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# Absorption of caffeine through isolated rat small intestine

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# ABSORPTION OF CAFFEINE THROUGH ISOLATED RAT SMALL INTESTINE

A Thesis

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Master of Science



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Member

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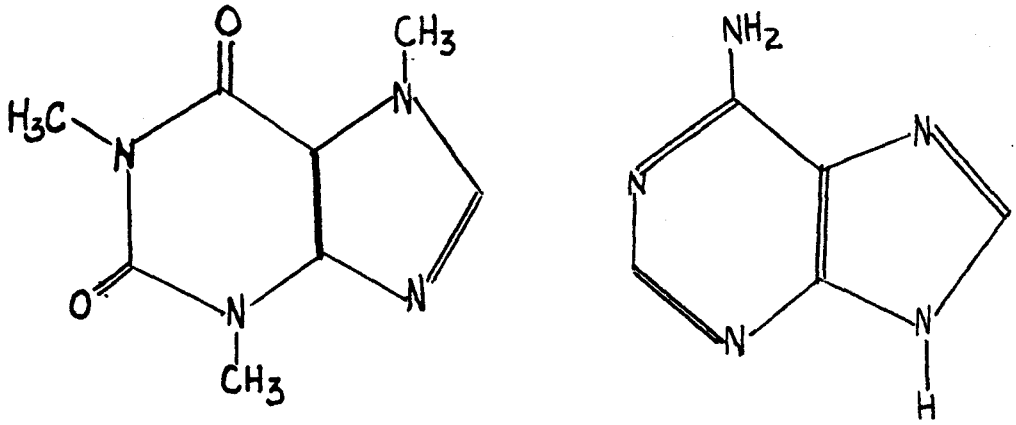
# INTRODUCTION

The absorption of caffeine into the blood stream from the lumen of the digestive tract is of interest for several reasons, some of which are listed below. Caffeine is a constituent of some beverages, including coffee and tea (Goodman and Gilman, 1970). Caffeine has a structure similar to adenine (Fig. 1). It inhibits the phosphodiesterase breakdown of 3'5' cyclic AMP (Haugaard and Hess, 1965), and consequently stimulates cyclic AMP related physiological functions (Sutherland and Rall, 1965) including processes in carbohydrate metabolism (Northrop and Parks, 1964), lipid metabolism (Hynie et al, 1966), and various hormonal activities (Turtle et al, 1967). These influences on biochemical pathways result in alteration of the functions of several organ systems, including the central nervous system (Nash, 1962), the heart and blood vessels (DeGubareff and Sleator, 1965), respiratory function (LeMessier, 1936), and the gastrointestinal system (Robertson et al, 1950).

When caffeine is introduced into the intestine by mouth (Schluger et al, 1957), or rectally by suppository (Waxler and Schack, 1950) the blood concentrations rise rapidly, but the mechanisms of absorption are not known. This thesis is about one study of transport of caffeine across the wall of the isolated rat small intestine. The introduction includes a review of properties of caffeine which could relate to its




Caffeine Adenine

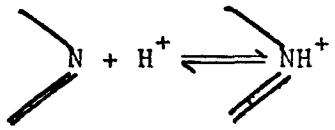
Fig. 1. The Structures of Caffeine and Adenine.

absorption through the intestinal wall, and a discussion of the methods which have been used to study intestinal absorption in general, and caffeine absorption in particular.

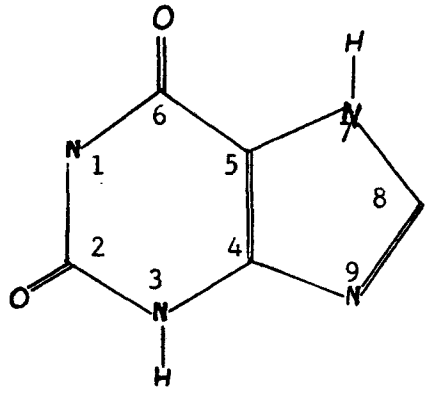
## CHEMISTRY OF CAFFEINE

Caffeine is a methylated xanthine and has properties similar to the other methylated xanthines theophylline and theobromine (Fig. 2 and Table 1)., Caffeine is not readily soluble in water (a 0.113 M solution is saturated) (USPXVIII, 1970), and for effective medical treatments it is usually combined with alkali benzoates, cinnamates, citrates, or salycalates (DiPalma, 1971).

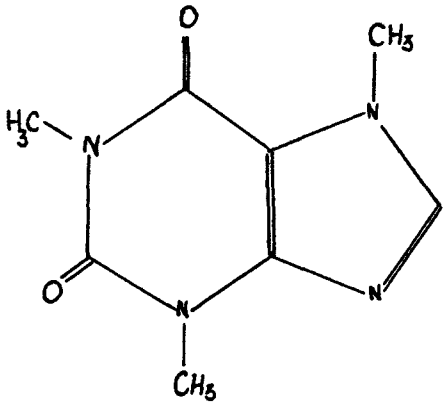
There is uncertainty in the literature about the properties of substituted xanthines as weak electrolytes. We should know more about this in order to develop a hypothesis of transport of these chemicals through the intestinal wall. Examination of xanthine structure suggests that these compounds would be weak bases by virtue of the ability of the ring nitrogen to accept a hydrogen ion:



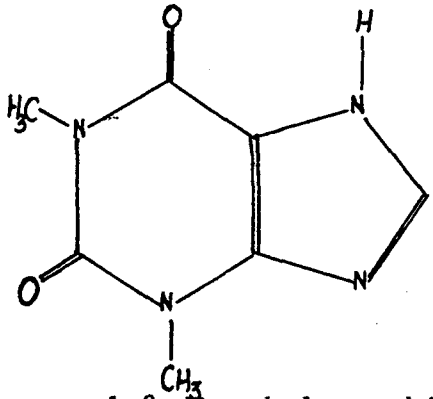

In one study, Ogston (1936) showed that some xanthines may behave as weak acids by virtue of the dissociation of the hydrogen at position 7 of the glycoxaline ring, or position



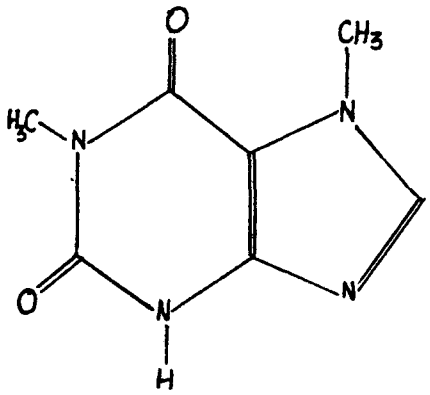
Xanthine



1,3,7-trimethyl xanthine
(Caffeine)



1,3-dimethyl xanthine
(Theophylline)



1,7-dimethyl xanthine
(Theobromine)

Fig. 2. The Structure of Xanthine and the Naturally Occurring Methylated Xanthines.

TABLE 1

SOLUBILITY AND pKa OF XANTHINES IN WATER

| Xanthine | Solubility gm/100ml $H_2O$ | Solution pH | pKa base | pKa acid |
|---|---|---|---|---|
| Caffeine | 1.46 | 6.9 | <1.0 | |
| Theobromine | 0.06 | 7.0 | <1.0 | 10.0 |
| Theophylline | 0.83 | 7.0 | <1.0 | 8.6 |

(From DiPalma, 1971)

1 or 3 in the pyrimidine ring (Fig. 3). In caffeine these are all substituted positions, and this dissociation cannot take place.

## TECHNIQUES FOR STUDYING INTESTINAL ABSORPTION

There have been two major kinds of techniques used in intestinal absorption studies, and these may be referred to as *in vivo* and *in vitro* procedures. *In vivo* techniques are the oldest and involve the use of a living animal. An *in vitro* procedure will use a portion of living intestine which has been removed from the animal. The three basic *in vivo* techniques which have been used are: (1) studying the chemicals in a test meal which are not absorbed (Cori, 1925; Visscher, 1943); (2) studying the quantity of chemicals which appear in the blood after they have been introduced into the lumen of the intestine (London, 1929); and (3) studying the urinary excretion of non-metabolized tracer compounds that have been introduced into the intestinal lumen. The latter test is part of the xylose tolerance test which is of clinical importance (McChance and Madders, 1930).

Absorption of any given compound from the intestine *in vivo* will be influenced by several factors such as specific transport mechanisms, solvent drag, diffusion, blood flow, degree of oxygenation, lumenal distribution of the substrate to be transported, and the pH of both the lumenal fluid and the blood. The identification of transport mechanisms requires

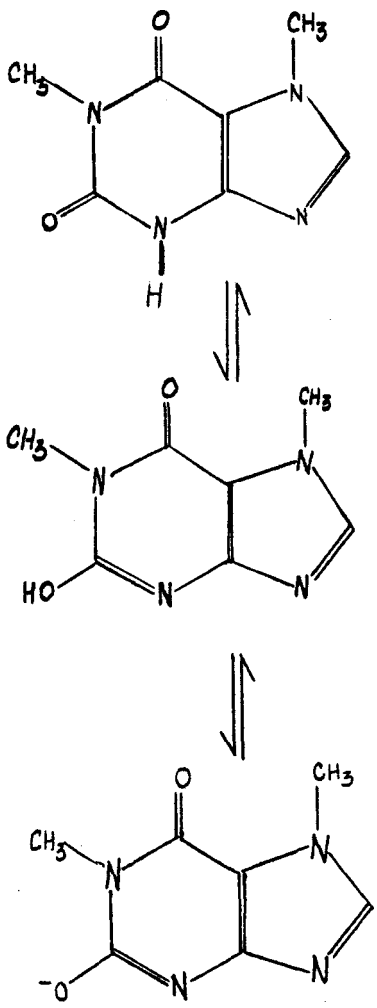


Fig. 3. Changes of Configuration in the Acidic Dissociation of Xanthines.

rigorous control of factors that may influence transport, such as blood flow or pH. Such control is best achieved by using *in vitro* preparations. It is recognized that information obtained from *in vitro* experimentation could introduce a factor not present in the intact animal. However, the mechanisms for transport identified in *in vitro* studies suggests possibilities for the mechanisms of absorption and provide a basis for the design and interpretation of *in vivo* studies. The advances of the last few years in the understanding of absorption mechanisms have been largely derived from the application of *in vitro* techniques. For example, *in vitro* studies of the mechanisms of hexose transport (Crane, 1960), amino acid transport (Christiensen et al, 1963), and disaccharide hydrolysis (Miller and Crane, 1961) have provided a basis for the understanding of defects of sugar and protein absorption (Sleisenger and Fordtran, 1973), and disaccharide intolerance (Crane, 1966).

Commonly used *in vitro* experimental techniques fall into two categories: (1) Polar studies in which (as well as possible) the normal function of the tissue is maintained while movements through the intestinal wall are measured, and (2) Non-polar studies of the accumulation of a substance within the intestinal wall. Polar *in vitro* intestinal studies were initiated in the nineteenth century (Jones, 1854), but were not put into widespread use until 1949 when Fisher and Parsons, and subsequently Darlington and Quastel (1953) independently devised apparatus to

perfuse the intestine. Both of these techniques require large amounts of saline on both sides of the tissue. Consequently substantial transport is required before significant transport may be observed. This drawback was overcome in the everted gut sac method of Wilson and Wiseman (1954) where small amounts of fluid are used and result in substantial changes in concentration when relatively small quantities of substrate are transported. A defect of this technique which is of particular importance in the study of transport of weak electrolytes is that significant pH changes occur in the medium during the course of the experiment (Wilson, 1956; Wilson and Kazyak, 1957). An important advancement in the investigation of epithelial function was the development of a chamber system described by Ussing and Zehran (1951). This procedure allows the analysis of a transport process in terms of opposed unidirectional fluxes, and is particularly appropriate to the study of electrical correlates of solute transport. Ussing and Zehran's technique has been adapted to the studies of solute transport in the intestine (Schultz and Zaluski, 1964), and has been utilized in a number of important studies of electrolyte, hexose, and amino acid transport mechanisms in the intestine (Schultz et al, 1966).

Agar, Hird, and Siddhu (1959) were the first to utilize a non-polar intestinal study technique. They cut the intestine into small pieces and measured the accumulation of amino acids

in the tissue. Using this technique, Crane (1960) has studied hexose transport and has concluded that organic solute transport processes are dependent on sodium ion transport.

For the research of this thesis a polar *in vitro* procedure was used. The method described by Fisher and Parsons was selected.

## CAFFEINE TRANSPORT

Two types of transport have been discussed in the literature with respect to the absorption of xanthines. Brodie and Hogben (1957) with *in vitro* preparations maintaining appropriately acidic pH levels describe caffeine absorption through the gastric wall in terms of a pH partition hypothesis (Travell, 1941; Hogben et al, 1957) in which the absorption characteristics of a weak electrolyte are determined by the pH of the bulk phases and the pKa of the weak electrolyte in question. This theory is based on the observation that the permeability characteristics of a membrane are determined by its lipid components and that a non-polar molecule diffuses through a membrane much more readily than an ionic species. For a weak electrolyte an equilibrium is achieved in which the concentrations of the non-ionized form in the two aqueous phases are equal to each other (Fig. 4). In each aqueous compartment the relationship between the ionized and non-ionized forms of a weak electrolyte is a

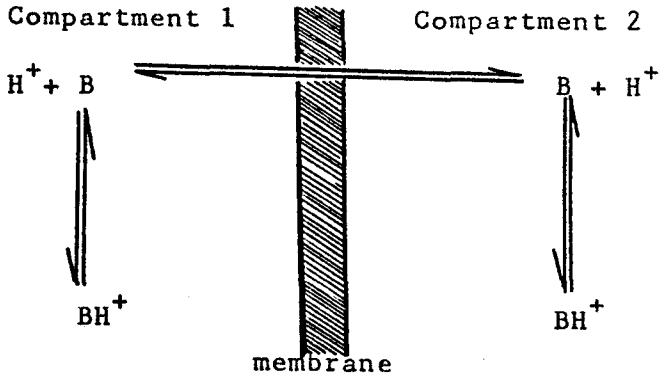



Fig. 4. The Relationship Between the Nio-Ionized and Ionized Forms of a Weak Base in Aqueous Bulk Phases Separated by a Lipid Membrane Barrier.

function of the pH values of the bulk phases. This relationship is described by the Henderson-Hasselbalch equation, and in the case of a weak base such as caffeine, this may be written:

$$[BH^+] = [B][10^{(pKa-pH)}] \qquad \text{eq. 1}$$

where $BH^+$ is the ionized form of the base and B is the non-ionized form. The total concentration $[C]$ of a weak base in aqueous solution is the sum of its ionized and non-ionized forms.

$$[C] = [B] + [BH^+] \qquad \text{eq. 2}$$

Thus, by adding $[B]$ to both sides of equation 1:

$$[C] = [B][1+10^{(pKa-pH)}] \qquad \text{eq. 3}$$

For a system under consideration consisting of two aqueous compartments separated by a membrane that is assumed to be permeable only to the non-ionized form, we write:

$$[C_1] = [B_1][1+10^{(pKa-pH)}] \qquad \text{eq. 4}$$

and

$$[C_2] = [B_2][1+10^{(pKa-pH)}] \qquad \text{eq. 5}$$

Thus at an equilibrium, when $[B_1]=[B_2]$, the ratio of weak base concentrations is given by:

$$\frac{[C_1]}{[C_2]} = \frac{[1+10^{(pKa-pH)}]}{[1+10^{(pKa-pH)}]} \qquad \text{eq. 6}$$

When $\frac{pH_1}{pH_2}$ is not unity, a net accumulation of weak base occurs in the aqueous compartment of lower pH.

Caffeine itself is highly soluble in lipid (U.S.P. XVIII, 1970) and is readily absorbed through the wall of the alimen-

tary canal. Blood concentrations after oral doses quickly approach levels attained through intravenous injection (Axelrod and Reichenthal, 1953). Quantities of caffeine introduced to the stomach by mouth are absorbed through the gastric wall in a manner that can be explained by the pH partition hypothesis (Brodie and Hogben, 1957; Schanker et al, 1957). Intestinal absorption is also known to occur, and this absorption has been suggested to be related to the dissociation constant (Schanker et al, 1957) because of the ability of caffeine to diffuse through the gut wall so readily.

Theophylline has been used extensively to test the pH partition hypothesis (Waxler and Moy, 1952; Broadwall, 1953; Schanker et al, 1957). As the lumenal pH increases, the transport of theophylline decreases. This is consistent with the prediction of the pH partition hypothesis for a weak acid. Because of the differences of the weak electrolyte characteristics of the molecules involved, application of the information obtained from theophylline is of questionable value in determining caffeine absorption.

In general terms, the pH partition hypothesis is applicable to weak electrolyte transport across the intestinal wall. But caffeine is such a weak base that at physiological pH ranges it remains largely in a non-ionized form. According to the pH partition hypothesis, the only transport mechanism necessary for weak electrolytes is diffusion (Brodie and Hogben,

1957). This is an oversimpliflication; other factors must also be taken into account (Jackson et al, 1974).

Fisher (1955) described the phenomenon of solvent drag when he stated "...that solutes are somehow entrained into the stream of absorbed water". Ussing and Anderson (1955) further noted that some solutes are pulled across the membrane by the osmotic movement of water. Lifson and Hakim (1959) proposed a model in which they describe the passive absorption of a non-ionized solute, urea, through the dog intestine in terms of both diffusion and a coupling of the solute to the movement of water through the intestinal wall.

Carrier-mediated transport is conceptualized as a sodium ion and a molecule of the transported substance attached to a protein carrier, and diffusing across the membrane (Crane, 1965). A sodium concentration gradient provides the energy source for a net transport against a concentration gradient (Crane, 1960). The normal cellular sodium concentration is low when compared to the extracellular concentration. And the integrity of these cellular "environments" is maintained by the active transport of sodium from the cell by $Na^+$-$K^+$ATPase. $Na^+$-$K^+$ATPase moves sodium ions out of the cell and potassium ions into the cell with the energy it derives from hydrolyzing ATP. Hadju and Leonard (1959) describe tha action of cardiac glycosides such as ouabain as inhibitory to $Na^+$-$K^+$ATPase.

Bonting and his associates (1961, 1962) studied ouabain activity in various tissues of several kinds of animals and found its inhibitory effects in all cases. By the use of ouabain in the study of intestinal absorption, it is possible to estimate the dependence of the substrate movement upon sodium gradients assuming that accumulation is accomplished by the use of a carrier which diffuses across the membrane complexed with sodium and the substrate (Crane, 1965).

## METHODS AND PROCEDURES

### IN VITRO PREPARATION

The procedure of these experiments followed that described by Fisher and Parsons (1949). Fifty-six male albino rats of the Holtzman strain weighing between 200 gm and 300 gm were fed and watered ad libitum until the time of the experiment. Each rat was sacrificed by a blow to the head. A midline incision was made through the abdominal wall, and the small intestine was gently removed and washed with 154 mM NaCl maintained at room temperature. The duodenum (defined as that portion of intestine bound by the ligament of Treitz) was cut, demesenterized, and attached to the perfusion apparatus (Fig. 5). Segments of intestine similar in length to the duodenum were cut from the jejunum and from the terminal ileum. The mesentery was removed from these segments, and they were also attached to a similar perfusion apparatus. The perfusion apparatus was simple (Fig. 5) and consisted of two reservoirs: A serosal reservoir which surrounded the intestine, and a mucosal reservoir which was a funnel attached to the intestinal segment. The perfusates were circulated through warmed tubing and lifted to the reservoirs by bubbles of 95% $O_2$, 5% $CO_2$. The volume of the fluid bathing the mucosal surface was 75 ml, and that of the serosal fluid was 25 ml.

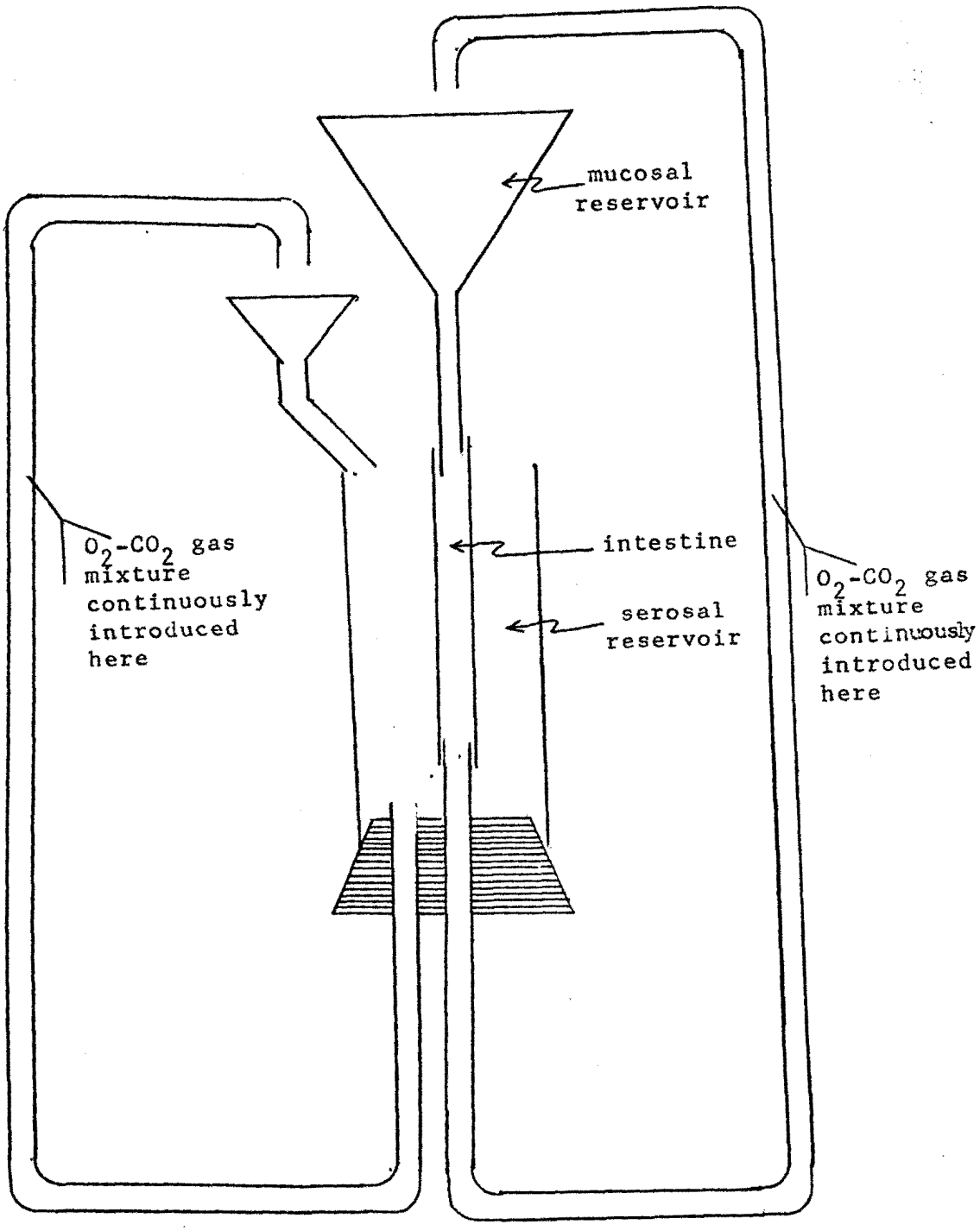



Fig. 5. Apparatus Used to Perfuse the Intestine

After one hour, 1 ml samples were taken from the serosal solution and analyzed for caffeine content.

## COMPOSITION OF SALINE

The saline used in these experiments was first described by Krebs and Henseleit (1932). The solution had the following composition in mEq/l.: $Na^+$, 143; $K^+$, 6; $Ca^{++}$, 5; $Mg^{++}$, 2; $Cl^-$, 128; $H_2PO_4^=$, 2; $SO_4^=$, 1; $HCO_3^-$, 25. Glucose was added to this solution to give a concentration of 5 mM. Reagent grade caffeine was added to this saline as described in this thesis. Sep rate experiments were performed in the presence of 0.01 mM ouabain to determine dependence of caffeine absorption on sodium gradients.

## ESTIMATION OF CAFFEINE

The analytical procedure used in the estimation of caffeine concentration was according to Englis and Miles (1954). 1 ml samples were taken at the end of the test period from the serosal solution, and extracted with 15 ml chloroform. The extract was evaporated to dryness, and redissolved in 3 ml chloroform. Caffeine concentrations were estimated by evaluating the optical density of this extract in a Beckman DU spectrophotometer at 272mu and comparison of the optical density of the sample with the standard curve (Fig. 6) of known concentrations of caffeine in chloroform. It was found that the recovery of caffeine by this method averaged 89.7±6.2%(n).

Some criticism could arise because other methylated xanthines have optical density at 272mu. However, the

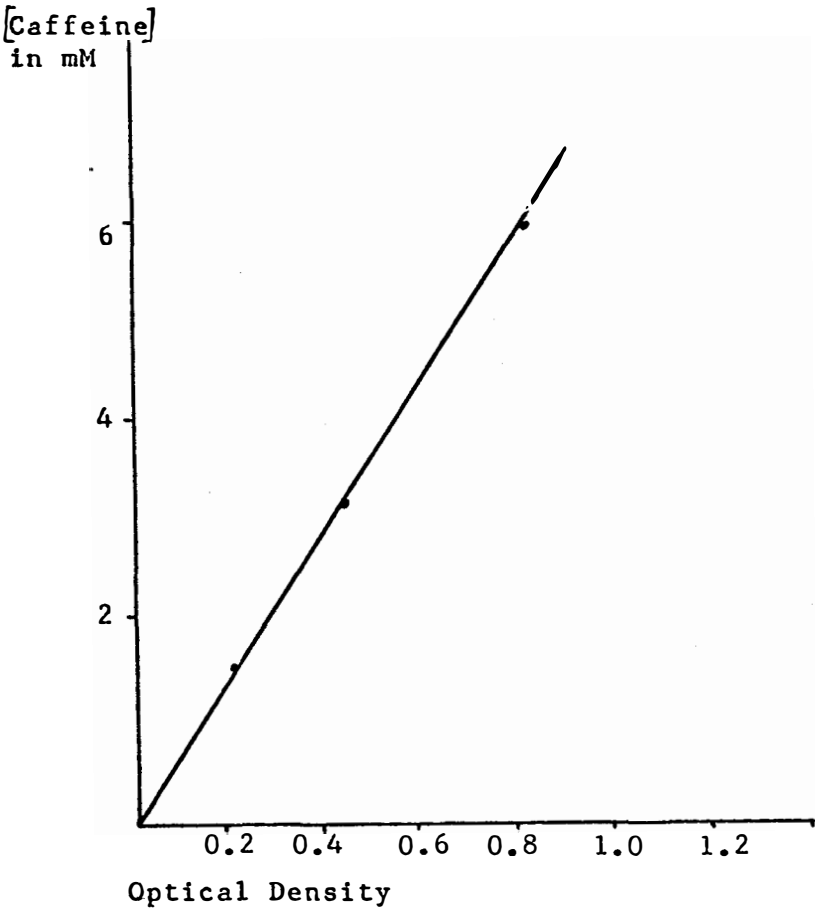



Fig. 6. Molar Absorbency of Caffeine at 272mu.

purpose of the present research is to determine whether or not caffeine absorption through the intestinal wall involves mechanisms other than simple diffusion. For a non-ionized molecule to be absorbed against a concentration gradient, a transport mechanism of sorts must exist. It is conceivable that one mechanism for accumulation is the metabolism of the accumulated substance. In this case, caffeine could be metabolized by demethylation to form another methylated xanthine, and the diffusion of caffeine and its metabolite may create the net movement of xanthine against a concentration gradient. The objective of these experiments was to examine the properties of caffeine transport in the intestine to see if a process other than simple diffusion may play a role in its absorption; and for this purpose, a non-specific method of caffeine estimation was considered adequate.

When no caffeine was added to the perfusion saline, the optical densities at 272mu taken at the end of a one hour test period were equivalent to less than 0.1 mM caffeine. It was concluded that the intestine did not contain quantities of substances that interfere with the the analysis of xanthines, and that the quantities determined indicated concentrations of exogenous xanthine.

# RESULTS

## SEROSAL CONCENTRATIONS OF CAFFEINE AFTER ONE HOUR OF ACCUMULATION

When the initial concentrations of caffeine in both the mucosal and serosal solutions were 3.0 mM, after one hour of accumulation the concentrations of the serosal solutions were:

| | |
|---|---|
| Duodenum | 6.53 ± 0.48 mM |
| Jejunum | 5.47 ± 0.51 mM |
| Ileum | 6.09 ± 0.37 mM |

(These values are the mean ± standard error)

It can be seen that in all portions of the intestine the final concentration of caffeine in the serosal perfusate was significantly greater than the mucosal concentration, indicating the transport of caffiene against a concentration gradient

## RELATION BETWEEN INITIAL CAFFEINE CONCENTRATION AND TRANSPORT

Figure 7 shows the relation of the concentration of caffeine and its transport in the duodenum, jejunum, and ileum. The relation between the final serosal and initial mucosal concentrations is not linear. When the initial perfusate concentrations were 1.5 mM and 3.0 mM, the final serosal concentrations were greater than the initial mucosal concentrations. Figure 7 includes a line drawn to indicate

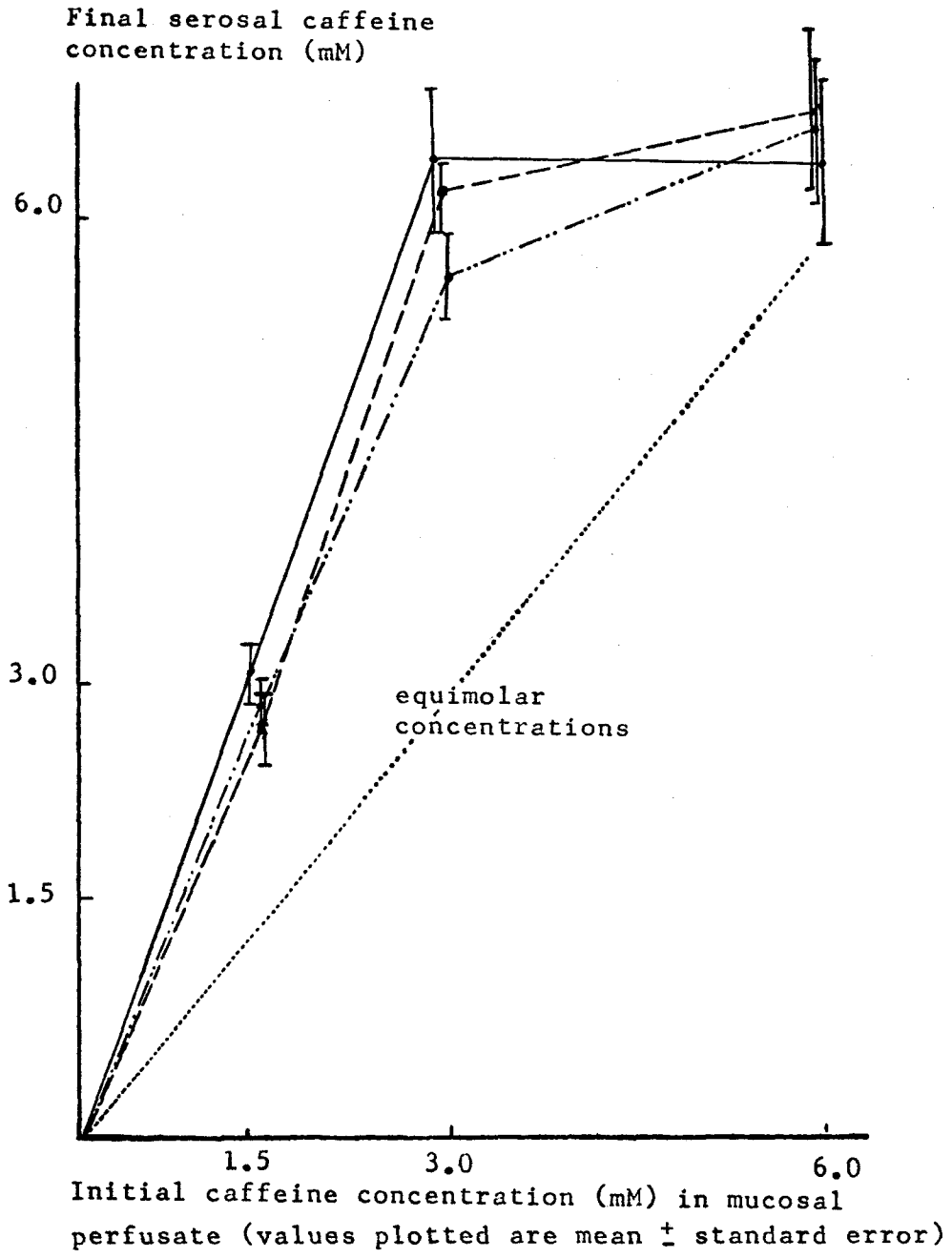



Fig. 7. Absorption of Caffeine through the Duodenum (——), Jejunum (—·—), and Ileum(— — —).

equal mucosal and serosal concentrations, and a comparison of the observed relations with this line is suggestive that the transport mechanism has an upper limit or threshold, a principle now referred to as saturation kinetics.

## EFFECT OF OUABAIN ON CAFFEINE TRANSPORT

Table 2 shows the effects of 0.01 mM ouabain on the distribution of caffeine. In the presence of ouabain, caffeine transport is significantly reduced in all parts of the intestine. In the duodenum and jejunum, the final serosal caffeine concentration is not significantly different than the initial concentration, and in the ileum, the final serosal concentration is slightly less than the initial concentration.

## RELATION OF CAFFEINE CONCENTRATION AND TRANSPORT IN THE PRESENCE OF OUABAIN

Figure 8 shows the results of experiments similar to those described above in which the effects of caffeine concentration were investigated. In the experiments shown in Figure 8, 0.01 mM ouabain was added to the perfusates. In no case did the final serosal concentration exceed the initial concentration of the perfusate; and in some experiments the final serosal concentration was significantly less than the initial concentration.

TABLE 2

MEAN SEROSAL CAFFEINE CONCENTRATIONS AFTER ONE HOUR OF INCUBATION IN THE PRESENCE OF 0.01 mMOLE PER LITER OUABAIN

| Portion of intestine | Initial mucosal and serosal caffeine concentrations were 3.0mMoles per liter | |
|---|---|---|
| | Control* | Ouabain* |
| Duodenum | 6.5±0.48 | 2.87±0.30 |
| Jejunum | 5.47±0.41 | 3.05±0.26 |
| Ileum | 6.09±0.37 | 2.58±0.17 |

All values listed are mMoles per liter.

*All values given are mean ± standard error

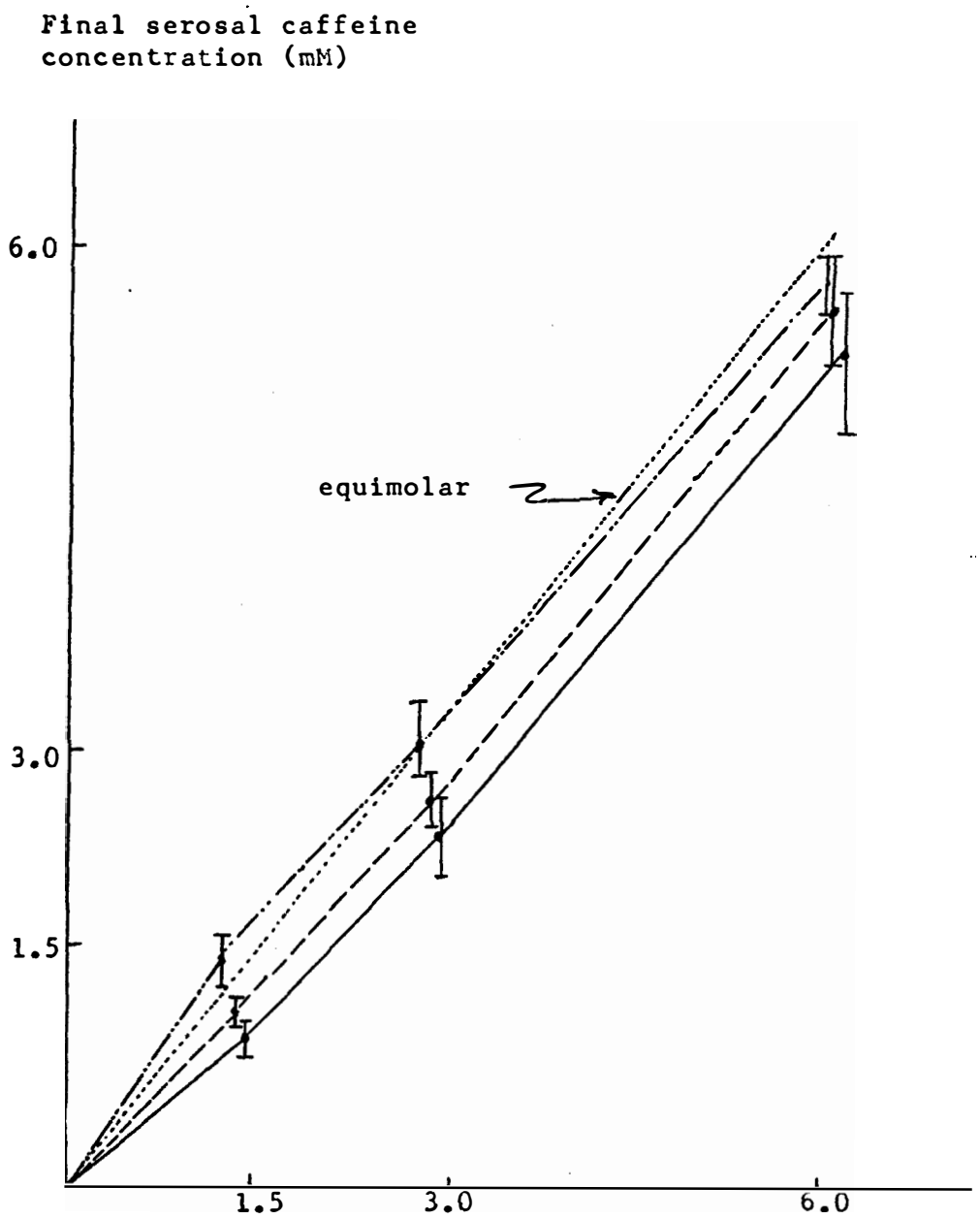



Fig. 8. Intestinal Absorption of Caffeine Through the Duodenum (——), Jejunum (—·—), and Ileum (– – –) in the Presence of 0.01 mMolar Ouabain.

## DISCUSSION

The objective of these studies was to examine some characteristics of caffeine transport through the wall of the small intestine to try to find if processes other than simple diffusion played any role in the transport of this compound. Caffeine was found to be transported against a gradient of concentration in all parts of the small intestine. There are several possible factors that could be involved in this transport. Caffeine is a weak electrolyte, and as such, its transport may be influenced by gradients of pH. The pH values of the mucosal and serosal fluids at the end of the incubation periods were not determined in these experiments. However, because:

$$\frac{C_1}{C_2} = \frac{1 + 10^{(pKa-pH_1)}}{1 + 10^{(pKa-pH_2)}} \quad ,$$

and because caffeine is such a weak base in an aqueous solution, the pH gradient necessary for the accumulation described here would exceed 14 pH units. It is considered that the pH partition hypothesis is an unlikely explanation of the observed phenomena.

Net transport against a concentration gradient can be caused by a solvent drag, where a solute molecule is pulled through a membrane by convection in the flow of solvent (Fisher, 1955). However, solvent drag is incapable of generating a

concentration gradient, and the maximal concentration that the solute may achieve in the moving stream of solvent is equal to that of the compartment of origin of flow (Lifson and Parsons, 1957).

The metabolism of caffeine within the epithelium of the mucosa could contribute to a concentration gradient across the intestine. For example, demethylation of caffeine within the epithelial cells could result in the serosal concentration of total xanthine (caffeine + demethylated product) being greater than the concentration of caffeine in the mucosal fluid. It is not known, however, if such a demethylation does indeed occur. Howard and others (1968) have discussed an analogous mechanism for the accumulation of glycerol intracellularly by the hydrolysis of glycerides. In addition, an asymmetry of distribution of metabolite may be associated with the flow of water through the cells (i.e. intracellular solvent drag).

Another process that cannot be discounted at this stage is that caffeine is transported "actively" by a carrier-mediated transport mechanism that derives the driving energy from the sodium gradient (Schultz et al, 1966). This hypothesis is consistent with the saturation kinetics exhibited, and the inhibition of the accumulation by ouabain.

## CONCLUSION

Evidence was obtained that caffeine can be transported against a concentration gradient by the rat small intestine. This transport showed saturation kinetics (that is an upper limit or threshold) and was inhibited by ouabain. The transport could be the result of the metabolism of caffeine or of a carrier-mediated active transport mechanism. To what extent other factors may be invloved remains to be investigated.

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# ABSORPTION OF CAFFEINE THROUGH ISOLATED RAT SMALL INTESTINE

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## ABSTRACT

Intestines of fifty-six albino rats of the Holtzman strain were removed and perfused with Krebs bicarbonate buffered solutions containing caffeine. After one hour of absorption, caffeine was extracted from the serosal fluids in chloroform and concentrations were determined spectrophotometrically. It was found that all portions of the small intestine transported caffeine against a concentration gradient. This transport mechanism demonstrated saturation kinetics and was inhibited by ouabain.

It was concluded that the movement of caffeine from the mucosa to the serosa of the small intestine cannot be the result of simple diffusion. Possible mechanisms by which the absorption of caffeine takes place are discussed.

COMMITTEE APPROVAL:

# VITAE

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