THE ABSORPTION CHARACTERISTICS OF SULFAPYRIMIDINES FROM THE SMALL INTESTINE OF RAT

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Dedicated to

BRUCE COPLAND

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ABSTRACT

An *in vivo* perfusion method for studying the intestinal absorption of selected sulfonamide drug has been devised. This method allowed us to follow the absorption of drug at various intervals and various concentrations of sulfonamide. The absorption data were related to the level of drug accumulated in various biological tissues.

At the same time an analytical procedure was designed to allow for rapid and accurate estimation of the drug in the various samples collected.

The data accumulated reflect, in general, the effect of the concentration of drug in the perfusion solution on the absorption profile of the drug and the level of the drug present in various biological tissues.

It is concluded that our results cannot be used to provide conclusive evidence for any one postulated transport mechanism but they would be useful in substantiating further *in vivo* and *in vitro* studies in this area of drug absorption. Possible areas of future study are suggested.

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INTRODUCTION

Modern studies on the transfer of various compounds across biological membranes have revealed and continue to reveal striking relationships between the type and the rate of transfer of the compound on one hand and certain physicochemical factors of both the membrane and the compound under study on the other hand.

When we consider that the classical studies of Intestinal Absorption by Thiry and Vella, (Wilson, 1962), were performed in the latter 19th century, it would seem that sufficient advancement in our concept of the phenomena of transport has been made since then and at present we can explain transport mechanisms in a much better way. But we have still a long way to go before the mechanisms can be fully understood.

Drug absorption from the gastrointestinal tract has been discussed by many investigators in recent years. Theoretical considerations on the mechanisms of drug absorption are becoming more valuable as they allow for more efficiency in drug administration.

Results obtained from *in vitro* studies will not always mean that absorption *in vivo* of a drug will be in the same proportion to that *in vitro*, e.g. concentration of drug *in vivo* will be constantly removed by circulation, whereas *in vitro* one will

observe the occurrence of high concentration gradients. The *in vitro* system will retain more heterogenous cellular elements.

Observations on the transport of many nutrient substances seem to indicate the involvement of specific transport sites directed from the mucosal to the serosal surface. These sites are able to transport molecules too large for the pores in the membrane.

A major difficulty in this field is the lack of our knowledge concerning the detailed polymolecular structure for the cell membrane. We are aware that the cell membrane has the unique ability to regulate ionic interchange in hostile and changing biological environments but little is known about the precise mechanism by which the cell utilizes its membrane to achieve this end and to maintain constant the physiological ionic level on either side. The problem of the mechanism of drug absorption might best be approached by considering the ways in which drugs may penetrate the epithelial lining of the intestine.

Since many drug molecules must be transferred across cell membranes before reaching their sites of action, it is becoming increasingly important to study the absorption pattern of various drugs, both *in vivo* and *in vitro* in order not only to determine the characteristics of that absorption, but also to see what factors,

physicochemical and/or biological, will modify the absorption pattern of a drug.

It would not be possible to cover all recent literature in the field of intestinal transport. Instead an attempt has been made to concentrate on publications which are relevant and which will help to clarify the properties of the intestinal mucosa and the mechanisms of transport across this barrier.

REASON FOR THIS STUDY

This study was undertaken to develop a biological and analytical system whereby various drug dosage forms could be studied, as well as to enable us to obtain a more clear picture of the absorption profile for a given drug than is available at present.

In order to standardize experimental methods, it was decided to choose a more frequently used class of drugs that could be readily estimated chemically in biological tissues with a reasonable degree of accuracy. The sulfonamides were among such early chemotherapeutic agents and are still of considerable importance as antibacterial agents. Although the sulfonamide drugs as a whole do not enjoy the popularity of earlier years, the sulfapyrimidines are still widely used in spite of the greater popularity of the newer antibiotics, (Neipp, 1964).

Sulfonamides with a free para-amino group are quantitatively estimated by the Bratton-Marshall Method, (Bratton and Marshall, 1939), which relies on the formation of a highly coloured azo-dye that obeys Beers Law.

At the same time, we wereaware of the studies of Schanker and his group on the Pyrimidine transport system for thymine. (Schanker et al, 1961, 1962 and 1963). It has been reported that

para-aminobenzoic acid was not transported against a concentration gradient *in vitro* by the rat and hamster intestine, (Spencer et al, 1966). On the other hand, mouse intestine demonstrated active phenomena, predictable on the basis of the electrochemical potential difference across the gut wall.

In this regard, we would expect that data collected would prove useful for future studies on the mechanism of Sulfapyrimidine Absorption.

Wilson, (1962), in his classical monograph entitled - "Intestinal Absorption" has commented on the paucity of quantitative studies available on the intestinal absorption of sulfonamides. We take a hopeful view that this study will provide us with some useful data. A computer kinetic analysis of the absorption of sulfamethazine in humans has demonstrated that the transfer of the drug from the intestine to the blood is a faster process than its disappearance from the blood, (Turco et al, 1966). These workers derived the term "Intestinal Weighing Function" which combines the many processes and compartments involved in the overall absorption of drug.

MEMBRANE CHARACTERISTICS

The cell membrane in general behaves as a lipid surface perforated by functional pores. Accordingly, this surface will have a great affinity for lipid-like molecules; the absorption of such substances occurring preferentially by diffusion through the bulk phase of the membrane due to the thermal kinetic energy of the permeant molecules. The cell membrane is a lamellar structure a few molecules thick.

The most common model proposed for the cell membrane at present is one consisting of a structured double layer of lipid molecules, (Danielli et al, 1964). Cholesterol and phospholipid molecules are arranged such that the nonpolar end of each molecule is directed inwards while its polar region faces out towards the surface of this bimolecular backbone. The phospholipids which make up the membrane are bound to one another hydrophobically, (Green and Tzagoloff, 1966). The essential properties of the membrane are derived primarily from the bimolecular phospholipid micelle sandwiched between two layers of protein.

Adsorbed upon the surface of this lipid membrane is a layer of protein which would impart greater elasticity and strength to the structure as well as having its own effects. The functional and polar discontinuities referred to as pores would penetrate the

structure in a random fashion and be filled with an aqueous medium.

The polar ends of the lipid molecules contain both fixed positive and negative charges, (Fordtran and Dietschy, 1966). These dipoles are oriented such that the negative charges are predominantly directed outwards towards the aqueous-lipid interface, whereas the positive charges point inwards towards the centre of the leaflet. The net result of this arrangement of charges is that there is a relative excess of mobile cations at the aqueous-lipid interface. In a similar fashion, there is a relative excess of mobile anions within the lipid structure. Therefore, the actual concentration of ions at the surface of the cell could be expected to vary considerably under a variety of environmental conditions.

There has been no direct evidence for the presence of pores thus far, but it is believed that the walls of the pores contain fixed charges which could affect the mobility of ions and small molecules capable of moving through these water-filled channels, (Dietschy, 1966). This influence would affect the relative passive permeability characteristics of the membrane. It is believed that these polar regions of the cell surface are responsible for the simple diffusion of ions across an epithelial surface in response to favourable electro-chemical gradients.

More or less specific "Carrier mechanisms" have been

postulated to exist as constituents of the cell membrane proper to account for the anomalous behaviour whereby certain ionic moieties penetrate the membrane more rapidly and with different kinetics than can be accounted for on the basis of diffusion through aqueous-filled pores alone. Complexation of the impermeable ionic entity with the carrier occurs at the outer surface of the cell membrane. The net result of this complexation is that the normally impermeable species can cross the barrier in concert with the carrier. The ionic species is then dissociated from the complex at the opposite side of the membrane and could partake in the subsequent stages of the transport process. The carrier would conceivably cross back by a passive process. By means of such a mechanism, transport phenomena would manifest substrate and steric specificity as well as saturation and competition kinetics.

The intestinal epithelial barrier can be considered as the major barrier to absorption since the intestinal capillary is known to be a relatively porous structure, (Mayerson et al, 1960). Recent studies have demonstrated what appears to be a longtitudinal gradient in intestinal pore size, (Soter et al, 1964). The larger pore sizes are found in the proximal intestine and allow passage of molecules having a molecular weight of 180, though not at a rapid rate.

A recent study of protein conformations in cellular membranes views the role of lipids to be more than just maintenance of membrane permeability and plasticity, whereas the genetic control of membrane structure and the biochemical regulation of membrane function are considered to reside mainly in the membrane proteins, (Wallach and Zahler, 1966).

More recent biochemical evidence points to a model of the membrane as a unimacromolecular film in which the repeating particles are lipoprotein macromolecules, (Green and Tzagoloff, 1966). These macromolecules would be identical or nearly identical in form and size, though comprised of a number of chemically and functionally different species. In this model the membrane is an expression of the component repeating units and of their pattern of alignment. Lipid is an intrinsic part of the repeating units and plays an important role in membrane formation.

Membrane pore size is subject to metabolic effects, such as hormone action, e.g. antidiuretic hormone (ADH), can regulate the pore size of relatively impermeable membranes as in the distal renal tubules. A recent study on the effect of ADH on intestinal epithelium demonstrated a paradoxical decrease in permeability, Soergel et al, 1965).

The intestinal mucosal cell membrane appears to be

lipophilic as do other membranes. The surface would tend to be acidic since the negative charges in the lipids would tend to accumulate hydrogen ions at the interface. These would then affect the permeability of substances by influencing the degrees of dissociation of the substance to be transported. This would explain why Schanker, (1958), and Hogben, (1959), believe that the true pH at the surface of the epithelial cell was lower than that of the bulk contents.

The diffusion of nonlipid soluble (polar) substances of molecular weight less than 180 can be correlated with molecular size, (Kruhoffer, 1961). Undeman and Solomon, (1962), have reconfirmed earlier studies and suggest the use of pores for the transport of this type of molecule. It should be noted that pores will not be numerous.

The epithelial cells lining the mucosa of the small intestine appear to be ideally designed for absorption. Their surface is lined by a brush-like border, (Crane, 1966). Crane, (1966), suggests that the entire process of sugar absorption is contained within the brush border region.

Thus, the functional properties of cellular membranes are a product of the molecular arrangements and the physical properties of the component molecules. Cell membranes have been

well discussed by Curran, (1963), and Hogben, (1963).

Pathological conditions could be expected to alter the normal membrane constituents which may lead to a state of malabsorption in the intestinal tract. High concentrations of drug present in the intestine could alter normal absorption rates across mucous membranes, not only by damaging the cellular elements, but also by an effect on cell membrane constituents and metabolic activity.

Recent progress in research on biological membranes has been the subject of an exhaustive symposium, (Lowenstein, 1966).

FACTORS AFFECTING THE RATE OF ABSORPTION OF DRUGS FROM THE INTESTINAL TRACT

1. Concentration:

When the absorption of other substances from the small intestine is negligible the rate of absorption of a given drug in a solution perfusing the intestine will depend mainly on the concentration of free drug and would be directly proportional to the total amount of drug in the intestinal lumen, multiplied by some absorption rate constant for the free drug.

Therefore, the number of drug molecules passing across a given unit area of intestinal wall from the lumen into the plasma in a unit of time will be proportional to the concentration of drug in the lumen. The total amount of drug molecules passing in this direction will depend on a permeability coefficient for the drug from the intestinal lumen into the blood.

It is difficult to properly estimate the true effective area of the intestinal mucosa which will be involved in the transport of the drug. The villous structure and the orientation of the intestine could vary widely during the course of an experiment as well as from animal to animal. The surface area of the lumen of the intestine is not constant throughout its length but exhibits a proximal distal gradient, (Schedl and Clifton, 1963).

2. Lipid Solubility:

Brodie, (1957), Schanker, (1958), and Hogben, (1959) have contributed much data to support the hypothesis that most drug absorption occurs by simple diffusion and can be accounted for by the lipid solubility of the drug and the lipoid nature of the cell membrane.

Their work demonstrated that drugs existing in their non-dissociated form would be preferentially absorbed across the gastro-intestinal epithelium due to their comparatively greater lipid solubility. Therefore, the degree of dissociation of any weak organic electrolyte in solution appears to be one of the most important factors in determining absoprtion of many drugs from the gastrointestinal tract. Evidence for lipid permeation is that lipid soluble drugs of high molecular weight are absorbed more rapidly than small lipid insoluble molecules like deuterium oxide (D2O), and urea, (Schanker, et al, 1958).

Further evidence that lipid solubility is the physico-chemical property governing the passage of uncharged molecules across the intestine-blood boundary was provided by the observation that the rates of absorption of a large number of weak organic acids and bases were approximately parallel to the organic solvent/water partition coefficients of the drug molecules, (Hogben et al, 1959).

It should be considered that the gastrointestinal tract epithelium will not be totally impermeable to the lipid "insoluble" dissociated form of a drug. That is, other mechanisms would allow partial penetration of the membrane by this form. This would mean that both dissociated and undissociated forms would have their own specific permeability coefficients. Totally ionized drug would still be absorbed by the passive diffusion of the very small proportion of lipid-soluble undissociated molecules.

In this regard, it has been demonstrated by Nogami and Matsuzawa, (1961, 1961a), that *in vitro* the rate of penetration of dissociated salicylic acid was one-sixth of the rate of penetration of the undissociated acid and the rate of penetration of dissociated aminopyrine was one-eleventh of the rate of penetration of the undissociated aminopyrine.

In a subsequent study *in vitro* it was demonstrated that an amphoteric compound such as sulfathiazole can penetrate the intestinal barrier in three forms: acid, alkaline, and undissociated, (Nogami, Hanano, and Watanabe, 1962). Absorption rate was greatest for the alkaline form and least for the undissociated form.

Lipid solubility appears to be a consistent explanation for the observed absorption of drugs having moderate or high lipophilic character as demonstrated by the studies of Brodie, (1957),

Schanker, (1958) and Hogben, (1959), referred to earlier.

Sulfonamides, as a rule, enjoy neither a high lipid-solubility nor a moderate water-solubility. Consequently, it may prove difficult to adequately explain the mechanism of sulfonamide absorption.

Generally, acidic and basic drugs with pK values between 2.9 and 8.0 are more rapidly absorbed from the intestine of rat than drugs whose pK values lie outside this range. (Schanker et al, 1958, Hogben et al, 1959). Therefore, luminal solutions having a low pH will favour the formation of un-ionized acids and will enhance the absorption of acidic drugs. Conversely, luminal solutions having a high pH value will favour the absorption of basic drugs by the same mechanism.

3. Hydrogen Ion Concentration:

As mentioned previously in the section concerned with lipid-solubility, the pH value of a solution will determine the degree of dissociation of weakly basic and acidic organic electrolytes. If this results in a high proportion of un-dissociated drug molecules then lipid solubility will be enhanced. The studies of Hogben et al, (1959), demonstrated that raising the pH of the drug solution increased the absorption of bases and decreased the absorption of acids. Compounds remaining essentially undissociated at

various pH values showed no change in absorption.

Although the pH of perfusate has been monitored during drug absorption studies and has demonstrated a decrease in pH values for buffered solutions in contact with intestinal mucosa, the altered value did not help to explain the intestinal absorption of aminopyrine, (Hogben, 1959). They observed a value of 6.6 pH units in the perfusate but calculated a virtual pH of 5.3 units at the micro-environment of the absorptive surface of the cell. The observed gut/plasma concentration ratios of drug agreed only when the effective or virtual pH value, at the site of absorption, of 5.3 was used. This hypothetical value then explained better the earlier data obtained by Brodie et al, (1957), and Schanker et al, (1958).

In the course of their investigation on the mechanism of penetration of sulfisomezole across the intestinal wall, Nogami, Hanano, and Watanabe, (1964), demonstrated transport of the sulfonamide against a concentration gradient _in vitro_. The other sulfonamides, sulfathiazole and sulfaguanidine did not demonstrate this specific mechanism. Part of the enhanced sulfisomezole transport was correlated with the rate of transport of fluid across the intestinal wall. No correlation was observed with the rate of transport of sodium ion. Their conclusion was that an effect by the

physiological pH mechanism at the absorptive surface to influence the degree of dissociation of the drug molecules at this surface would explain the uphill transport as well as would an active form of transport.

4. Effect of Cations and Metabolic Inhibitors:

It is widely observed that the transport rates of sugars and amino acids are significantly reduced whenever lithium or potassium ion are substituted for sodium ion during *in vitro* experiments, (Czaky, 1960). More recently, the concentration of sodium ion in the medium has been correlated with the transport of actively transported sugars, (Bihler and Crane, 1962).

In a more recent study, Nogami, Hanano and Aruga, (1966), have demonstrated that a potassium chloride-isotonic solution reduces the intestinal absorption of sulfisoxazole *in vitro*. Studies *in vitro* by these workers demonstrated that the transport of sulfisoxazole, sulfanilamide and sulfisomezole was reduced to 61%, 78% and 44% respectively in sodium-free media. Only the compound sulfisomezole had its transport rate decreased significantly when the metabolic inhibitor 2,4-dinitrophenol was present in the sodium.

When the medium was glucose-isotonic, the transport of sulfanilamide, sulfisoxazole and sulfisomezole was decreased to 69%, 64% and 39% respectively, in comparison to transport in sodium

chloride-isotonic solution. In media where sodium was originally absent, the restoration of reduced transport to maximal levels was achieved on replacing sodium ion. These workers went on to show that the level of sodium ion was correlated with the observed transport of sulfonamide directly. Similar results were obtained when the experiments were repeated by means of an intestinal tissue incubation method.

Although these studies were perhaps the first to probe the mechanisms of intestinal absorption for sulfonamide, they did not explain the difference in absorption characteristics of the two very physicochemically similar compounds - sulfisoxazole and sulfisomezole.

5. Water and Electrolyte Transport:

The physicochemical properties of the epithelial membrane described will limit the passage of water and electrolytes across the intestine. Rapid transport of these substances appears to require passage through pores or specific pump mechanisms as in the case of sodium ion.

The epithelial cells of the gut mucosa are described to be polar, (Schedl, 1966). Thus, the rate of active sodium transport would be greater at the serosal than at the luminal pole and permit net transport across the cell, and the movement of water and electrolytes across the gastrointestinal tract mucosa would be

bidirectional.

It is suggested that water movement is not independent of solute movement, (Schedl, 1966). The view appears to be that water moves as a consequence of solute movement, with an approximately iso-osmotic solution being transported. The absorption of water and electrolytes from isotonic saline has been demonstrated to occur at approximately equal rates per unit of mucosal area in proximal and distal small intestine, (Whalen et al, 1966). The absorbed fluid was hyper-tonic and thus caused the total solute concentration of the test solution to decrease during its passage through a segment of intestine. Perhaps this occurrence merits examination for its possible effects in perfusion experiments where the drug solution was constantly recirculated.

Nutrient transport (monosaccharides and amino acids) appears to be associated with a significant part of the net sodium and water transport at the mucosal border, even though the latter transport processes are isolated, (Schedl and Clifton, 1963a). The intestinal absorption of amino acids is well reviewed by Saunders and Isselbacher, (1966).

Studies with tritiated water have demonstrated absorption rate and blood concentration changes can be explained by changes in intestinal blood flow, (Winne, 1966). Increased blood flow resulted

in decreased blood level of tritiated water. It has been shown that increased blood volume increases tritiated water transfer, while decreased blood volume decreases the transfer of tritiated water, (Ochsenfahrt, et al, 1966).

5a. Transport of Organic Ions: The rates at which organic anions and cations cross the intestinal barrier and enter the circulation are extremely slow when compared to the rates of passage of uncharged molecules, (Schanker et al, 1957; Schanker, 1959). It was suggested that the penetration of these substances would result in a number of ways:

1. By very slow diffusion through the lipoid areas of the membrane
2. By diffusion through a limited number of large pores or through the spaces which might exist between the epithelial cells
3. By diffusion through the membrane in the form of a less polar complex formed with some material normally present in the intestine proper
4. By transport across the epithelial barrier by a specialized process involving membrane carriers or ion-exchange mechanisms.

Levine and her group, (1955), (1957), have studied the poor absorption of a number of quaternary ammonium ions by measuring their disappearance from isolated intestinal loops in the rat. In

general, the rate of absorption appeared to decline with time. At first it was believed that complexation with some component of mucin caused the decrease in absorption rate, but the removal of mucous by gentle washing of the intestine in a later study resulted in a decrease rather than an increase in the absorption of these organic ions, (Levine, 1959). Many normal biochemical intestinal constituents were added to the drug solutions but they had no effect on the absorption of the drugs.

6. Donnan Effects:

It is conceivable that charged unabsorbed macromolecules could enhance the absorption of certain drugs, (Doluisio and Swintosky, 1965). The increase in absorption would be due to a Donnan effect and would occur when the macromolecule and drug were of similar charge. The charged drug would have to be capable of penetrating the intestinal membrane. Since the Donnan equilibrium demands a balance of charges on each side of the membrane, the repulsion of charged drug molecules by the impermeable charged macromolecule could hasten the transfer of like charged transportable drug molecules.

7. Intestinal Circulation:

Several studies relating intestinal blood flow and absorption have been well reviewed, (Jacobson, 1967). In general,

large decrements in intestinal blood flow are necessary before absorptive processes, both passive and active types, are impaired. Whether changes in absorption have an effect on local blood flow does not appear to have been investigated to date. There does not appear to be sufficient data accumulated to determine the effect(s) of motility on intestinal blood flow. The flow of plasma at a site of absorption will assist the maintenance of a concentration gradient by constant removal of absorbed drug.

8. Effect of Hormones:

Insulin has been shown to control the accumulation and release of drugs in tissues, both *in vivo* and *in vitro*, (Danysz and Wisniewski, 1965). In a subsequent study the influence of posterior pituitary hormones increased the velocity of penetration, distribution, and concentration of Isonicotinic Acid Hydrazide into various tissues, (Danysz and Wisniewski, 1966). Riggs, (1964) has adequately reviewed the influence of hormones on transport of nutrients across cell membranes.

9. General Factors:

Some drugs which appear to be poorly absorbed could be unstable in the gastrointestinal contents and thus be adversely affected with regard to their absorption, although *in vitro* studies

may indicate good absorption characteristics.

Other compounds, especially acidic drugs, would be more susceptible to precipitation in the fluids of the gastrointestinal tract and thus be absorbed slowly. In this instance their rate of dissolution would become a factor limiting the rate of absorption.

TRANSPORT MECHANISMS

Transport refers to the net difference between opposing unidirectional fluxes across a membrane. The fluxes themselves may be high, but the net transport could be small. Absorption of any substance from the intestine proper consists of the pattern of movement across the following biological compartments: the epithelial surface, the interior of the mucosal cell, the basement membrane, the connective elements, and ultimately an endothelial surface before ending up in a capillary or lymph vessel. One advantage of getting an insight into transport mechanisms for a drug would be that this knowledge might be suitably utilized to enhance the likelihood of possibly increasing the availability of a drug.

A permeant species must be influenced by some driving force resident in the cell to achieve transmembrane movement. As mentioned previously, membrane lipids, water-filled pores and specialized carriers will determine the permeability characteristics of the epithelial cell.

Forces known to influence the movement of ions across intestinal epithelium have been discussed recently, (Fordtran and Dietschy, 1966). These are listed as follows:

1. Cellular Metabolic Energy - the details of coupling to the absorption process remain unknown

2. Activity Gradients - ionic activity gradients may be the reverse of the concentration gradients
3. Electrical Gradients - these can cause passive ionic movement against a concentration gradient
4. Hydrogen Ion Concentration Gradients - when these are favourable to un-ionized moiety, it will equilibrate rapidly across the mucosal cell layer by a passive mechanism
5. Solvent Drag - water flow across a membrane can be expected to influence the movement of solute.

Some of the results involving ions in transport studies across the intestinal mucosa are that one can discuss and define the phenomena with respect to:

1. The specific forces which provide energy for movement
2. The structural features of the cell membrane which permit or facilitate this movement

Some more experimental data are needed to definitely categorize these different transport phenomena. Most of the current work in this field is attempting to amplify the mechanisms involved in intestinal absorption. No one mechanism is intended to explain comprehensively the transfer of a given compound across a biological membrane.

Some of the different transport mechanisms proposed to

date can be listed as follows:-

1. Solvent Drag

As mentioned briefly in a previous section, the bulk flow of water across intestinal epithelium by way of aqueous-filled channels or pores in the membrane can result in a driving force. Equations have been derived to predict the magnitude of the fluxes set up, (Solomon, 1961). It is assumed that the bulk flow of solvent and the passive movement of the permeant species occur through the same aqueous-filled channels. The movement of fluid in bulk would thus drag along small solute molecules through the pores. Solvent flow could also occur due to localized osmosis in addition to being a codiffusion process secondary to some form of solute transport.

Undoubtedly, such bulk flow of water can be expected to occur in the intestine and thereby influence the movement of solute molecules across the mucous membrane. As bulk movement of fluid occurs across the membrane, it is conceivable that a "sieving" of solute molecules by the membrane will result. This could be another means of achieving a potential difference across biological membranes, (Dietschy, 1966).

2. Simple Passive Diffusion:

The influencing force for the passive diffusion of

ionized and non-ionized moieties is believed to be the result of the existence of physical electro-chemical gradients across the intestinal mucosa. These gradients would cause an ionic moiety to diffuse passively through the aqueous-filled channels while non or very weakly ionized acids and bases are presumed to diffuse passively through the lipid barrier by solution in the lipid structure of the membrane proper. From the nature of the existent electrochemical gradients, the direction and the rate of diffusion would presumably be related in a linear fashion to the magnitude of these gradients. Solvent drag can be expected to modify this type of diffusion. Elaborate experiments are usually designed to minimize electrochemical gradients and reduce bulk solvent flow.

Passive diffusion of solute can often be explained by Fick's Law of Diffusion, i.e. the rate of diffusion is directly proportional to the surface area of the absorbing membrane and the difference in solute concentration across the membrane (the concentration gradient), and inversely proportional to the thickness of the membrane, (Kruhoffer, 1961).

3. Facilitated Diffusion:

This type of transport is believed to result from passive forces, the main driving force being a favourable electrochemical gradient across the membrane. However, in contrast to simple

diffusion, the actual movement across the mucosa has been suggested to be facilitated by means of complexation with a membrane carrier. As a result, the kinetics of this movement are not just of the first order as occur with simple passive diffusion but tend to resemble those features, characteristic of an active carrier-mediated transport system, viz.

a) Substrate structural and steric specificity

b) Saturation behaviour

c) Competitive inhibition between related substances capable of transport

This mechanism has been suggested to operate in the absorption of certain sugars from the intestinal tract, (Wilson, 1962).

4. Active Transport:

The basis of this form of transport is the suggestion that the driving force involved derives its energy directly from cellular metabolism by some form of coupling to a carrier mechanism. Net movement by this mechanism is characterized by the ability of substrate to move against an electrochemical gradient and demonstrates a form of kinetics typical of a saturable carrier system. In addition, this type of transport is sensitive to inhibitors of eellular metabolism and temperature changes. Therefore, active

transport resembles enzymatically mediated reactions in many respects.

Active ionic transport has been associated with the development of significant transport potentials, (Dietschy, 1966). This results in the serosal surface becoming positively charged relative to the mucosal surface. The uptake in the active absorption of hexoses, (Crane, 1965), and amino acids, (Saunders and Isselbacher, 1966), by the mucosal cell is a step which is believed to be sodium-dependent in the overall transport process. Receptors and enzyme carrier systems have been suggested in the active transport mechanism of methionine and sodium butyrate by Hart and Nissim, (1965).

5. Exchange Diffusion:

In this form of transport, the movement of one substance against an electrochemical gradient is synchronous with the movement of another substance with the electrochemical gradient in the opposite direction. This type of diffusion may result in rapid exchange of substances across a membrane. On the whole, the net absorption of substances by this process will not be significant.

Exchange diffusion was postulated to explain the observed rapid bidirectional fluxes of isotopes across membranes at rates greater than what could be explained on the basis of the known

passive permeability characteristics of the epithelial surfaces, (Dietschy, 1966).

It is thought that a carrier system present in the membrane, is able to move one particular ion from the mucosal to the serosal surface and move another similar ion in the opposite direction on the return of the carrier to the mucosal surface, (Kruhoffer, 1961). A counterflow exchange system has been described by Kruhoffer, (1961), in his review of transport mechanisms. In this type of mechanism a sugar moving in one direction is accumulated in the cell and is able to facilitate movement of a different sugar in the opposite direction.

6. Pinocytosis:

Intestinal epithelial cells have been shown to possess the ability to engulf particles or dissolved materials by a process of vesiculation, (Wilson, 1962b). It has also been shown that some of these vesicles are discharged into the lymphatic vessels after being formed at the base of the microvilli projecting from the surface of the cell. Experiments with proteins and lipids have indicated that this phenomena enjoys a high degree of substrate specificity. However, the process appears to operate far too slowly to account for the rapid cellular uptake of natural substrates. It could account for the uptake of small amounts of protein and other macromolecules.

EXPERIMENTAL

METHOD

1. Introduction:

In order to study the characteristics of absorption of the sulfapyrimidines it was decided to utilize an *in vivo* method for various reasons. The major consideration here is that such a study allows absorption to occur exclusively through the mucosa of the small intestine, while maintaining the flow of blood in the intestinal wall. In addition, an *in vivo* approach allows one to investigate the process of absorption in a state closer to physiological normality. The perfusion technique allows the opportunity to investigate the relationship between the rate of drug absorption and the increase in plasma levels of drug, by presenting the drug directly to the absorption sites and eliminating such factors as gastric emptying time, transit time, and the influence of digestive factors. Analytically, the advantage is that one is allowed to perform multiple sampling on the same preparation.

A drawback of this method is that unlike *in vitro* methods it is more difficult to demonstrate active phenomena across cell membranes. The experimental technique we utilized, was similar to the *in situ* cannulation and perfusion of the intestine adopted by Schanker, Brodie, and their associates, (1958), in their

classical studies on gastrointestinal absorption. There were modifications to the original method by us for the various reasons mentioned in an effort to improve the technique.

As a result of the diversity of experimental methods used to study the mechanism of gastrointestinal absorption of drugs, there is a large amount of data in the literature. However, a common basis for the comparison and evaluation of this data is, in many cases, lacking.

Quantitative estimates of the rate of absorption of drugs and nutrients are difficult. By minimizing and/or eliminating physiological variables and administering drugs in true solution, we can better relate the important physicochemical properties of drug molecules which determine the rate of absorption to membrane and transport characteristics.

Although no one method for studying absorption of drugs is definitive and free of disadvantages, the intraluminal perfusion *in situ* of an intact segment appears to be a more rational approach to this problem. This method allows one to compare the rates of absorption of a wide variety of compounds by measuring directly the disappearance of drug from the intestinal tract. It has been reported that intestinal perfusion experimental procedures can in certain instances contain experimental errors which may be of the

same magnitude as the actual absorption rates under investigation, (Whalen et al, 1966).

The hematocrit has been observed not to vary widely when up to 30 ml of blood was sampled from rabbits during *in vivo* intestinal absorption studies, (Mehran and Blais, 1966).

We chose the Sprague-Dawley White Laboratory Rat because we have had much success with this species in previous work. Young adult males, weighing from 250/350 grams, were utilized. Pre-operative fasting consisted of leaving the animal without food for approximately 20 hours, but not without water. The basic diet consisted of Laboratory Chow.[R] A study of the effects of manipulation and fasting on the gut in the *in vivo* intestinal absorption of quaternary ammonium compounds has shown that fasting did cause significantly increased levels of absorption whereas the effect of mild physical manipulation of the intestine resulted only in a minor decrease in absorption in fasted rats, (Levine and Pelikan, 1960).

Standard surgical instruments were used for the operative technique. All sutures were made with No. 10 white mercerized crochet cotton employing standard surgical needles. We found the following monographs helpful in our experimental surgical technique: Lambert, (1965) and Farris and Griffith, (1949).

R - Ralston Purina Company, St. Louis, Missouri

2. Anaesthesia:

Diethyl ether was chosen as the anaesthetic agent because of the simplicity of its administration and its lesser metabolic effects in comparison to commonly used Barbiturate anaesthetics. Kojima, (1966), and his associates, have shown that the absorption of several drugs in experiments utilizing the in vivo perfusion technique was suppressed as much as 50% when using pentobarbital as the anaesthetic agent. It was suggested that this effect would occur only with strong acids and bases.

Small intestinal absorption in the immediate two hour post-operative period has been examined in dogs, (Glucksman et al, 1966). These workers used pentobarbital as the anaesthetic agent and showed that glucose and $Na^{22}Cl$ absorption were reduced from 49% to 21%, and from 68% to 50% respectively. They also detected a tendency for the gastrointestinal tract motility to increase above control values.

An intratracheal cannula was connected to a respiration pump to administer the anaesthetic and maintain respiration in order to minimize the potential danger of respiratory failure and excessive bronchial secretions.

Prior to securing the animal to the operating table, the animal was anaesthetized in a covered beaker using 0.2 volume % of

the ether soaked onto a screen covered with cotton and positioned at the top of the beaker.

Approximately one minute after the animal was completely immobilized, it was removed from the beaker and fixed to a Brodie[R] Operating Table. The table was fitted with a heating device and provided warmth to the animal. The skin directly above the trachea was shaved lightly with an animal clipper.

While the animal was under this preliminary ether anaesthesia a midline incision was made over the trachea. The muscles covering the trachea were separated by blunt dissection to expose the trachea. An opening was made in the trachea by a transverse incision between two cartilage-like rings below the cricoid cartilage. A glass or polyethylene cannula was inserted gently through the opening in the trachea and secured in position by a ligature. The cannula was connected by rubber latex tubing to a Brodie[R] Anaesthetic Bottle which was in turn connected to the respiration pump. This anaesthetic bottle allowed us to control the level of anaesthesia accurately, and permitted changes in concentration of ether in the inspired air readily during an experiment.

Respiration was maintained artificially by an Ideal[R] Pump Assembly operating at 85 strokes per minute. The stroke volume was adjusted for each cannula such that 2-2.5 cc of air was inspired

R - C.F. Palmer (London) Ltd., London, England

and expired with each breath.

Before proceeding to the operation proper, the level of anaesthesia was stabilized at the correct stage and the heart and respiratory rate were monitored physically for a short period. Constant checks were made throughout the experiment. Once we were satisfied that the animal was responding well to the anaesthetic and positive pressure artificial respiration, the abdominal wall was prepared for further procedure.

3. Operative Procedure:

a) Opening of the Abdominal Cavity -

The operative field was confined to the anterior abdominal region. The area was shaved and wiped clean before proceeding further. An incision was made with great care and strictly adhering to the midline of the abdomen. The incision was extended from below the xyphisternum to a point just above the symphysis pubis. The abdomen was opened by cutting through the muscles and peritoneum along the linea alba.

Clean gauze swabs soaked in warm normal saline were arranged around the incision to minimize shock. The border of the swab adjacent to the incision was folded over the cut edge of the abdominal wall and clamped to the skin and muscle by means of tissue forceps. The forceps were turned back to better expose the

abdominal cavity. Other swabs soaked in normal saline were placed over the exposed viscera as protection from contact with the air. Care was taken not to disturb or manipulate the intestine unduly at this and subsequent stages.

b) Surgery of the Intestine:

In all intestinal surgery great care was taken to avoid any contact of the intestinal contents with the abdominal cavity and the organs contained therein. Traction on the wall of the intestine was avoided as much as possible to minimize shock and the incidence of a hematoma due to rupture of small blood vessels. The tips of the fingers were moistened with saline for all manipulations involving direct contact with the intestinal tract. Movements inside the abdominal cavity were executed carefully, especially when separating the coils of the intestine. When necessary, the stomach was mobilized indirectly by way of the greater omentum.

Incision of the wall of the intestine was executed with fine straight-pointed scissors. Larger blood vessels were avoided to minimize haemorrhage at the site of incision. Incision was also avoided over the location of Peyers Patches on the gut. Saline moistened swabs were used to wipe off any oozing of blood and to remove any unwanted matter coming out from the incised intestine.

In the majority of cases, it was not necessary to lift the intestine completely out of the abdominal cavity. Whenever it was necessary to lift the gut outside the abdominal cavity, it was covered with saline moistened gauze, in order to keep the intestine moist and warm, and to protect it from the air.

We avoided squeezing the gut to remove any particulate matter, down and out of the experimental segment. We rather preferred to let particles remain in the segment until the initial perfusion was commenced. Any blocking of the tubulature was then corrected by injecting a small quantity of air part way up the efferent tube to dislodge the interfering particle into the efferent tube. Carefully executed, this procedure resulted in no excessive increase in intraluminal pressure.

The proximal portion of the small intestinal segment (Jejenum) was localized just distal to the duodeno-jejenal flexure.

Two ligatures were then passed carefully through the intestinal border of the mesentery to avoid the mesenteric vessels at the proximal site. The site of entry of the perfusion tube into the intestinal lumen was such that the direction and position of the intestinal perfusion segment upon cannulation would be as close to normal as possible.

One ligature was then tied around the loop of intestine

to obliterate the lumen, but not tight enough to injure or cut the tissue. This ligature marked the proximal end of the loop of intestine to be perfused. The second ligature was left as such to be used later to secure the inflow tubing into the lumen of the perfused loop.

Commencing at the fixed proximal end we measured off approximately 20 cm of intestine distally, taking every precaution not to stretch or disturb the segment. During this direct and mild physical handling of the intestine, we did not choose a length of tissue greater than that supplied by one vascular mesenteric arch. Two ligatures were passed in a similar fashion at the distal point. One ligature was tied to set the distal limit of the perfusion segment. This procedure isolated the anatomical continuity of the intestinal tract but the isolated loop retained its blood and nerve supply intact. The site of each ligature was chosen such that the experimental segment would continue to receive optimal circulation from direct and collateral vessels. Direct handling of intestinal vessels was avoided to minimize the incidence of pulsatile haematoma which would jeopardize blood supply to a segment.

We found that the length of 20 cm was most suitable for our experiments. Flow occurred uniformly and the incidence of

obstruction was not great in a loop of this size. Determination of the length of the experimental segment on termination of the test indicated an error of the order of 10%. As this variation occurred predominantly in the distal region, the change in area and weight of intestine did not occur in a region of high absorption activity.

Having thus isolated a segment of the small intestine for perfusion purposes, we then proceeded to cannulate the proximal and distal ends of the isolated loop for the inflow and outflow of the perfusion drug solution.

For the perfusion tubing we chose Silastic[R] Medical Grade Tubing with an inner diameter of 1.98 mm and an outer diameter of 3.75 mm. The end of the tubing was bevelled to facilitate introduction into the lumen of the intestine.

c) Cannulation of the Intestinal Loop:

A small incision was made in the longtitudinal plane by means of fine straight scissors with pointed tips at the anti-mesenteric border of the proximal end of the isolated loop just distal to the first closed ligature, care being taken to avoid visible blood vessels. Gentle pressure was applied to the wall of the intestine at the incision to minimize any leakage of blood from collateral vessels and the tube was inserted gently into the lumen

R - Dow Corning Corporation, Medical Products Div., Midland, Michigan

to a distance of about 5 mm inside. This portion of the loop with the tubing inside was secured in position while the assistant tied the second ligature around the segment of intestine and over the tube to fix the tubing at the proximal end such that it did not slip out of the lumen while at the same time being careful not to partially reduce or obliterate the internal diameter of the inflow tubing. This securing ligature also served to control any haemorrhage. In very few cases a second ligature was placed adjacent to this ligature to ensure complete haemostasis.

The inflow tubing was then securely anchored to the chest wall by a ligature passed through the skin to hold the tube in a proper position in order that no twisting or tension was transmitted to the intestinal loop.

A short piece of tubing was cannulated at the distal end of the isolated loop in a similar manner to serve as the outflow or collection tube. The viscera disturbed during the operation were then returned to their normal residence in the abdominal cavity in a more or less normal orientation to ensure a constant mean flow rate during the perfusion. The inflow tube had previously been filled with drug solution and was now attached to the perfusion pump. The collecting tube was placed into a 10 ml graduated cylinder to collect the perfusate.

d) Closure of the Abdominal Wall:

Once the necessary tubing was secure the abdominal wall was closed. We preferred to close the abdomen in two layers. Interrupted stitches were passed through the muscle and peritoneal layers and tied. Next, the skin was apposed by means of interrupted stitches. The suture material was Size 10 mercerized white cotton thread. Before complete closure of the abdominal a small quantity of warmed normal saline was put inside the peritoneal cavity. Gauze pads, soaked with warm saline, were arranged over the closed incision for the duration of the experiment.

A heat lamp was positioned over the animal at this stage to further minimize the chance of shock occurring and to provide extra warmth. Temperature checks at intervals by means of a termistor probe inserted rectally indicated only very minor body temperature variations when the above mentioned were employed.

e) Duration of Operation:

The abdominal operation described was completed in approximately 30 minutes. The most common delaying factors encountered at this stage were variation in the efficiency of respiration and the depth of anaesthesia.

Whenever it was obvious that the operative procedures employed had caused undue shock and thus resulted in the alteration

of necessary physiological and metabolic functions in the experimental animal, the animal was discarded.

f) Collection of Blood Samples:

The zero hour blood sample was collected by amputation of the tip of the rat's tail with a sharp razor blade. The blood was then allowed to flow freely into a heparinized 5 ml beaker until just slightly more than 0.2 ml was collected. The required volume of 0.2 ml of blood was then pipetted quickly into a centrifuge tube. The perfusion pump was started immediately to commence perfusion of the isolated segment.

Subsequent samples of blood were collected at the specified times till the termination of the experiment.

g) Termination of Experiment:

Once the final blood sample was collected the peristaltic pump was switched off. The ether concentration in the inspired air was adjusted to a lethal level and the stitches in the abdominal wall were removed and the abdomen opened.

Artery forceps were clamped over the isolated loop of intestine at the proximal and distal sites to include a small bit of the terminal portion of the tubing inside the lumen. At this stage the heart beat was very feeble and the mesenteric circulation quite sluggish. Detachment of the perfused intestine from the

mesentery at this stage caused very little bleeding. The segment was freed from its mesentery quickly with scissors, care being taken to avoid puncturing the loop filled with drug solution. On removal of the loop, it was then quickly rinsed, measured, and emptied of its contents into the final collection cylinder. The last traces of fluid were removed by means of a moderate flow of compressed gas introduced into the segment.

The gut segment was then attached to a separatory funnel system filled with cool saline. This arrangement produced a pressure of 45 cm of water and was found to be satisfactory for washing the excised intestinal segment free of the last traces of drug not inside the tissue. Approximately 900 ml of the saline was allowed to percolate through the intestinal segment and was collected in a litre flask by means of a funnel plugged lightly with acid-washed glass wool. The last traces of fluid were removed by means of compressed air, blotted, dried between filter papers and weighed. After this, the intestinal tissue was ground up in a Duall[R] Tissue grinder for the purpose of estimating the tissue content of drug. We found it better to avoid cutting intestinal tissue when attempting to determine the amount of drug in the tissue with accuracy. Tissue samples were generally homogenized on the same day. Whenever tissue was left for a short period, it was kept

R - Kontes Glass Laboratory, Vineland, New Jersey

moistened with a slight amount of saline and placed over ice. On very few occasions it was necessary to store tissues in a freezer for a few days before drug could be estimated.

The kidneys and the liver were also excised quickly and rinsed with saline. They were stored over ice in a cover evaporating dish while blotting and weighing of the tissue was performed. The appropriate amount of tissue was cut from the middle portion of the respective organs and placed in a separate tissue grinder, and made ready for homogenization for the estimation of the drug contained in 1 gram of tissue respectively. Urine was withdrawn from the bladder by means of a tuberculin syringe fitted with a 22 gauge needle. The bladder was punctured and emptied carefully. The total volume was noted and 0.2 ml were then delivered into a centrifuge tube.

g) Examination of Intestinal Tissue:

As a histological examination of the intestine was not possible in our laboratory, we had to resort to a macroscopic examination of the perfused intestinal segment. This examination was carried out regularly in our preliminary experiments during standardization of the method. Gross examination of the mucosal layer with a magnifying glass demonstrated very little, if any, damage of the epithelium at the tips of the intestinal mucosa.

Damage of this type was correlated with the amount of mucosal debris and cloudiness, present in a given perfusate sample. The amount of mucosal debris present was negligible in all samples, a further indication that minimal stress was imposed on the isolated intestine in our experiments. The viability of canine gastrointestinal epithelium subject to perfusion at rates of 4-5 ml/minute has been examined histologically and functionally, (Dritsas and Kowalewski, 1966). The preparation appeared to remain viable following 16 hours of perfusion. Intraluminal perfusion of the rat intestine *in vivo* to study the effect of inhibitors on intestinal absorption were performed by Hart and Nissin, (1965). At rates of perfusion of 1-2 ml/minute their histological examination of intestinal tissue indicated breakdown only at high concentrations of drugs.

METHOD

1. PERFUSION OF ISOLATED INTESTINE

The intestinal segment was perfused for a period of 2 hours with approximately 100 ml of the appropriate drug solution, heated to 40°C by means of a condenser arranged 5 cm from the end of the inflow tube. Periodic checks of the system indicated that the temperature of drops of drug solution delivered at the tip was 38°C ± 0.5.

We did not carry out prolonged initial washing or cleansing of the intestinal segment with drug solution in order to minimize the incidence of damage, potential shock to the intestine, and removal of normal intestinal contents, as a result of prolonged perfusion. The presence of intestinal mucoid material in drug solutions has been shown to result in a uniform decrease in the amount of benzomethamine absorbed from an *in vivo* loop of intestine, (Levine and Pelikan, 1960). There would be some stress imposed by our technique on the biological system during the course of an experiment. The washing of the segment at the conclusion of the experiment removed a significant amount of mucous-like material.

Our method did not involve continuous closed circulation of a given drug solution. Intestinal perfusion experiments where there is a continuous recirculation of solution would acquire some

biological substances in the perfusion medium which could influence consequent absorption of the drug. Schanker and his co-workers, (1958), reported that the binding of drugs to intestinal mucosa in 3 hours of this type of perfusion technic was negligible.

We chose to perfuse drug solution from a stock bottle and collected 10 ml aliquots of perfusate for analysis. We felt that a steady-state was achieved readily and would always maintain a constant concentration gradient across the intestinal wall.

Our drug solution did not incorporate phenol red or any other poorly absorbed marker as a volume reference indicator. Preliminary experiments indicated that phenol red concentration remained constant on passage through the segment of intestine, i.e. volume changes of drug solution were minimal.

Our initial flow rate was approximately 1.4 ml/minute. This was maintained until 20 ml of perfusate was collected to equilibrate the concentration gradient in the intestinal segment and to wash out the larger particles. The first 10 ml of perfusate was sufficient to wash out bile and was discarded as it added to the absorbance during analysis.

Increasing the duration of the equilibration period has been demonstrated to increase the accuracy and decrease the variability of absorption rate, (Fordtran, 1966b). However, such

a step would reduce the number and length of studies possible with a given preparation. In addition, there would be considerable absorption of the test substance. We feel that in view of the length and size of our intestinal segment, that mixing and equilibration were more or less satisfactory under the conditions mentioned.

Our flow rate was lower than that reported by previous workers. Our preliminary experiments demonstrated that intraluminal pressure varied little at a perfusion rate of 0.7 ml of fluid per minute. Ochsenfahrt et al, have utilized a perfusion rate of 0.7 ml/minute to study the effect of various catecholamines on intestinal circulation and transfer of tritiated water. Direct observation of the gastrointestinal tract motility indicated a seemingly normal tone and movement pattern during perfusion. The incidence of plugging in the experimental segment was very low.

The effect of changes in intestinal lumen diameter on absorption does not appear to have been evaluated. At low flow rates the volume of the intestine will increase more or less in direct proportion with increasing flow rates. Of course, there will be limits beyond which an intestinal segment is resistant to dilatation and an increase in flow rate or volume will be handled by a proportionate decrease in transit time.

It is important to maintain a constant mean flow rate, an equal mean concentration or osmotic gradient in the experimental segment to minimize any net fluxes of ions and fluid across the intestine.

We chose Kreb-Ringer Bicarbonate Buffered solution as the vehicle for the drug solutions since it is isotonic with rat serum and bicarbonate is the main buffer system in the body. Hypo-, and hyper-tonic vehicles were shown to cause pronounced net exchange of water across the intestinal wall, (Koizumi et al, 1964). The osmotic effect of the drug was not corrected for in the total concentration, since at the highest concentration of drug used this would be about 2 percent of the total.

Addition of endogenous fluid or substances into the perfused experimental segment will cause deviation from an ideal steady state condition. This would also cause a continual mixing and dilution pattern to exist at some points in the segment. Bile appeared to be completely washed out very early in the experiment. Schanker and his associates, (1958), did not demonstrate any influence due to the presence or absence of bile on the absorption of the compounds studied.

Schanker and his group, (1958), reported that there was a decrease in the recovered volume of the perfusion solution

employed, of the order of 3% from the initial volume. Our recovery of perfused volume was decreased beyond this value in only a few experiments. On the whole, recovery volumes varied between 1-2% less than the volume delivered from the stock flask. This does not preclude that there was water exchange across the intestine.

a) Perfusion Pressure:

Measurement of the pressure developed during perfusion indicated a maximum hydrostatic pressure of 10-15 cm of water at the proximal end of the perfused segment. This appeared to result in sufficient filling of the loop to produce a moderate degree of distension of the intestine so as to give a good exposure of the mucosal membrane surface to the perfused solution. It has been reported that at hydrostatic pressures beyond 60 cm of water, the intestinal tissue becomes damaged, (Wiseman, 1961).

We timed the collection of the 10 ml perfusate in order to give us a better idea of the pressure-motility-transit time relationships. On the whole, there was uniformity in the time taken for 10 ml of drug solution to perfuse through the segment. Lacking adequate pressure sensing devices, we found the time relationships extremely helpful in this regard. With the rate of perfusion being 0.7 ml/minute and the delivery of fluid coming in peristalic pulses from the inflow tube, the gut would have certain

regular periods in which to equilibrate for any increase in fluid volume and thus minimize the build-up of excessive pressure. Dumont et al, (1960), used a peristaltic pump to deliver 0.5 ml of fluid per minute at a hydrostatic pressure of not more than 5 cm. Under these conditions, they were able to record small variations in water absorption.

In the vast majority of cases, the increase in intraluminal pressure was due to plugging of the intestinal segment, particularly at the opening of the distal tube in the loop. As noted elsewhere, this blocking was overcome readily by injecting a small amount of air part of the way up the distal tube via the exit point to dislodge the offending particle as gently as possible.

b. Sampling:

Larger sample volumes would be more representative of the fluid which has perfused through a segment, but would not reflect changing patterns. Too rapid a sampling rate may distort results, if there are sudden variations in flow rates and movement of fluids across intestinal epithelium. Staggering of the sampling time would tend to increase the accuracy of the method, especially during the course of short experiments. This staggering was achieved somewhat in our experiments by the variation in time taken for any 10 ml portion of drug solution to be collected. We selected

15 minute intervals for the purpose of comparison as this value was an intermediate value for the collection of 10 ml of perfusate.

c. Influence of Flow Rate on Absorption:

Although our pump flow rate remained constant, except for the initial equilibration-wash period, there was some variation in the time taken for individual 10 ml portions to perfuse through the isolated segment. This variation would result from the influence of several factors:

1. Plugging of the outflow tubing by mucous and intestinal debris
2. Closure of the outflow aperature by intestinal tissue
3. Variation in the overall tone of the intestinal musculature causing volume changes in the isolated loop proper
4. Movement of the intestinal segments resulting in temporary closure by kinking or invagination of some segments

Motility has been shown to alter the flow rate by a factor of 2 under conditions of constant average flow rate in the human, (Fordtran, 1966b).

d. Influence of Volume Change in Perfusion Solution:

A balance sheet was kept in which was recorded the starting and finishing volume of drug solution. By adding the volume recovered to the finishing volume of drug we were able to determine what percentage of the volume perfused was lost in the

system. In the majority of experiments the change in fluid volume was minimal. The technique was such that dead space in the system was reduced to a low figure.

The problems one may encounter on using poorly absorbed markers as volume indicators has been discussed fully by Schedl, (1966a). It would seem that as data on this subject becomes more sophisticated, that the previous use of these markers has been somewhat indiscriminate, Fordtran, (1966b), has also commented on these difficulties and proposes that it would be best to utilize an internal marker whose absorption rate was dependent solely on surface area. Under these circumstances, one could then relate the absorption of other substances directly to the mucosal surface area.

Criteria for a reference marker substance would ideally be that it should be -

1. Strictly nonabsorbed or adsorbed
2. Not subject to degradation in the intestine
3. Not trapped by mucous or other intestinal contents
4. Not influential on intestinal motility
5. Measurable with accuracy while present in low concentration
6. Similar in physical and solubility characteristics to the test substance
7. Not influencing the biochemical handling of the test substance

Thus, this aspect of the technique would require a formidable substance and it appears that no work to date has thrown any light on all of the criteria listed. Perhaps work directed towards obtaining a universal perfusion vehicle would be more beneficial.

Several workers have used various non-absorbed reference marker substances such as phenol red to detect volume changes, (Koizumi et al,(1964); Reynell and Spray, (1956), and Schanker et al, (1958). However, as their data indicated that their procedure resulted in little or no fluid shift when perfusing with solutions having an isotonic (.154 mole/litre) composition, we chose to avoid the use of a marker. In addition, the estimation of phenol red is made at a wavelength close to 545 millimicrons. Koizumi and his associates, (1964), demonstrated that there was a slight increase in sulfanilamide absorption when the phenol red factor was increased, but that for a larger molecule such as sulfamerazine, there was little or no change in the absorption rate for increase in phenol red factor. Nogami and his workers, (1963), concluded in similar experiments that for all purposes the volume of the perfusion solution remained constant. Water absorption was shown to occur from a lumen that is 20% hypertonic with respect to the fluid in which the segment was bathed, (Parsons and Wingate, 1961).

e. Influence of pH:

Since we chose Kreb-Ringer Bicarbonate solution our perfusion experiments were performed at a relatively constant pH value. Constant check of the drug solutions used indicated that this value varied between 7.1-7.4 pH units before perfusion of the loop.

We also checked the pH of various perfusate samples and noted that the pH value tended in the range 6.5-6.8 units. This shift in pH was also noted by Koizumi and his associates, (1964). These workers studied sulfanilamide absorption over a range of pH values and concluded that the maximum absorption rate occurs when the sulfonamide molecule is non-ionized. However, they observed that for some reason maximum absorption in their experiments did not occur at a pH where the non-ionized fraction is present in a maximal concentration, even if pH shift is taken into account. Their data indicated that absorption rate was maximal beyond pH values of 5.0 units for most sulfonamides.

2. PREPARATION OF SAMPLES FOR ANALYSIS:

The outlfow tubing, the excised loop of intestine, and the instruments used for handling the perfused loop were rinsed with normal saline. The rinses were collected and kept in a flask until they could be added to the gut wash flask, (refer to section 2(e)). Analysis of control washing of the perfusion

tube showed that no detectable amount of drug was retained either on or inside the wall of the perfusion tubing.

We noted no decomposition of the drug during the perfusion period, at least this was not measurable if any had occurred. Furthermore, ultraviolet scanning of filtered perfusates indicated almost negligible shift in the absorption peaks of the sulfonamides when compared with control standards. This would confirm that the concentration of drug remained constant during an experiment.

We concluded on the above basis that the difference in the concentration of the sulfonamide in 10 ml of the perfusate from the concentration of sulfonamide in 10 ml of drug solution before and at the conclusion of the experiment could be regarded as the actual amount of drug absorbed. As mentioned previously, changes in the volume of the drug solution perfused were negligible.

a) Perfusate:

To 10 ml of collected perfusate in a 10 ml graduated test tube designed to minimize loss of fluid from evaporation was added 5 ml of a solution (20 percent w/v) of Trichloracetic Acid (TCA). If after acidification the perfusate sample remained clear it was carefully transferred to a 100 ml volumetric flask. The perfusate test tube was thoroughly washed out with distilled water and the final volume was adjusted to 100 ml to ensure accurate dilution of

the sample.

If on adding TCA solution there was precipitation of protein or other matter, the whole sample was centrifuged after careful rinsing of the test tube into a 50 ml centrifuge tube. The precipitate was centrifuged down at 5000 x g and the supernatant added to a 100 ml volumetric flask. The pellet was washed with 0.1 N HCl and centrifuged a total of three times, the volume kept under a 100 ml to allow making up to 100 ml in the dilution flask.

One ml of the diluted sample was then pipetted into another 100 ml volumetric flask for assay purposes. To this flask and its contents was next added 10 ml of 4 Normal HCl. This flask was now ready for the analysis of accurately diluted sulfonamide originally contained in the perfusate collection tube.

b) Blood:

To 0.2 ml of heparinized blood sample previously delivered into a 50 ml centrifuge tube was added 9.8 ml of distilled water and the mixture was thoroughly stirred on a vortex mixer. Haemolysis was allowed to proceed to completion and the proteins were then precipitated by adding 10 ml of TCA (20% w/v solution) to the contents of the tube.

The precipitation in the tube was then centrifuged down

at 10,000 x g for 15 minutes. Fifteen ml of the supernatant were transferred to a graduated tube and 1 ml of 4 N HCl was added. The tube was then placed in a boiling water bath for 30 minutes for the purpose of acid hydrolysis of any conjugated sulfonamide.

The tube and contents were removed, cooled to room temperature, and the volume adjusted accurately to the 15 ml mark with distilled water. The contents were thoroughly mixed with a vortex mixer. Ten ml of this hydrolyzed sample were pipetted into a 25 ml volumetric flask for analysis. To this flask was added 2.5 ml of 4 N HCl. This flask now contained a suitable amount of sulfonamide for accurate determination in the range of 0-20 mg% of drug per ml of blood.

c) Urine:

To 0.2 ml of urine sample in a centrifuge tube was added 19.8 ml of TCA solution (20% w/v). The mixture was centrifuged at 10,000 x g for 15 minutes to sediment precipitated protein.

Fifteen ml of the supernatant were acid hydrolysed as described under the procedure for Blood, (refer to Section 2 (b)). Ten ml of the hydrolysed supernatant were then pipetted into a 100 ml volumetric flask for assay. Ten ml of 4 N HCl was then added to the flask before proceeding further for analysis. Although our urine data were quite variable and showed wide ranges, the procedure

described above was the most satisfactory that we could devise.

d) Tissue:

The different tissue samples were blotted dry on clean filter paper and not more than 2 grams of each were weighed for the purpose of homogenization. The weighed samples were placed in separate grinding tubes and stored in crushed ice. Sufficient 0.5 Normal Perchloric Acid (PCA) was then added to each tube just before homogenization. Grinding of the tissue was carried out by rotating the pestle with a heavy-duty electric motor and firmly pressing the pestle against the tissue by means of constant up and down movements uniformly inside the grinding tube until the tissue sample was reduced to a fine homogenate. The grinding tube was emptied of its contents into a graduated cylinder. The grinding apparatus was rinsed carefully with more 0.5 N PCA, the rinses being added to the cylinder carefully to make up 20 ml of suspended homogenate. This amount was then transferred in total to a 50 ml centrifuge tube. The reduced particles of tissue were centrifuged down at 15,000 x g for 15 minutes.

Fifteen ml of the supernatant were then subject to acid hydrolysis in a boiling water-bath in a manner similar to that utilized for blood and urine samples. The contents of each tube were then adjusted and mixed on a vortex shaker prior to removing

10 ml for analysis. The 10 ml of sample were pipetted into a 200 ml volumetric flask, acidified with 20 ml of 4 N HCl and set aside for subsequent estimation of the level of drug present in one gram of fresh tissue. As we were primarily interested in the level of drug utilized by a gram of given tissue, we did not repeatedly extract the tissue particles to remove all the drug present in the tissue. Preliminary experiments indicated that if repeated extraction of the homogenized tissue was carried out then contamination of the sample used for analysis became increasingly severe with each extraction. This step of the analysis would have to be modified before any attempt of quantitating the amount of drug present in a gram of tissue was successful.

e) Gut Wash:

Prior to homogenization of the excised segment of the intestine, cool normal saline was percolated through the lumen of the gut sample. Removal of the drug remaining free or bound to solid intestinal contents in the lumen of the intestine was achieved by attaching one end of the excised segment to the stem of a separatory funnel and allowing approximately 900 ml of cool saline to pass through the lumen at a moderate rate. This wash fluid was allowed to run into a litre flask through a glass wool plug in a glass funnel. Any solids washed out of the gut were retained on

the plug. The external tissue was rinsed with a stream of saline periodically.

The rinses collected previously were now transferred via the funnel into this flask. The funnel and plug were then rinsed with 75 ml of 4 N HCl and the gut wash was set aside for analysis. Further washing of the excised loop was not necessary as very little or no drug was measured if the washing procedure was repeated.

It was judged that for all purposes the free drug trapped by the numerous crypts and villi in the intestine as well as bound to solid intestinal contents was effectively removed by the procedure. The whole method rarely took more than 15 minutes.

f) Drug Perfusion Solutions:

Samples of the drug solution contained in the perfusion flask were removed immediately before and after the experiment by pipetting 10 ml of solution into a 100 ml volumetric flask. These samples were then treated in the same way as the perfusate and prepared for estimating the amount of drug contained in a 10 ml of perfusion solution.

g) Recovery Experiments:

The results of recovery experiments were collected and have been tabulated in Table I-A.

TABLE I-A - Results of Recovery Experiments Expressed as Percent of Drug Recovered ± the Percentage Range

Drug	Concentration (milligram %)	Intestine	Liver	Kidney	Blood	Urine
		± %D	± %D	± %D	± %D	± %D
SF	25	96.8±5.2	97.3±6.4	94.5±10.1	99.2±2.1	84.4±10.2
SF	75	98.3±4.8	96.7±6.9	92.1±12.3	98.7±2.3	79.6±12.7
SD	50	97.7±4.4	97.6±7.1	89.7±9.7	99.1±3.2	81.1±13.9
SD	100	98.9±4.1	97.9±4.5	88.2±14.6	98.6±1.9	78.5±16.3
SMET	25	95.6±3.9	98.2±5.6	91.4±13.7	97.9±2.6	82.9±11.8
SMET	75	98.1±5.6	96.5±6.3	90.9±15.5	98.5±2.5	77.7±15.5
SMER	50	95.5±4.3	97.4±7.5	87.3±16.4	98.2±3.0	79.3±14.1
SMER	100	98.6±3.8	96.9±8.0	89.8±18.9	98.9±2.7	76.4±17.8
	MEAN	97.4±4.5	97.3±6.5	90.5±13.9	98.6±2.5	79.9±14.0

Note: All Blood recovery experiments were in the range 0-20 mg% of the sulfonamide

%D: Refers to the percent deviation from the mean value

The values were determined and calculated for each drug at various concentrations. These values were compared with suitable separate controls prepared in saline along with the particular sample. On the whole, the results were satisfactory except for the kidney and especially the urine samples.

3. PREPARATION OF DRUG SOLUTIONS

With the exception of sulfanilamide, all other sulfonamides dissolved in the perfusion medium with much difficulty. The powders did not wet readily on contact with the liquid and tended to remain suspended as particles on the surface and the walls of the flask when preparing sulfadiazine, sulfamerazine and sulfamethazine solutions.

The procedure adopted to dissolve the required amount of drug in the perfusion solution, was to stir contents of the flask with a glass rod at 35°C for a minimum of 24 hours. This resulted in some loss of drug by decomposition when compared to solutions prepared by first dissolving the powder in a small quantity of sodium hydroxide. This problem was encountered to a lesser degree at lower concentrations of drug. The difficulty encountered in attempting to dissolve the sulfonamides was unexpected since on checking the solubilities of the drugs in solutions of pH 7.4 in various reference texts, our concentrations were seen to be significantly below the limit of solubility published. We checked the purity of the samples by noting the melting points of the compounds regularly. A further puzzling factor was that only on rare occasions did crystallization of solids occur when dissolved samples were stored in the cold.

The actual amount of drug in 100 ml of drug solution for perfusion was determined chemically and the difference from the theoretical amount noted. The results of these determinations are contained in Table I-B.

TABLE I-B - Data Indicating the Accuracy in Preparing Drug Solutions for Perfusion

Drug	Theoretical Concentration(mg%)	Actual Concentration(mg%)	% Error
SF	25.0	26.06±1.27	4.2
SF	50.0	51.77±1.14	3.5
SF	75.0	75.76±1.69	1.0
SF	100.0	101.11±1.43	1.1
SD	25.0	24.29±1.41	2.8
SD	50.0	48.33±1.75	3.3
SD	75.0	72.14±2.62	3.8
SD	100.0	94.76±4.37	5.2
SMET	25.0	24.22±1.36	3.1
SMET	50.0	47.35±1.99	5.3
SMET	75.0	72.58±3.11	3.2
SMET	100.0	97.29±3.90	2.7
SMER	25.0	24.28±4.05	2.8
SMER	50.0	51.64±2.15	3.3
SMER	75.0	73.19±1.38	2.4
SMER	100.0	97.76±3.73	2.2

Drug solutions were generally prepared for use shortly after dissolution of the compound, but whenever storage was necessary for a few days, the solutions were stored in full glass-stoppered vessels in a refrigerator.

Prior to commencing an experiment 500 ml of drug solution was measured and transferred accurately to a two-neck 500 ml round-bottom reaction flask. This flask was positioned on a hot plate-magnetic stirrer and clamped securely. A small magnetic-stirrer was in continuous motion throughout the experiment and heat was maintained at 30°C or less.

One neck of the flask was utilized to bubble a fine stream of oxygen (95%) - carbon dioxide (5%) through the drug solution during the experiment. The other neck was fitted to accept the free end of the inflow tubing. All joints were ground-glass to ensure a good fit in order to minimize the effects of evaporation on the drug solution.

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ANALYTICAL

The Bratton-Marshall method formed the basis for all our chemical estimation of sulfonamides, (Bratton and Marshall, 1939). This is a colourimetric method wherein the colour is generated by diazotization of the free arylamino group and coupling with a suitable agent after neutralization of the excess of the diazotizing agent, sodium nitrite. The theory and application of diazotization and coupling of aromatic amines for their colourimetric analysis has been discussed fully by Connors, (1965).

Compounds other than sulfonamide drugs which contain the amino group attached para to the benzene ring are also determined by this procedure, e.g. para-aminobenzoic acid and procaine. The coloured compounds formed all exhibited a characteristic absorption peak at 545 millimicrons in the visible absorption spectrum.

There is no assurance that the original substrate and the final dye-complex will be identical in concentration. Recovery experiments seem to indicate that the recovery of sulfonamide, with the exception of kidney and urine, ranged from 95% to 100% of the starting material.

The final pH of the diazotized and coupled sulfa solutions was always 1.25-1.35 by virtue of the acid added earlier to obtain the greatest optical density. Generally, the concentration of drug

was not high in any assay flask and instability of the colour due to the precipitation of high concentration of dye was negligible. Whenever the final colour was too strong, appropriate dilution was made and the reading taken quickly. No coloured solutions were allowed to remain beyond 15 minutes before taking an optical density reading. It has been reported that extinction coefficients decrease with increasing concentration of sulfonamide during the estimation of sulfaquinoxaline, (Dux and Rosenblum, 1949).

All samples extracted from biological tissues and fluids were hydrolyzed in a boiling water bath for 30 minutes in the presence of 1 ml of 4 normal hydrochloric acid. This was carried out in order to free the acetylated and/or conjugated amino group and allow us to estimate the total amount of sulfonamide present in a given sample. We did not differentiate between acetylated and non-acetylated drug.

The reagents required for the quantitative development of colour were made up fresh daily just prior to commencing the analysis of samples from a given experiment. The coupling agent N-(1-Naphthyl)-ethylenediamine Dihydrochloride (NED) was stored in a dark coloured glass bottle to minimize photdeterioration.

1. Determination of Absorbance Values:

The instrument used for the analysis of the samples

collected was the Bausch and Lomb Spectronic 20[Ri] Colourimeter/ Spectrophotmeter, regulated model.

All solutions were determined against a reagent blank in a Celvac[Rii] semi-automatic flow-through cuvette, having an external diameter of 14 millimeters. The cuvette was set in place in the instrument and remained in a given position until all samples were analysed. A given coloured assay sample was flushed through the cuvette a minimum of three times, The sample remained in the cuvette for at least one minute before the absorbance reading was noted.

The wavelength of maximum absorption for all compounds was 545 millimicrons and it was at this wavelength that the instrument was set for the period of this investigation.

2. Effect of Temperature on Colour Development:

It has been reported that the effect of temperature on this assay procedure is considerable over a range of 10-60 degrees centigrade, (Colaizzi et al, 1965). We did not consider temperature to influence our results as the temperature in our laboratory (24°C) did not vary more than one degree throughout an assay procedure. This variation in temperature was encountered only on rare occasions. In addition, the instrument was cooled continuously from within by means of a motor driven fan. As a criterion for indication of constant temperature in the cuvette, we allowed

Ri - Bausch and Lomb, Rochester, New York
Rii - Echols Products Co., Houston, Texas

coloured standard solutions to remain in the cuvette for at least ten minutes. In all cases absorbance readings remained constant during this specified time and were the same as those recorded at the one minute interval.

3. Stability of Colour Development:

We encountered early in our analytical investigation some difficulty in the neutralization step and the colour development stage of the Bratton-Marshall (B-M) method. Accordingly we modified the procedure as follows -

Stage	Bratton-Marshall, (1939)	Our Method
Diazotization	Sodium Nitrite 0.1% (1 ml) (NaNO2)	NaNO2 0.15% (3 ml)
Neutralization	Ammonium Sulfamate 0.5% (1 ml) (NH4SO3NH2)	NH4So3NH2) 0.65% (3 ml)
Colour Development	N-(1-Naphthyl)-ethylene-diamine Dihydrochloride (NED) 0.1% (1 ml)	NED 0.25% (3 ml)

This modification resulted in little or no difficulty in subsequent assay procedures. There was rapid and complete neutralization of the excess sodium nitrite by the ammonium sulfamate.

We increased the amount of the chromogenic compound NED to increase the rate at which the final colour was developed. With the amount we used full colour developed within 15 seconds.

The increase in volume of reagent added was found to be an asset in that it precluded lack of full colour development whenever

the stoichiometric amount of drug in a sample exceeded the amount of reagent contained in 1 ml of reagent as happened in early analytical procedures.

4. Development of Standard Colour:

For the preparation of standard curves, powdered samples of the drugs obtained from the manufacturers listed in the appendix were employed. All powdered sulfonamides were stored in a dessicator under vacuum.

Spectral-Absorbance curves for the various sulfonamide-diazo coupled chromogen were obtained to get the wavelength most suitable for absorbance readings. This wavelength was similar for all the sulfonamides with variation occurring mainly in the intensity of absorption of light at 545 millimicrons due to the different molar quantities for each drug involved. Shifts in absorption maxima were never more than 2 millimicrons when detected.

We determined the absorbance of tissue, urine, blood, and perfusate extracts at 545 millimicrons and found that absorbance was contributed to the final colour by liver and kidney extracts to a minor degree. In any case, where some trace of other colour, particularly a yellow colour in tissue extracts was present the subsequent dilution minimized the interference due to this form of contamination. It has been reported that analysis of sulfonamide

tablets coloured with a yellow dye suffered from negligible interference of the yellow dye on dilution, (Colaizzi, et al, 1965).

5. Effect of Solvent of Reagents on Colour Development:

Since our solvent system in all samples consisted only of acid-water, we encountered no difficulty attributable to differences in the solvent system among the samples. We avoided introducing non-aqueous solvents which would influence absorbance values, via an effect on the solvent system. We found that the concentration of the B-M reagents present in the various flasks did not influence absorbance readings. As a precaution, we reduced the volume of NED from 3 ml to 1.5 ml for the blood samples as this was the only assay carried out in a flask smaller than 100 ml, and the influence of the agent could be significant.

6. Effect of Ionic Strength on Colour Development:

The relative amount of various inorganic electrolytes was judged to be non-influencing on the final colour since dilution of all samples was considerable prior to the final assay. Thus, any deleterious effect of high concentrations of inorganic ions was avoided.

7. Effect of pH on Colour Development:

Our analysis was carried out in sufficient hydrochloric acid to give a final pH of about 1.2 in the assay flask. To deter-

mine the effect of hydrogen ion concentration on absorbance, we plotted the absorbance of a 100 mg solution of sulfanilamide as a function of pH range of 0.75 to 1.75 units. It was demonstrated that the method was only slightly sensitive to small variations in pH in the range chosen.

8. Determination of Sulfonamides in Low Concentrations:

All absorbance readings recorded for calculation of the concentration of the drug were below 0.600 absorbance units. Where necessary, the appropriate dilution of a given sample was made if the final colour exceeded 0.600 absorbance units.

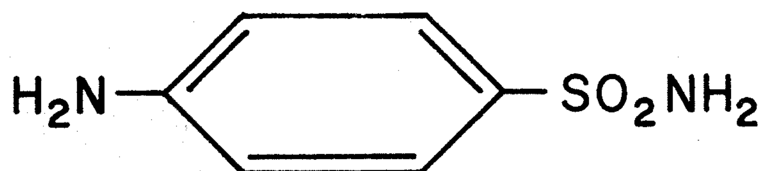
We did not resort to the low absorbance method of precision spectrophotometry for low concentration of drug, (Reilly and Sawyer, 1961). Instead we prepared linear standard calibration durves for the concentration ranges encountered in our analysis. By repeating these determinations periodically, we amassed sufficient data to give very linear calibrations curves for a particular range of concentration. We then calculated the equivalent amount of absorbance produced by 1 microgram of drug in a specified assay.

9. Performance of Instrument:

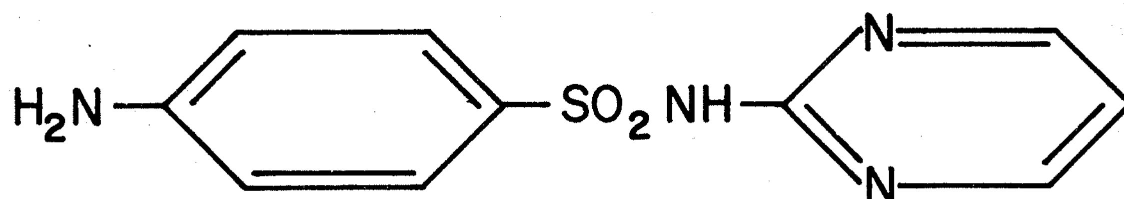
The instrument was calibrated regularly by reading the absorbance of a 2.2% w/v solution of cobalt chloride at 510 millimicrons.

TABLE NO. 11

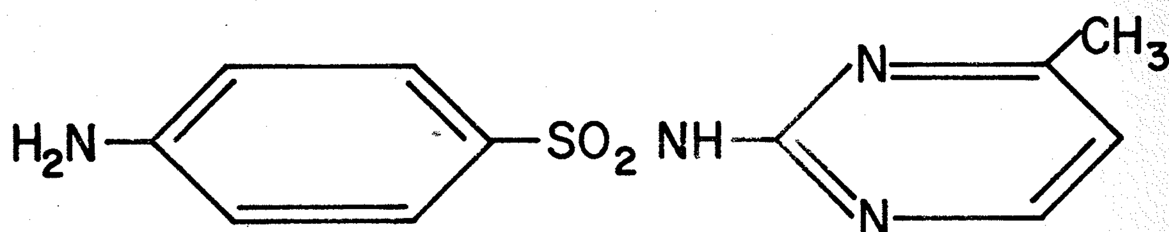
CHEMICAL NAMES AND STRUCTURES OF SULFONAMIDE COMPOUNDS EMPLOYED



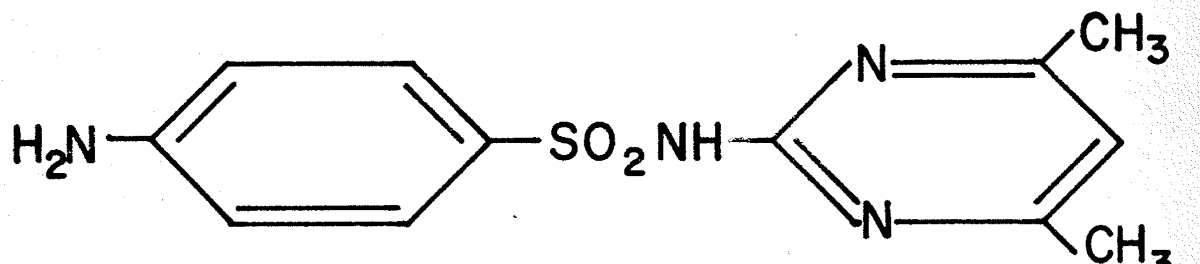
p-AMINOBENZENESULFONAMIDE
(SULFANILAMIDE, SF)



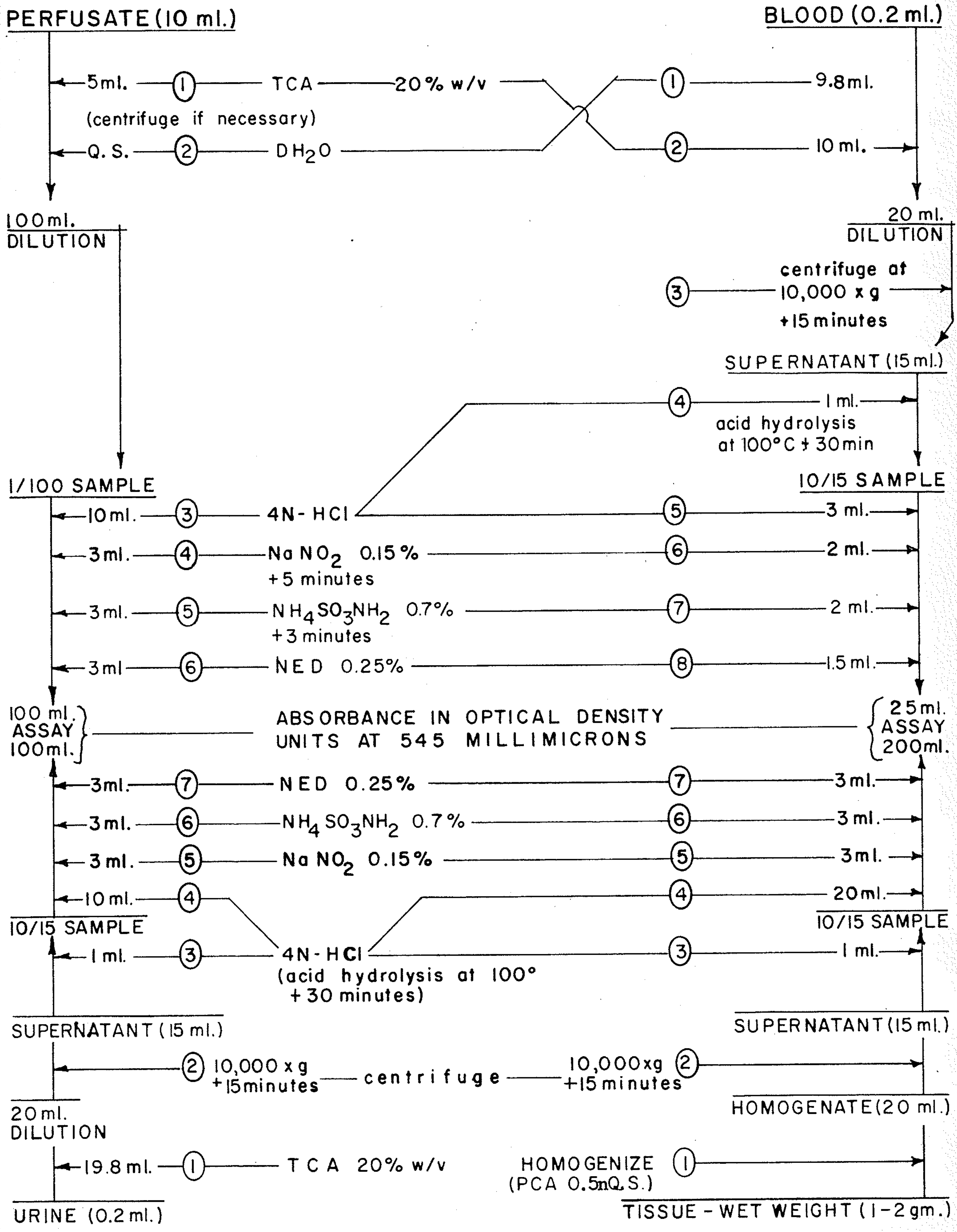
N'-2-PYRIMIDINYLSULFANILAMIDE
(SULFADIAZINE, SD)



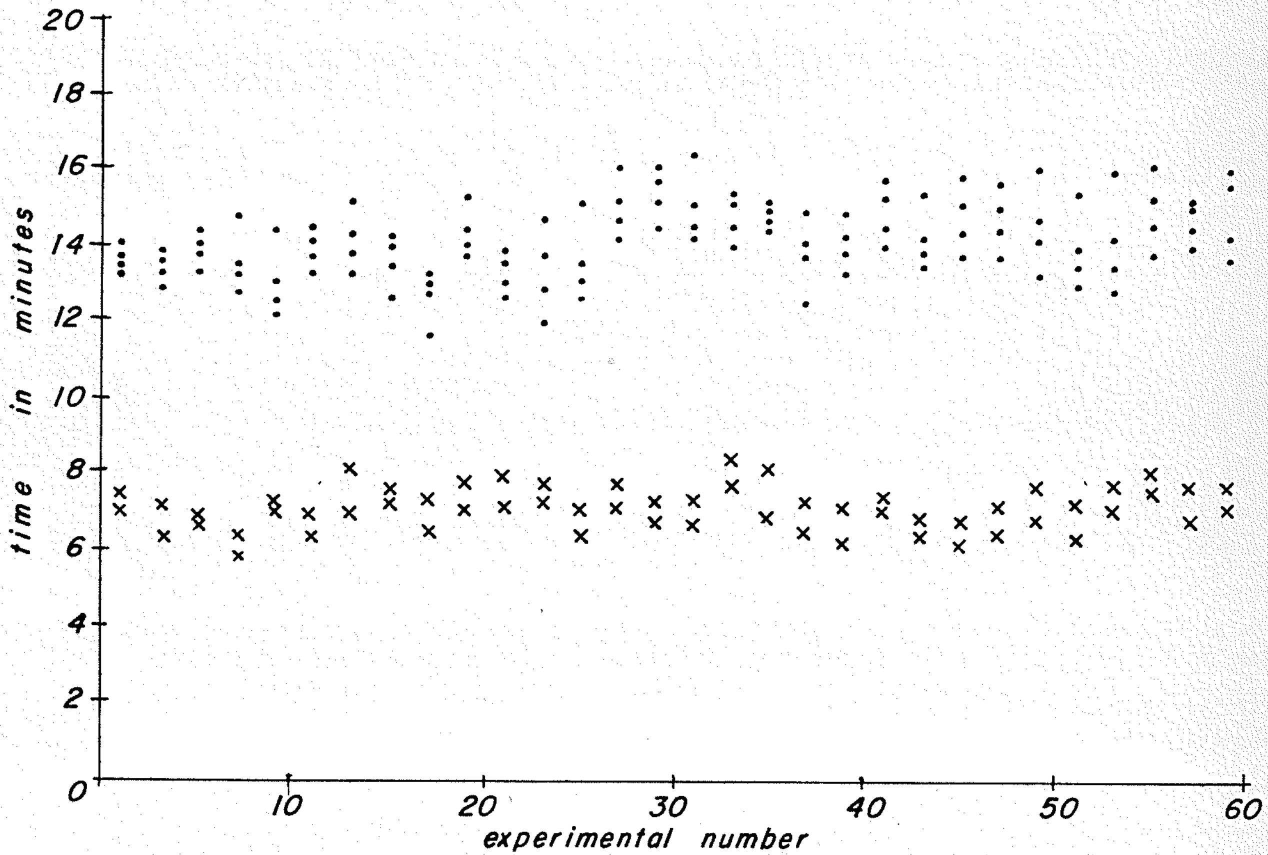
N'-(4-METHYL-2-PYRIMIDINYL)SULFANILAMIDE
(SULFAMERAZINE, SMER)



N'-(4,6-DIMETHYL-2-PYRIMIDINYL)SULFANILAMIDE
(SULFAMETHAZINE, SMET)


FLOW SCHEMATIC - ANALYTICAL PROCEDURE (in numerical sequence)
PERFUSATE (10 ml.)
BLOOD (0.2 ml.)
5ml.
1
TCA
20% w/v
1
9.8ml.
(centrifuge if necessary)
Q. S.
2
DH2O
2
10 ml.
100ml.
DILUTION
20 ml.
DILUTION
3
centrifuge at
10,000 x g
+15 minutes
SUPERNATANT (15 ml.)
4
1 ml.
acid hydrolysis
at 100°C + 30min
1/100 SAMPLE
10/15 SAMPLE
10 ml.
3
4N-HCl
5
3 ml.
3 ml.
4
Na NO2 0.15%
+5 minutes
6
2 ml.
3 ml.
5
NH4SO3NH2 0.7%
+3 minutes
7
2 ml.
3 ml
6
NED 0.25%
8
1.5 ml.
100 ml.
ASSAY
100ml.
ABSORBANCE IN OPTICAL DENSITY
UNITS AT 545 MILLIMICRONS
25ml.
ASSAY
200ml.
3ml.
7
NED 0.25%
7
3 ml.
3 ml.
6
NH4 SO3NH2 0.7%
6
3 ml.
3 ml.
5
Na NO2 0.15%
5
3ml.
10 ml.
4
4
20ml.
10/15 SAMPLE
10/15 SAMPLE
1 ml.
3
4N-HCl
(acid hydrolysis at 100°
+30 minutes)
3
1 ml.
SUPERNATANT (15 ml.)
SUPERNATANT (15 ml.)
2
10,000 x g
+15minutes
centrifuge
10,000xg
+15 minutes
2
20 ml.
DILUTION
HOMOGENATE (20 ml.)
19.8 ml.
1
TCA 20% w/v
HOMOGENIZE
(PCA 0.5nQ.S.)
1
URINE (0.2 ml.)
TISSUE - WET WEIGHT (1-2 gm.)

GRAPH NO. 1.



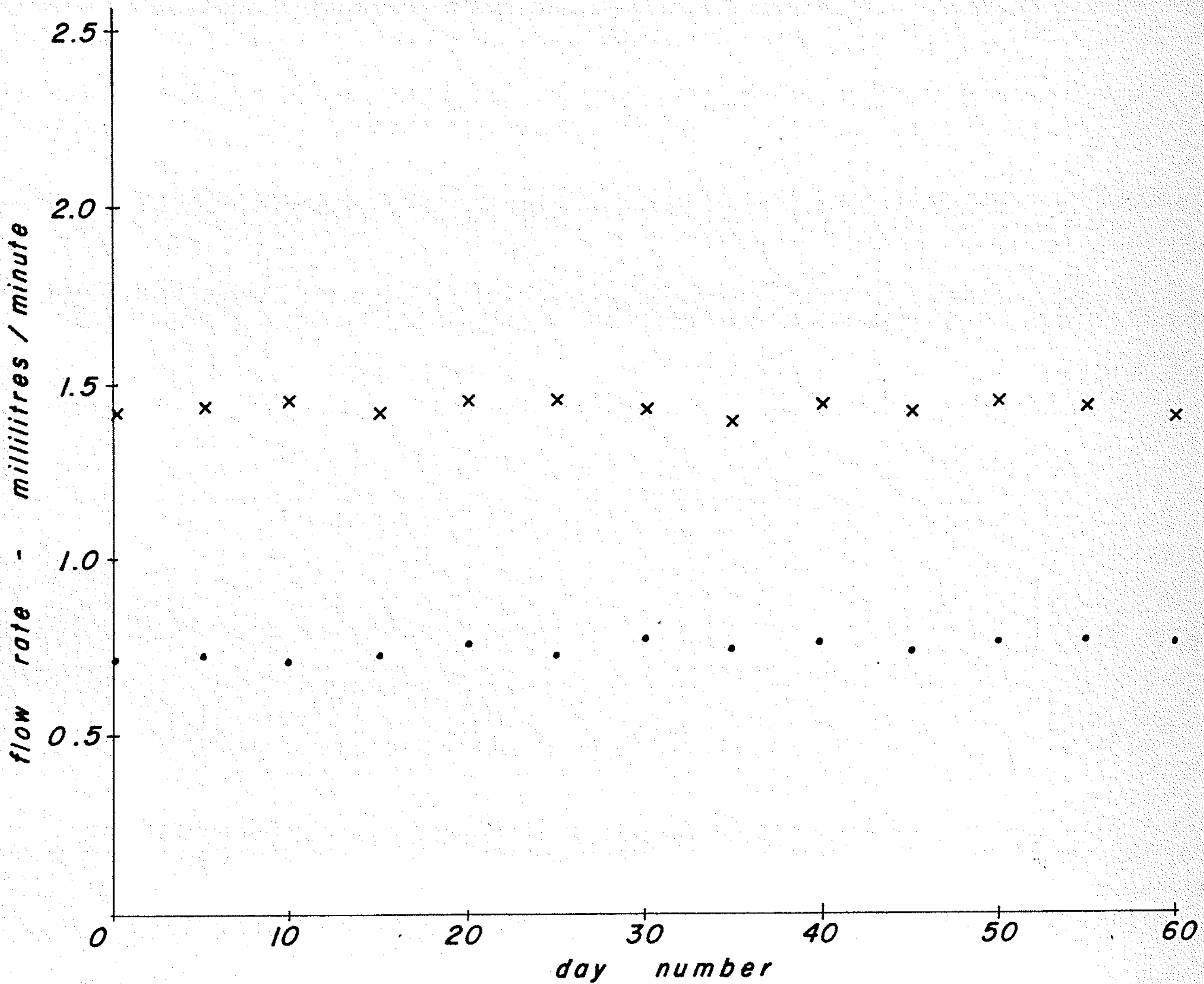
GRAPH SHOWING TIME TAKEN FOR PERFUSION OF 10 MILLILITRES OF DRUG SOLUTION.

× — INITIAL WASH & EQUILIBRATION AT 12 R.P.M. PUMP SPEED

• — EXPERIMENTAL PERIOD AT 6 R.P.M. PUMP SPEED

(Data selected at random from experiments throughout this study)

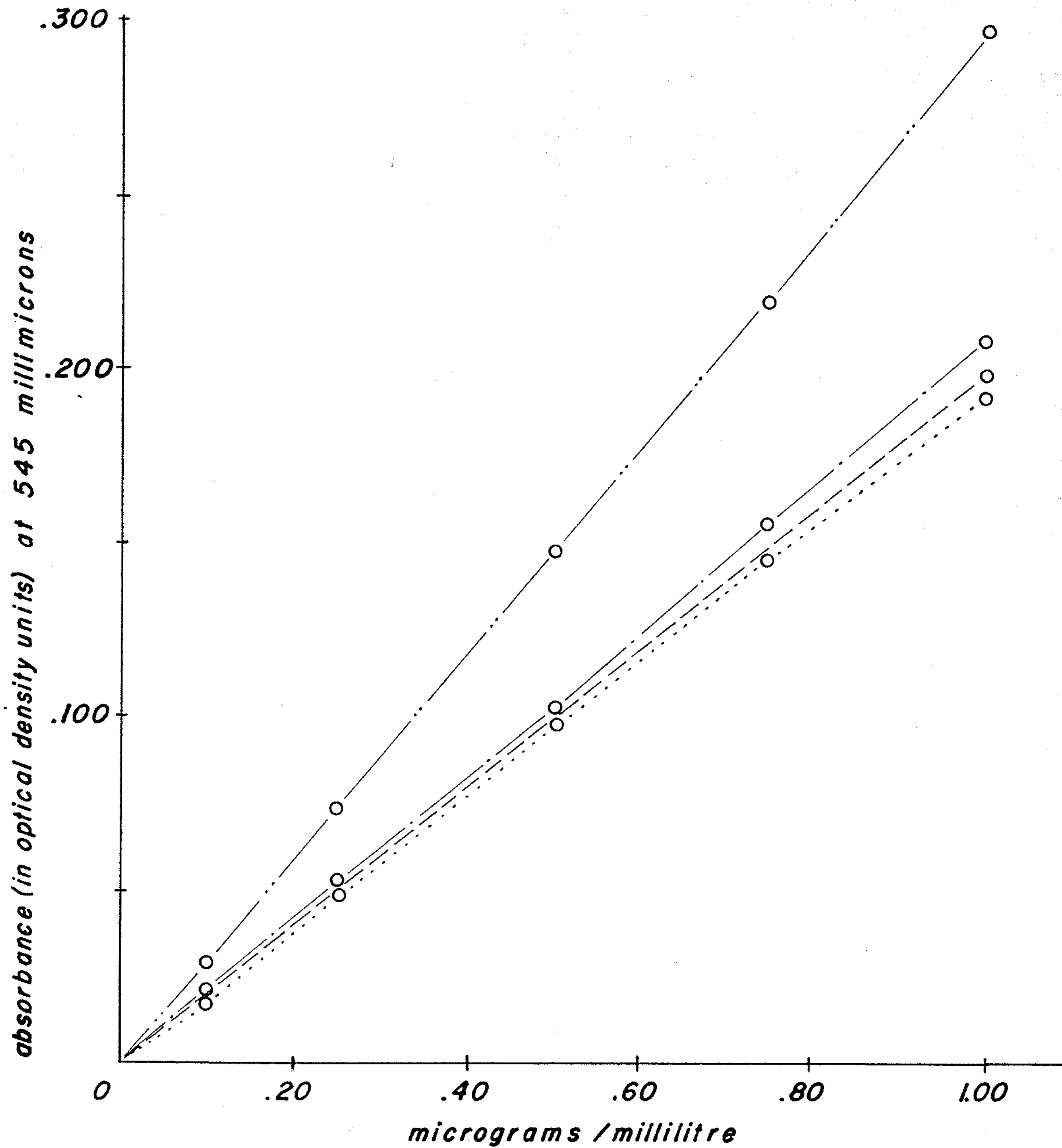
GRAPH NO. 2.



GRAPH SHOWING FLOW RATE FOR PERFUSION TUBING (1.98mm. I.D. x 3.75mm. O.D.)

• - PUMP SPEED NO. 4 (6 rpm)
x - PUMP SPEED NO. 3 (12 rpm)

GRAPH NO.3.

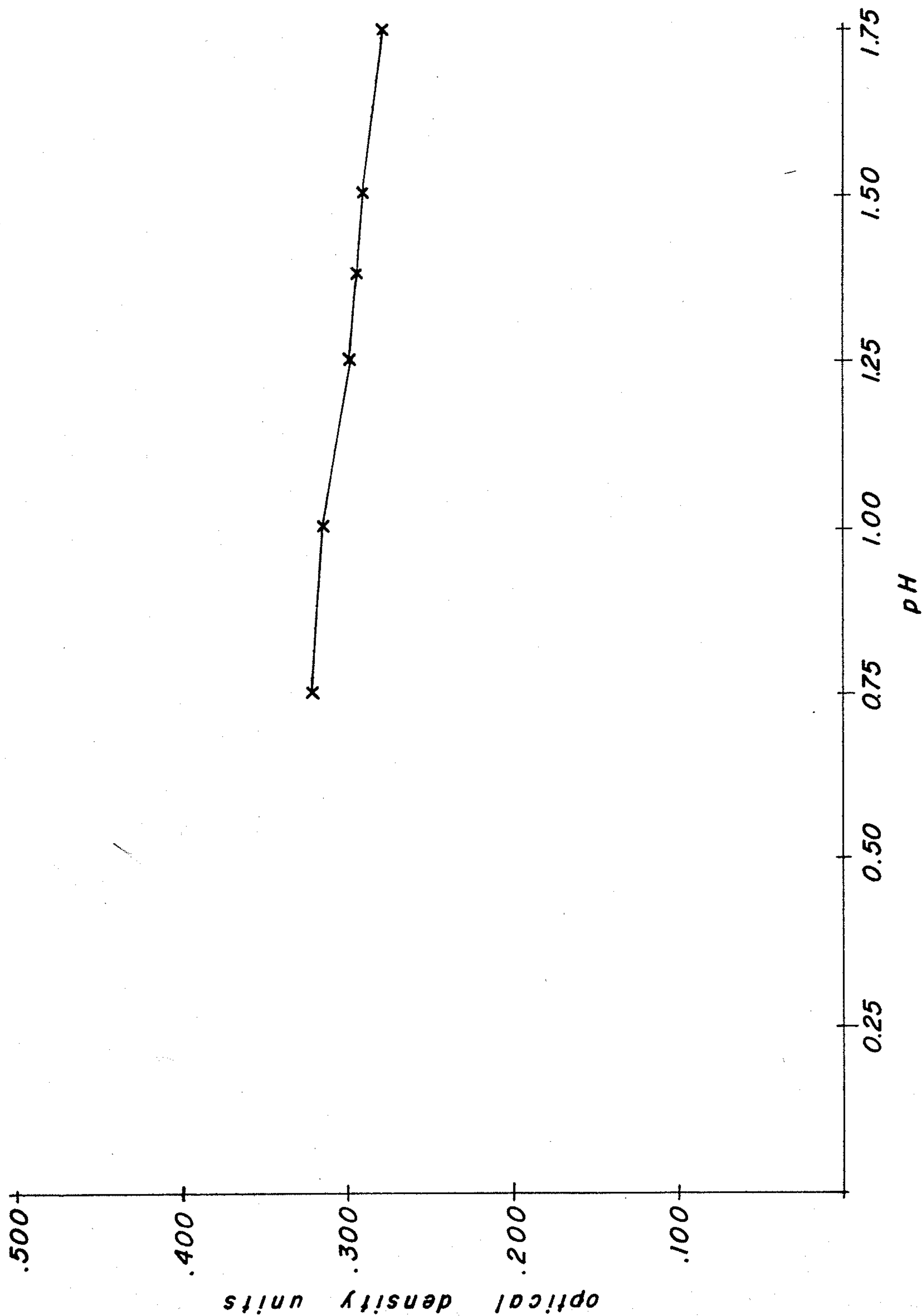


STANDARD CURVES RELATING OPTICAL DENSITY AT 545 MILLIMICRONS AND SULFONAMIDE CONCENTRATION.

(Colour development is seen to be a linear function of sulfonamide concentration in the range 0.1 - 1.0 micrograms/millilitre.)

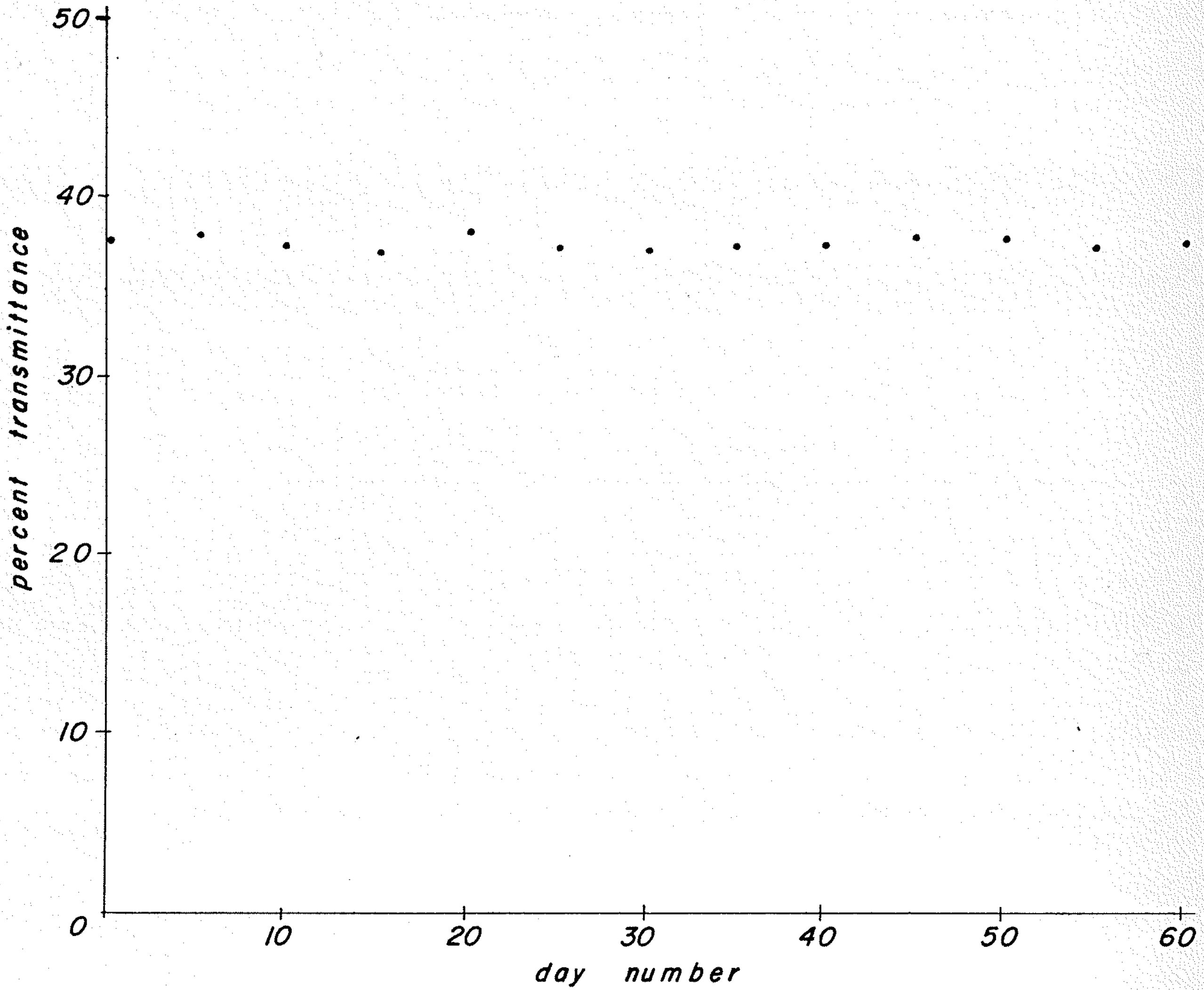
—·— SULFADIAZINE
· · · · · · SULFAMETHAZINE
—··— SULFANILAMIDE
— — — — SULFAMERAZINE

GRAPH NO.4.



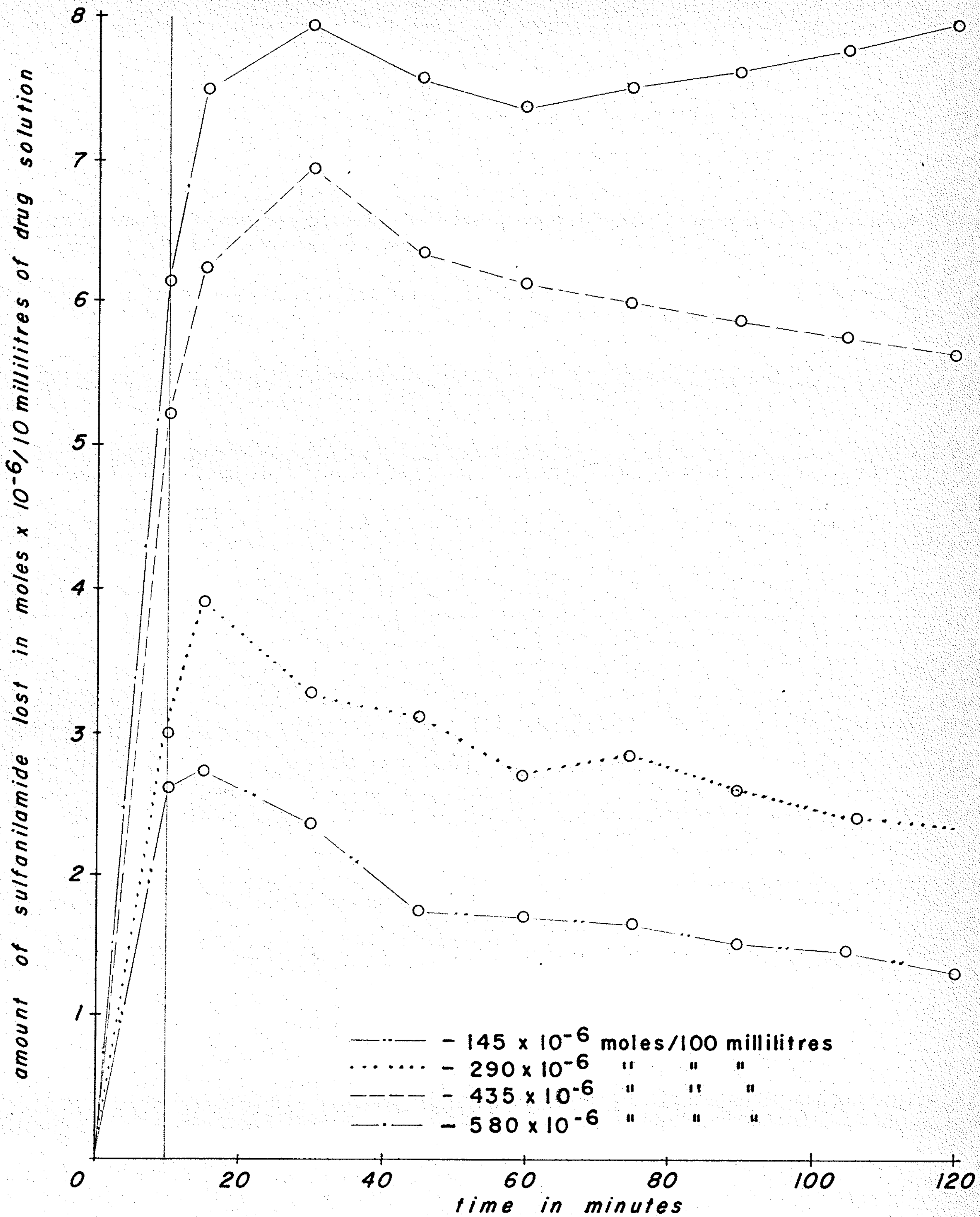
ABSORBANCE OF SULFANILAMIDE (100mg%) AS A FUNCTION OF pH AT A GIVEN IONIC STRENGTH.

GRAPH NO. 5.



GRAPH SHOWING PERFORMANCE OF SPECTROPHOTOMETER OVER A THREE-MONTH PERIOD AT 510 MILLIMICRONS.
(Checked with cobalt chloride 2.2% W/V in 1% HCl)

GRAPH NO. 6.



SULFANILAMIDE ABSORPTION PROFILE

GRAPH RELATING CONTINUOUS LOSS OF SULFANILAMIDE FROM 10 MILLILITRE ALIQUOTS OF VARIOUS CONCENTRATIONS PERFUSED THROUGH AN ISOLATED INTESTINE IN SITU.

GRAPH NO. 6A.

log$_{10}$ of concentration of sulfanilamide lost in moles x 10^{-6} per 10 millilitres

time in minutes

GRAPH RELATING ABSORPTION PROFILE OF SULFANILAMIDE ON A SEMI-LOG$_{10}$ OF CONCENTRATION SCALE AGAINST TIME

- —··— 145×10^{-6} moles/100 millilitres
- ········ 290×10^{-6} " " "
- — — — 435×10^{-6} " " "
- —·— 580×10^{-6} " " "

GRAPH NO. 7A.

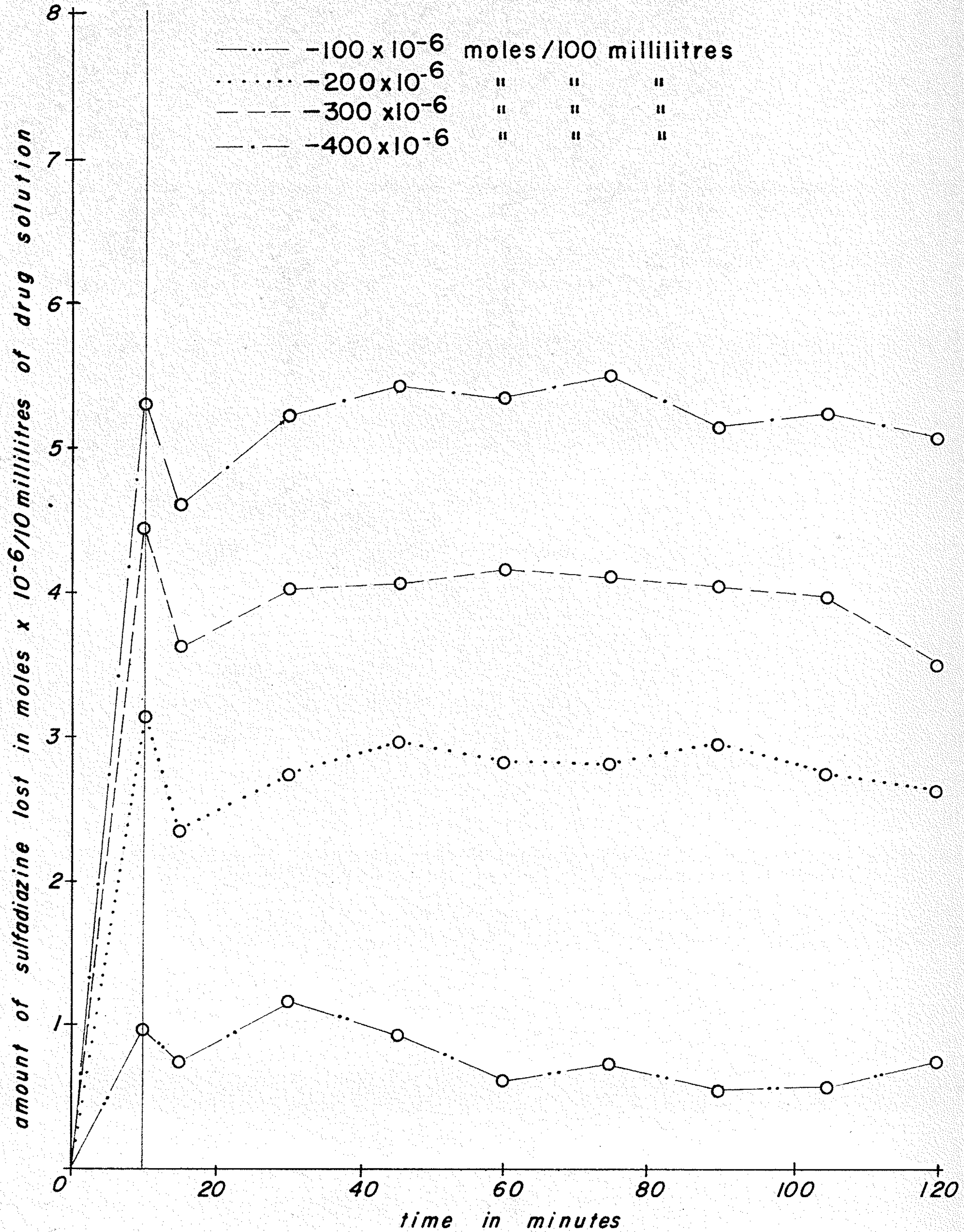
log$_{10}$ of concentration of sulfadiazine lost in moles x 10^{-6} per 10 millilitres

time in minutes

GRAPH RELATING ABSORPTION PROFILE OF SULFADIAZINE ON A SEMI-LOG$_{10}$ OF CONCENTRATION SCALE AGAINST TIME

- —··— 100×10^{-6} moles/100 millilitres (data too low for range of values)
- ········ 200×10^{-6} moles/100 millilitres
- — — — 300×10^{-6} " " "
- —·— 400×10^{-6} " " "

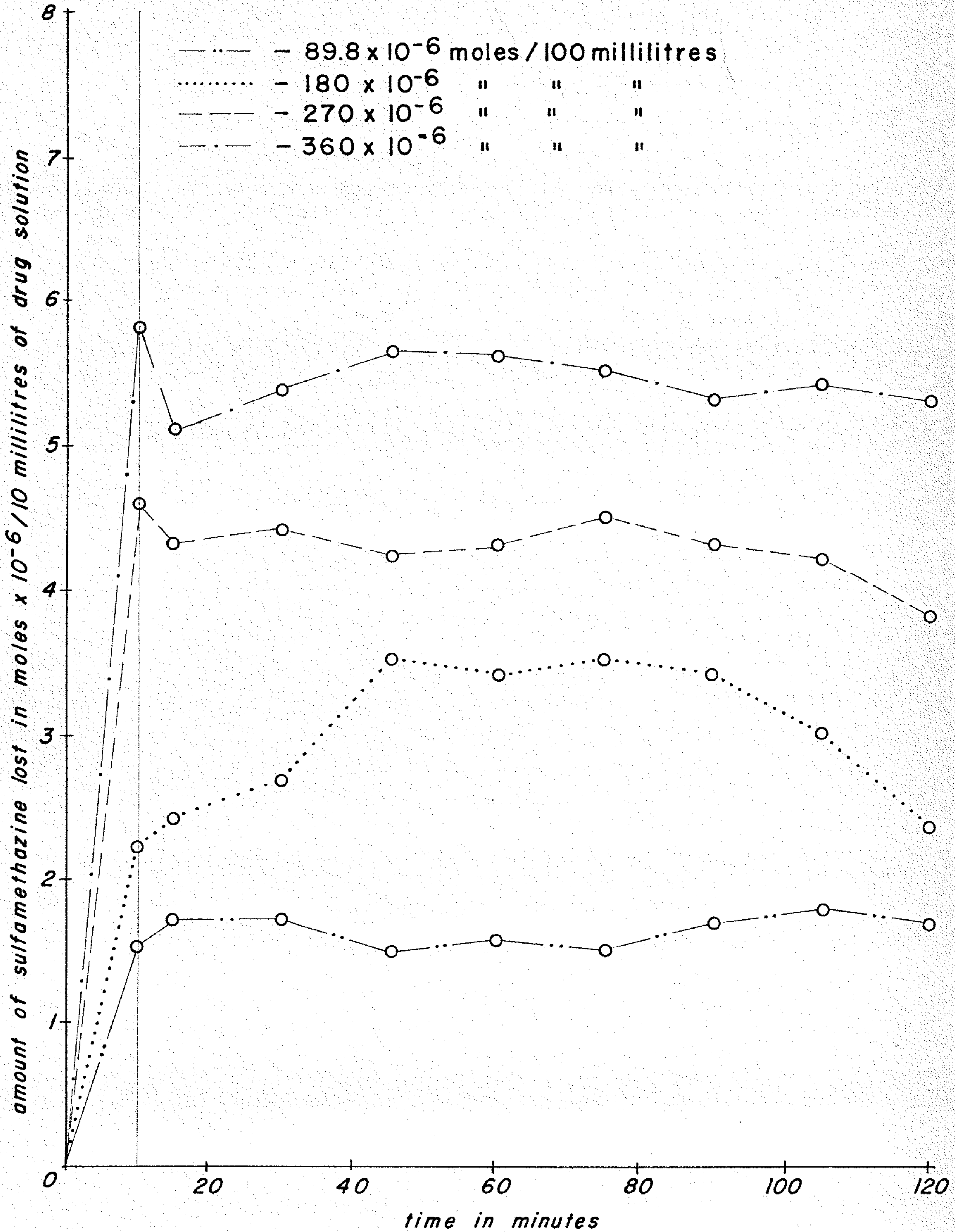
GRAPH NO 7.



SULFADIAZINE ABSORPTION PROFILE

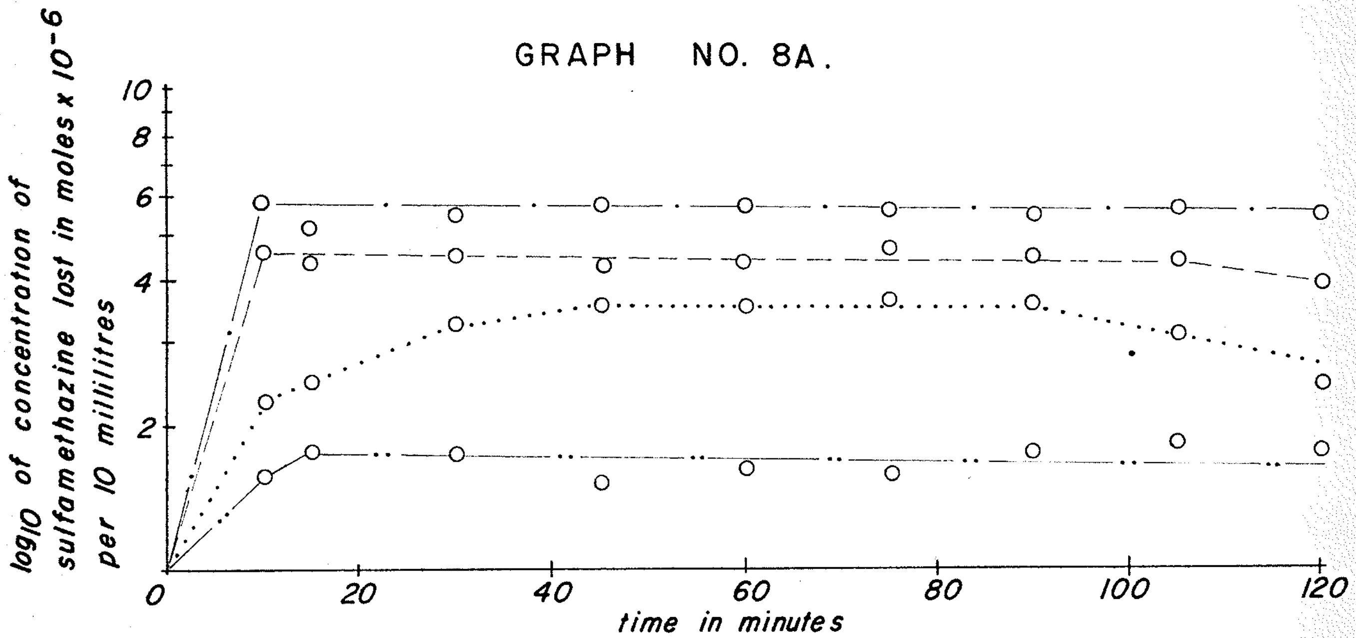
GRAPH RELATING CONTINUOUS LOSS OF SULFADIAZINE FROM 10 MILLILITRE ALIQUOTS OF VARIOUS CONCENTRATIONS PERFUSED THROUGH AN ISOLATED INTESTINE IN SITU.

GRAPH NO. 8.



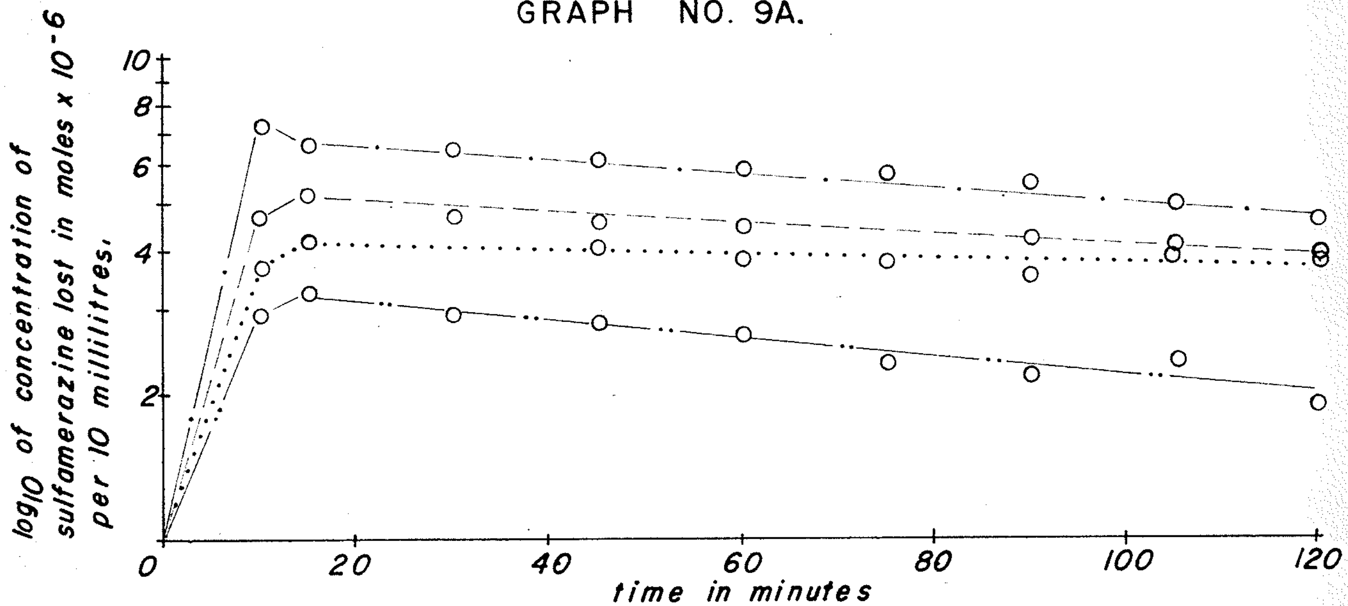
SULFAMETHAZINE ABSORPTION PROFILE

GRAPH RELATING CONTINUOUS LOSS OF SULFAMETHAZINE FROM 10 MILLILITRE ALIQUOTS OF VARIOUS CONCENTRATIONS PERFUSED THROUGH AN ISOLATED INTESTINE IN SITU.



GRAPH RELATING ABSORPTION PROFILE OF SULFAMETHAZINE ON A SEMI-LOG_{10} OF CONCENTRATION SCALE AGAINST TIME.

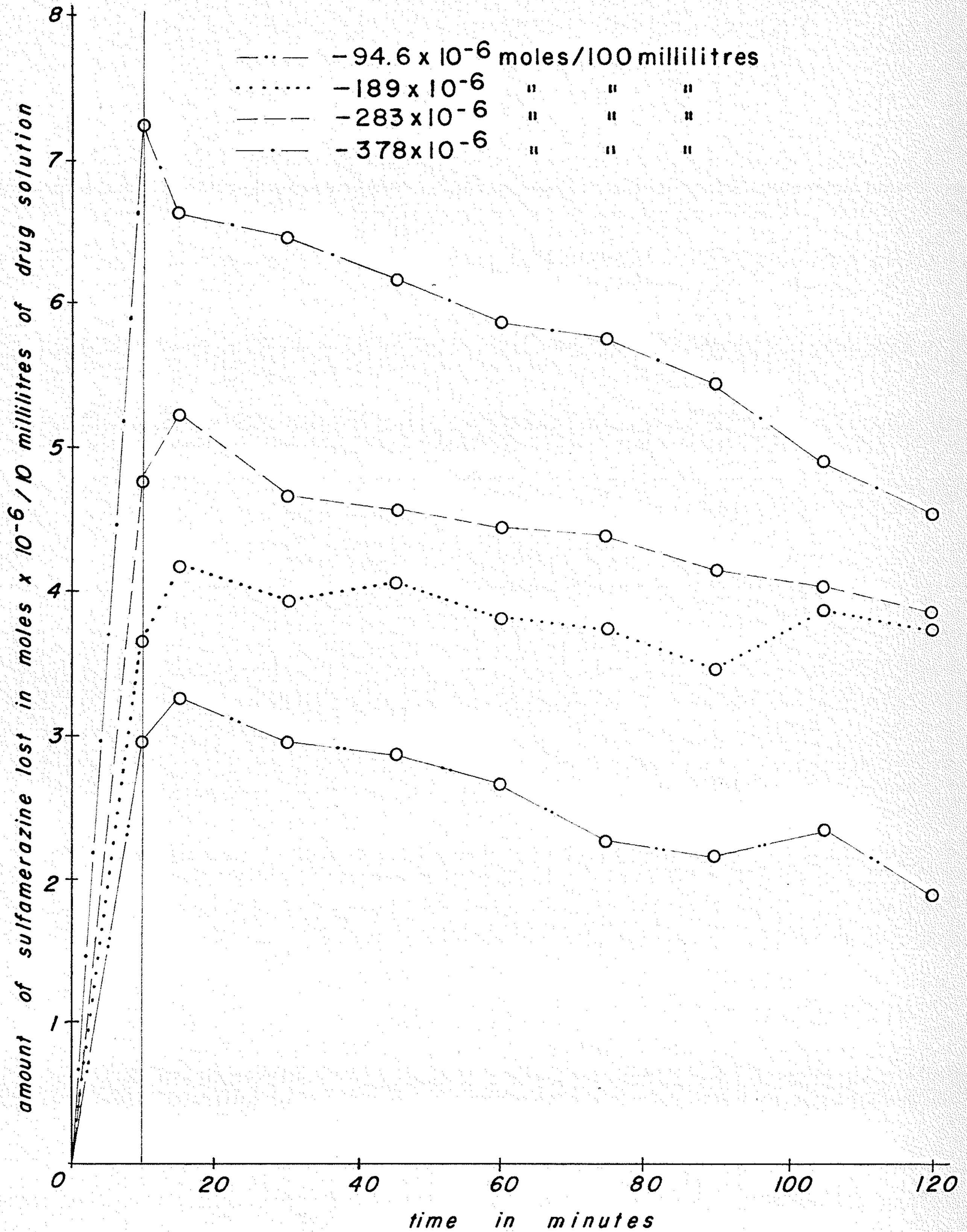
—··— - 89.8 x 10^{-6} moles/100 millilitres
······ - 180 x 10^{-6} " " "
— — — - 270 x 10^{-6} " " "
—·— - 360 x 10^{-6} " " "



GRAPH RELATING ABSORPTION PROFILE OF SULFAMERAZINE ON A SEMI-LOG_{10} OF CONCENTRATION SCALE AGAINST TIME.

—··— - 94.6 x 10^{-6} moles/100 millilitres
······ - 189 x 10^{-6} " " "
— — — - 283 x 10^{-6} " " "
—·— - 378 x 10^{-6} " " "

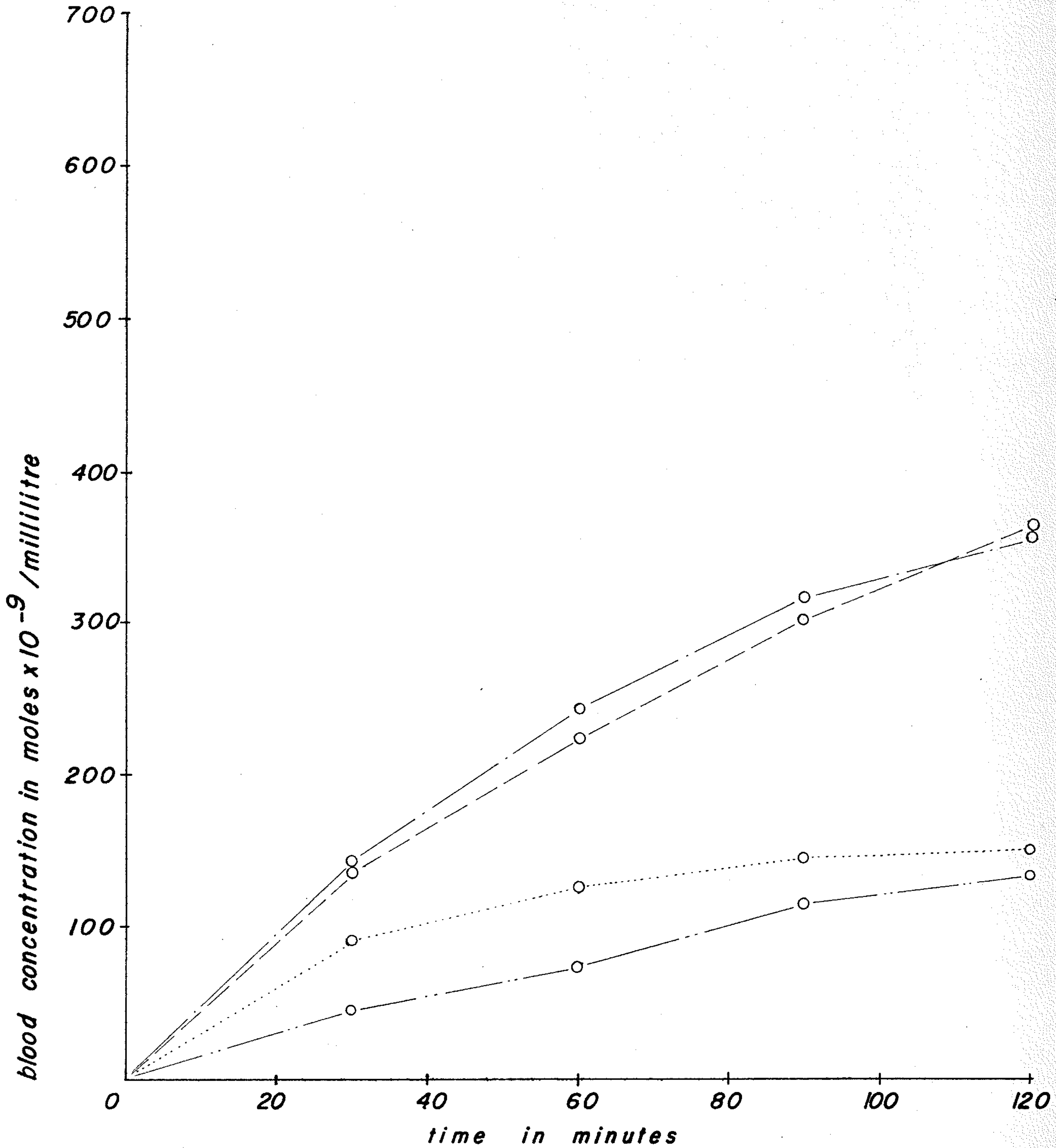
GRAPH NO. 9.



SULFAMERAZINE ABSORPTION PROFILE

GRAPH RELATING CONTINUOUS LOSS OF SULFAMERAZINE FROM 10 MILLILITRE ALIQUOTS OF VARIOUS CONCENTRATIONS PERFUSED THROUGH AN ISOLATED INTESTINE IN SITU.

GRAPH NO. 10



GRAPH SHOWING INCREASING BLOOD LEVELS OF SULFANILAMIDE ON PERFUSION OF THE FOLLOWING AMOUNTS:

—··— 150×10^{-6} moles / 100 millilitres
········· 240×10^{-6} " " "
– – – 435×10^{-6} " " "
—·— 580×10^{-6} " " "

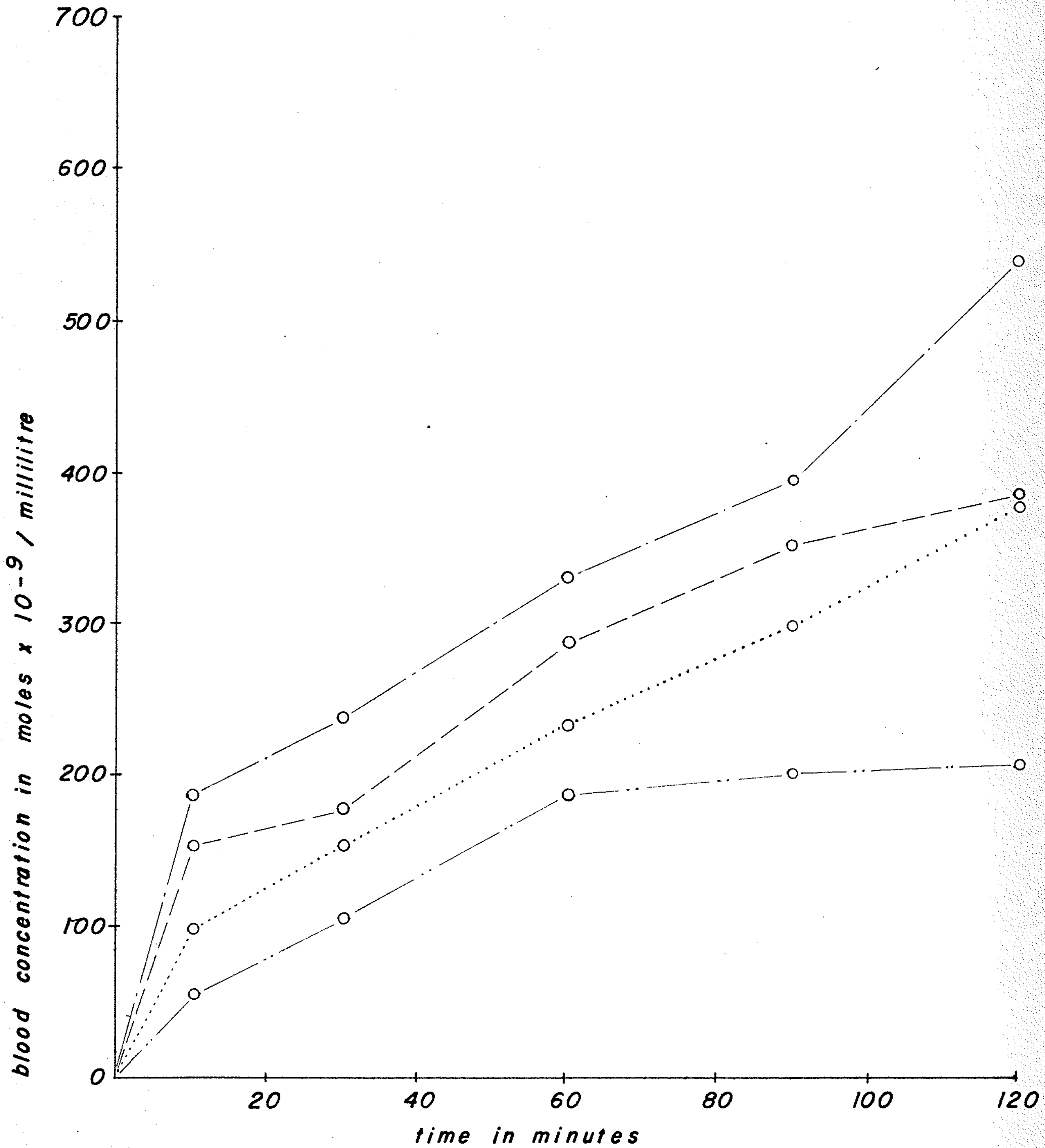
GRAPH NO. 10A

GRAPH RELATING BLOOD LEVEL OF SULFANILAMIDE ON A SEMI-LOG_{10} SCALE FOR SULFANILAMIDE PERFUSED AT VARIOUS CONCENTRATIONS

GRAPH NO. 11A.

GRAPH RELATING BLOOD LEVEL OF SULFADIAZINE ON A SEMI-LOG_{10} SCALE FOR SULFADIAZINE PERFUSED AT VARIOUS CONCENTRATIONS

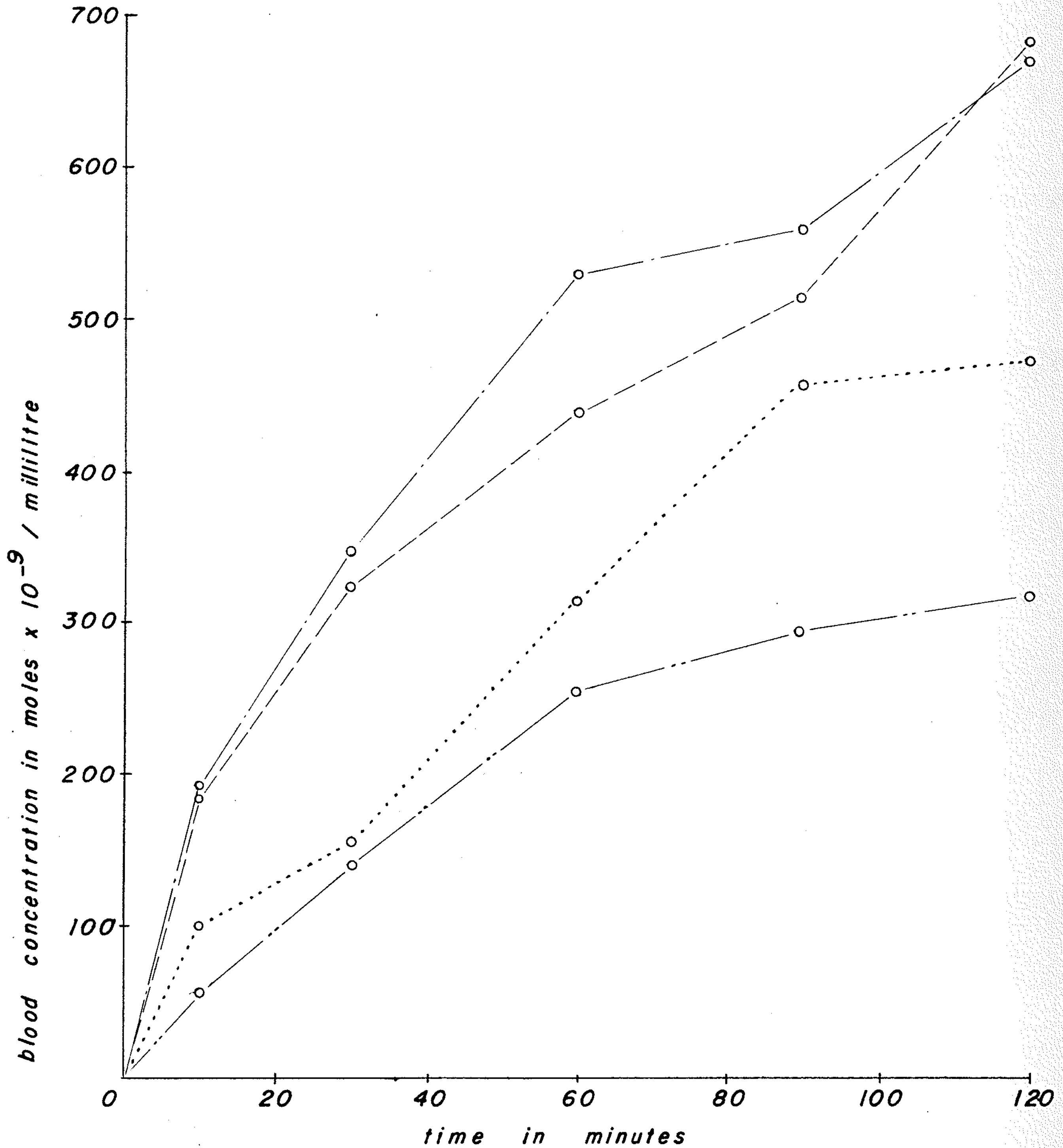
GRAPH NO. II



GRAPH SHOWING INCREASING BLOOD LEVELS OF SULADIAZINE ON PERFUSION OF THE FOLLOWING AMOUNTS :

—··— 100×10^{-6} moles / 100 millilitres
········ 200×10^{-6} " " "
— — — 300×10^{-6} " " "
—·— 400×10^{-6} " " "

GRAPH NO. 12



GRAPH SHOWING INCREASING BLOOD LEVELS OF SULFAMETHAZINE ON PERFUSION OF THE FOLLOWING AMOUNTS:

—··— 89.8×10^{-6} moles / 100 millilitres
········ 180×10^{-6} " " "
— — — 270×10^{-6} " " "
—·— 360×10^{-6} " " "

GRAPH NO. 12A.

level of sulfamethazine in moles x 10^{-6} (log 10) per millilitre of blood

.800 .600 .400 .200 .100 .080 .060 .040 .020

— ·· — - 89.8 x 10^{-6} moles/100 millilitres
· · · · · · - 180 x 10^{-6} " " "
— — — - 270 x 10^{-6} " " "
— · — - 360 x 10^{-6} " " "

GRAPH RELATING BLOOD LEVEL OF SULFAMETHAZINE ON A SEMI-LOG_{10} SCALE FOR SULFAMETHAZINE PERFUSED AT VARIOUS CONCENTRATIONS

GRAPH NO. 13A.

level of sulfamerazine in moles x 10^{-6} (log 10) per millilitre of blood

.800 .600 .400 .200 .100 .080 060 .040 .020

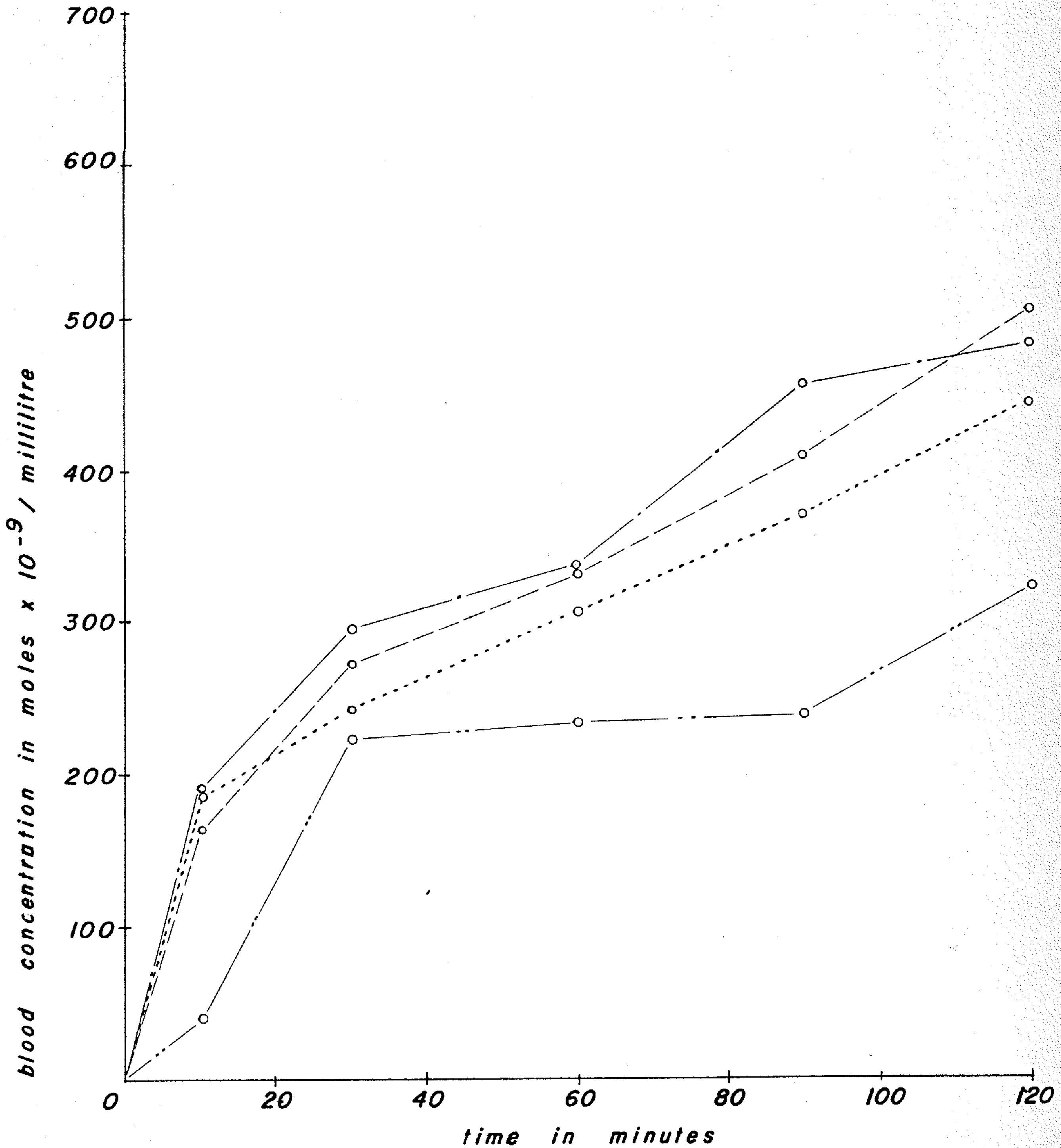
— ·· — - 94.6 x 10^{-6} moles/100 millilitres
· · · · · · · - 189 x 10^{-6} " " "
— — — - 283 x 10^{-6} " " "
— · — - 378 x 10^{-6} " " "

GRAPH RELATING BLOOD LEVEL OF SULFAMERAZINE ON A SEMI-LOG_{10} SCALE FOR SULFAMERAZINE PERFUSED AT VARIOUS CONCENTRATIONS

20 40 60 80 100 120

time in minutes

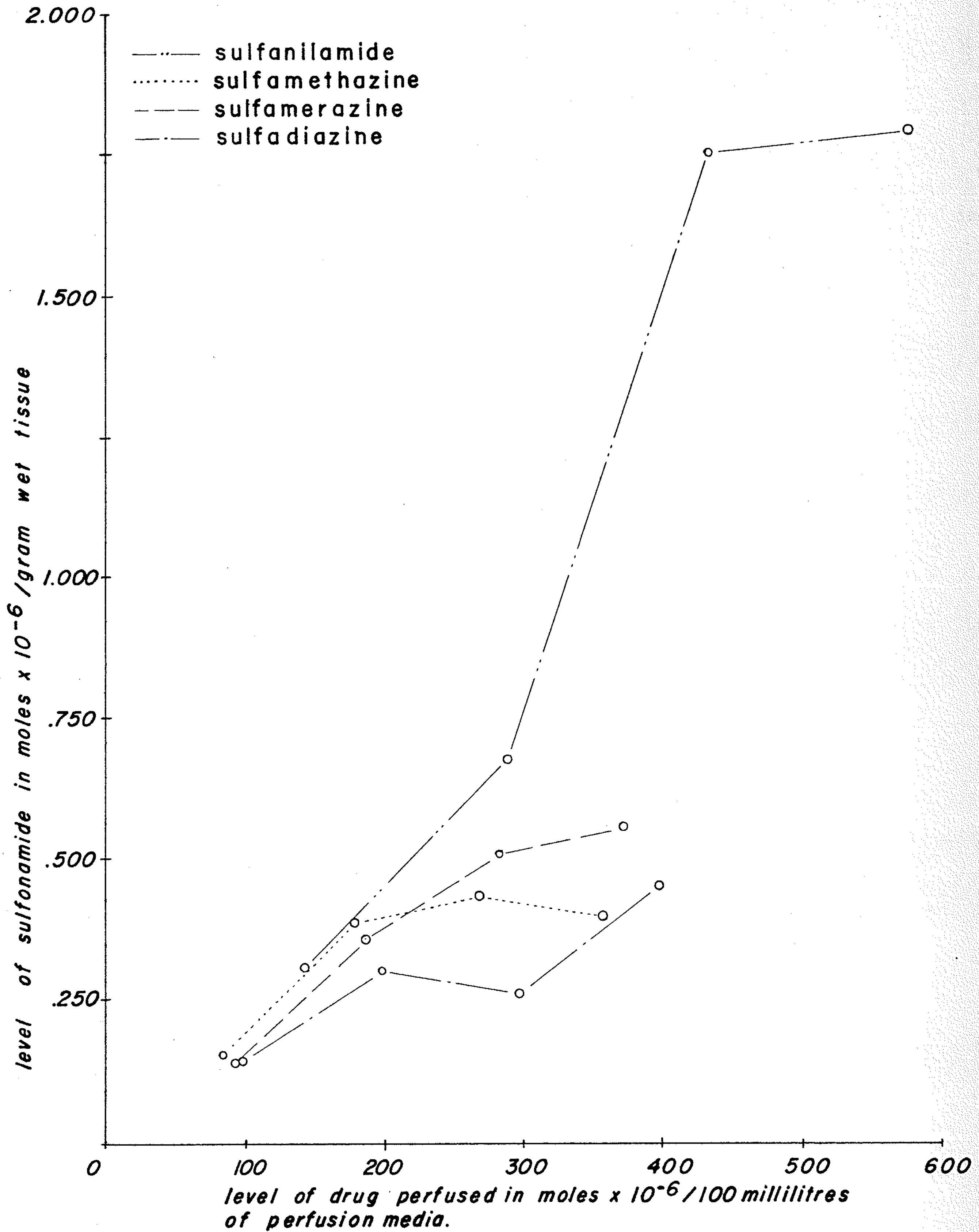
GRAPH NO. 13.



GRAPH SHOWING INCREASING BLOOD LEVELS OF SULFAMERAZINE ON PERFUSION OF THE FOLLOWING AMOUNTS:

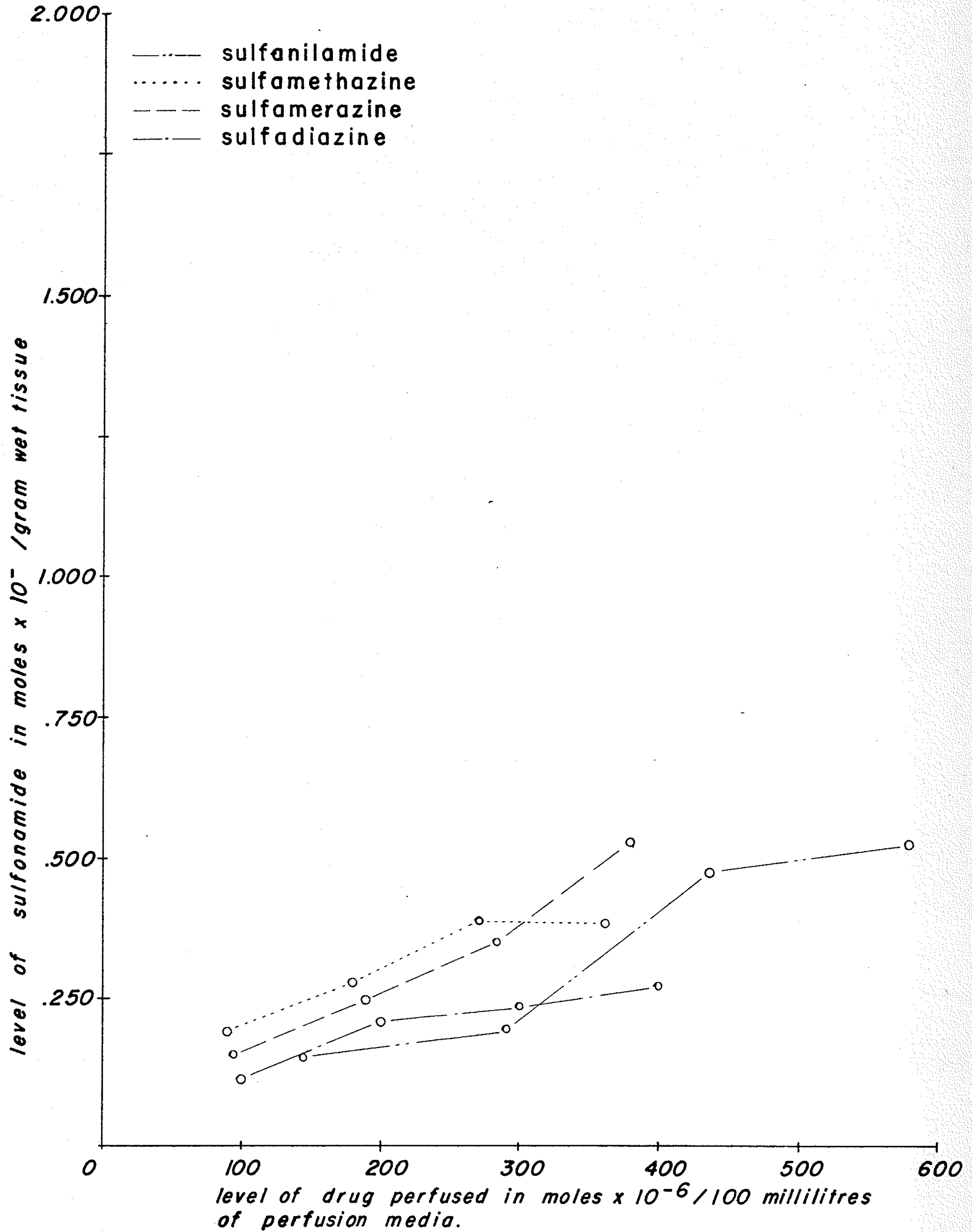
—··— 94.6×10^{-6} moles / 100 millilitres
········ 189×10^{-6} " " "
— — — 283×10^{-6} " " "
—·— 378×10^{-6} " " "

GRAPH NO. 14



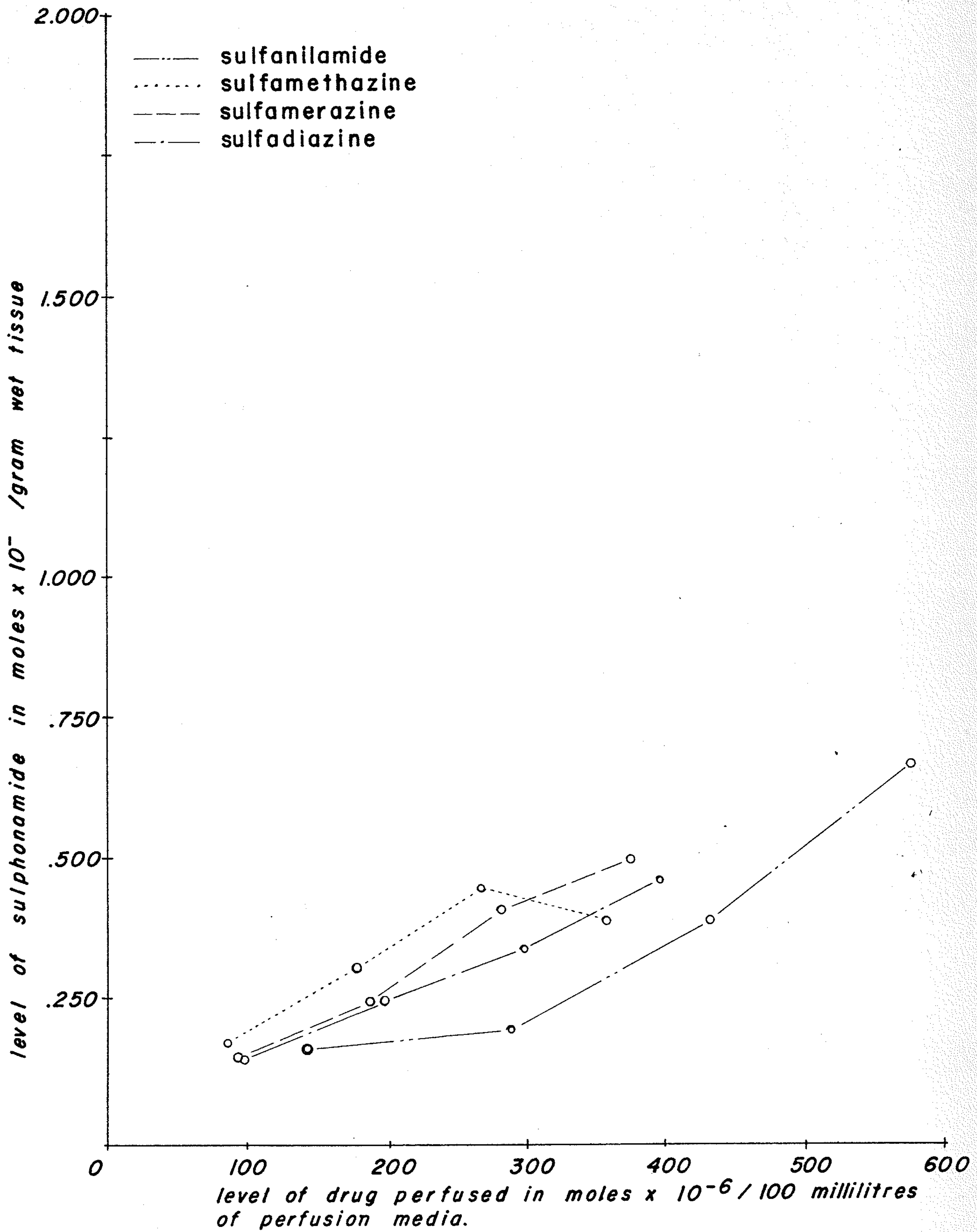
GRAPH RELATING UPTAKE OF SULFONAMIDE BY INTESTINAL TISSUE

GRAPH NO. 15



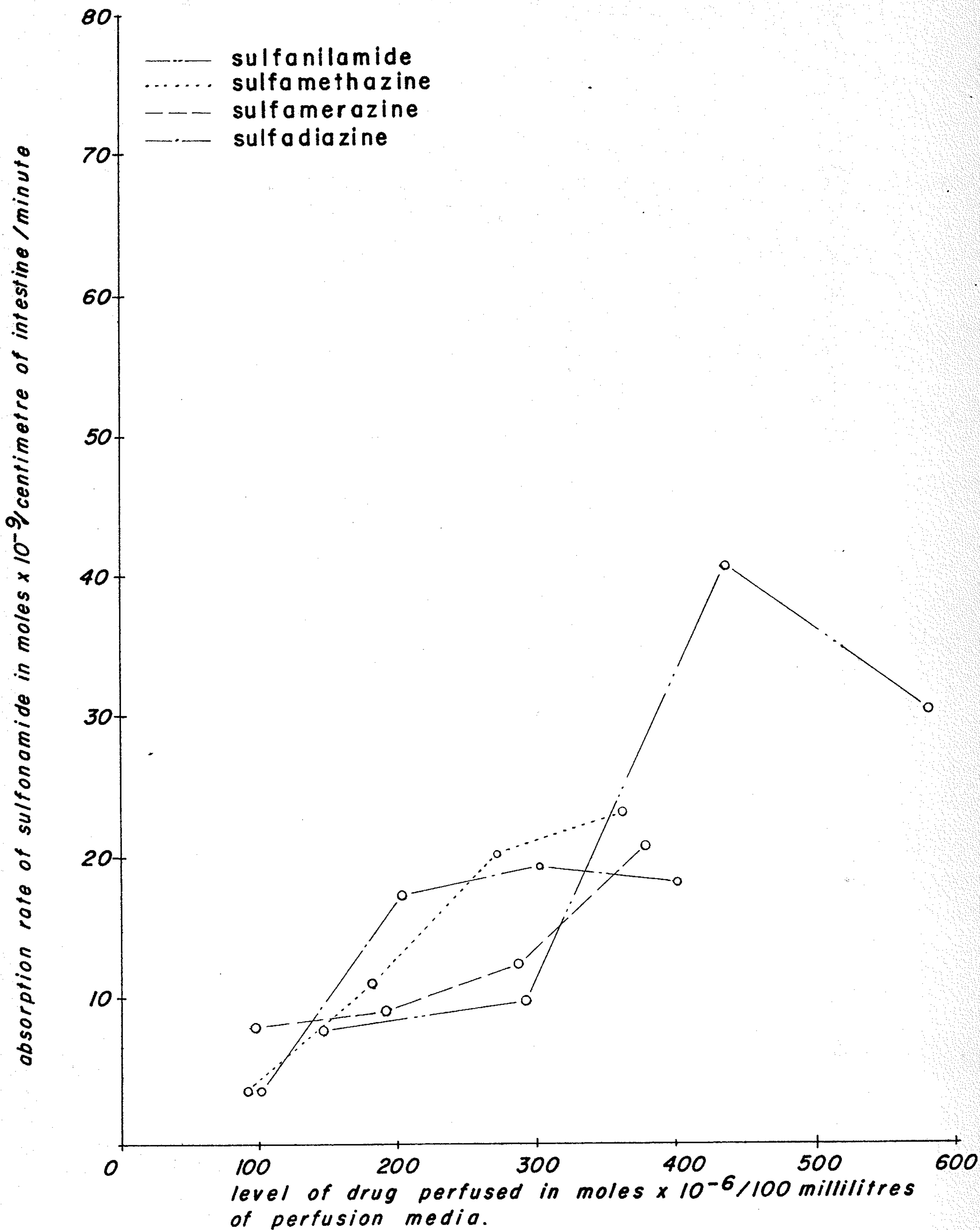
GRAPH RELATING UPTAKE OF SULFONAMIDE BY LIVER TISSUE

GRAPH NO. 16



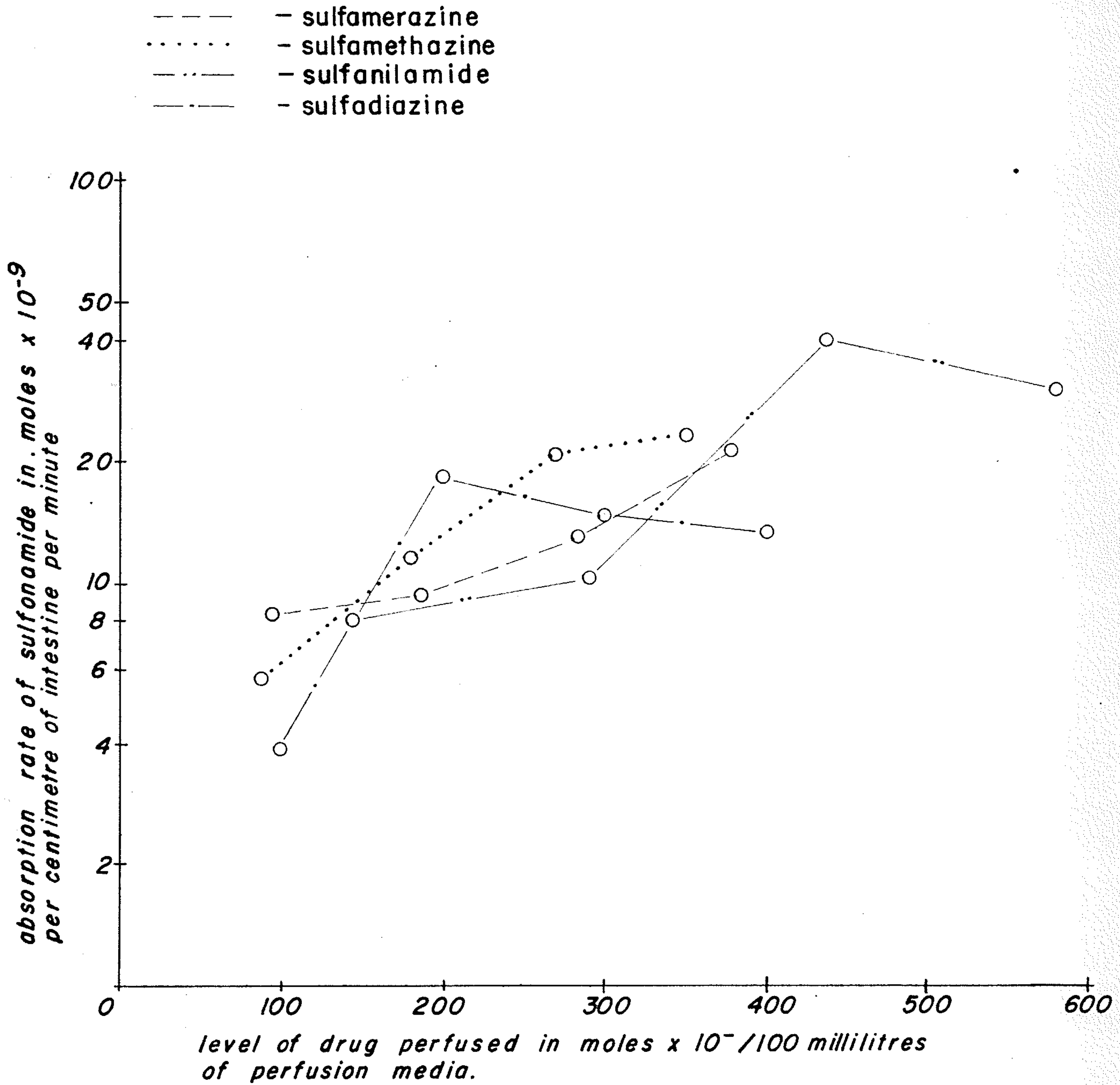
GRAPH RELATING UPTAKE OF SULFONAMIDE BY KIDNEY TISSUE

GRAPH NO. 17



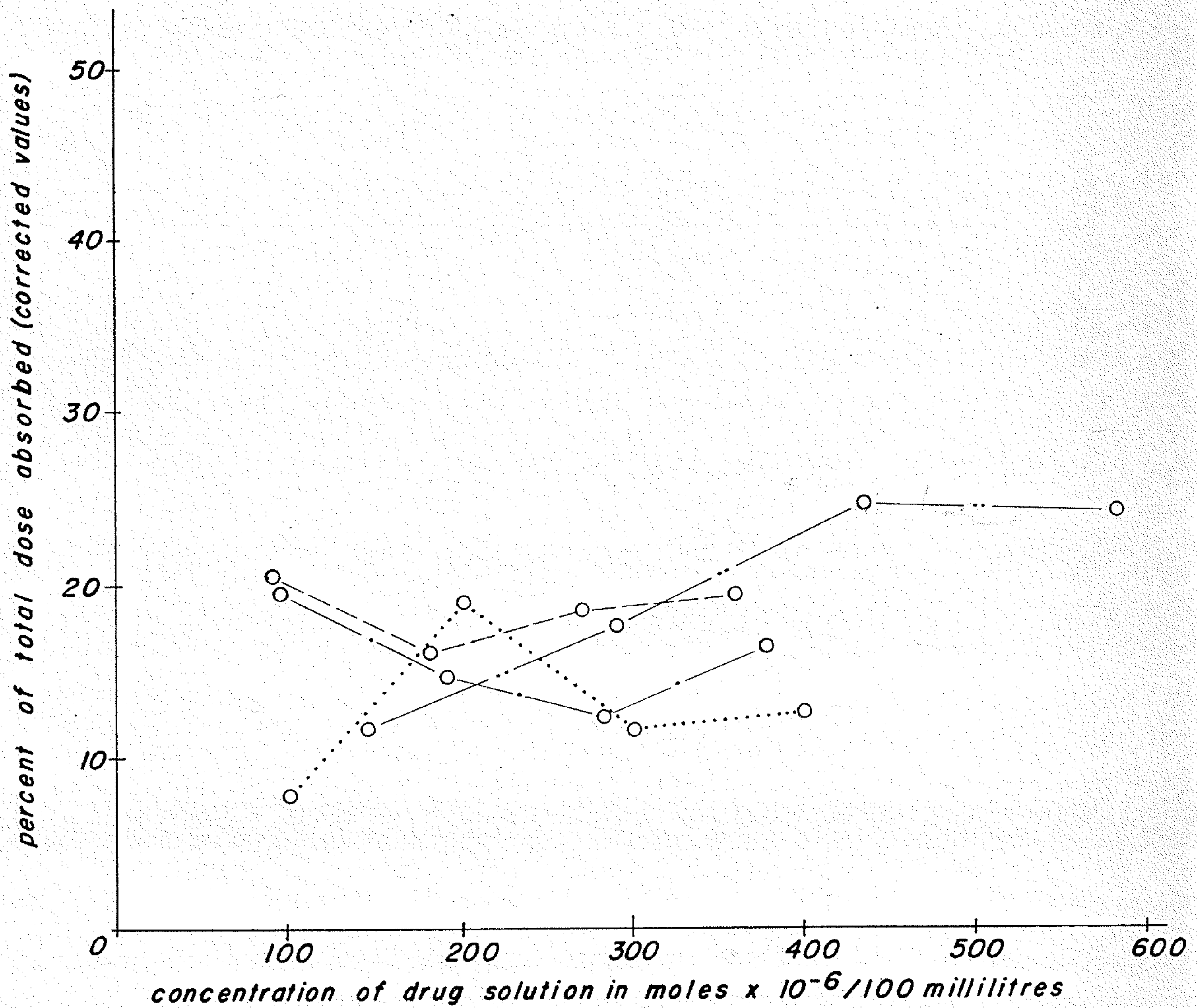
GRAPH RELATING ABSORPTION RATE OF VARIOUS SULFONAMIDES AT DIFFERENT CONCENTRATIONS.

GRAPH NO. 17A.



GRAPH RELATING ABSORPTION RATE ON A SEMI-LOG_{10} OF VARIOUS SULFONAMIDES AT DIFFERENT CONCENTRATIONS PERFUSED THROUGH AN ISOLATED INTESTINAL SEGMENT.

GRAPH NO. 18



GRAPH RELATING PERCENT OF TOTAL DOSE OF SULFONAMIDE ABSORBED IN 120 MINUTES BY AN ISOLATED INTESTINE.

TABLE III - Absorption of Various Sulfonamides Indicated by Disappearance of Drug in Moles x 10^{-6} from 10 Millilitres of the Perfusion Solution at Various Concentrations

			TIME OF SAMPLE IN MINUTES (APPROXIMATE)							
DRUG	LOAD	WASH	15	30	45	60	75	90	105	120
S1	145(2)	2.63±.21	2.74±.24	2.39±.31	1.77±.33	1.70±.36	1.67±.30	1.54±.39	1.50±.28	1.34±.21
S1	290(3)	3.03±.19	3.94±.47	3.28±.39	3.11±.42	2.70±.33	2.85±.46	2.62±.41	2.41±.46	2.36±.28
S1	435(3)	5.25±.33	6.26±.58	6.95±.55	6.37±.68	6.13±.65	6.03±.49	5.87±.52	5.77±.57	5.65±.54
S1	580(5)	6.16±.54	7.50±.62	7.94±.76	7.56±.59	7.36±.74	7.50±.77	7.63±.68	7.79±.73	7.95±.69
% Variation		7.44	9.34	9.78	10.74	11.63	11.19	11.33	11.68	9.94
S2	100(2)	0.97±.13	0.76±.19	1.19±.16	0.92±.22	0.62±.21	0.74±.12	0.56±.15	0.56±.18	0.75±.21
S2	200(3)	3.14±.35	2.36±.23	2.75±.20	2.97±.27	2.85±.22	2.82±.38	2.97±.41	2.77±.36	2.63±.33
S2	300(3)	4.47±.48	3.67±.34	4.04±.31	4.09±.39	4.18±.34	4.10±.39	4.05±.09	3.97±.33	3.50±.45
S2	400(3)	5.33±.62	4.60±.47	5.24±.39	5.45±.46	5.37±.40	5.50±.46	5.17±.44	5.25±.41	5.09±.38
% Variation		11.36	10.8	8.02	9.98	8.99	10.26	8.55	10.2	11.45
S3	89.8(3)	1.60±.15	1.76±.21	1.75±.16	1.50±.13	1.61±.19	1.55±.12	1.72±.14	1.80±.22	1.70±.11
S3	180(3)	2.24±.20	2.47±.17	3.21±.19	3.57±.27	3.45±.18	3.56±.24	3.46±.33	3.02±.24	2.39±.28
S3	270(4)	4.63±.49	4.33±.32	4.45±.27	4.26±.38	4.31±.26	4.55±.35	4.32±.45	4.24±.30	3.87±.43
S3	360(4)	5.84±.64	5.17±.38	5.42±.41	5.69±.55	5.66±.49	5.55±.42	5.34±.47	5.45±.65	5.34±.56
% Variation		10.34	7.87	6.95	8.85	11.95	7.43	9.37	9.72	10.38
S4	94.6(3)	2.95±.25	3.25±.29	2.96±.32	2.88±.39	2.68±.32	2.28±.29	2.16±.37	2.33±.31	1.90±.23
S4	189(4)	3.68±.21	4.18±.34	3.94±.37	4.07±.46	3.83±.44	3.76±.41	3.48±.29	3.89±.44	3.75±.35
S4	283(4)	4.79±.36	5.24±.38	4.67±.50	4.57±.33	4.46±.39	4.44±.46	4.15±.45	4.02±.36	3.86±.41
S4	378(4)	7.23±.59	6.62±.62	6.48±.56	6.18±.51	5.86±.55	5.75±.62	5.44±.61	4.89±.60	4.54±.58
% Variation		7.56	8.45	9.70	9.55	10.1	10.97	11.29	11.3	11.17

S1 = Sulfanilamide; S2 = Sulfadiazine; S3 = Sulfamethazine; S4 = Sulfamerazine

LOAD - Refers to the concentration of Sulfonamide in Drug Solution in Moles x 10^{-6}/100 Millilitres
WASH - Refers to the initial equilibration period
% VARIATION - Refers to the percent of the range
(No.) - Refers to the number experiments

TABLE IV - Blood Level of Sulfonamide in moles x 10^{-6}/millilitre of Blood Resulting on Perfusion of Various Concentrations of Drug

Drug	Load	10 ±D	30 ±D	60 ±D	90 ±D	120 ±D	120 (mg %)
		Time of Sample in Minutes					
SF	145(2)		0.046±.017	0.076±.014	0.115±.015	0.131±.015	2.5
SF	290(3)		0.091±.013	0.127±.022	0.146±.018	0.151±.018	3.1
SF	435(3)		0.135±.029	0.227±.033	0.302±.042	0.367±.047	6.3
SF	580(5)		0.151±.008	0.244±.018	0.319±.017	0.354±.053	6.1
% Deviation			±15.84	±12.91	±10.43	±13.26	
SD	100(2)	0.064±.007	0.104±.008	0.188±.011	0.198±.016	0.203±.018	5.1
SD	200(3)	0.099±.010	0.154±.013	0.237±.025	0.296±.041	0.376±.043	9.4
SD	300(3)	0.153±.018	0.178±.024	0.287±.029	0.352±.043	0.381±.047	9.5
SD	400(3)	0.188±.016	0.238±.021	0.332±.036	0.391±.045	0.540±.062	13.5
% Deviation		±10.12	±9.79	±9.67	±11.72	±11.33	
SMET	89.8(3)	0.059±.016	0.147±.015	0.261±.033	0.298±.036	0.320±.042	8.9
SMET	180(3)	0.103±.019	0.157±.022	0.319±.037	0.460±.046	0.476±.051	13.2
SMET	270(4)	0.189±.027	0.327±.011	0.441±.053	0.520±.037	0.685±.058	16.2
SMET	360(4)	0.194±.031	0.352±.038	0.533±.033	0.560±.066	0.676±.080	15.8
% Deviation		±.17.06	±8.75	±10.04	±10.07	±10.71	
SMER	94.6(3)	0.047±.010	0.229±.038	0.238±.033	0.243±.041	0.327±.037	9.2
SMER	189(4)	0.191±.051	0.247±.056	0.311±.068	0.373±.039	0.448±.054	11.8
SMER	283(4)	0.170±.039	0.275±.047	0.338±.050	0.411±.053	0.508±.025	13.3
SMER	378(4)	0.193±.017	0.298±.033	0.340±.021	0.459±.064	0.485±.069	12.8
% Deviation		±19.47	±16.59	±14.02	±13.26	±10.46	

Load - refers to the concentration of Sulfonamide in moles x 10^{-6} /100 millilitres of solution

% Deviation - refers to the percent deviation from the mean value

TABLE V - Data Indicating the Amount of Intestine Perfused, the Percent of the Dose Retained in the Intestine, the Percent of Absorption Determined, and the Absorption Rate Calculated from Isolated Intestinal Loops

Drug	Load	Length Of Gut (cm.)±R	Weight Of Gut (Mean)±R	% of Dose In Gut (Mean)±%D	% of Dose In Gut Wash (Mean)±%D	% of Absorption ±%D	Absorption Rate ±%D
SF	145(2)	19.0±1.5	1.43±.31	0.24±12.1	0.26±15.3	11.8±12.4	8.1±21.5
SF	290(3)	18.5±1.5	1.37±.25	0.32±9.8	0.42±21.6	17.7±19.9	10.6±17.4
SF	435(3)	20.5±2.0	1.56±.32	0.63±16.5	0.43±13.8	24.6±21.3	41.5±31.9
SF	580(5)	21.5±2.5	1.59±.38	0.43±20.4	0.34±17.5	24.0±17.6	31.3±27.2
Mean		19.8±1.5	1.49±.32	0.41±14.7	0.36±17.1	±17.8	±24.5
SD	100(2)	20.0±1.0	1.51±.20	0.20±12.6	0.17±10.2	7.5±10.9	3.9±16.0
SD	200(3)	19.5±2.5	1.62±.38	0.26±19.7	0.18±12.4	18.9±11.7	18.4±21.3
SD	300(3)	21.5±2.0	1.75±.35	0.13±23.2	0.22±19.9	11.8±9.8	14.9±18.5
SD	400(3)	21.0±2.5	1.78±.44	0.21±16.6	0.22±17.6	12.4±4.6	13.7±15.1
Mean		20.5±2.0	1.67±.34	0.20±18.0	0.20±15.0	±9.2	±17.7
SMET	89.8(3)	22.5±2.5	1.95±.52	0.40±17.4	0.62±18.7	20.5±18.1	5.9±12.6
SMET	180(3)	22.0±0.5	1.81±.27	0.36±15.3	0.27±16.1	16.2±19.3	11.8±14.9
SMET	270(4)	21.5±2.0	1.83±.39	0.27±19.0	0.34±20.7	18.5±15.5	21.0±5.5
SMET	360(4)	19.0±1.5	1.46±.36	0.16±24.5	0.35±22.5	19.1±25.0	23.9±34.2
Mean		21.2±1.6	1.76±.39	0.30±19.1	0.39±19.5	±19.5	±16.8
SMER	94.6(3)	18.5±3.0	1.29±.32	0.17±22.9	0.35±18.2	19.5±14.4	8.5±19.3
SMER	189(4)	23.0±1.5	2.02±.35	0.53±23.5	0.72±21.9	22.0±24.1	9.4±32.8
SMER	283(4)	18.0±2.5	1.31±.37	0.24±14.6	0.20±15.2	12.3±23.7	13.1±7.7
SMER	378(4)	21.0±2.0	1.73±.41	0.23±12.4	0.26±17.8	16.2±24.3	21.4±5.8
Mean		20.1±2.3	1.59±.36	0.29±18.4	0.38±18.2	±21.6	±16.4

R = Refers to the range of values; %D - refers to the percent deviation from the mean

Absorption Rate - is expressed in moles x 10^{-9}/cm/min; Load - refers to moles x 10^{-6}/100 ml

% Absorption - refers to the % of the dose absorbed

TABLE VI - Tissue Levels of Sulfonamide in moles x 10^{-6}/gram of Wet Tissue

Drug	Load	Intestine ± %D	% of Dose	Liver ± %D	% of Dose	Kidney ± %D	% of Dose	Blood Level ± D
SF	145(2)	0.3138±13.5	1.88	0.1562±28.8	0.93	0.1766±18.2	1.06	0.131±.015
SF	290(3)	0.6892±21.4	3.14	0.2096±19.5	0.96	0.2169±39.5	0.97	0.151±.018
SF	435(3)	1.7644±17.0	1.70	0.4896±27.1	0.47	0.4069±29.5	0.39	0.367±.047
SF	580(5)	1.8024±38.1	2.41	0.5425±28.3	0.72	0.6905±48.6	0.92	0.354±.053
Control		0.0328±5.4		0.0945±12.9		0.1027±22.6		
Mean			2.28		0.77		0.84	
SD	100(2)	0.1517±19.2	1.76	0.1216±26.7	1.41	0.1648±39.2	1.91	0.203±.018
SD	200(3)	0.3007±12.7	0.75	0.2236±20.4	0.56	0.2629±35.3	0.66	0.276±.043
SD	300(3)	0.2723±9.8	0.67	0.2432±23.7	0.60	0.3531±24.0	0.86	0.381±.047
SD	400(3)	0.4573±12.5	1.38	0.2864±25.9	0.86	0.4786±29.8	1.44	0.540±.062
Control		0.0313-7.5		0.1122-15.4		0.0980-16.4		
Mean			1.14		0.86		1.22	
SMET	89.8(3)	0.1504±15.0	0.79	0.2051±25.2	1.08	0.1896±20.1	0.99	0.320±.042
SMET	180(3)	0.4023±22.6	1.24	0.2919±21.9	0.90	0.3237±13.1	1.00	0.476±.051
SMET	270(4)	0.4400±16.7	0.97	0.4071±19.1	0.82	0.4701±25.6	0.95	0.685±.058
SMET	360(4)	0.4143±12.3	0.77	0.4021±14.7	0.75	0.4101±19.3	0.76	0.676±.080
Control		0.0320±4.8		0.0920±10.7		0.1003±19.5		
Mean			0.94		0.89		0.93	
SMER	94.6(3)	0.1485±9.4	0.72	0.1627±12.5	0.79	0.1721±27.5	0.84	0.327±.037
SMER	189(4)	0.3873±13.7	1.08	0.2672±17.1	0.75	0.2595±21.0	0.73	0.448±.054
SMER	283(4)	0.5163±17.1	1.75	0.3712±16.9	1.26	0.4254±29.4	1.44	0.508±.025
SMER	378(4)	0.5687±10.8	0.89	0.5403±18.4	0.84	0.5164±30.7	0.81	0.485±.069
Control		0.0309±8.2		0.0891±21.4		0.0968±25.4		
Mean			1.11		0.91		0.96	

Blood Level - refers to concentration in moles x 10^{-6}/ml at 120 minutes

RESULTS

The absorption data in Table III indicates the mean value obtained for each drug at a particular concentration at regular time intervals. The figures recorded were obtained from experiments where the range in values was not beyond 15 percent of the mean. In a good number of experiments the loss of drug from the isolated loop either varied greatly from interval to interval or all the data were far removed from other experiments in that group. This meant that the number of suitable experiments that could be tabled and plotted was reduced.

An examination of the values in Table III indicates that the sulfanilamide (SF) absorption profile agreed better with the absorption profile of sulfamerazine (SMER) than it did with any of the other sulfonamides. SMER was better absorbed at lower concentrations than SF, however, the loss of SMER from the loop was more regular and steady once equilibrium washing of the loop was completed. Sulfamethazine (SMET) and Sulfadiazine (SD) absorption was 1-2 moles x 10^{-6} per unit less for the same intervals. The absorption of SF at low concentrations tended to fall off more rapidly than with SMER. Whereas the absorption of SF and SMER had a tendency to fall off with time, that of SD and SMET was seen to proceed at a more or less constant rate. The absorption rate for

SD and SMET was somewhat higher and more regular than for SF and SMER.

Only SF demonstrated an increase in the percent of dose absorbed with increasing concentration of drug. The percent of SMER absorption tended to decrease in a regular fashion, with some increase at the highest dose. The percent absorption of SMET remained more or less constant at all concentrations and was of the order of 20%. The percent absorption of SD was a bit erratic at the various concentrations of drug used, but was on the whole considerably less. With SF the level of 24% of absorption, agrees with the data obtained by Schanker et al, (1958).

On checking the blood levels obtained with the various drugs, it is seen that SF had the lowest concentration of drug, even at perfusion concentrations twice that of the other sulfonamides.

SMET achieved the highest blood levels of 16 mg% which would agree with its generally constant and higher absorption rate. SD blood levels become greater than those for SF rapidly at higher concentrations of drug. Both SMER and SD produced blood levels of approximately 13 mg% in a steadily increasing fashion. SF blood levels exceeded 6 mg% only by a small amount.

Except for blood levels at low concentrations, the blood levels when plotted on an arithmetic scale appeared to be increasing

quite steadily with time. However, on a semi-log scale the blood levels appear to be much steadier at 2 hours.

Studies on the absorption of the long-acting sulfa, sulfamethoxydiazine demonstrated that a blood level of 13-15 mg% was preferred for therapeutic effectiveness, (Chew et al, 1965). Randall et al, (1959), reported slightly higher blood levels in rats for sulfadimethoxime in rats after oral administration. Kidney levels rose to 4 mg/gram, while liver tissue contained a little less.

When we compare the handling of the sulfonamides by the various tissues examined, we note that SF tended to be accumulated in the intestinal wall much better than any of the other sulfonamides. The accumulation of SMER and SMET was quite uniform with increasing doses and was about 0.3 percent of the dose. SD was accumulated the least by the intestine.

Liver accumulation of all the compounds was reasonably similar with the levels increasing almost in a parallel fashion with the increasing doses. SF was accumulated to a slightly lower amount.

Kidney accumulation of SD, SMER and SMET was similarly parallel. However, SF levels in kidney were considerably lower. Unfortunately, the only data collected for urine studies that was adequate was that for SF. In a given 3 hour period, animals

dosed orally with SF consistently excreted more urine and drug than did any of the animals dosed with the other compounds at increased concentration of drug.

The absorption rates were determined by simply dividing the amount of drug absorbed in moles x 10^{-9} by the length of intestine and by 120 minutes. Although this resulted in rather an arbitrary figure, it does afford some basis for comparison. The absorption rate calculated on this basis is reflected in the blood levels.

On the whole, doubling the concentration of drug resulted in a similar increase in absorption rate, and percent of drug absorbed with SF. The absorption rate for SMET increased in a parallel manner with increasing dose, but this was while the percent absorbed remained fairly constant. SD absorption on the other hand reached a maximal absorption rate of about 15 x 10^{-9} moles/cm/min, while the percent of drug absorbed varied between 8 to 12 percent.

The absorption rate of SMER increased in a linear fashion while the percent of drug absorbed varied between 12-22 percent.

Although the gut wash data were never greater than 0.4% of the total amount of drug perfused, they do indicate that this amount of drug did not appear to be involved in the drug absorption process. The wash values obtained were subject to wide variation

for a given drug, but on the whole were reproducible. Considerable difficulty in standardizing the flow rate for washing no doubt added to the error.

The tissue levels of sulfonamide were obtained in an effort to see if there was any correlation between the amount of drug accumulated in a tissue and the concentration of drug perfused. If these values reflect handling of the drug by an organ then variation from a reference figure could possibly be an indication of variation in the metabolic activity of the organ and thus help to possibly explain discrepancies in blood level.

DISCUSSION AND CONCLUSIONS

At the outset of this investigation it was hoped that the data obtained would allow a modest advance in the broad field of intestinal transport. However, as the study progressed many difficulties presented themselves and in some cases were only partly solved. The range of values was in some cases very wide, such that we had to discard many experiments. This reduced the number of animals per group to as low as two in some cases. It is felt that more work will have to be carried out, particularly in cases where the relationship between one group and the next is less evident. It was felt that at the conclusion of the experiments reported here the state of technique was such that little difficulty would be encountered in obtaining these figures.

The total blood level in no case ever reached the level of drug present in a corresponding volume of perfusion solution.

When considering the absorption of SF we note that the percent absorption became similar at concentrations beyond 290 x 10^{-6} moles/100 ml (50 mg%). Therefore, it can be suggested that concentrations greater than 50 mg% of SF are absorbed by means of a first order process, (Yamada and Yamamoto, 1965). Thus, at these elevated concentrations the drug is probably crossing the

intestinal epithelium by means of simple diffusion, rather than an active saturable transport mechanism. This does not exclude that an active mechanism could also be operating under these conditions, it is just that the main mechanism is passive diffusion. Additional evidence on this aspect would best be obtained by noting the effect of another drug in the perfusion solution and the absorption rate of the given drug. If the second drug failed to alter the absorption rate of the first drug then this would be evidence in favour of a passive process involving the lipid areas of the intestinal absorption barrier.

On the other hand, a decline in the absorption rate at higher concentration of drug could be accepted as evidence in favour of an active saturated mechanism. There appears to be some decline in absorption with SF at higher concentration of drug but more intermediate data would be required before a firm statement could be made in this regard.

The difference in rate of absorption for SF at concentrations below 50 mg% could be due to the involvement of a carrier mechanism of low specificity, resulting in transport of an active type. At higher concentrations simple passive diffusion would predominate.

In addition, the fact that the log of absorption rate vs. concentration graph (17A) is not a single linear function through-

out the entire range of concentrations used is an indication that the possibility of more than one transport mechanism is operating. One explanation here could be that sodium and water transport could influence the SF transport. If the transport of SF were related independantly to each of these mechanisms then the SF transport profile could be expected to change when either sodium or water transport changed. Sognen, (1965b) has demonstrated that sodium and water transport were susceptible to decreased rates at increased rates of calcium in the perfusion solution.

The increase in blood level for increasing SF concentrations seems, at first, to be not in keeping with the increase in absorption rate for each successive concentration. However, Despopoulos and Callahan, (1962), have demonstrated that of 52 sulfonamides studied, SF is bound 10% to protein, which is considerably lower than that of the other sulfonamides: (SD - 17%; SMER - 39%; SMET - 61%). As a result, more SF will be free for excretion and thus lower blood levels will result. In terms of the number of moles, SF compares better with SD, for blood levels, but it is still lower. Most clinical data for the antibacterial efficacy is expressed as mg%.

The accumulation of SF by intestinal tissue beyond concentrations of 50 mg% was almost four times that for the other sulfo-

namides. It would seem that SF was somehow better incorporated into the intestinal tissue since its protein binding in blood is considerably lower than that of the other compounds tested. We are aware that the antibacterial action of SF is due to the incorporation of SF into bacterial cell wall, (Neipp, 1964). Whether a similar factor could be involved here is not clear. In any case this accumulated drug will not have been involved in the whole of the transport process.

It is to be expected that as the experimental period is prolonged that water would probably be transported out of the intestine, and this would contribute towards masking any possible effect on transepithelial transfer of drug. However, by using the whole of the perfusate sample for analysis and adding warm normal saline to the peritoneal cavity we feel that water transport from the intestinal loop was minimized.

Sognen, (1965), in an elaborate and comprehensive study on the effect of calcium binding substances on intestinal transport has reported that sulfanilamide was passively transported *in vitro* when conditions, where suitable, supported active transport of glucose. Therefore, one would expect that the compound would accumulate first in the intestinal wall before being removed via the blood and lymph.

Sognen, (1965a), also demonstrated that a damaged mucosa was less permeable to sulfanilamide than the intact one, and that absorption of sulfanilamide was favoured by the presence of glucose in the perfusion solution. The passive sulfanilamide transport appeared to be independent of water transport and occurred at a greater rate than water transport.

However, Sognen employed sulfanilamide at much lower concentrations and his perfusion technique was quite different from that of ours.

When we consider the sulfapyrimidines proper, SD, SMET, and SMER, we note that as a rule their absorption profiles are quite similar. SMER appeared to demonstrate higher initial losses of drug from the perfusion solution, but as time progressed these rates of loss of drug dropped off, whereas those of SD and SMET were better maintained at their maximal levels. On plotting the absorption rates (Graph 17 and 17A) we note that they are approximately similar in many respects. Perhaps more data would increase the degree of similarity. It is also felt that more data would establish better linearity in the percent of the total dose absorbed relationship, (Graph 18).

The highest blood levels were achieved with SMET, (Table IV) which would be explained in part by its optimal protein binding.

The accumulation of the sulfapyrimidines by tissue was quite similar in all cases, with SD tending to be accumulated to a lesser degree. Kidney levels in particular indicate that the renal handling of the sulfapyrimidines is uniform. Once again more data will be necessary before a clear picture of the organ utilization of the compounds is obtained.

It was unfortunate that the urine data were not obtainable with a good degree of accuracy for these sulfonamides as the urine excretion would further aid in the correlation with the absorption data.

It is unfortunate also that we did not switch to identical molar quantities for each drug early in the study. However, perhaps by having a spectrum of concentrations our observations were made more representative. We corrected figures only for the percent of the dose absorbed (Table V) and the tissue levels, where we had an indication what amount of our determined values were not involved in the transport process. The use of correction factors could be misleading when factors like protein binding and excretion are high. Therefore, we avoided further use of correction factors.

Although our results can not be used to provide conclusive evidence of one or another mechanism of transport they could be used to back up further _in vivo_ and _in vitro_ studies in

this area of drug transport.

Some comment on sulfonamide transport mechanisms at this point could be useful. Despopoulos and Callahan, (1962), have concluded from the renal excretion of SF and the sulfapyrimidines studied here that these sulfonamides are not actively transported across the renal epithelium.

The renal transport of sulfonamide in excretory processes has been suggested to involve a postulated intracellular receptive molecule, (Despopoulos and Callahan, 1962). The necessity for three specific physicochemical structural features in the sulfonamide molecule to permit active transport was discussed. Though the sulfonamide transport mechanism was demonstrated to be independent, its relation to the renal hippurate transport system was recognized.

Their study concluded that sulfonamides would have to be present as anions for active transport. The pKa values for sulfapyrimidines reported were as follows:

a) sulfadiazine 6.5 (SD)
b) sulfamerazine 7.1 (SMER)
c) sulfamethazine 7.4 (SMET)

Thus, at the range of pH values that we would encounter in our experiments, these compounds would be only partly ionized and would have to rely mainly on passive diffusion of the unionized portion for transport.

If we consider the partition coefficients obtained betweenn chloroform and water by Kakemi and his group, (1964), viz:

A) SF 0.04
B) SD 1.16
C) SMER 2.40
D) SMET 5.40

then it appears that lipid solubility is important for sulfapyrimidine absorption since SMET had higher absorption rates and a consistently higher percentage of the total dose absorbed, whereas SMER had intermediate values, and SD had the lowest absorption rates and percentage of total dose absorbed in our studies.

When we compare and contrast SF to the sulfapyrimidines it would appear that lipid solubility is not the dominant factor in its transport mechanism. Since the molecular weight of sulfanilamide is below 180 then perhaps the pore route is more significant in its transport, as its absorption rate and percentage of dose absorbed compare favourably with those of the sulfapyrimidines.

The dissolution rate of the compounds studied in the Kreb-Ringer perfusion solution was a major problem in this study, so that perhaps a similar study involving more soluble derivatives of the sulfapyrimidines would indicate if water solubility is an important consideration.

During the course of these experiments we assume that the absorption mechanisms of the intestine remain operative at optimal

levels. However, in an organ such as the intestine, the very fact of its dynamic nature could result in rapid development of malabsorption, (Weser, 1966), or malabsorption could have been present in the animal to begin with.

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APPENDIX A

COMPOSITION OF KREB-RINGER BICARBONATE PERFUSION MEDIUM

(Umbreit, Burris and Stauffer, 1964)

Ingredient	Moles/Litre	Parts in Perfusion Medium Solution
Sodium Chloride (NaCl)	0.154	100
Potassium Chloride (KCL)	0.154	4
Calcium Chloride (CaCl2)	0.110	3
Potassium Dihydrogen Phosphate (KH2PO4)	0.154	1
Magnesium Sulfate (MgSO4.7H2O)	0.154	1
Sodium Bicarbonate (NaHCO3)	0.154	21

The mixture was gassed for 10 minutes with oxygen (95%) and carbon dioxide (5%).

This solution was prepared fresh at least twice weekly and stored in glass stoppered flasks in the cold. The pH of solution required was checked daily. Under the experimental conditions we rarely encountered precipitation of perfusion medium constituents.

APPENDIX B

LIST OF CHEMICAL COMPOUNDS AND ABBREVIATIONS

Compound	Manufacturer	Lot Number
Sulfanilamide, B.P.C. (SF)	British Drug Houses	31264
Sulfadiazine, B.P., U.S.P. (SD)	" " "	30215
Sulfamerazine, U.S.P. (SMER)	" " "	31294
Sulfamethazine, B.P. (SMET)	" " "	29362
Sodium Nitrite (NaNO2)	Fisher Scientific Co.	760813
Ammonium Sulfamate (NH4SO3 NH2)	" " "	741695
N-(1-Naphthyl)-ethylene diamine Dihydrochloride (NED)	" " "	760987
Cobalt Chloride, A.C.S.	" " "	765081