated Mucosal and Musculature Fractions of Intestinal Weight.

served with fasting.

The observed loss in musculature weight that follows fasting may imply that the mesenteric vascular system is decreased in size due to fasting and that the decreased absorption rates may be a function of both reduced blood flow and lowered mucosal cell number.

Several investigators (35,36) have noted that fasting causes a decrease in both the number and the size of the mucosal epithelial cells. It has also been observed that following fasting, a significant decrease in de novo protein synthesis occurs (37). Newey (3) and others (8,38,39) have noted that glucose metabolism in rats, as well as general enzyme activity, was reduced with fasting. Similar studies in humans have related protein-calorie malnutrition with depressed intestinal enzyme activity and reduced absorption of essential amino acids (40,41).

Summary

Fasting has been shown to reduce the intestinal tissue weight in a fairly linear manner, affecting both the muscosal and musculature fractions about equally. At the same time, the apparent intestinal absorption rates of ionized salicylate and non-ionized antipyrine were reduced significantly under fasting conditions. The literature provides evidence for reduced carbohydrate and amino acid absorption and reduced enzyme activity with fasting.

Thus it seems apparent that there are at least three interrelated factors involved in the intestinal absorption of drugs under the influence of fasting: intestinal mucosal cell number, mucosal cell viability, and mesenteric blood flow. If mucosal cell mass and viability are important factors in absorption, fasting effects could have some clinically important aspects. Some disease states mimic the effects of fasting in terms of morphological changes in mucosal tissue. In ulcerative colitis, cell damage is first noted in the epithelial crypt areas. With tropical sprue, epithelial cells often remain cuboidal and fail to differentiate into columnar cells with accompanying patterns of nutritional deficiencies (42). In human patients suffering from kwashiorkor, a nutritional disease, Cook and Lee (43) have observed lowered absorption rates for carbohydrates as well as reduced intestinal enzyme function. Stanfield, et al. (44) have reported that the intestinal epithelium is altered in kwashiorkor.

In subsequent sections the effects of metabolic inhibitors, certain disease states and blood flow on drug absorption rates will be investigated.

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CHAPTER 3

THE EFFECTS OF ADDITION OF INHIBITORS AND NUTRIENTS ON THE INTESTINAL ABSORPTION OF DRUGS

Introduction

Drugs may enter the vascular system from the gastrointestinal tract by one or more of several mechanisms. A drug may be transported across the intestinal barrier coupled to active processes such as the Na-hexose-amino acid transfer mechanisms, by concentration dependent passive diffusion, by solvent drag, or by a combination of these methods. It has been reported that most drugs enter the general circulation from the intestine by passive diffusion of the non-ionized species present in the intestinal lumen (1). Until recently, little consideration has been given to the possible importance of drug ion absorption and the mechanisms involved. Although there is currently some interest in defining these absorption mechanisms, at present they remain unresolved (2).

The introduction of certain metabolic inhibitors and nutrients into an in vivo intestinal perfusion system (as described in Chapter 2) containing a test drug might lead to further elucidation of the mechanisms by which drugs, and particularly drug ions like salicylate, are absorbed from the intestinal tract. With this aim in mind, a series of experiments was designed where various agents were added to the system while absorption measurements were in progress. The effects of these additives on the apparent absorption rates of the drugs were observed.

Experimental

i. Materials

Chemicals - Phlorizin, ouabain, acetazolamide, and probenecid were chosen as specific metabolic inhibitors. 2,4-Dinitrophenol (DNP) was chosen as a general metabolic inhibitor. Sodium taurocholate was selected as a natural bile salt normally found in the g.i. tract, and 3-O-methyl-glucose (3 MG), D-glucose, and D-fructose were chosen as nutrients or

stimulants of metabolic function. The effect of salicylate on its own absorption rate was also studied. The buffers and drug solutions used were identical to those described in Chapter 2, as was the preparation of the in vivo perfusion system. The drugs studied were salicylate and antipyrine.

ii. Methods - The substances to be added to the intestinal perfusate were weighed out prior to the experiment on a Mettler analytical balance and were diluted with the perfusion fluid immediately following the withdrawal of the 50 min. sample from the reservoir. Sampling was then continued at 5 min. intervals for an additional 50 minutes. Assays of salicylate and antipyrine and data reduction were carried out as described in Chapter 2.

Control studies were carried out in which no substance was added to the perfusate at 50 minutes. The rates for the 0-50 minute period and the rates for the 50-100 minute period were then compared to show the consistency of rates over 100 minutes. No change in slope, as calculated by a t test for slopes (3), was taken as showing that the viability of the membrane remained constant over the time of the experiment.

Results and Discussion

Table IV shows the comparison of initial rates (0-50 min) and final rates of absorption (50-100 min) in experiments where no treatment was applied. The results show that the absorption rates for salicylate and antipyrine are essentially constant within experimental error ($\pm$ 5%) over the duration of the experiment (100 minutes).

Both the paired t test for comparing treatments versus no treatment, and the t tests for comparison of individual slopes showed no significant difference between rates up to 50 min. and rates from 50 min. onward.

Table IV

Comparison of Initial and Final Rat Intestinal Absorption Rate Constants for Salicylate and Antipyrine with No Perturbing Agent Added

Agent Added	Drug	Absorption Rate Constants (min^{-1} cm^{-1} 10^4) Initial	Final	Percent Change from Initial	Significance*
None	Salicylate (17mM)	3.00	3.07	+2.3	
		2.75	2.70	-1.8	
		1.76	1.67	-5.1	NS
		0.78	0.73	-6.4	
		1.05	1.12	+6.3	
		1.53	1.49	-2.6	
		1.81/(0·80)	1.80/(0.91)	-	
None	Antipyrine (5.3mM)	1.46	1.45	-0.7	
		1.56	1.54	-1.3	
		0.99	1.00	+1.0	NS
		1.31	1.29	-1.5	
		1.33(0.25)	1.32(0.24)	-	

* Significance tested by paired t test between groups (3) at p = 0.05.

The wide variation in initial rate constants reported in Table IV results from selection of experiments in which some rats were fasted for various periods of time and others were non-fasted.

i. Effect of Acetazolamide on the Absorption Rate of Salicylate.

The effect of the addition of 0.9 mM acetazolamide on the apparent absorption rate of salicylate is shown in Table V. An increase of from 5-13% in the apparent absorption rate of salicylate was observed. Metabolic acidosis may be the cause for the slight increases in transfer rates. Hill (4) has reported that acetazolamide in rats lowers blood pH, raises plasma and tissue concentrations of salicylate and increases the toxicity of salicylate. In his study, the administration of acetazolamide with salicylate increased by 4-fold the brain salicylate concentrations. These increased brain concentrations could be explained by the pH drop that occurred in the blood. According to the pH-partition hypothesis of Shore et al. (5) a lower plasma pH would allow for the existence of higher plasma concentrations of non-ionized drug which could have resulted in the observed higher brain and tissue concentrations. Hill showed that prevention of acidosis by the introduction of bicarbonate into the blood prevented the increased tissue concentrations.

From our experiments, it was presumed that since the salicylate absorption rate was not impeded by acetazolamide the transport of salicylate was not coupled directly to the bicarbonate mechanism. The observed results could be explained by acetazolamide-induced acidosis.

ii. Effect of Probenecid on the Absorption Rate of Salicylate.

Probenecid, a competitive inhibitor of weak acid active transport shows no consistent effects on the apparent absorption rate of 17 mM salicylate as shown in Table V. Two rates were increased variably and

Table V

The Effect of Addition of Acetazolamide, Probenecid, and Salicylate on the Intestinal Absorption Rate Constants for Salicylate in Rats

Agent Added	Drug	Absorption Rate Constants (min^{-1} cm^{-1} 10^4) Initial	Final	Percent Change from Initial	Significance[1]
Acetazolamide (0.9mM)	Salicylate (17mM)	1.31	1.38	+5.3	Sig.
		1.27	1.46	+13.0	
		1.53	1.73	+11.6	
Probenecid (1.3mM)	Salicylate (17mM)	2.08	2.10	+1.0	
		1.98	1.76	-11.0	NS
		1.76	2.56	+45.0	
Salicylate (17mM)	^{14}C-Salicylate (7.4×10^{-3}mM)	2.08	1.61	-22.6	
		2.16	1.99	-7.9	Sig.
		2.15	1.95	-10.3	

[1] Calculated by paired t test (3) at p = 0.10.

one rate was decreased by 11%. It has been well documented (6) that an active transport system for weak acids exists in kidney epithelia, and similar systems have been suggested for the "blood-brain barrier" (7-9), and for the intestinal barrier by Stevens and associates (10) who proposed an intestinal transepithelial transport mechanism for weak electrolytes. Binder et al. (11) have demonstrated that probenecid inhibits the active transport of several amino acids across hamster g.i. tract. These workers found that probenecid did not effect Na transport in hamster gut preparations, but that chlorothiazide did inhibit Na movement. These authors noted that there were many functional similarities between the kidney and the small intestine. Jusko and Levy (12) using riboflavin as a model have suggested that probenecid may inhibit the intestinal absorption of agents that are absorbed by site specific and saturable transport processes. Frederick and coworkers (13) noted that 5 mM probenecid did not inhibit the Na-independent transfer of lysine in intact intestinal sac preparations in vitro, but that 0.4 mM DNP did inhibit the lysine transfer.

In our experiments, tremor in the forelimbs and apparent convulsive behavior were noted in the probenecid treated rats 10 to 20 minutes after the addition of the probenecid to the intestinal perfusate. These observations would seem to support the contention of González (9) that probenecid causes elevated plasma and brain levels of salicylate and increased toxicity.

The results in Table V for the effect of probenecid on salicylate absorption do not provide evidence for involvement of salicylate with an intestinal weak acid pump mechanism. The implication that salicylate is passively absorbed at the concentrations studied is supported by Lanman et al (14) who studied the intestinal absorption of several other organic

acids known to be actively transported in the kidney tubule, namely para-amino-hippuric acid, sulfanilic acid, hippuric acid and phenol red. They concluded that these compounds were passively absorbed from the intestinal tract.

If there were an active process involved, however, it may be that the concentrations of salicylate used in our study (17 mM) were in such excess compared to some maximal concentration for active transport, that the passive component of absorption was very much greater than an active component. If this were the case with salicylate, no effect of the inhibitor could be seen, i.e. very low Tm.

An example supporting this idea comes from the work of Smith (15), who studied folic acid absorption from the intestine. He showed that folic acid is actively transported from rat jejunum at concentrations of 10^{-7} M/L or less. This transport was shown to be inhibited by methotrexate. At concentrations greater than 10^{-6} molar, the passive component of folic acid absorption was far in excess of the active component, and the effect of inhibition by methotrexate could not be detected.

iii. Effect of 17mM Salicylate on the Absorption Rate of Trace Salicylate

The addition of 17 mM salicylate to a perfusate solution containing only pH 7.4 K-H buffer and a trace of ^{14}C-salicylate, caused a significant decrease in the apparent first order absorption rate of the trace ^{14}C-salicylate as noted in Table V. In other studies from our laboratory (16) of salicylate absorption across the in vitro rat intestinal mucosa, stripped according to the method of Wolfe et al (17), the addition of 17 to 30 mM salicylate to a perfusate containing only trace ^{14}C-salicylate, caused a decrease in the transfer rate of the ^{14}C-salicylate of 15 to 31%. The decrease might be explained by competition for absorption sites be-

tween the trace ^{14}C-salicylate and the cold (unlabeled) salicylate. However, if this were so, the concentration of unlabelled salicylate is in such excess of the concentration of ^{14}C-salicylate that the apparent absorption of the trace ^{14}C-salicylate would be completely inhibited and the rate would approach zero.

Inhibition of its own absorption, perhaps by uncoupling oxidative phosphorylation, or by some direct effect on the cell membrane permeability would be a more likely explanation for the decrease in apparent rates of absorption of salicylate.

iv. Effect of Bile Salt on the Absorption Rates of Salicylate and Antipyrine.

Sodium taurocholate (Table VI) at luminal concentrations of 5 mM, caused a consistent increase in the transfer rates of both salicylate and antipyrine. The increases are not significant by the paired t test because of the small number of experiments. Bile salt enhancement of absorption has been reported in rat intestine for salicylate by Gibaldi and associates (18,19), and for sulfaguanidine by Kakemi et al (20). It is suggested by these authors that the increased rates of drug transfer are due to a direct effect of the bile salt on the permeability of the mucosal membrane. Ganeval and coworkers (21) have shown that the addition of bile salts to perfused jejunal loops in rats, caused a net increase in transfer of Na^+, K^+ and Ca^{++} ions from blood to intestinal lumen. They also noted net increases in the blood to gut transfer of urea, creatinine and uric acid. The bile salt used, sodium deoxycholate, increased the permeability of the mucosal barrier along a blood to gut concentration gradient for these substances. The effects were reversible and no histo-

Table VI

The Effect of Addition of Sodium Taurocholate on the Intestinal Absorption Rate Constants of Salicylate and Antipyrine in Rats

Agent Added	Drug	Absorption Rate Constants (min^{-1} cm^{-1} 10^4) Initial	Final	Percent Diff. from Initial	Significance[1]
Sodium Taurocholate (6mM)	Salicylate (17mM)	1.52	2.42	+59.0	
		1.89	2.01	+6.3	NS
		1.71	2.22		
		(.26)	(.29)		
Sodium Taurocholate (6mM)	Antipyrine (5.3mM)	1.37	1.44	+4.9	
		2.07	2.43	+17.4	NS
		1.72	1.93		
		(.49)	(.7)		

[1] Calculated by paired t test (3) at p = 0.10.

logical changes were noted. In our histological studies, no apparent change in the appearance of the villi or the epithelial border was noted. Feldman et al. (45) have reported that the effects of sodium taurodeoxycholate on the in vitro rat intestinal membrane can be related to an increase in membrane permeability in which there is an observed efflux of membrane components and increased transfer of phenol red. They suggested that this surfactant interacts with protein or phospholipid components of the membrane to produce the increased permeability. It would appear then from the work reported in the literature and the results in Table VI, that bile salts increase the permeability of the mucosal barrier to the passive movement of ions and molecules in both directions.

v. Effect of Phlorizin on the Absorption Rate of Salicylate.

Phlorizin has been shown to inhibit D-glucose transfer across in vitro intestinal sacs (22) and the isolated brush border of rat intestine (23). In the latter study, it was demonstrated that the brush border membrane contains the glucose transport system and that the active transfer of glucose is Na dependent. It was also noted that higher than usual extracellular concentrations of Na^+ led to increased rates of glucose transfer.

The results of the phlorizin experiments are shown in Table VII. Phlorizin (1 mM) showed no consistent change in the absorption rates of salicylate with the rate changes varying from +8% to -7.2%. The inability to demonstrate consistent alterations in the transfer rate of salicylate with phlorizin would tend to support the opinion that this drug anion is passively transferred across the intact rat intestine. Sanford (24) has shown that phlorizin inhibits aerobic oxidation in mitochondria, with the result that the general metabolic well-being of the intestinal epithelial

Table VII

The Effect of Addition of Phlorizin, Ouabain, and Dinitrophenol on the Intestinal Absorption Rate Constants for Salicylate in Rats

Agent Added	Drug	Absorption Rate Constants Initial	Final	Percent Change from Initial		Significance[1]
Phlorizin (1mM)	Salicylate (17mM)	1.86	2.01	+8.1		
		2.50	2.32	-7.2		NS
		1.80	1.95	+8.3		
		2.57	2.51	-2.3		
Ouabain (1mM)	Salicylate (17mM)	1.77	2.14	+21.0	•	
		1.77	1.90	+7.3		NS
		2.30	2.12	-7.8		
Ouabain (1mM)	^{14}C-Salicylate (7.4×10^{-3}mM)	1.68	2.15	+28.0	Sig.[2]	
		1.67	2.25	+34.7		
		1.65	1.49	-9.7		NS
		1.25	1.67	+33.6		
2,4-DNP (1mM)	Salicylate (17mM)	1.78	1.96	+10.1		
		2.00	2.21	+10.5		Sig.

[1] Calculated by paired t test (3) at p = 0.10.

2 Sig. at p = 0.10 when all seven experiments combined.

cells may be interfered with. Further, the cells may be deprived of glucose (and hence energy for enzyme processes) in the presence of phlorizin since this agent has been shown by Crane (25,26) and other (22,27) in perfusion studies, to reversibly inhibit sugar transport. They concluded that this inhibitor exerts its effect on a glucose transport system located on the luminal side of the epithelial cells.

vi. Effect of Ouabain on the Absorption Rate of Salicylate

Ouabain (Strophanthin-G) is a cardioactive glycoside which has been shown to passively enter the intestinal mucosal cells (28). Ouabain has been shown to inhibit mucosal to serosal transfer of sodium in several types of tissue (29-31) when applied to the serosal side of the tissue. The inhibition of Na transport by this drug has been linked to inhibition of the activity of a Na -K dependent ATP-ase found in intestinal mucosal epithelial cells (32). Curran (31) and others (33,34) have also demonstrated that ouabain inhibits sodium-sensitive active hexose transport and the active transport of several amino acids. The effect of the addition of ouabain to the intestinal perfusate containing salicylate (i.e., on the mucosal side) caused a general but not completely consistent increase in the rate of absorption of salicylate. As shown in Table VII, the salicylate absorption rates were increased in 5 out of 7 experiments when ouabain was present.

Lyon and Crane (35) have shown that ouabain, added to the serosal side of in vitro rat intestinal preparations, depresses the transmural potential difference (PD). This depression is consistent with the known inhibition of cation transport by this cardiac glycoside. However, when these workers placed the ouabain on the mucosal side of the preparation the PD was in-

creased, implying an increase in cation transport. Lyon and Crane (35) concluded that mucosal ouabain increases the influx of sodium ion. The observed increase in salicylate transport rate with ouabain could be explained by an increase in the concentration gradient and/or solvent drag as water absorption has been shown to obligatorily follow Na^+ absorption (36). The single decreased rate and the great variability seen in the increased rates for 17 mM salicylate with ouabain may be a result of variability in the nutritional states of the intestinal mucosa at the time of the experiments since the presence or absence of glucose may alter the sodium flux (35).

Table VII also shows the effect of addition of ouabain (1 mM) to a perfusate solution consisting of pH 7.4 K-H buffer with only a trace amount of ^{14}C-salicylate present. The initial concentration of the labelled salicylate was calculated to be 7.4×10^{-6} M/L. The results show an increase in the absorption rate of the salicylate (33%) in two of three experiments. The third experiment shows a decrease in salicylate rates after the addition of ouabain. These results are similar to those obtained with transfer rates from 17 mM salicylate solutions when ouabain was added to the perfusate. The 17 mM salicylate solutions contained approximately 2000 times the amount of salicylate in the trace studies.

Effect of Addition of 2,4-Dinitrophenol (1mM) on the Absorption of Salicylate.

As noted in Table VII, DNP added to the intestinal perfusate caused a 10% increase in the transfer rates of salicylate in both experiments. These preliminary results are different from those of Winne (37) who reported that 2,4-dinitrophenol (DNP), an oxidative phosphorylation uncoupler, had no influence on the active intestinal absorption of L-phenylalanine

across the in vivo rat jejunum. Curran (38), however, has shown that DNP inhibits the mucosal to serosal flux of sodium in rat ileum as well as intestinal amino acid transport (31,39). Inhibition of water absorption was seen to follow the net inhibition of solute transport. Kunze et al. (40), on the other hand, reported results similar to ours, showing that DNP addition increased the intestinal absorption rates of salicylate from 0.5 mM luminal solutions. DNP is known to cause an increase in both aerobic and anaerobic glycolysis. Thus a temporary increase in the metabolic activity of the epithelial cells under the influence of DNP may be responsible for the observed increase in salicylate transfer rates. Alternately, the general inhibitory effects of DNP on normal cell function may have increased the cell membrane permeability to polar compounds like salicylate. Plasma samples taken during these experiments indicate considerable absorption of DNP and systemic effects may be partially responsible for observed increased rates.

vii. Effect of 3-O-Methylglucose (3 MG), D-Glucose, and D-Fructose on the Absorption Rate of Salicylate

The addition of the actively transported but non-metabolized 3-O-methylglucose (3 MG) at 25 mM concentrations, increased the transfer rate of salicylate by 21 and 63% in two out of three experiments as shown in Table VIII. There was an inconsistency in that one salicylate absorption rate was depressed in the presence of 3 MG. Na transport has been shown to be stimulated by 3 MG (29) and the observed increases in salicylate absorption rates may be only a consequence of increased nutrition supplied to the mucosal cells.

When 3 MG (25 mM) was added to a perfusate containing only trace

Table VIII

The Effect of Addition of 3-O-Methylglucose, D-Glucose, and D-Fructose on the Intestinal Absorption Rate Constants for Salicylate in Rats

Agent Added	Drug	Initial	Final	Percent Change from Initial	Significance[1]
3-O-Methylglucose (3 MG)	Salicylate (17mM)	1.47	2.40	+63.3	
		1.67	1.07	-36.0	NS
		2.29	2.77	+21.0	
3 MG (25 mM)	^{14}C-Salicylate (7.4×10^{-3} mM)	1.81	2.17	+19.9	
		1.19	1.80	+51.3	Sig.
		1.31	1.97	+50.4	
D-Glucose (25mM)	Salicylate (17mM)	2.01	2.57	+27.9	
		1.63	2.07	+27.0	Sig.
		1.57	2.24	+42.0	
D-Fructose (25mM)	Salicylate (17mM)	1.19	2.13	+44.0	
		1.88	2.63	+39.9	Sig.
		1.59	1.89	+18.9	
		0.87[a]	1.20	+27.5	

[1] Calculated by paired t test (3) at $p = 0.05$.

[a] animal was fasted for 12 hours prior to experiment

^{14}C-salicylate (7.4×10^{-6} M/L), the effect was to significantly increase the absorption rates of salicylate from 20 to 51% over control values (Table VIII). These results are similar to those obtained when 3 MG was added to the 17 mM salicylate solutions and support the suggestion that an enhanced nutritional and metabolic state in the tissue may be responsible for the observed increased transfer rates.

Glucose (25 mM) which is metabolized and has both an active, sodium-sensitive component and a passive component of transport, caused a significant increase in salicylate absorption rates (Table VIII). A probable mechanism for this rate increase may also be ascribed to the general increase in metabolic activity resulting from absorption of nutrients.

These in vivo results with glucose on the rate of salicylate absorption appear to be in complete disagreement with the results obtained from in vitro intestinal sacs by Mayersohn and Gibaldi (41) who demonstrated that with in vitro gut sacs, the introduction of glucose reduced the rates of transfer of salicylate, sulfanilamide, and riboflavin. A possible mechanism for the results observed by Mayersohn and Gibaldi (41) might be as follows: Glucose enters the mucosal cells, sodium absorption is stimulated, and water follows sodium and glucose osmotically into the cells. Since there is no blood supply in their in vitro preparation, and the possibility of poor oxygenation of the interior of the tissue exists, flux of sodium, water, and glucose serosally out of the cells may be slowed considerably, swelling may then occur resulting in the observed decrease in mucosal to serosal transfer of riboflavin, sulfanilamide, and salicylate.

D-fructose is a metabolizable hexose that is reported to be passively absorbed (42). MacRae and Neudoeffer (43), however, have observed that the absorption of fructose can be inhibited by: low temperature, DNP and

sodium fluoride. They concluded that there appears to be an active component of transport for fructose separate from that of glucose. Recently, Gracey and coworkers (44) have also noted that fructose could be accumulated against a concentration gradient across in vitro rat intestine (Km = 0.9 mM) by a mechanism which was Na dependent, energy dependent, and inhibited by phlorizin.

In our study, fructose (25 mM) showed a significant increase in transfer rates of salicylate as shown in Table VIII. This evidence and the observed effects of 3 MG and glucose on salicylate absorption listed in Table VIII suggest that there is a direct stimulation of salicylate absorption rates related to a general increase in metabolic function where glycolysis, ATP production, and Na transport are increased. In vitro studies show glucose to increase transmural PD and short circuit current with no apparent increase in salicylate transfer rates (16). These salicylate rate increases observed in vivo with glucose (Table VIII) are in disagreement with the in vitro results but lead to a still to be tested hypothesis that stimulation of endogenous ion transport causes a concommitant increase in drug ion transport.

In the fructose experiments (Table VIII), the animal subjected to an overnight fast (24 hrs) had a lower initial rate of absorption than the nonfasted animals (as was also found in fasting experiments in Chapter 2). However, the fasted animal showed a percent increase in salicylate absorption rate similar to that seen with the nonfasted animals. Although this is only one experiment, it may suggest that the addition of fructose can cause a certain finite stimulation of metabolic activity irregardless of the baseline nutrient concentration in the mucosal cells.

Summary:

The significant increase in transport rates of salicylate seen with the metabolizable sugars, glucose and fructose, coupled with the observations that 3 MG, a nonmetabolizable, actively transported sugar, also stimulated salicylate transport in 5 out of 6 cases, suggests that the mechanism of stimulation of transfer is not direct through any active transport system but is indirect via increased metabolic activity and increased Na absorption provided by the sugars. Further, the observations of no consistent change in intestinal transfer rate of salicylate with specific inhibitors, such as probenecid, phlorizin and ouabain, would lend support to the supposition that salicylate ion, at the concentrations studied, is passively absorbed from the intestinal lumen of rats, and is not involved with a weak acid pump mechanism, nor coupled directly to a Na-hexose transport mechanism.

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CHAPTER 4

THE EFFECTS OF EXPERIMENTAL DIABETES ON THE RATES OF INTESTINAL DRUG ABSORPTION IN RATS

THE EFFECTS OF EXPERIMENTAL DIABETES ON THE RATES OF INTESTINAL DRUG ABSORPTION IN RATS

Introduction

Jervis and Levin (1) noted that alloxan diabetes in rats produced stunted growth, distended stomachs and hyperphasia and hyperplasia of the small intestine. More recently, the literature contains much information concerning the effects of experimental diabetes on intestinal mucosal growth in rats. Using wet and dry weights, Schedl and Wilson (2) showed that the mucosal tissue growth rate in diabetic rats was nearly twice that of their non-diabetic controls. Hexose and amino acid transport in diabetic rats was also shown to be increased over controls (3-6). Other studies have shown that intestinal disaccharidase activity is increased 2 to 3 times when compared to controls (7,8). This increase was much greater than could be explained by the observed 17% increase in intestinal mass. Rosenberg and Schultz (9) noted enhanced Na^+ transport and oxygen consumption in the intestine of diabetic rats. Schneider and Schedl (10) have demonstrated increased fat absorption in diabetic rats. These workers and others (11) also noted decreased duodenal calcium absorption and abnormal calcium metabolism which correlates with the observed decreased body growth associated with diabetes.

Commenting on the induced hyperphasia and small bowel hypertrophy caused by conditions such as hyperthyroidism, insulin injections, high lactose diets, as well as experimental diabetes, Dowling (12) stated that "... either small bowel enlargement or mucosal hypertrophy will increase the absorptive surface area and one might expect to find enhanced absorption in these situations."

If mucosal cell number is an important factor in the intestinal absorption of drugs, it would seem that the increase in mucosal mass in diabetes would result in increased absorption of drugs that are passively absorbed. If an active process is involved, it would seem probable that more "pumps" would be available for transport of the drug, providing that the intestinal hypertrophy results in normally structured mucosal cells with normal metabolic function.

Surprisingly, there is little information in the literature regarding the absorption rates of drugs from the diabetes modified intestine. Mild diabetes can often be controlled by the use of orally ingested hypoglycemic agents such as the sulfonylureas. Furthermore, diabetic patients are frequently troubled with secondary complications such as infections, for which oral medications are often prescribed. For these reasons, it would seem pertinent to investigate whether or not the intestinal transfer of drugs would be enhanced by this disease state as has been shown for sugars and amino acids. To do this, the absorption model described in Chapter 2 was used to study the absorption of salicylate and antipyrine from the intestines of normal and diabetic rats.

EXPERIMENTAL

1 - Materials:

The diabetogenic agents used in this study were alloxan and streptozotocin. Alloxan (2, 4, 5, 6 (1,3) - pyrimidenetetrone) as the monohydrate was used (Sigma Chemical Co.). An intraperitoneal dose of 200 mg/Kg (3) generally ensures production of uncontrolled diabetes through pancreatic β cell destruction. Streptozotocin (Ben Venue Laboratories), an antibiotic complex isolated from Streptomyces achromogenes, has also been

successfully used to produce experimental diabetes (2).

ii. Methods:

Male albino rats of the Sprague-Dawley strain (Simonsen) initially weighing 300-350 grams were used. The rats were randomly divided into two groups. Rats in the control group were weighed and injected with 0.5 ml of distilled water intraperitoneally (IP). Those rats to be made diabetic were weighed and injected IP with freshly prepared solutions of either alloxan monohydrate (200 mg/Kg) or streptozotocin (100 mg/Kg). Standard rat chow (Ralston-Purina Co.) and water were allowed ad libitum. The animals were individually housed in metabolic cages following injection of the drugs. Total food and water consumption was measured from the 5th to the 10th day as was the 24 hour urine output. Daily measurements of urine glucose and urine ketones were carried out using Keto-Diastix (Ames Company, Elkhart, Ind.).

iii. Criteria for Diabetes:

Olsen & Rogers (7) reported that glucosuria levels of 500 mg/100 ml or greater corresponded to hyperglycemic blood sugar levels of 300-500 mg/100 ml. In this study, only animals that had glucosuria levels measured by a glucose oxidase paper method (Keto-Diastix) of 1.0% or greater were employed. Daily urine ketone formation was generally moderate to heavy in the rats that were both glucosuric and polyuric. Blood samples were taken at the time of the experiment to assure adequate hyperglycemia. Criteria for diabetes in the treated rats were: glucosuria, retarded weight gain, polyuria, and hyperglycemia. Drug transport studies were carried out following the 10th day post-exposure to the diabetogenic agents. The experimental methods and drug assays used were the same as those described in Chapter 2. Following each experiment, the intestinal

loop was carefully separated from the mesentery and the mucosa stripped from the underlying musculature as described in Chapter 2. The mucosa and musculature were separately dried to constant weight.

Referring to Tables A-4 to A-10 in the Appendix, one can see that during the onset of experimental diabetes, several of the rats demonstrated polydipsia as well as the usual polyuria and glucosuria.

Most of the diabetic rats showed light to moderate ketone formation (ketonuria) in contrast with the results of Rakieten et al. (13). Moderate to heavy ketonuria tends to mask some of the color development in the glucose oxidase test thus the observed glucosuria may have been more severe than reported above.

OBSERVATIONS

i. Macroscopic Observations:

At the time of the experiment, the diabetic rats appeared emaciated with distended abdomens. Their coats were unkempt and they were generally quite lethargic. During surgery, it was noted that the small intestine was visibly thickened as compared to controls. These effects are similar to those noted by Jervis & Levin (1). The mesenteric blood vessels appeared to be engorged as though the blood supply was increased or as though peripheral resistance in the intestinal vascular bed was increased.

ii. Microscopic Observations:

Sections of jejunum taken from the diabetic rats were fixed and stained with hematoxylin-eosin stain. Under examination, the intestinal mucosa showed pronounced increased growth. The villi appeared larger and much more numerous than those in control rats.

RESULTS AND DISCUSSION

Initial body weights were in a range of 300-350 grams for both the control and diabetic groups as noted in Table IX. The mean body weight of the diabetic rats, 10 days after the injection of the diabetogenic agents, decreased by 22%. This is similar to the 10 to 20% weight loss reported by Schedl and Wilson (2) for measurements 5 to 8 days post-treatment. The control rats gained an average of 10% body weight over the same period. The environmental stress of the metabolic cages may have slightly exaggerated the weight loss in the diabetic group.

Net water absorption has been reported to be increased in diabetic rats compared to control rats, due in part to the dehydration that accompanies diabetes, and in part to the serum osmolality which is elevated in diabetes due to hyperglycemia (10).

Daily urine output and water intake by the diabetic group exceeded the values reported by Schneider and Schedl (10) for control animals. Urine output in the control group in this study was not monitored due to an insufficient supply of metabolic cages.

Table X shows a comparison of the two diabetogenic agents used in this study. At the 95% confidence limit, there is no difference between treatments for initial or final weights. The level of disruption of β cells seems to be about the same with each agent based on glucosuria levels. These results compare favorably with those of Rakieten et al (13) who have shown that streptozotocin and alloxan are equivalent in their ability to produce experimental diabetes in rats.

Table XI shows the mass changes that occur as a result of experimental diabetes, for the total intestine, the mucosal fraction and the intestinal musculature fraction. The mucosal mass/length increased 33% with

TABLE IX

Body Weights and Glucose Levels in Normal and Diabetic Nonfasted Rats

	Control	Diabetic[1]
Number of rats	14	7
Body weights (gm)		
Initial weight	349 (8)*	323 (29)
Final weight	386 (12)	252 (16)
Difference (%)	+10%	-22%
Urine Glucose (%)	0	1%
Blood Glucose (mg/100 ml)	189 (10)	343 (13)
Water Intake (ml/day)	30+	90
Urine Output (ml/day)	13+	35

1 10-13 days post-treatment with either alloxan or streptozotocin

* Values are means ($\pm$ SD)

+ Values reported by Schneider & Schedl (10)

TABLE X

A Comparison of Diabetogenic Agents

	Alloxan	Streptozotocin	Significance[+] (P = .05)
Number of rats	3	4	
Body weights (gm)			
Initial weight	303 (6)[*]	333 (33)	(NS)
Final weight	247 (20)	256 (15)	(NS)
Urine Glucose (%)	1%	1%	-
Blood Glucose (mg/100 ml)	335 (10)	352 (16)	(NS)

[*] Values are means ($\pm$ SD)

[+] Paired t test calculation.

TABLE XI

A Comparison of the Mucosal, Musculature and Total Intestinal Weight Per Unit Length in Normal and Diabetic Rats

	Control	Diabetic[+]	% Change from Control
Number of rats	14	7	
Dry intestinal weight (mg/cm)			
Total weight/length	14.90(0.3)[*]	13.5(1.1)	-9.4
Mucosa weight/length	5.49(1.1)	7.3(1.2)	+33.0
Musculature weight/length	9.42(1.5)	6.2(0.7)	-34.0
Mucosa as a % of total weight	36.8(4)	53.5(6)	+44.0

[*] Values are means (± SD)

[+] 10-13 days post-treatment with diabetogenic agent

diabetes while the musculature mass/length decreased by 34% compared to control values. Schneider and Schedl (10) observed a 39% increase over controls for mucosa and only a 9% decrease from controls for musculature mass. In the present work, the total mass/length of diabetic intestinal loop was only 9.4% less than controls while the whole diabetic animal weight-loss over the same period was 22% of initial weight. Some of these differences are probably due to the age and weight differences between our rats and the rats used by Schneider and Schedl.

Figure 10 shows pictorially the intestinal mass changes that occur as a result of the experimental diabetes. These growth data can be explained from the viewpoint that glucose can enter the mucosal epithelial cells without insulin, but that penetration into muscle and adipose tissue is severely inhibited in the absence of insulin (14). The nutrients entering the intestinal lumen are available only to tissues which can absorb glucose without insulin (e.g. lens, retina, nerve, kidney, blood vessel, and mucosal tissues)(14). The underlying intestinal muscle tissue is nutrient starved in the absence of insulin and weight loss in this tissue occurs. Hyperplasia and hyperphasia in mucosal tissue may occur in response to a condition in the rest of the body resembling nutritional starvation.

The apparent rate constants for intestinal absorption of salicylic acid and antipyrine in control and diabetic rats are presented in Table XII. The absorption rate for salicylate in diabetic rats, is reduced by 25% from control values while antipyrine rates remain unchanged. Theoretically, one might anticipate that the intestinal absorption rates of these compounds would increase due to the increased number of mucosal cells present in the diabetic rat (12).

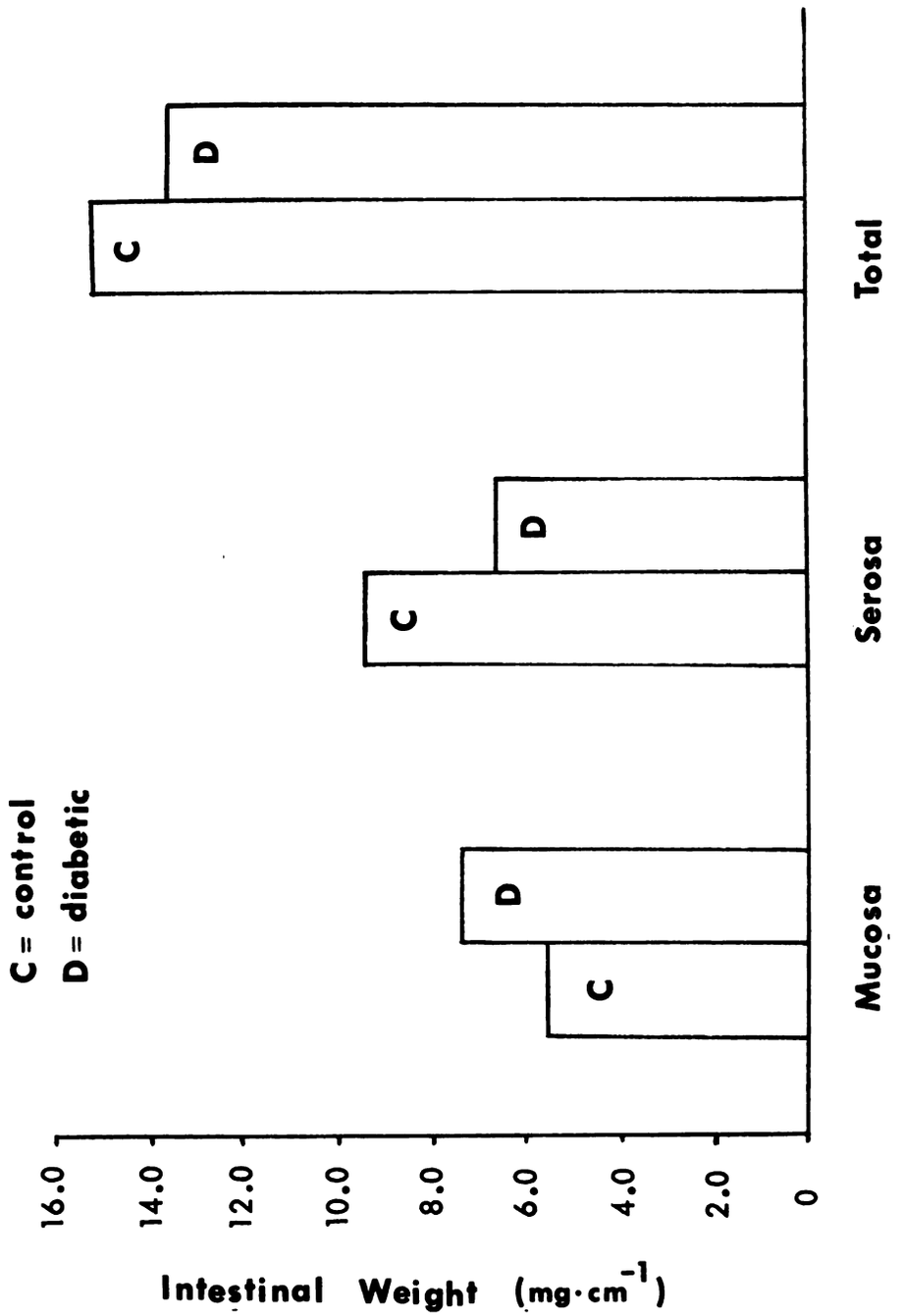

Figure 10. Effect of Experimental Diabetes on the Intestinal Weight Per Unit Length in Rats.

TABLE XII

A Comparison of Intestinal Absorption Rate Constants for Salicylate and Antipyrine in Normal and Diabetic Rats[1]

	Control	Diabetic[2]	% Change from Control
Number of rats	8	3	
Apparent drug absorption rate constant (min^{-1} cm^{-1} 10^4)			
Salicylate	2.38(0.68)*	1.78(0.08)	-25.2
Antipyrine	1.40(0.10)	1.46(0.16)	+ 4.3

* Values are means ($\pm$ SD)

1 All values are from non-fasted animals.

2 10-13 days post-treatment with diabetogenic agent.

One reason for the decreased salicylate transport rate and the unchanged antipyrine transport rate may be related to the increased Na^+ (9) and water uptake into the mucosal cells (10). This could result in smaller extracellular spaces and, therefore, in smaller "pores" for the transport of ionized substances like salicylate. Hyperphasia of the intestinal epithelial cells may also be a major factor in reducing the size of the zonula occludens or tight junctions, thus retarding the absorption of ionized salicylate. The absorption rate of the non-ionized antipyrine, on the other hand, was unaffected. The passage of antipyrine may be mainly through the membrane and only secondarily along the "aqueous pores" as discussed previously in Chapter 2. Thus antipyrine rates would not be as adversely affected as salicylate by cell swelling or reduced cell number (15,16, Chapter 2).

A major complication of diabetes mellitus is reduced vascular circulation. This may progress to a level where peripheral ischemia can seriously retard mesenteric vascular bed perfusion with significant arteriovenous shunting occurring. Thus, the observed hyperplasia in diabetic intestinal tissue is not necessarily paralleled by an increase in blood supply to this organ. In fact, the presence of peripheral vascular disease that accompanies diabetes (18-20) and the rates reported in Table XII indicate that the opposite may be true. Further, the observed 22% decrease in total body weight (Table IX) and the 34% decrease in intestinal submucosal musculature weight (Table XI) may be reflected in a coincident drop in intestinal blood flow due both to a smaller total blood volume and cardiac output, as well as to reduced mesenteric vascular bed size (smaller submucosal tissue mass). Reduced blood flow to the mesenteric capillary bed may be one of the reasons that salicylate rate constants are decreased

since Ochsenfahrt and Winne (17) have shown that absorption of salicylate is blood flow dependent. Decreased oxygenation of the mesenteric vascular bed resulting in cellular hypoxia and thickened capillary walls (18-20) may also contribute to reduced rates of transfer of salicylate.

Summary

The results of this study indicate that the possibility of increased drug absorption rates due to mucosal cell mass proliferation in the diabetic state appears to be neutralized or overwhelmed by reduced mesenteric vascular bed size and reduced blood perfusion. These effects seem to be more important for ionized drugs like salicylate than for non-ionized drugs like antipyrine. Further studies on blood flow effects will be described in the next section.

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CHAPTER 5

THE EFFECT OF THE HYPERTENSIVE STATE ON THE INTESTINAL ABSORPTION RATE OF DRUGS IN RATS

THE EFFECT OF THE HYPERTENSIVE STATE ON THE INTESTINAL ABSORPTION RATE OF DRUGS IN RATS

Introduction

Forsyth and coworkers (1) have shown that angiotensin infusion in monkeys reduces the fraction of cardiac output that perfuses the small intestine by 50%, while the fraction to the kidneys and skin is reduced by 30-40% and that to the liver by 20-30%. Forsyth has also shown (2) with monkeys that pressor areas in the hypothalamus have some control over the regional distribution of the cardiac output to organs without changing systemic arterial pressure, pulse rate, or the total cardiac output. The blood flow to the small intestine was reduced to 22-28% of normal mean values under hypothalamic stimulation. Khanna (3) has demonstrated in rats that the mucosa is the most seriously affected region of the intestine under conditions of ischemia produced by reduced blood flow, following mesenteric arterial occlusion. It was theorized that blood flow is redistributed via arterio-venous shunts and the resultant cellular hypoxia in the mucosal tissue seriously reduces normal cellular function. Arteriovenous shunting may become chronic with hypertension as Short (4) in studies in humans and others (25,26) studying rats noted decreased vascularity of the small intestine and diffuse narrowing of submucosal arteries in conditions of chronic and malignant hypertension.

Corday (5), studying regional circulation in dogs, observed that blood pressure falls rapidly and drastically with hemorrhage and shock. Portal venous flow decreased by about 83% due to increased total peripheral resistance in the mesenteric vasculature. This would be reflected by a parallel decrease in the mesenteric arterial flow rate. Hoffbrand

and associates (6) have shown that as a result of acute hemorrhage with 50% blood loss, non-anesthetized monkeys showed significant decreases in pancreatic, renal, splanchnic, muscle, and mesenteric blood flow rates. Sutherland et al. (7) studied hemorrhagic shock and ischemia produced by mesenteric arterial occlusion in rats. They noted that mucosal sloughing resulted with cell replacement occurring within 48 hours. The new cells were abnormal in that they were permeable to curare. Saperstein and associates (8) noted in studies with rats that hemorrhage lowered the cardiac output to levels that were 12 to 50% of normal while the blood pressure fell from 121 to 28 mm. Hg. (a 75% decrease). The organ fraction of the cardiac output was altered by hemorrhage in a manner similar to other studies (1,6), with the heart and brain receiving a larger fraction of available blood. At the same time the gut and splanchnic bed blood flow fell to 57% of normal rates under conditions of mild hemorrhage. With severe hemorrhage, the blood flow decreased to 12% of normal values. The peripheral resistance in these two organs was increased compared to normal measurements by 30% and 97% for mild and severe hemorrhage respectively.

The relationship between intestinal blood flow and intestinal absorption rates has been established in animals by several workers for many drugs and biological compounds. This relationship may have important human clinical implications. Goldberg and Fine (9) compared the intestinal absorption of glucose in normal dogs with glucose absorption in dogs in hemorrhagic shock. They noted a pronounced deficiency in the ability of the intestine to absorb glucose under shock conditions. Similarly, Guthrie and Quastel (10) using guinea pigs, noted that glucose transport was depressed 69% with tourniquet shock and observed that these changes in glucose transport were similar to those produced under anaerobic condi-

tions. Amino acid transport was also substantially reduced. These workers found that shock resembled anoxia in its depressant effect on cellular metabolism. McArdle and co-workers (11) studied hemorrhagic shock in dogs and recorded a 90% depression of adenine uptake into ATP. Amino acid transport was arrested for 3 days and oxygen uptake was depressed. However, the hexose monophosphate shunt was apparently unaffected since this pathway of metabolism requires no ATP. Mucosal cells were observed to be fragmented, vacuolized, or shed into the lumen of the intestine under these stressful conditions. Williams and associates (12) noted that glucose absorption was significantly reduced when mesenteric blood flow in dogs was reduced by 50%.

Winne (13) and Ochsenfahrt (14) noted that an 85% decrease in jejunal blood flow rate caused a 52% decrease in the intestinal transfer rate of actively absorbed L-phenylalanine, while the passive transfer rates of antipyrine and salicylate were decreased by 62% and 32% respectively. In stop-flow studies in our laboratory (see Table A-3, Appendix) when blood flow was arrested by euthanasia, transfer rates for antipyrine and salicylate were decreased by 73% and 81% respectively.

Apart from hemorrhage and shock, there are disease states in which decreased mesenteric blood flow can occur and which may be associated with reduced intestinal absorption. Two such diseases are diabetes mellitus and essential or idiopathic hypertension. Brod (15), in a classical treatise on essential hypertension in man, pointed out that the resting regional hemodynamics in patients with essential hypertension are analogous to the hemodynamics of the acute pressor reaction where blood is shifted from the kidneys, splanchnic area, gut and skin, to muscle tissue. Total peripheral resistance depends on a balance of vasoconstrictor action

in the viscera and skin, and the vasodilator action in muscle. Total peripheral resistance will increase if visceral vasoconstriction exceeds musculature vasodilation. Such is the case in conditions of essential hypertension.

With the development of a strain of spontaneously hypertensive rats (15,25), an animal model of hypertension that is not surgically induced became available for the study of drugs. This strain of Wistar rats was developed by Professor Kozo Okamoto at Kyoto University in Japan. These rats have been primarily used for the screening of potential hypotensive agents, since the hypotensive state exhibited by these animals closely resembles essential hypertension as observed in humans. These SH rats exhibit blood pressures double that of normal rats of the same strain and sex, increased total peripheral resistance, peripheral arterial narrowing, reduced fraction of the cardiac output to the gastrointestinal tract, as well as hypertensive retinopathy often leading to blindness (25).

Few reports have been published regarding absorption patterns in hypertensive patients, and fewer still have compared hypertensive patients to normal persons in drug absorption studies (17). If the factors related to hypertension alter intestinal absorption, this strain of hypertensive rats should be useful for pharmacokinetic studies of orally ingested drugs. The reduced peripheral blood flow and increased intravascular pressure that are characteristic of hypertension may adversely affect the absorption rates of drugs in general as well as drugs used particularly to treat the hypertensive condition. For these reasons, we investigated the _in vivo_ absorption rates of two model drugs, salicylate and antipyrine in spontaneously hypertensive rats. These studies may help to elucidate the effects of this disease state on intestinal absorption of drugs.

Experimental:

Materials and Methods

Thirteen-week old spontaneously hypertensive (SH) female Wistar rats were obtained from Carworth (a division of Becton, Dickinson & Co, N.Y.). The Carworth colony consists of brother-sister mated offspring which are direct descendants of the original Okamoto strain. Company supplied information on the indirect mean arterial blood pressure (tail-cuff method) of these rats, reports values of 170-180 mm. Hg. Normotensive rats are reported to have mean arterial pressures of 116 mm. Hg. (18). The SH rats obtained from Carworth were allowed to acclimatize in our cages for 4 days before experiments were carried out. Nine week old normotensive female wistar rats of similar weights were obtained from Simonsen and used as controls. It was not possible to weight-match exactly the controls to the hypertensive rats. Normal rats at the same age as the SH rats are somewhat heavier and this increased weight might possibly have introduced differences in absorption rates related to physical development and not to the hypertensive versus normotensive states alone.

Five-week weight gain studies of normotensive and spontaneously hypertensive rats (19) showed parallel growth development for the two groups, with the SH rats averaging somewhat smaller weights at each age. Hypertensive rats appear to have lower birth weights but develop in a normal fashion (19).

The heart rates of spontaneously hypertensive rats were on the average 10-20% higher than the heart rates of the normotensive rats.

Surgical procedures, in vivo perfusion techniques, intestinal mass determinations and drug analyses were carried out as described in Chapter 2.

RESULTS AND DISCUSSION

The results of the intestinal mass and drug absorption rate comparisons in normotensive and hypertensive rats are presented in Table XIII. Note that the average total body weights for the control and SH rats in Table XIII are different by 18%. Although the SH rats are two weeks older, they weigh less than the controls. These data agree with the weight-age profiles on SH and normotensive rats supplied by Carworth (19), which show that SH rats have lower average birth weights than normotensive animals but both groups mature in a parallel fashion.

Although the average mass/length of intestine for the hypertensive rats was not significantly different from the controls (by paired t test), the decrease in absorption rates for the hypertensive rats was 18% lower for salicylate (Sig. at $P = 0.10$) and 28% lower for antipyrine (Sig. at $P = 0.05$) compared to controls (Table XIII). Thus, it appears that the decrease in absorption rates with hypertension is greater than can be explained by intestinal weight differences alone. The fact that the average total body weight difference of 18% between controls and SH rats is the same as the rate difference for salicylate between these groups is probably fortuitous since our fasting studies showed that there was no correlation between total body weight and absorption rates for salicylate in rats ranging in weight from 302 to 375 gm., a 24% difference in weights.

It is interesting to note that the mass/length values and transfer rates in normotensive female Wistar rats were lower, but within the observed range of values found for the S-D male rats in the fasting studies (See Table I), suggesting similar drug absorption rate patterns between these two species of rat. Idiopathic or essential hypertension in humans is manifested by an increase in total peripheral resistance in most or-

TABLE XIII

A COMPARISON OF INTESTINAL WEIGHT AND DRUG ABSORPTION RATES IN NORMAL AND SPONTANEOUSLY HYPERTENSIVE* FEMALE WISTAR RATS

	Wistar Control	Wistar Hypertensive	% Change from Control	
Mass Study				
Number of rats	10	10	-	
Age	11 wks	13 wks	-	
Total body weight	194 (8.5)+	158 (12)	-18.5	Sig.[1]
Dry intestinal weight/length (mg/cm)	12.07 (1.55)	11.79 (1.35)	-2.3	N.S.
Intestinal Absorption Study				
Number of rats	3	3		
Apparent drug absorption rate constant ($\overline{kap}$) ($min^{-1}cm^{-1} \times 10^{+4}$)				
Salicylate	2.03 (0.10)	1.67 (0.35)	-18	Sig.[2]
Antipyrine	1.09 (0.07)	0.78 (0.08)	-28	Sig.[1]

* Obtained from Carworth - Div. of Becton Dickinson & Co., New York

+ Values are means $\pm$ (SD)

[1]Calculated by paired t test at P=0.05.

[2]Calculated by paired t test at P=0.10

gans in the body. Cardiac output usually remains unchanged so that blood flow to most organs is normal (1). However, mesenteric vascular bed perfusion may be reduced through arterio-venous shunting as has been observed in retinopathy associated with diabetes (20) and with hypertension (17).

If a diminution of mesenteric blood flow to the vascular bed perfusing the mucosal tissue is occurring with hypertension, the noted decreases in the drug absorption rates in the hypertensive rats could be related to blood flow and cell viability.

First, decreased drug concentration gradients across the mucosal membranes may result in drug build-up in the mucosal cells following reduced blood flow. This is the "mechanical" influence of blood flow on passive transfer of drugs as described by Winne (21,22).

Secondly, the mucosal cell hypoxia that would follow lowered blood perfusion of these tissues would tend to cause a decrease in the general viability of the cells. Cellular hypoxia has been demonstrated to lower the penetration rates of actively absorbed substances such as glucose (23). The absorption rate results in Table XIII are different than those obtained by Lehner *et al.* (24) who studied the effects of hypothyroidism, insulin deficiency and hypertension on the intestinal absorption of cholesterol in primates. These workers were not able to discern any difference in the extent of absorption between controls and hypertensive animals, with both groups absorbing about 70% of the administered dose of cholesterol. However, these workers did not report on the rates of absorption of cholesterol in normals and hypertensives and differences may have occured with respect to this parameter.

Williams (12) has commented that a lack of adequate methods of investigation is probably responsible for the dearth of information concerning

the mesenteric circulation in health and disease.

Summary

From these preliminary studies it would appear that the disease state of hypertension with possible accompanying vascular changes, tends to adversely alter the absorption rates of passively transported drugs such as salicylate and antipyrine. One explanation is that if changes in the blood flow patterns to the vascular bed of the intestinal mucosa result from hypertension, these changes could affect absorption in two ways. First, reduced blood perfusion to the absorbing mucosal cells can adversely affect the absorption rates of drugs that are blood flow limited and, second the reduced oxygenation of the cells can produce a loss in cell viability and function which can result in lowered transfer rates.

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OVERALL SUMMARY

The purpose of this work was to study the influence of several factors on the intestinal absorption rates of an ionized acidic drug, salicylate and a non-ionized basic drug, antipyrine in an attempt to further delineate the mechanisms by which drugs penetrate the intestinal barrier, *in vivo*. An absorption model, in which the small intestines of rats were cannulated and perfused *in situ* was used to study the absorption rates of the two model drugs under a variety of experimental conditions.

Since most drug absorption is assumed to occur by passive processes following Fick's law principles, absorptive surface area was investigated as a major factor influencing drug absorption rates. Various periods of fasting were utilized to study surface area effects on absorption since fasting has been shown to reduce the mucosal epithelial cell population and hence the absorptive surface area. Absorption rates of salicylate and antipyrine were reduced by up to 63 & 17%, respectively, under the influence of fasting. At the same time, fasting caused a reduction in the intestinal weight per unit length which showed a linear relation to the length of time of the fast.

In mucosal tissue separation studies, it was shown that weight loss for both the mucosal and musculature fractions of the total intestinal mass was at about the same rate under the influence of fasting. It was surmised that mucosal cell surface area is not by itself the most important factor influencing drug absorption. To further investigate mucosal epithelial surface effects, drug absorption rates from the intestines of rats exhibiting experimental diabetes were studied. In this disease state, mucosal cell number is significantly increased over normal values and it

was predicted that the increased surface area for absorption would result in increased drug absorption rates. The absorption rate for antipyrine was unchanged and the absorption rate for salicylate was not increased in diabetic rats, in fact the salicylate rate was decreased by 25% compared to the rate from non-diabetic control rats. This decrease was noted in spite of an increase of 33% in the mucosal fraction of the total intestinal weight. While the mucosal cell mass was increased, the intestinal musculature mass was decreased by 34%. These results infer that possible increases in absorption rates with increased mucosal surface area are more than offset by reduced musculature mass with accompanying reduced mesenteric vascular bed blood perfusion. Further, mucosal cell hypoxia following reduced blood perfusion to the mesenteric capillary bed would also decrease normal mucosal cell metabolic function. These results led to a consideration of factors other than mucosal cell surface area that could be important in affecting intestinal drug absorption.

Preliminary studies on normal mucosal cell function employing changes in perfusion buffer constituents had shown that replacement of Na^+ with K^+ caused a severe reduction in the *in vitro* and *in vivo* absorption rates of salicylate. Mucosal cell swelling under the influence of the K^+ rich medium with concommitant reduction of intercellular spaces was considered to be the reason for the reduced drug absorption rates. Thus, the ionic milieu of the mucosal cells was deemed to be important for optimal absorption of compounds like salicylate.

The effect of mucosal cell viability and cellular metabolic processes on drug absorption, and the possibility of co-transport of drug molecules were investigated. Glucose, fructose and 3-O-methylglucose were employed as metabolic and transport stimulants, while acetazolamide, probenecid,

ouabain, phlorizin, and dinitrophenol were chosen as metabolic and transport inhibitors. Results showed that the absorption rate of salicylate ion was stimulated in the presence of the sugars but was not depressed in the presence of the metabolic inhibitors. In fact ouabain stimulated salicylate transfer. This infers that the absorption of salicylate is not directly linked to an active transport process but is readily influenced by the level of metabolic activity or nutritional state of the intestinal mucosal cells.

Preliminary blood-flow experiments in which blood flow was stopped, showed that intestinal absorption rates of salicylate and antipyrine were strongly dependent on intestinal blood flow rates. For this reason a second disease state model, the spontaneously hypertensive rat was employed to investigate the possible effects of hypertension on the intestinal absorption rates of drugs. Ancillary effects associated with hypertension involve changes in total peripheral resistance and probable changes in the volume of blood perfusing the mesenteric vascular bed. Salicylate and antipyrine absorption rates from hypertensive rats were reduced 18% and 28% respectively compared to normotensive rats of the same species and sex. Arterio-venous shunting in the mesenteric vascular bed may be the reason for the depressed rates, since intestinal mass per unit length studies showed no difference in mass between hyper- and normo-tensive animals.

The conclusions drawn from these studies are:

1. that salicylate and antipyrine appear to be absorbed from the intestines of rats by passive processes.
2. that salicylate appears to be absorbed as both the ionized and non-ionized species.

3. that the nutritional state of the mucosal tissue is a factor in intestinal drug absorption and is easily modified by fasting or by added nutrition.
4. that disease states may seriously alter intestinal drug absorption rates.
5. that mucosal cell viability, cell number, and mesenteric blood flow are major factors influencing the intestinal absorption of drugs.

APPENDIX

Table A-1

Intestinal Perfusate Buffer Formulae

a. Krebs-Henseleit pH 7.4 Buffer [1]

Constituent	mM/liter
NaCl	118.6
$NaHCO_3$	25.0
$CaCl_2$	2.5
KCl	4.8
KH_2PO_4	1.2
$MgSO_4$	1.2

Adjusted to pH 7.4

b. Sodium Phosphate pH 7.4 Buffer[2]

Constituent	mM/liter
$NaH_2PO_4 \cdot H_2O$	22.2
Na_2HPO_4	103.5

Adjusted to pH 7.4

c. Potassium Phosphate pH 7.4 Buffer[2]

Constituent	mM/liter
KH_2PO_4	22.6
K_2HPO_4	105.4

Adjusted to pH 7.4

[1]Reference 21, Chapter 1.
[2]Reference 7, Chapter 1.

Table A-2

The Effect of Replacement of Na^+ With K^+ in the Perfusion Buffer on the *In Vivo* Intestinal Clearance of Salicylate and Acetanilide in Rats

Drug	Intestinal Clearance ($ml \cdot hr^{-1} \cdot cm^{-1}$)		Percent Difference	Significance[1]
	Sodium Phosphate Buffer[2]	Potassium Phosphate Buffer[2]		
Salicylate	0.387 (0.12)	0.117 (0.024)	-70	Sig.
Acetanilide	0.354 (0.06)	0.270 (0.027)	-24	Sig.

[1]Calculated by paired t test (Reference 3, Chapter 3) at P=0.05.

[2]Buffers prepared at pH 7.4 according to Reference 7, Chapter 1.

Table A-3

Effect of Blood Flow Changes on the Intestinal Absorption Rate Constants for Salicylate and Antipyrine

Drug	Number of Rats	Absorption Rate Const. in ($min^{-1} \cdot cm^{-1} \cdot 10^4$) Normal Blood Flow	Zero Blood Flow	Percent Change from Normal	Significance †
Salicylate	3	1.88 (0.44)	0.33 (0.08)	-81	Sig.
Antipyrine	3	1.37 (0.03)	0.36 (0.01)	-73	Sig.

† Calculated by paired t test (Reference 3, Chapter 3) at P=0.05.

Table A-4

Water Consumption, Urine Output, and Urinalysis

for Diabetic Rat # A-4-0

Diabetogenic Agent: Alloxan

	Number of Days Post-Treatment					
	5	6	7	8	9	Average/24hr
Urine Volume (ml/24hr)	24+	25	14	25	25	23
Urine Glucose (%)	0.5	0.5	1.0	1.0	1.0	-
Urine Ketones	trace	+	+	trace	+	-
Water Consumption (ml/24hr)	80	60	60	40	60	60

Table A-5

Water Consumption, Urine Output, and Urinalysis

for Diabetic Rat # SA-6-0

Diabetogenic Agent: Steptozotocin

	Number of Days Post-Treatment					
	5	6	7	8	9	Average/24hr
Urine Volume (ml/24hr)	25	37+	70+	50	90	54+
Urine Glucose (%)	1.0	1.0	1.0	1.0	1.0	-
Urine Ketones	+++	+++	++	++	+++	-
Water Consumption (ml/24hr)	160	140	40	50	80	94

Table A-6

Water Consumption, Urine Output, and Urinalysis

for Diabetic Rat # SA-6-X

Diabetogenic Agent: Streptozotocin

	Number of Days Post-Treatment					
	5	6	7	8	9	Average/24hr
Urine Volume (ml/24hr)	22	12	35	40	60	34
Urine Glucose (%)	1.0	0.5	1.0	1.0	2.0	-
Urine Ketones	trace	+	0	0	0	-
Water Consumption (ml/24hr)	100	60	140	110	120	106

Table A-7

Water Consumption, Urine Output, and Urinalysis

for Diabetic Rat # SA-7-0

Diabetogenic Agent: Streptozotocin

	Number of Days Post-Treatment					
	5	6	7	8	9	Average/24hr
Urine Volume (ml/24hr)	42	45	15	50+	45+	40+
Urine Glucose (%)	1.0	0.5	0.5	1.0	1.0	-
Urine Ketones	+++	+++	trace	+	++	-
Water Consumption (ml/24hr)	150	140	25	200	200	142

Table A-8

Water Consumption, Urine Output, and Urinalysis

for Diabetic Rat # SA-8-X

Diabetogenic Agent: Streptozotocin

	Number of Days Post-Treatment					
	5	6	7	8	9	Average/24hr
Urine Volume (ml/24hr)	17	20	45	42+	70	39
Urine Glucose (%)	1.0	0.5	0.5	1.0	1.0	-
Urine Ketones	+++	+++	+	trace	+	-
Water Consumption (ml/24hr)	140	150	180	100	80	130

Table A-9

Water Consumption, Urine Output, and Urinalysis

for Diabetic Rat # SA-9-X

Diabetogenic Agent: Alloxan

	Number of Days Post-Treatment					
	5	6	7	8	9	Average/24hr
Urine Volume (ml/24hr)	15	15+	20	60	50	32
Urine Glucose (%)	1.0	0.5	0.5	1.0	1.0	-
Urine Ketones	+++	+++	+	+	+	-
Water Consumption (ml/24hr)	25	20	40	80	120	57

Table A-10

Water Consumption, Urine Output, and Urinalysis

for Diabetic Rat # SA-10-0

Diabetogenic Agent: Alloxan

	Number of Days Post-Treatment					
	5	6	7	8	9	Average/24hr
Urine Volume (ml/24hr)	18	17+	10+	37+	20+	20+
Urine Glucose (%)	1.0	0.25	0.25	1.0	1.0	-
Urine Ketones	+	+	+	++	+	-
Water Consumption (ml/24hr)	40	45	40	25	20	34

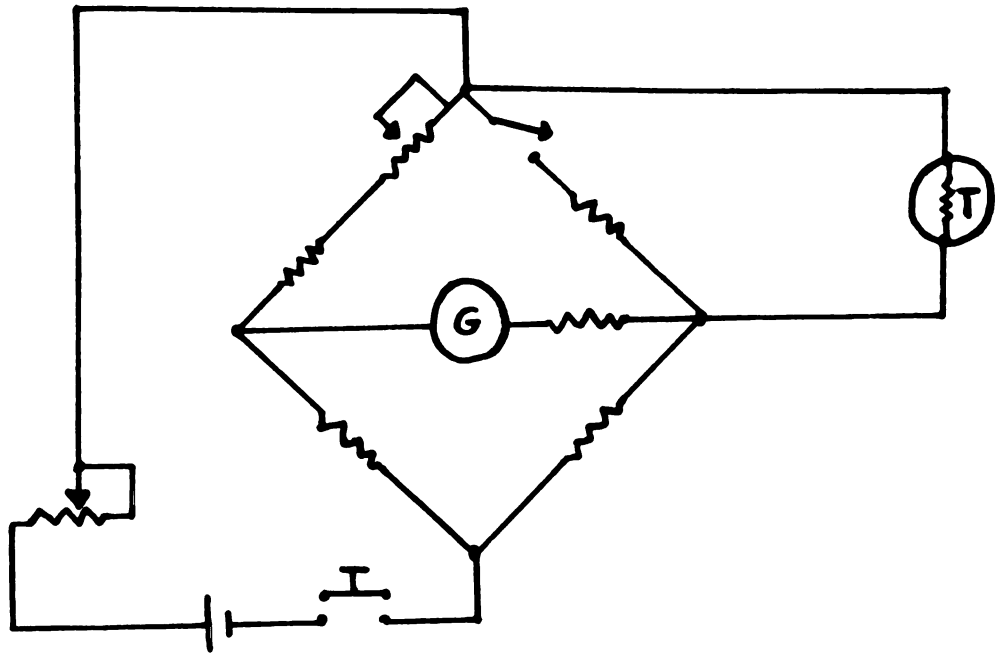

Figure A-1. Schematic Diagram of Thermister Thermometer.

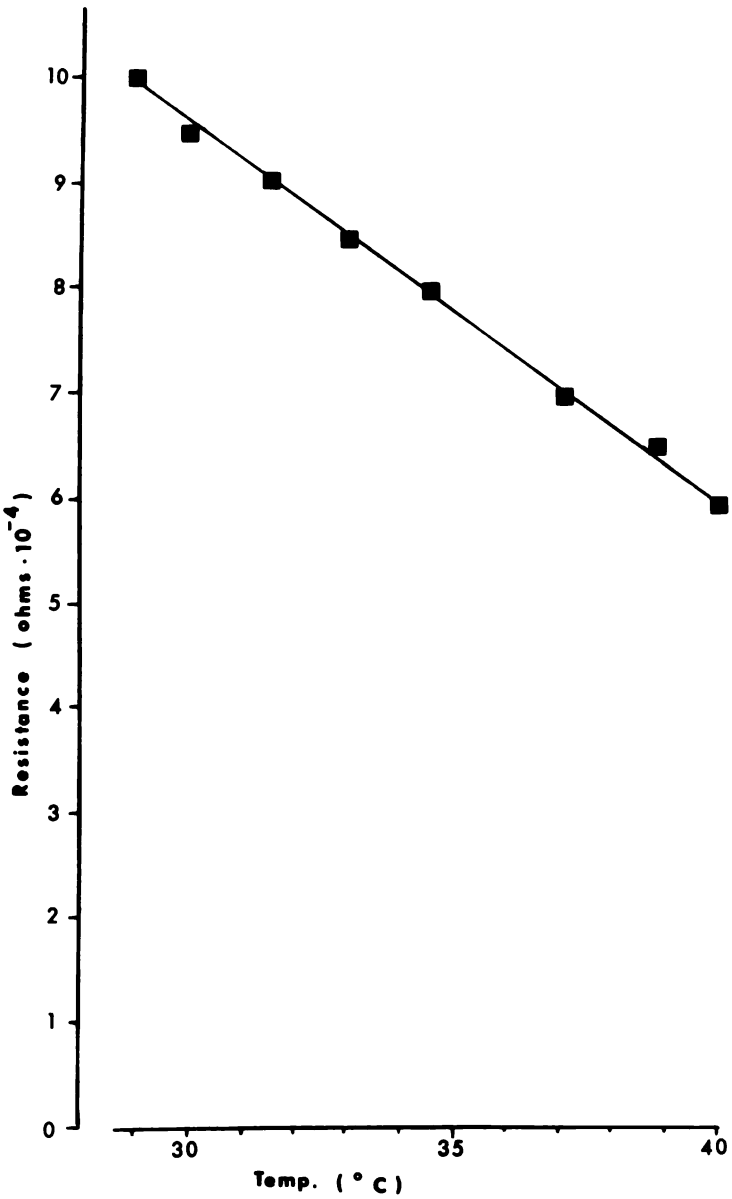

Figure A-2. Calibration Curve for Thermister Thermometer.

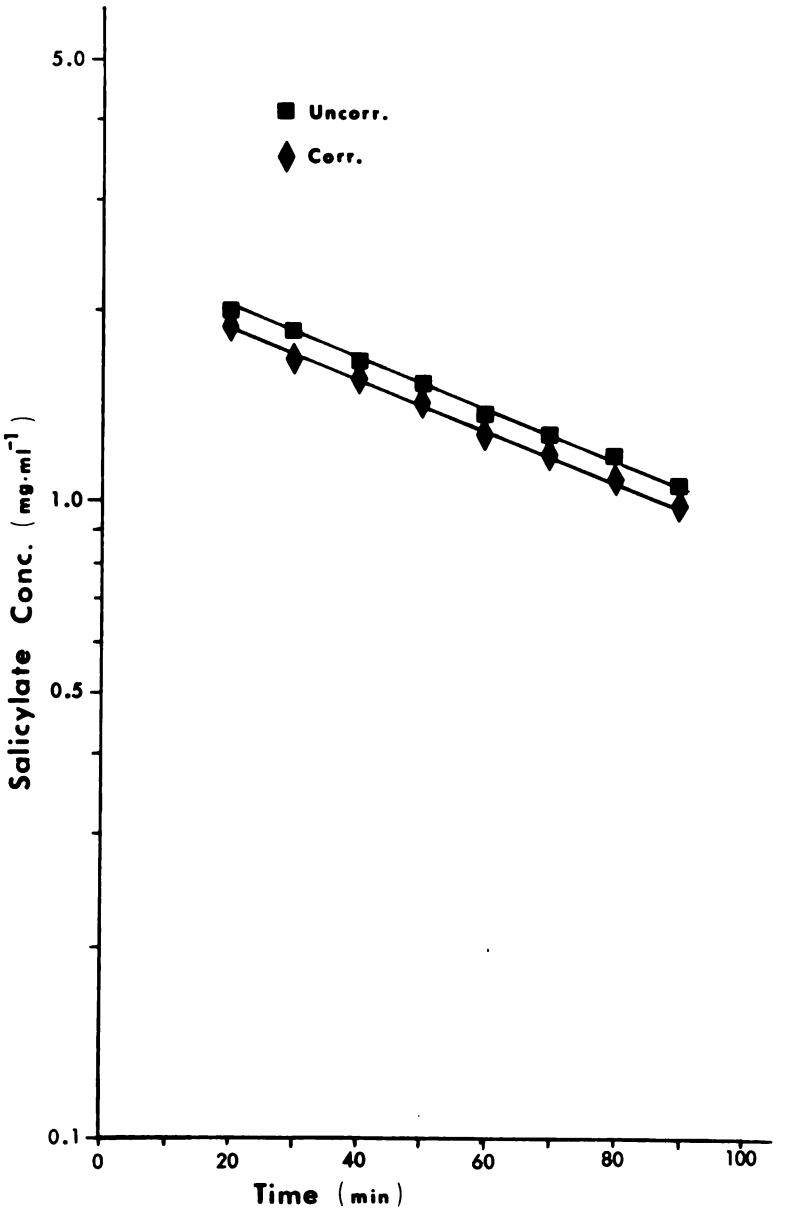

Figure A-3. Sample Semilogarithmic Plot of Salicylate Concentration in the Intestinal Perfusate Uncorrected, and Corrected for Water Flux Versus Time.

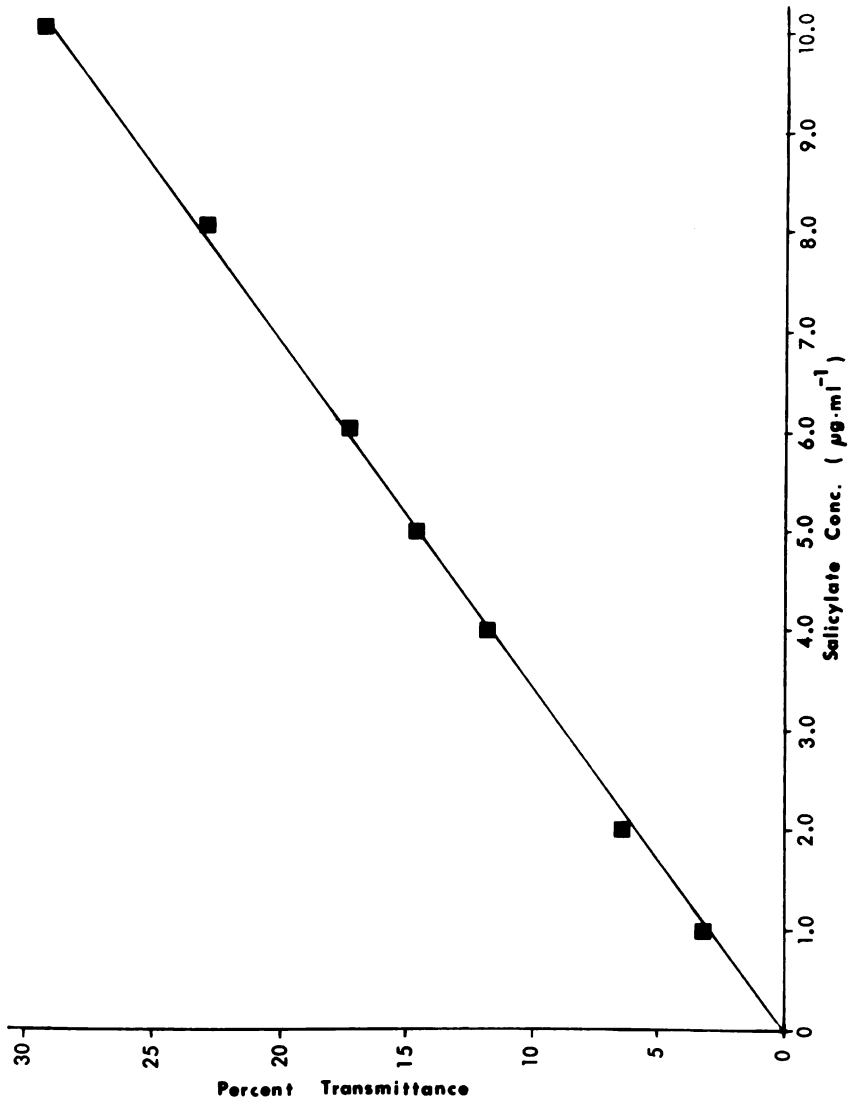

Figure A-4. Sample Standard Curve for Fluorescent Assay of Salicylate.

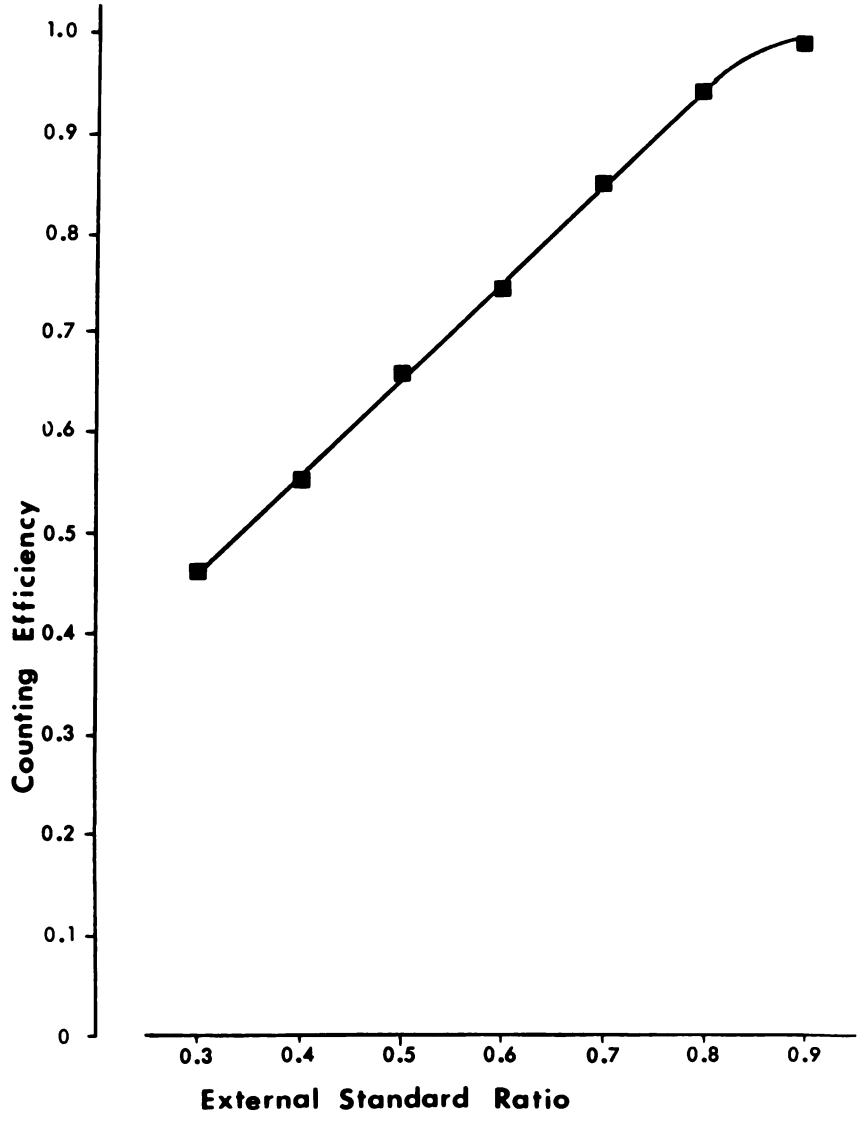

Figure A-5. Sample Plot of Counting Efficiency Versus External Standard Ratio for Liquid Scintillation Assay.