

INTERRELATIONS IN THE ABSORPTION OF SUGARS,

WATER, AND IONS

BY THE SURVIVING SMALL INTESTINE

by

MICHAEL L. G. GARDNER

Department of Biochemistry,
University of Edinburgh
Medical School,
Teviot Place,
EDINBURGH EH8 9AG.

Ph.D. Thesis
University of Edinburgh
August, 1971



DECLARATION OF ORIGINALITY

I hereby certify that I personally composed this thesis, and that, with the exception of the work described in Chapter VII, the contents are my own work.

The work described in Chapter VII was performed with the collaboration of Dr. R. J. Bywater, Department of Veterinary Pharmacology, Royal (Dick) School of Veterinary Studies, University of Edinburgh.

Michael L. G. Gardner

Michael L. G. Gardner
August, 1971

ACKNOWLEDGEMENTS

I am deeply indebted to my supervisor, Professor R. B. Fisher, C.B.E., for his guidance and encouragement from which I have derived much benefit and pleasure.

I am also grateful to many friends and colleagues for their stimulating discussions; particularly I wish to thank Dr. Ian Nimmo for his valued criticism of my draft thesis, Dr. Gordon Atkins for his advice on computing matters, Dr. Robin Bywater whose collaboration I enjoyed during the work with deuterium oxide, and Niall Campbell with whom I have had many profitable discussions.

My grateful thanks are also due to Miss E. M. Middleton for her enthusiastic technical assistance in the laboratory, and to Mr. A. Purdie and the staff of the workshops of the Department of Biochemistry who have skilfully constructed, maintained, and repaired many items of specialised apparatus.

I acknowledge a research grant to Professor Fisher from the Medical Research Council from which part of the expenses of this work were met, also the award by the University of Edinburgh of the James Traquair Scholarship (1969-70) and a Faculty of Medicine Graduate Research Scholarship (1970-71).

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ABSTRACT

The work falls into two parts: the development and evaluation of new methods for the simultaneous study of intestinal transport and metabolism, and their application to the investigation of the interrelation between sodium ions and the mechanism for glucose absorption.

Two new isolated preparations of rat small intestine with luminal perfusion have been developed. The perfusate is segmented with bubbles of oxygen so that adequate oxygen can be supplied even at perfusion rates which are low enough to permit accurate measurement of absorption. The stability of both preparations is excellent as judged from rates of absorption of glucose and water, and permits both experimental and control measurements to be made on the same segment of intestine. The net absorption rates compare very favourably with data in the literature, and large glucose concentration ratios (mucosal:serosal) demonstrate the activity of the organ.

In one preparation the mesentery is stripped off, so that the fluid transported across the mucosa appears as droplets on the serosal surface and drips off for collection and analysis (secretion). When the luminal glucose concentration was changed, the rate of glucose absorption changed abruptly but the glucose concentration in this secretion changed only slowly. The kinetics of the attainment of the steady state in the secretion fitted to a one-compartment system, and the secretion appeared to be a sample of the sub-mucosal tissue fluid. The kinetics of deuterium oxide secretion conformed to a single-exponential model whose rate constant allowed estimation of the ratio of the unidirectional flux rates from lumen to tissue

fluid and vice versa. There was evidence that the tracer and water were absorbed at indistinguishable rates.

Although the glucose absorption rate fell suddenly when the luminal glucose concentration was reduced, this was not so when the luminal sodium was replaced by choline. Instead glucose absorption fell gradually. At least in part this was owing to reflux of sodium ions to the lumen from the tissue fluid whose composition slowly changed.

In order to allow exact control of the composition of the tissue fluid, the second new technique - 'arterial infusion' - was introduced. An infusate was pumped into the superior mesenteric artery of a segment of intestine with intact vasculature, as for vascular perfusion. Instead, however, the superior mesenteric vein was ligated, so that the infused fluid had to cross the capillary walls, exchange rapidly with the tissue fluid, and escape via the lymphatics.

Replacement of the sodium in the arterial infusate by choline had no effect on glucose absorption, although it affected the rate of response to removal of the luminal sodium. Ouabain in the arterial infusate inhibited glucose and water absorption, but this strongly depended on the sodium concentration in the infusate. Although it appeared that ouabain inhibition was secondary to an increase in the intracellular sodium level, no antagonism was found between ouabain inhibition and the effect of depletion of the luminal sodium. Luminal dinitrophenol inhibited glucose absorption even in conditions where the intracellular sodium level probably remained normal.

Active glucose absorption probably depends directly on metabolic energy, rather than on 'co-transport' with sodium ions by an allosteric carrier as Crane (1965) has proposed. Maintenance of a normal intracellular sodium concentration appears to be important for glucose absorption, although the role of luminal sodium per se is not yet understood. Several hypotheses are discussed.

CHAPTER I

GENERAL INTRODUCTION

At the end of the last century, E. Weymouth Reid, first professor of Physiology at University College, Dundee, reported that intestinal absorption of glucose was stimulated by sodium ions (Reid, 1900, 1902). At that time his crucial observations appear to have been overlooked, perhaps because other workers were concerned mainly with whether 'vital processes' had to be invoked in explanations of the mechanisms of intestinal absorption (Goldsmicht, 1921). Later several accounts were published showing that death of adrenalectomised animals could be avoided if the diet contained sodium chloride (Verzar & MacDougall, 1936; Althausen, Anderson & Stockholm, 1939; Clark & MacKay, 1942). Death was attributed to impaired intestinal absorption consequent on electrolyte imbalance. Recent work suggests that this reasoning was, at least in part, correct (Levin, Newey & Smyth, 1965).

These early studies were hampered by the lack of satisfactory experimental techniques. Preparations of intestine in vivo were commonly used, and these are influenced by innumerable factors which are outside the control of the operator. Work with isolated segments of intestine was even less satisfactory, because of the difficulties in maintaining the organ in a viable condition. Experimenters either made no systematic attempt to supply oxygen to the tissue, or they trusted that oxygen could diffuse sufficiently rapidly across the serosa and muscle coats, etc. (see Parsons, 1968). Then, in 1949, Fisher and Parsons in Oxford demonstrated that isolated segments of intestine from the rat could be maintained in relatively good condition, as judged from their absorptive activity and histological evidence, provided that suitable precautions were observed.

These authors realised the vital need of intestine for an adequate supply of oxygen and they supplied this to the mucosal face of the epithelial cells by perfusing rapidly through the intestinal lumen a solution saturated with oxygen. Further, the intestine was never even momentarily deprived of oxygen. While the segment was still in situ in the anaesthetised animal, they cannulated the lumen and passed through it a well oxygenated solution of a physiological saline containing glucose. Then the intestine was removed from the animal, the adhering mesentery was stripped off gently, and the segment was transferred to a chamber containing a second well oxygenated medium which was continuously recirculated and re-oxygenated. Hence warm oxygenated perfusates were always in contact with both mucosal and serosal sides of the intestine respectively. With this preparation, Fisher & Parsons were able to measure the dependence of glucose absorption rate on the intra-luminal concentration of glucose, the linear gradient of absorptive activity for glucose and water down the length of the small intestine, and the rates of glucose utilisation by the intestine (Fisher & Parsons, 1949, 1950).

The success of this new technique marked the beginning of a new era in intestinal physiology, and stimulated the development of many further preparations of intestine in vitro. For example, Darlington & Quastel (1953), Wiseman (1953), Smyth & Taylor (1957), Csaky & Thale (1960), Gilman & Koelle (1960 a, b), Parsons & Wingate (1961) and others have described modifications to the Fisher & Parsons (1949) basic technique of perfusion.

The everted sac method, probably introduced by Reid (1901), was re-invented by Wilson & Wiseman (1954a) and has gained much popularity, possibly on account of its apparent simplicity. The

segment of intestine is everted on a glass rod, is tied into a sac with the mucosal surface inside, and is incubated in an oxygenated medium. With this preparation transport of, say, glucose from mucosa to serosa against a concentration gradient can be demonstrated (e.g. Wilson & Wiseman, 1954a; Wilson & Crane, 1958). However the act of eversion or the time lag between death of the animal and incubation almost certainly damages the tissue; Baker, Watson, Long & Wall (1969) have shown that the electrical potential difference and the short circuit current across the intestinal wall are severely reduced by eversion. This probably reflects damage to the mechanisms for sodium ion transport. In setting up the preparation it is impossible to avoid temporary severance from oxygen and nutrient supply while the tissue is taken from the animal, and this is probably detrimental. Furthermore, Pietra & Capelli (1970) have evidence which suggests that it is impossible to satisfy the oxygen requirement of an everted sac from oxygen dissolved in the surrounding medium.

Agar, Hird & Sidhu (1954) introduced the technique of 'tissue accumulation' in which slices, rings, or other fragments of intestine are incubated with shaking in an oxygenated medium. Solutes in samples of the medium and of the tissue water can be estimated, and active transport can be demonstrated if the tissue:medium concentration ratio exceeds unity. However, often it is not possible to interpret unequivocally these results in terms of the flux rate of the solute or of water. The mucosal cells, exposed on both faces to the incubation medium, lose their polarity and trans-cellular concentration gradients must be reduced or even abolished thereby. Because it is generally difficult or impossible to estimate loss by metabolism these studies are often restricted to

the transport of substances which are not metabolised, such as 3-deoxyglucose. The intestine then is not provided with an exogenous nutrient, so that a falling off in activity results. Further, these preparations cannot be set up without temporary interruption of the oxygen and nutrient supply. Robinson (1966) demonstrated that tissue accumulation of amino acids was very much reduced if the tissue was removed from an animal which had been dead for only 15 minutes than if the animal was living when the intestine was removed. Fell (1964) suggested that epithelial cells were shed from sheep intestine within ten minutes of death, probably owing to anoxia. These matters are discussed in more detail in Chapter V.

Sheets of excised tissue in flux chambers (Ussing & Zerahn, 1951; Schultz & Zalusky, 1964a) and isolated cells (Harrer, Stern & Reilly, 1964; Clark & Porteous, 1965; Sognen, 1967; Kimmich, 1970a) have also been used extensively with some success, but are open to similar criticisms.

In their now classic paper, Riklis & Quastel (1958) re-discovered the effect of sodium ions on glucose absorption from guinea pig intestine in a modified Fisher & Parsons apparatus. They found that the presence of Na in the luminal perfusion medium was obligatory for glucose absorption. This finding was confirmed and extended by other workers who showed that the requirement for sodium ions was absolute in that no other ion could replace sodium. Similar effects of sodium on the absorption of amino acids have been reported (see review by Christensen, 1970) and on the absorption of many other substances including acetamide (Esposito, Faelli & Capraro, 1969).

As judged from the prolific literature now published about the effects of sodium ions on non-electrolyte absorption, this topic is now considered to be of central importance in gastro-enterological

and membrane transport research. Workers are unanimous in concluding that sodium ions are closely involved in the mechanisms of intestinal absorption, but controversy and speculation are attached to the interpretation of the existing data. The main object in the present work has been to shed light on the interrelations between the sodium ion and the mechanisms for glucose absorption. Current views on water absorption have not been tested in detail. However as the determination of water absorption is essential in the calculation of solute absorption rates, the data have been collected and some are given in this thesis.

One theory of sugar transport which has attracted substantial attention is that of R. K. Crane (Crane, 1962, 1965; Crane, Miller & Bihler, 1961). Crane proposes that the driving force for uphill glucose transport comes from an activity gradient of sodium ions across the mucosal membrane. Glucose and sodium ions are claimed to enter the epithelial cells combined with an allosteric carrier; as the sodium ions diffuse (facilitated diffusion) into the cell down their electrochemical gradient, glucose is carried up its concentration gradient into the cell. Release of glucose from the carrier is alleged to be facilitated by the relatively low intracellular sodium concentration, since the apparent K_m for glucose transport is sodium-dependent in several species (Crane, Forstner & Eicholz, 1965). Continued absorption therefore depends on the maintenance of an intracellular sodium level below that in the luminal perfusate. Crane, Miller & Bihler (1961) originally postulated a sodium 'pump' extruding sodium ions out of the cell and back into the intestinal lumen. Schultz & Zalusky (1964b) upheld the general system but suggested that the sodium pump was located at the serosal membrane of the epithelial cell, and that

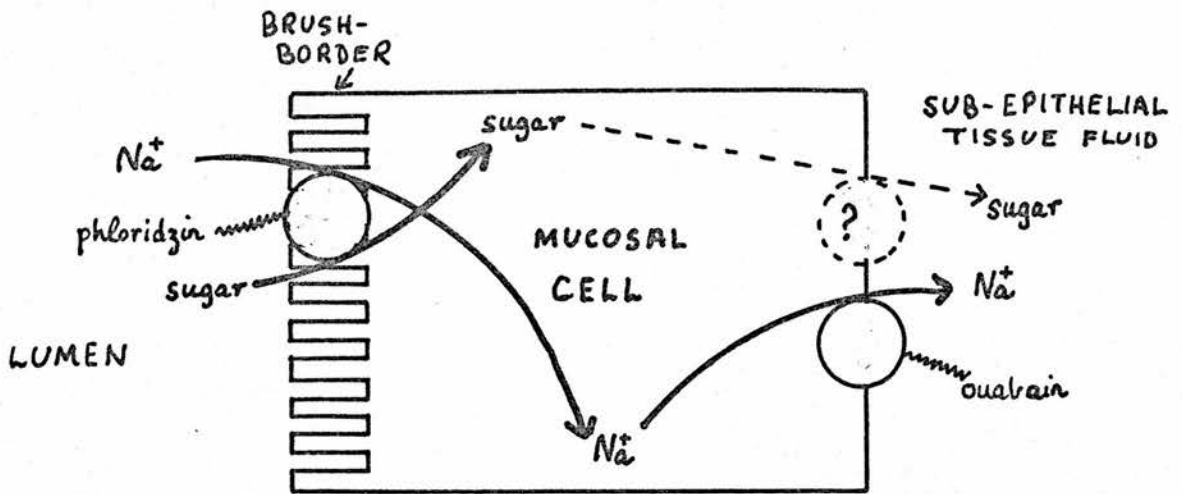


FIGURE 1

Diagramatic representation of Crane's 'gradient-coupled', 'co-transport', or 'ternary complex' model for glucose absorption (after Schultz & Zalusky, 1964b)

this pump used metabolic energy to maintain a low intracellular sodium level. However the exact location of the pump is unimportant to Crane's argument. Figure 1 summarises diagrammatically the essence of the proposed model. Peters & Visscher (1939) had already considered a similar mechanism viz.:- "... combination with some second substance, in or at the membrane phase, whose concentration gradient is maintained by continued removal or hydrolysis on the opposite side of the membrane". However theories of carrier-mediated transport had not then been developed (see Wilbrandt & Rosenberg, 1961; Bowyer, 1957) and they rejected this mechanism because nothing was known to combine with sodium chloride in the cell.

Crane (1964) summarised the evidence for his hypothesis as follows:

1. Entry of actively transported sugars into the epithelial cells under conditions of limited energy supply (i.e. in the presence of o-dinitroresol) is sodium-dependent (Bihler, Hawkins & Crane, 1962).
2. Accumulation of sugar and entry of sodium ions across the mucosal membrane are inhibited similarly by any one of a number of monovalent cations (Bosackova, 1963; Bosackova & Crane, 1965).
3. The addition of an actively transported sugar to the medium bathing the mucosal side of the tissue in vitro increases the transmural potential difference (Barry, Dickstein, Matthews & Smyth, 1961; Schultz & Zalusky, 1963) and the net flux of sodium ions from the mucosal to the serosal side of the preparation (Schultz & Zalusky, 1964a).

To this list can be added several other observations which have been cited in support of Crane's theory.

- 7
4. Cardiac glycosides, which are inhibitors of the active transport of cations, inhibit active transport of sugars in hamster and frog intestine (Crane, 1962).
 5. The similarity between the effects of Na-lack and phloridzin on the distribution of the products of disaccharide hydrolysis suggested to Crane (1968) that the sites of sodium activation and phloridzin inhibition are identical.
 6. Kinetic analysis of sugar absorption data at several sodium concentrations appears to be consistent with an allosteric carrier system (Semenza, 1967).
 7. Crane (1964) claimed to demonstrate uphill 'counter-transport' of sugar out of epithelial cells, induced by reversal of the normal electrochemical gradient of sodium ions.

These, the main body of evidence in favour of Crane's theory of 'gradient-coupled', 'co-transport', or 'ternary complex' mechanism for glucose absorption, have been reviewed in some detail by Schultz & Curran (1970) who agree with the conclusions. Stein (1967, Chapter 5) also accepts that "... all these observations and many others have been succinctly explained by the admirable model put forward by Crane ...".

An alternative hypothesis, which has received favourable interest until recently, is that of Csaky (1963). He holds that the intracellular concentration of sodium ions is critical for the maintenance of normal metabolism. Removal of the sodium ions from the intestinal lumen is suggested to result in depletion of the intracellular pool of sodium. Consequently, production of energy within the cell becomes restricted and active absorption of glucose slows down. Downhill absorption of sugar (i.e. down its concentration

gradient from relatively concentrated luminal solutions) is unaffected by the presence or absence of sodium ions (Csaky, 1963). Csaky further argued that, since sodium ions affect similarly the absorption of a number of unrelated non-electrolytes (e.g. amino acids and uracil), the sodium ions interact with a non-specific part of a transport system, such as the source of metabolic energy, rather than with a carrier system. He suggested that, when the intracellular sodium level fell below a certain critical level, Na-K-sensitive ATPase enzymes were inhibited. One of Csaky's early findings (1963) which appears to have been overlooked is that the rate of glucose absorption in the rat and the toad changed only slowly in response to a step change in the intraluminal sodium ion concentration. This suggests that the actual site of the sodium/glucose interaction is not the luminal surface itself, as Crane's theory seems to predict. This will be amplified in Chapter VIII.

Several items which have been taken to support Crane's theory can be interpreted equally well in terms of Csaky's theory. For example, cardiac glycosides cause an increase in intracellular sodium concentration subsequent to the inhibition of cation transport (e.g. Schultz, Fuisz & Curran, 1966). The change in sodium level rather than the actual cessation of sodium transport could be responsible for the inhibition of glucose transport. Further, the similarity between the effects of sodium deprivation and phloridzin does not necessarily mean that their primary actions are on the same site. The phloridzin could affect the carrier system and the intracellular sodium level could regulate the availability of energy to that carrier system.

Schultz & Zalusky (1964a) showed clearly that the addition of an actively transported sugar to the mucosal medium in a flux chamber

TABLE 1

Unidirectional fluxes of sodium ions across
isolated rabbit ileum

(Data of Schultz & Zalusky, 1964a - their Table 1, page 572)

	J_{ms}^{Na}	$\mu\text{mol}/\text{cm}^2 \cdot \text{hr}$ J_{sm}^{Na}	J_{net}^{Na}
Glucose present	9.6 ± 0.3 (39)	5.7 ± 0.2 (33)	3.9 ± 0.4
Glucose absent	9.5 ± 0.5 (42)	6.7 ± 0.3 (33)	2.8 ± 0.4

(The number of observations is indicated in parentheses.)

Note: J_{ms}^{Na} is the unidirectional flux of sodium ion from mucosa to serosa.

J_{sm}^{Na} is the unidirectional flux of sodium ion from serosa to mucosa.

J_{net}^{Na} is the net flux of sodium ion to serosa.

caused a large and very rapid increase in the net flux of sodium ions from mucosal to serosal surface. Because they noted that sugars which were metabolised, but not actively transported, failed to affect the sodium flux Schultz & Zalusky thought that these sugars and the sodium ions interacted at the transport stage. Again, because the effect was complete within a few seconds of adding the sugar, these authors considered that it was unlikely that the sugar/sodium interaction was intracellular. But these conclusions do not seem to be justified. For example, Gilles-Baillien & Schoffeniels (1965) and Wright (1966) used microelectrodes to show that the addition of sugar to the mucosal medium affected the potential difference across the serosal membrane of epithelial cells but not across the mucosal membrane*. While caution is needed in interpretation of any microelectrode studies, it seems that the sugar/sodium interaction is unlikely to be at or outside the mucosal membrane.

Further, Schultz & Zalusky's (1964 a, b) data have been interpreted repeatedly by themselves and other authors as showing that the influx of sodium into the cells is closely coupled with the influx of the actively transported sugar. Certainly their results do show that the net transmural flux of sodium ions increased when sugar was added to the mucosal medium. However, Table 1 is reproduced from Schultz & Zalusky (1964a, their Table 1), and it is clear that the addition of the sugar did not increase the undirectional influx of sodium ions into the cell. The increase in the net flux rate was solely due to a reduction in the serosal to mucosal backflux of sodium ions. Thus, these results cannot be accepted as support for a co-transport model of sodium ions and glucose.

* These experiments were on the intestine of the tortoise. Schultz & Curran (1970) mention experiments on the rabbit where contradictory results were obtained.

TABLE 2

6-deoxyglucose outflow induced by reversal of the Na gradient
(Experimental data of Crane, 1964)

<u>Experiment</u>	<u>Conditions of incubation</u>	<u>Concentration (mM)</u> <u>of 6-deoxyglucose</u>	
		<u>Medium</u>	<u>Tissue</u>
all expts.	Initial conditions	1.60	0
A	5 min @ Na 120 mEq/litre	1.52	2.06
D	10 min @ Na 120 mEq/litre	1.51	1.72
B	5 min @ Na 120 mEq/litre, then 2 min @ Na zero	1.61	1.02
C	5 min @ Na 120 mEq/litre, then 5 min @ Na zero	1.63	0.76

One of Crane's strongest pieces of evidence is the demonstration of uphill counter-transport of sugar, induced by reversal of the sodium electrochemical gradient (Crane, 1964). However inspection of his data which are reproduced in Table 2 reveals several discrepancies. Experiments A and D show that, under normal conditions, there was less tissue accumulation after 10 minutes incubation than after 5 minutes incubation - i.e. some backflux of sugar was already occurring, even before the sodium gradient was reversed. Also, it is hard to interpret these data in detail, since no information is given as to the number of experiments or the estimated error in the results. Further, no water flux rates are given.

Superficially in favour of Crane's mechanism is the observation of 1:1 stoichiometry of 3-methylglucose to sodium influxes in rabbit ileum under several different conditions (Goldner, Schultz & Curran, 1969). In contrast, Barry, Smyth & Wright (1965) could find no stoichiometric relation between sodium and glucose fluxes in rat jejunum. Although Stein (1967, page 183) has suggested that the demonstration of a tight stoichiometry between the two co-transported solutes is powerful evidence in favour of the co-transport model, this is not necessarily true. Such a condition would be fulfilled only if glucose and sodium can only enter via the one mechanism.

These controversial aspects make it clear that the nature of the interaction between the sodium ion and the mechanism for sugar (and other non-electrolyte) transport is still unknown. The principles of intestinal transport are likely to be relevant to similar problems in other tissues, and Crane (1968) has proposed that his model may be widely applicable.

In the section which follows, the layout of this thesis is described in relation to the problem which has been tackled.

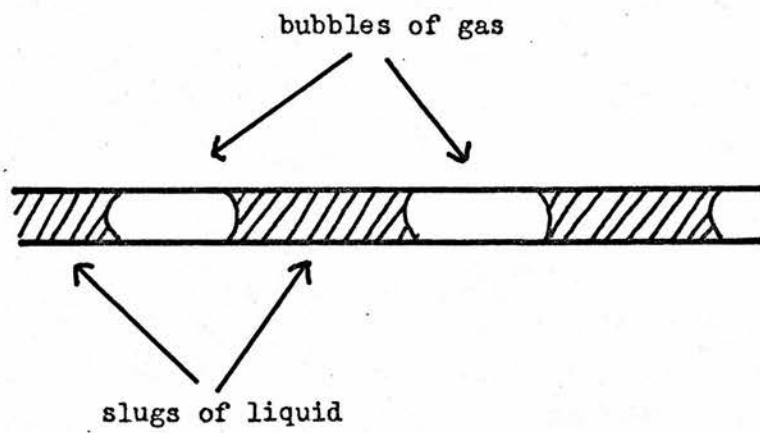


FIGURE 2

Segmented flow

Chapter II describes the choice of the experimental preparation, the materials and apparatus, and the setting up of the intestine. In all the current work, the laboratory white rat has been used, mainly because much data already exist, the animals are easily obtainable and relatively cheap, and the intestine is of a convenient size for this work (see below). The method which was adopted is based on a modification of Fisher & Parsons' (1949) preparation of perfused small intestine. It was devised by Professor Fisher in 1968, and I am grateful to him for placing it in my hands for development and evaluation. Adequate oxygenation of the tissue is possible, even at low rates of luminal perfusion, owing to the introduction of oxygen bubbles into the perfusate stream so as to form a 'segmented flow' - see Figure 2. Fluid which is transported across the intestinal mucosa is allowed to drip from the serosal surface of the experimental segment of intestine as in the technique of Smyth & Taylor (1957).

Chapters III & IV are devoted to analytical methods. Glucose has been the experimental sugar used throughout this work, for three main reasons. (1) It is metabolised readily by rat small intestine, and so can serve as nutrient. (2) Active absorption of glucose in the rat proceeds rapidly, and can be measured with relative ease. (3) Excellent methods are available for the estimation of glucose. In Chapter III an automated method for analysis is discussed, and tests are described which show that the method is capable of high precision. Chapter IV describes the estimation of cations, with special reference to sodium which is of prime importance in this investigation.

Many of the important characteristics of the new preparation have been explored, and these are discussed in Chapters V and VI.

These experiments provide the facts on which the succeeding investigations were based. The transport activity and stability of the preparation were found to be excellent, and confirmed the hopes that the new preparation would prove superior to existing ones for this investigation. Serial measurements of rates of water and glucose absorption from the intestinal lumen, of glucose secretion (i.e. net translocation on to the serosal surface of the intestine), and of glucose utilisation were made. These showed that a steady-state could be established in the lumen effluent very rapidly, but that the composition of the fluid translocated on to the serosal surface took over an hour to become steady. This lag appeared to be a consequence of the relatively large sub-mucosal extracellular space, and a theoretical model was derived to characterise the attainment of the steady-state. The observed kinetics were in good agreement with the predictions made from the compartmental analysis, and it appeared that the fluid which appeared on the serosal surface of the intestine is a fair sample of the sub-mucosal tissue fluid.

Chapter VII describes the predicted kinetics of the net transport of deuterium oxide from the lumen to the serosal surface, and experiments which supported the validity of the theoretical treatment. A colleague required to test the suitability of deuterium oxide as a tracer for water, and the present preparation appeared to afford an ideal opportunity to do so. The conclusion was that the rates of tracer and water movement were indistinguishable. This investigation was a useful extension of the compartmental analysis already referred to, and the exponential rate constant gave an estimate of the ratio of the uni-directional flux rates of water across the mucosa. The analysis developed here was also applied in Chapter VIII to the two-directional wash-out of sodium ions from the intestinal tissue fluid.

The work of Chapter V had already shown that the rate of glucose absorption from the intestinal lumen changed rapidly when the intraluminal glucose concentration was abruptly changed. Therefore it was desirable to find out how rapidly glucose absorption declined following an abrupt decrease in the intraluminal sodium concentration. Subject to certain qualifications (discussed later) it could be predicted from Crane's co-transport model that the rate of glucose absorption would fall as rapidly as did the intraluminal sodium concentration. Conversely, Csaky's hypothesis suggests that the rate of glucose absorption would change only gradually. Experiments described in Chapter VIII showed that, indeed, the latter was the case and that the rate of glucose absorption fell no more rapidly than did the concentration of sodium in the sub-mucosal tissue fluid. Interpretation of these results was complicated by the fact that with this intestinal preparation one has no direct control over the composition of the tissue fluid which in some instances could vary continuously throughout an experiment.

Consequently attention was directed to methods of controlling this tissue fluid. Preparations in which the serosal surface is bathed in a medium (e.g. Fisher & Parsons, 1949) were not considered because there is no evidence that the composition of the bathing medium is that of the tissue fluid. Indeed, there is some evidence that the converse is true (e.g. Csaky & Hara, 1965). Methods of vascular perfusion were considered to be the most suitable of existing preparations, but they suffer from disadvantages. On the whole, numerous attempts in the literature to produce satisfactory preparations of intestine with vascular perfusion have been abortive, especially in the rat (see comments by Parsons & Pritchard, 1968; Windmueller, Spaeth & Ganote, 1970). To some extent poor

oxygenation of the tissue may have been responsible for part of the recorded failures. A major disadvantage attached to such preparations is that the intestinal vasculature of the rat is prolific in arterio-venous anastomoses, and so the space accessible to the vascular perfusate is unpredictable, and probably variable. In an attempt to overcome this, a novel technique of 'arterial infusion' has been developed. The intestine is set up as for a vascular perfusion, but once the blood has been displaced from the vessels the superior mesenteric vein is tied off. Hence, the infusate which is pumped into the superior mesenteric artery cannot escape via the venous system. Instead the fluid crosses the permeable capillary walls, exchanges rapidly with the interstitial water, and escapes via the lymphatics. This infusate simply serves to control the composition of the fluid bathing the serosal pole of the epithelial cells, and to carry away the products of absorption etc. The organ receives its oxygen from the segmented flow of luminal perfusate, just as in the preparation already described. Chapter IX describes this new technique of arterial infusion, and the subsequent Chapter deals with its properties and some experiments designed to test the effect of sodium depletion. Although the properties of this new preparation have not been shown to be superior to those with vascular perfusion, they in general fulfilled the requirements of the present work. The stability was excellent over one hour, and steady-states could be established in both lumen and tissue fluid within quite short times. With direct access to both sides of a single layer of epithelial cells many valuable experiments are now possible. As with the previous preparation the stability is so good that each intestine can be used for control measurements before making the experimental measurements.

The possibility that the intracellular sodium concentration is critical for normal glucose absorption was then considered.

Finally the sensitivity of rat intestine to ouabain, a cardiac glycoside, was tested (Chapter XI) since this drug is thought to be able to alter the intracellular concentrations of sodium and potassium ions. Glucose absorption was inhibited by ouabain at low concentrations in the arterial infusate, but this inhibition strongly depended on the sodium concentration in the arterial infusate. This suggested that the effects of ouabain on glucose absorption might be secondary to an increase in the intracellular level of sodium,^{*} as has often been speculated. Consequently, the possibility of antagonism between ouabain inhibition and sodium depletion was tested, but not found.

In conclusion the General Discussion in Chapter XII summarises the advantages of the new preparations and discusses models which may account for the complex interrelations between glucose absorption and sodium ions.

* No measurement has been made of intracellular sodium concentration in the current work (see p. 170).

CHAPTER II

A PREPARATION OF SURVIVING SMALL INTESTINE

FOR THE STUDY OF ABSORPTION AND METABOLISM

CHAPTER II A PREPARATION OF SURVIVING SMALL INTESTINE FOR
THE STUDY OF ABSORPTION AND METABOLISM

Introduction

A number of experimental preparations of intestine have already been described in the General Introduction, where it was concluded that major disadvantages are associated with most techniques hitherto adopted. The development of an improved type of preparation therefore became desirable. In this Chapter, the main features required of such a preparation are laid out, and a potentially suitable preparation together with associated apparatus is described. Chapter V will deal with some properties of the new preparation.

In vivo studies are of limited utility owing to the large number of variable factors which are outside the control of the experimenter. For example, it is widely accepted that hormones, nervous stimuli, and blood flow rate are among the many factors which can influence the absorption of fluids and solutes from the intestinal lumen (e.g. see Levin, 1969; Verzar & McDougall, 1936). It is desirable to eliminate extraneous variables, and so it becomes essential to work with isolated intestinal tissue.

The intestine requires an adequate supply of oxygen and nutrients to sustain the cells. As is well known and will be shown later, the mucosal cells are capable of transporting actively certain solutes against large concentration and electrical gradients. Therefore, a large oxygen consumption might be expected; reported values for the Q_{O_2} of rat small intestines are as high as 20 $\mu\text{l O}_2/\text{mg dry weight/hr}$, (Bronk & Parsons, 1965), and although published estimates vary widely it is possible that this is an underestimate. It is probable that metabolic energy is required to maintain the organisation, and hence the integrity, of the mucosal cells and of their transport

mechanisms. As will be shown later, this can only be fulfilled if the tissue is continuously supplied with oxygen, and thorough precautions are taken to avoid even temporary hypoxia. Such precautions are not possible in many widely used preparations including everted sacs (Wilson & Wiseman, 1954a), tissue accumulation slices or rings (Agar, Hird & Sidhu, 1954; Crane & Mandelstam, 1960), sheets of excised tissue mounted in flux chambers (Ussing & Zerahn, 1951; Schultz & Zalusky, 1964a), or isolated cells (Harrer, Stern & Reilly, 1964; Kimmich, 1970a).

Luminal perfusion (or superfusion) techniques have at least five potential advantages. Firstly, the oxygen can be supplied to the tissue from the luminal perfusate since oxygen can readily cross the luminal membrane. Secondly, luminal perfusion can be established before the animal's blood supply is interrupted. Thirdly, conditions can be maintained almost constant since the medium bathing the mucosal pole of the epithelial cells can be continuously replenished. Fourthly, the composition of the luminal medium can be rapidly changed; hence control and experimental measurements can be made on one and the same segment of intestine provided that the preparation is stable. Fifthly, continuous or serial measurements of absorption and metabolism are possible. Therefore one can study both the steady-state conditions and the kinetic approach to the steady-state.

Although perfusion methods have been used from the earliest days of intestinal physiology (e.g. Carpenter, 1869) credit for the first preparation of an isolated segment of intestine maintained in a relatively viable condition is generally ascribed to Fisher & Parsons in 1949 (e.g. see Smyth, 1967). They realised the vital need for adequate oxygenation, and showed that the oxygen could be

supplied from solution in the lumen. Earlier workers seem to have either ignored oxygenation of the organ or attempted to supply oxygen via the serosal surface. Of course, the thickness of the serosal muscle coats militates against such oxygen diffusing sufficiently rapidly to the working mucosal cells. Fisher & Parsons (1949) furthermore never interrupted the blood supply to the segment of intestine until after they had established a rapid flow of perfusate saturated with oxygen through the lumen. Hence the organ was never even momentarily deprived of oxygen.

However, the method of Fisher & Parsons (1949) has major drawbacks. It allows only a single estimate of solute absorption rate and of solute secretion rate. Also, the conditions during any experiment are not constant, since the compositions of the fluids bathing both mucosal and serosal surfaces of the intestine are continuously changing. Finally, no reliable estimate of water transport is possible. The first two objections can be overcome if the perfusion fluids are not recirculated, but instead are passed once only through the intestinal lumen or through the organ chamber.

Smyth & Taylor (1957) modified Fisher & Parsons' technique by removing the fluid bathing the serosal surface of the intestine so that the intestinal segment was suspended in an atmosphere of moist air. The fluid transported by the epithelial cells then exuded in drops as a 'sweat' from the exposed serosal surface and could be collected, weighed, and analysed.

Gilman & Koelle (1960ab) introduced a further modification by passing the luminal perfusate, under gravity, once only through the lumen instead of recirculating this fluid as Fisher & Parsons and Smyth & Taylor had done. Gilman & Koelle stated that at their perfusion rate of about 5 to 10 ml/min through the lumen the

intestine was receiving ample oxygen. However, this assumption may not be justifiable. If the intestinal Q_{O_2} is taken to be $20 \mu\text{l } O_2/\text{mg dry weight/hr}$ (Bronk & Parsons, 1965), and if the dry weight is taken to be 12 mg/cm (see Table 13), then a forty centimetre segment of intestine will require some $160 \mu\text{l } O_2$ per minute. Aqueous perfusates at 37°C in equilibrium with 95% O_2 contain only $23.6 \mu\text{l } O_2$ per ml (Lange, 1952); therefore the intestine's oxygen requirement cannot possibly be satisfied at perfusion rates of less than about 8 ml/minute if all the oxygen is to come from solution.* At a perfusion rate as high as this, it would be difficult to measure with precision the rates of absorption of most solutes. For example, a 40 cm length of upper jejunum will absorb about 2 mg of glucose and about 0.12 ml of water per minute from a luminal perfusate containing 5 mg/ml glucose (Fisher & Parsons, 1949; see also Table 10). At a perfusion rate of 8 ml/minute , the intestine would be presented with 40 mg of glucose per minute, and measurement of the absorption of such a small fraction of the infused glucose could not be accurate. Conversely, accurate measurements of absorption rate are favoured when very low perfusion rates are employed, as in the *in vivo* studies of Schanker, Tocco, Brodie & Hogben (1959) with a flow rate of only 1.5 ml/minute . As has already been stressed, such low rates of perfusion militate against adequate oxygenation in most *in vitro* studies, and so can normally be applied only to *in vivo* experiments such as those of Schanker et al (1959).

For this work, a novel technique has been adopted so that adequate oxygen can be supplied to the tissue even at rates of luminal perfusion which are low enough to permit accurate measurement

* This assumes complete abstraction of the oxygen from solution. Further, it is probable that the supply of oxygen to the mucosa might be limited by the radial diffusion through the bulk perfusion medium.

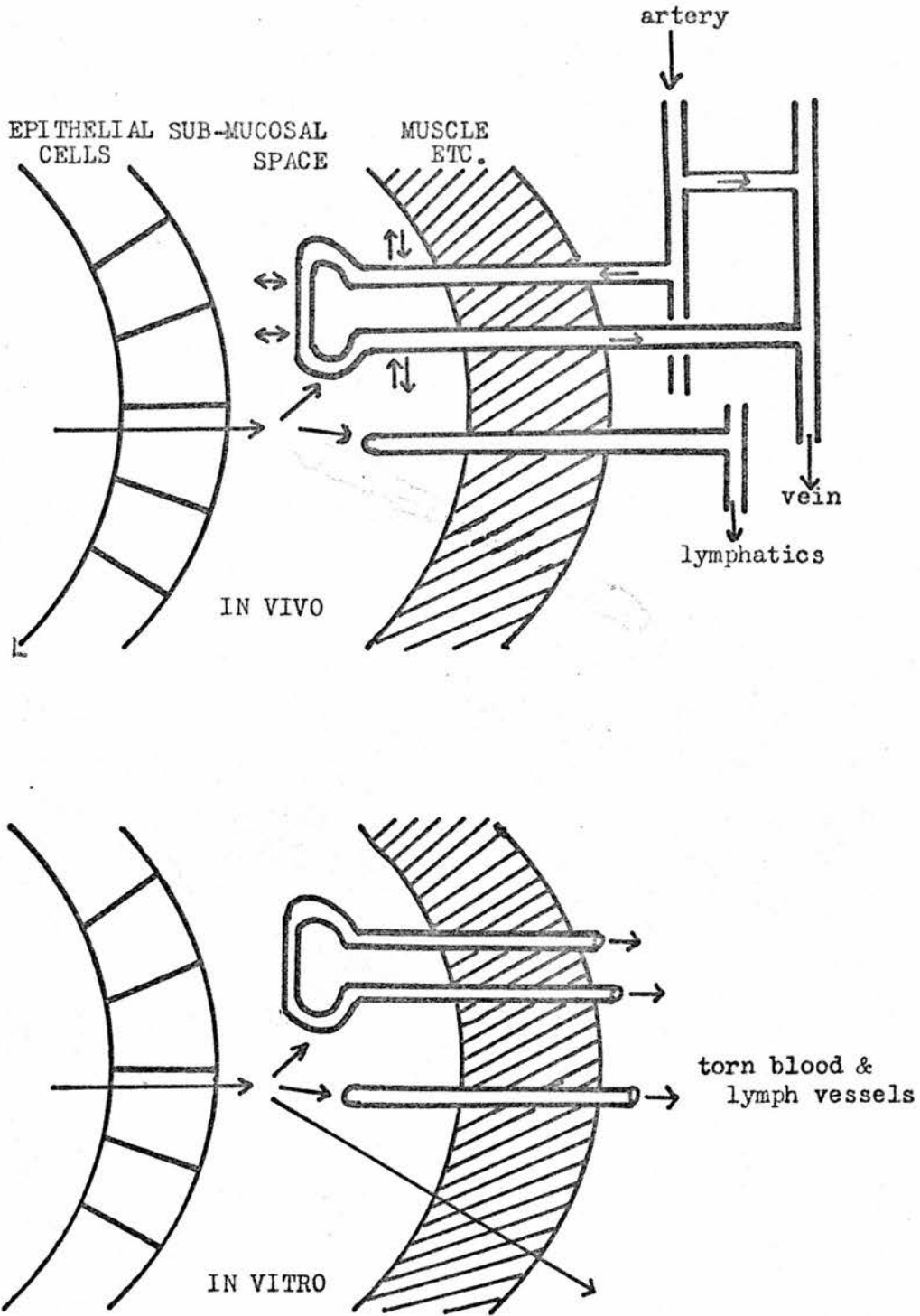


FIGURE 3

A diagrammatic comparison between the intestine in vivo and a preparation in vitro with torn mesentery

of absorption rates. The principle of the new technique was first suggested by a chance observation made by Professor Fisher when he was using a stream of oxygen to displace liquid from the lumen of a segment of intestine in situ. He observed that as soon as the oxygen was passed into the lumen, the blood in the arcadian vessels of the mesentery suddenly became noticeably arterialised. This suggested that gaseous oxygen rather than oxygen dissolved in the perfusate could cross the mucosa so readily that it would suffice to oxygenate the tissue. Experiments to be described later have now confirmed the validity of this suggestion.

Warm perfusate saturated with oxygen is pumped to an inlet cannula tied into the intestinal lumen. Into this stream of perfusate is pumped oxygen so as to form a sequence of slugs of liquid separated by bubbles of oxygen. This is subsequently referred to as 'segmented flow'. In addition to providing the oxygen supply to the tissue, this segmented flow has valuable hydrodynamic properties which are described in Chapter V. The perfusate issuing from the lumen is taken via a resistance to a collecting vessel. The resistance serves to distend the intestine, so that the unfolded mucosal surface is uniformly exposed to perfusate and to oxygen. The fluid which is transported across the intestine, and which appears as droplets on the serosal surface, drips off as in the preparation of Smyth & Taylor (1957) and is also collected. Throughout this thesis, this exuded fluid is referred to as 'intestinal secretion', or simply as 'secretion'. The mesentery and blood vessels are torn off from the intestine, as in Smyth & Taylor's preparation. The secretion can drip from the cut ends. Figure 3 shows a diagrammatic comparison between this preparation in vitro and the intestine in vivo.

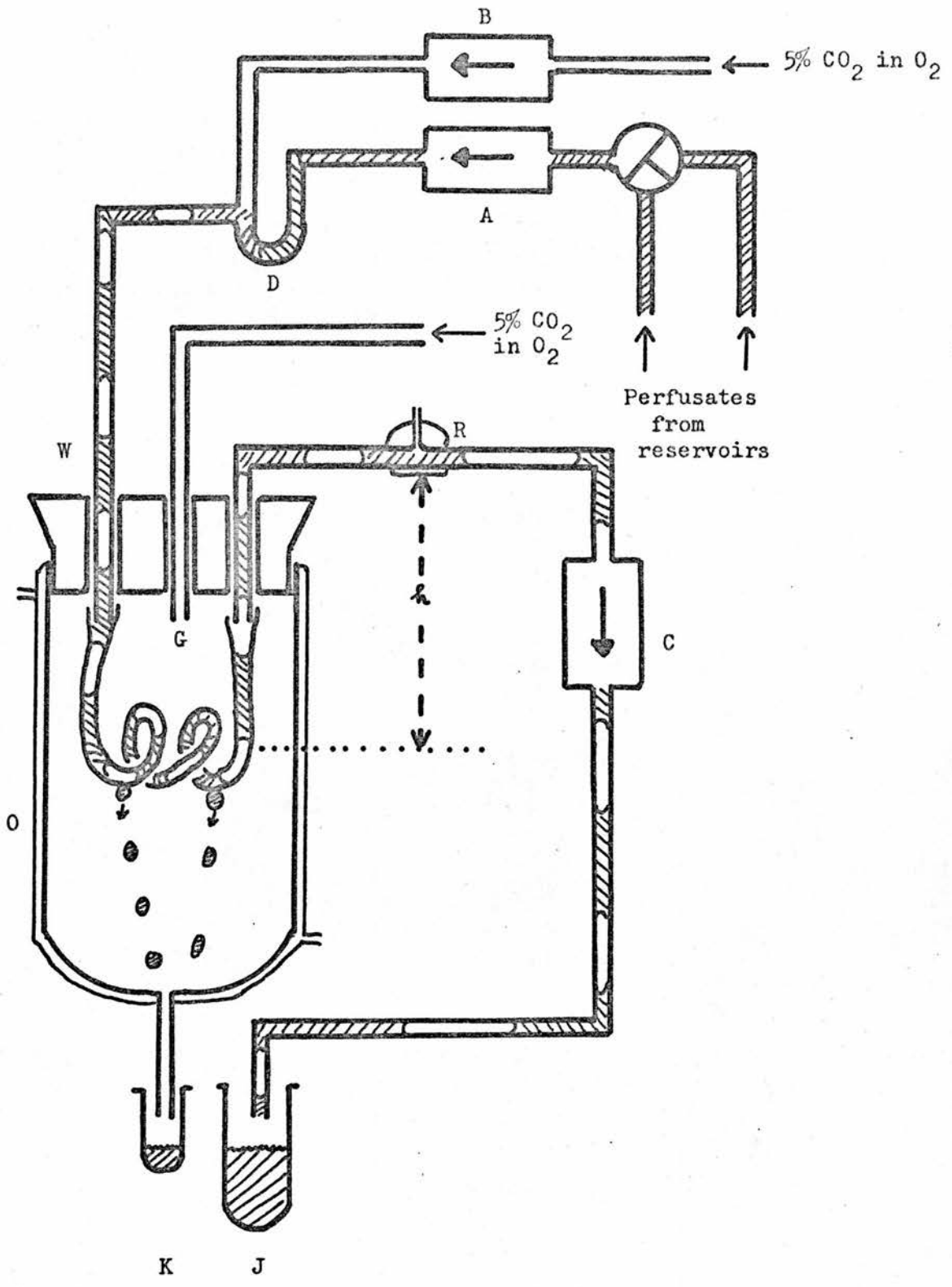


FIGURE 4

The perfusion apparatus

The following sections are devoted to the details of the apparatus, the animals, the perfusion media, the experimental procedure for setting up a segment of intestine, and finally the calculation of the experimental results.

The Perfusion Apparatus

The essential components of the apparatus are shown diagrammatically in Figure 4.

Warm moist gas (5% CO₂ in O₂ - subsequently referred to as 'gas') is pumped by a pump, B, into a stream of warm oxygenated perfusate pumped by another pump, A. The gas and liquid flows converge at D, which is a glass U-tube with a side arm. The resulting segmented flow is delivered through a flexible water-jacketed tube, W, to a glass inlet cannula which is ligated into the intestinal lumen.

The outflow from the intestine is taken from another glass cannula to a resistance unit R, which maintains an intra-luminal distension pressure of about 40 cm water. Pump C impels the outflow from the resistance, R, to a set of tared collecting tubes at J.

The segment of intestine is hung in a water-jacketed glass chamber, O, so that the organ hangs in a series of naturally formed loops on a sling of nylon wool. Gas, at 37°C and partly saturated with water vapour, passes into the organ chamber at G. This serves to oxygenate the serosal surface of the intestine and to force the drops of secretion along the exit tube to a second set of tared collecting tubes at K.

Perfusates, the compositions of which will be described below, are contained in reservoirs immersed in a thermostatted water bath. These reservoirs are of glass, and have a capacity of 500 ml or 1 litre. They have a narrow neck into which fits a rubber bung

carrying gas lifts, gas exit tube, and a narrow bore tube through which the perfusate can be withdrawn by the pump. The temperature of the water bath is maintained at 41°C by a 'Circotherm' circulating heater unit (Shandon Scientific Co.); this temperature was experimentally chosen so that the temperature of the perfusate entering the intestine would be $37^{\circ}\text{--}38^{\circ}\text{C}$. The 'Circotherm' also circulates water at this temperature through the water-jackets which surround the organ chamber and the tubing at W. The perfusates are equilibrated with 5% CO_2 in O_2 by means of pairs of gas lifts which carry about 100 ml of gas per minute into each reservoir. The perfusate within each reservoir is circulated in intimate contact with the gas in these gas lifts.

The pumps A, B and C are peristaltic pumps mounted on a common rotor. This unique type of peristaltic pump was designed by Professor Fisher, and built by Mr. A. Purdie and Mr. W. Tait in the workshops of the Department of Biochemistry. It has been fully specified under U.K. Patent Application No. 60942/68. The pump tubes are of 'Tygon' P.V.C. tubing 0.110 inch O.D. (supplied by Technicon Instrument Co.) bonded by adhesive No. EC847 (Minnesota Mining and Manufacturing Co.) into a tube harness made from terylene tape. This type of harness makes it possible to pump against considerable and varying pressures at a constant rate; it also gives the tubes a protection from wear. Tubes have been successfully used for some 500 hr of pumping before replacement was necessary. The small diameter rollers on the pump which compress the tubing give a much more uniform and pulse-free flow than could be possible with a pump such as the Technicon AutoAnalyzer model which unfortunately has rollers of much larger diameter. The power supply to the pump motor is electronically regulated by a silicon controlled

rectifier circuit. This control unit receives a feedback signal from a tachogenerator driven by the pump motor, and thus a high output torque is achieved; consequently, the pump speed remains constant. As the properties of silicon controlled rectifiers (thyristors) are thermally dependent, the control unit is always kept switched on. When the pump is not in use the output from the controller is switched into a dummy load. The speed of pumping can be continuously varied over a wide range by means of a helipot with vernier dial. The normal rate of perfusion is 4 ml/minute of each of perfusate and gas - i.e. 8 ml/minute total fluid flow.

The water-jacketed organ chamber, O, is similar to that used by Fisher & Parsons (1949). It is cylindrical and of glass, with internal dimensions approximately 12 cm X 6.5 cm diameter. It was made by Mr. D. MacDonald in the Glass Workshops of the Department of Biochemistry.

The resistance, H, is achieved by raising the plastic tubing which carries the effluent from the intestinal lumen to an appropriate height, h, above the level of the intestine. Throughout this work h has been approximately 40 cm. To prevent syphoning there is a small plastic chamber open to the atmosphere placed in series with this effluent tube at its highest point. However, an inverted Y-tube would be equally satisfactory.

The water-jacket at W is constructed from P.V.C. tubing. The tube carrying the segmented flow of perfusate passes into this jacket through rubber bungs in each end of the water-jacket. It is flexible, so that the bung of the organ chamber carrying the attachments for the two intestinal cannulae can be lifted from the organ chamber to the adjacent operating table. Thus, the luminal perfusion can be established before the blood supply to the intestine in situ is interrupted.

Most of the tubing carrying perfusate to and from the intestine and secretion from the organ chamber is of P.V.C. (especially 'Tygon transmission' tubing supplied by Technicon Instrument Co.) and joints are effected by cyclohexanone which is a good solvent for this tubing.

Warm oxygenated bicarbonate-saline for washing out the segment of intestine before its connection to the perfusion apparatus is pumped by an independent peristaltic pump at about 80 ml/minute. A by-pass line with a relief valve allows saline to return to the reservoir if the intra-luminal distension pressure reaches about 20 cm of water. This relief valve is of perspex, and operates on the principle of the 'Starling resistance'. A detailed description has been given by O'Brien (1969). Thus, the danger of occluding the blood supply by excessive intra-luminal distension while the preparation is being set up is minimised.

The test tubes which receive the effluent from the lumen and the secretion from the serosal surface are held in a 'Central' fraction collector (supplied by Gallenkamp Ltd., London). The collector table was modified by drilling a second set of holes adjacent to the existing holes and on common radii, so that two liquids can be received simultaneously into two separate tubes - viz. a 50 ml boiling tube for lumen effluent and a 10 ml centrifuge tube for secretion. The delivery tubes are held about 1 cm below the mouths of the receptacles so as to eliminate loss of liquid owing to spluttering. When the fraction collector table rotates, the delivery tubes are raised briefly by a solenoid operated mechanism. This solenoid and the fraction collector are actuated by a Londex IMP Mark 2 synchronous type auto-reset process timer which is continuously variable from 15 seconds to 15 minutes. The timer has been modified by Mr. A. Purdie so that it is automatically reset

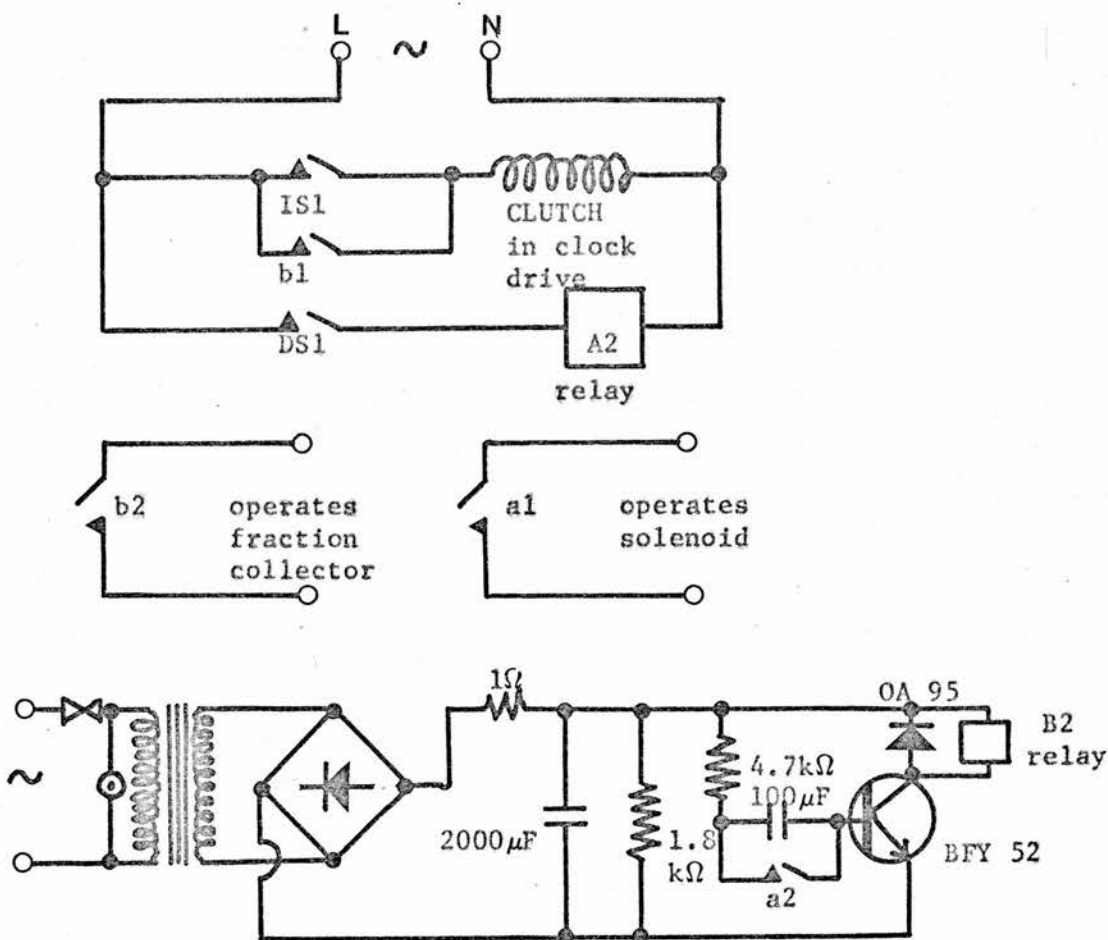


FIGURE 5

Londex timer unit with modification for automatic restart

1. Start timer
2. Timer runs to end of preset time, then contacts DS1 close energising relay A2
3. Contacts a1 are closed and fraction collector solenoid is actuated. Contacts a2 are closed and energise relay B2
4. Contacts b2 are closed and fraction collector is actuated, while contacts b1 close and reset timer
5. Relay A2 is now de-energised, and contacts a2 open
6. After a delay (ca 1 sec), while the cut off bias capacitor charges, relay B2 is de-energised
7. Contacts b1 close, and timer is restarted.

and restarted after each cycle. Figure 5 shows the circuit diagram of the timer unit.

Animals

Throughout this work female albino rats^{*} were used. They were of a local strain, bred at the University Animal Centre, Small Animal Breeding Research Organisation, Bush, near Penicuik, Midlothian. They were kept for at least 7 days before experiment in the animal houses of the Department of Biochemistry, under conditions of controlled temperature and day-length (12 hours light and 12 hours dark) with free access to water and a commercial 'normal' diet for small animals (Diet 86, supplied by Oxoid Ltd., London).

The time of day at which animals were used for experiment was also recorded.

Anaesthesia

Surgical anaesthesia was induced by placing the animal in a glass desiccator which contained a pad of cotton wool, soaked in ether, and was maintained by ether inhaled from a mask. An assistant helped with the anaesthesia.

Perfusion Media

The normal perfusate pumped through the intestinal lumen is a bicarbonate saline solution, modified from the medium of Krebs & Henseleit (1932). This has a composition similar to that of mammalian plasma, except that the calcium concentration has been halved to allow for binding to the plasma proteins (Greene & Power, 1931). Likewise the magnesium concentration should have been halved, but in practice it has been quartered throughout this work owing to an error in communication. An experiment will be described below which suggests that this discrepancy is of little consequence.

* Weighing about 190 - 230 g

TABLE 3

The composition of the normal perfusion medium

	Concentration in stock solution		Volume of stock solution per litre of perfusate ml	Final concentration in perfusate mM
	g/l	M		
NaCl	138.503	2.37	50.0	118.50
NaHCO ₃	41.798	0.498	50.0	24.88
KCl	35.30	0.473	10.0	4.73
KH ₂ PO ₄	16.10	0.118	10.0	1.18
CaCl ₂ .6H ₂ O	27.80	0.127	10.0	1.27
MgSO ₄ .7H ₂ O	14.60	0.0592	5.0	0.30
Phenol red	1.00	0.0028	50.0	0.14

Plus glucose 5 or 1.5 mg/ml (27.8 or 8.3 mM)

The final osmolarity of the perfusate with 5 mg/ml glucose was 318 mOsm/litre.

The saline used to rinse out segments of intestine prior to their connection to the perfusion apparatus has the following composition:

NaCl 118 mM

NaHCO₃ 25 mM

In doubly distilled water, and equilibrated with 5% CO₂ in O₂.

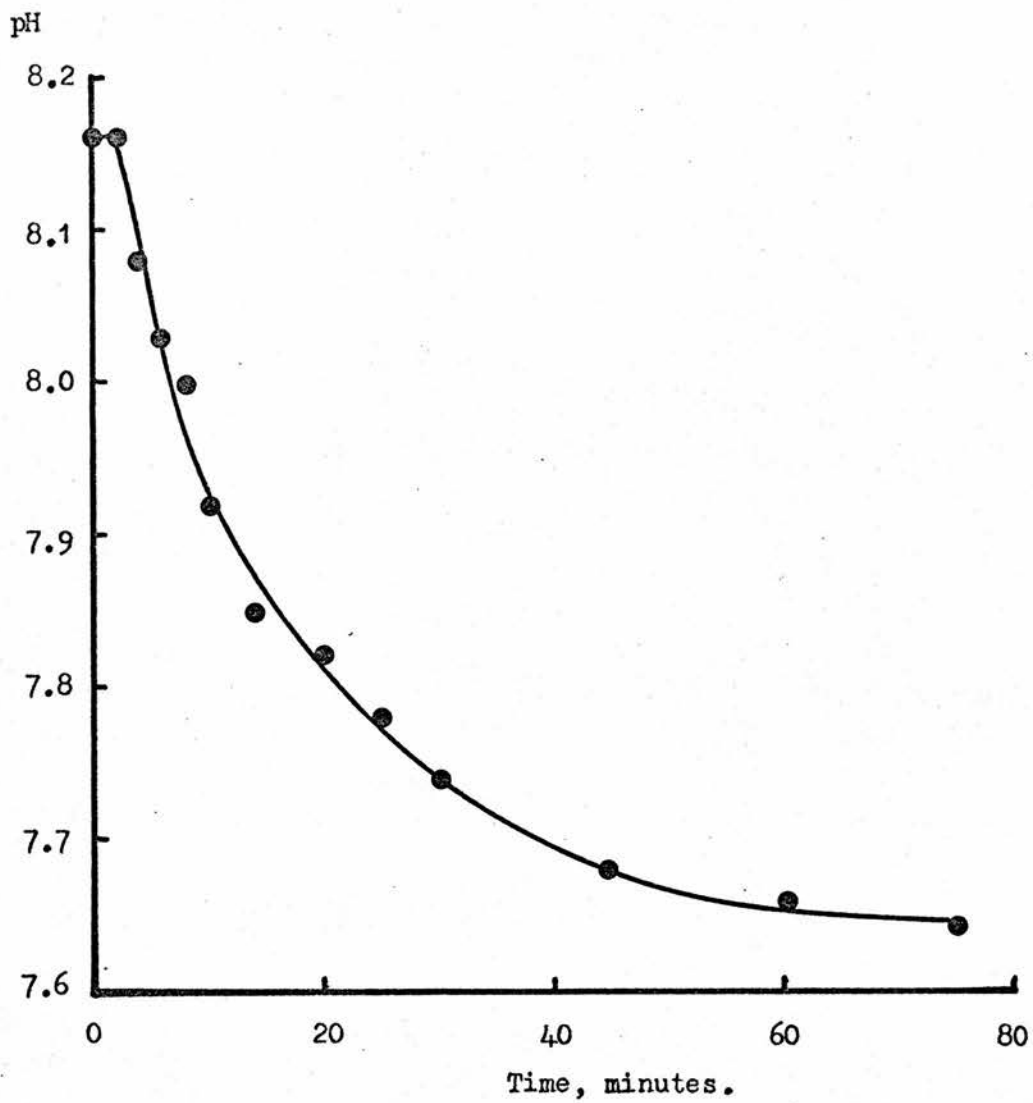


FIGURE 6

Time-course of equilibration of perfusate

The perfusate also contains phenol red (50 $\mu\text{g}/\text{ml}$) to act as an indicator of leaks, and glucose (5 mg/ml ; 28 mM , unless otherwise stated). It is freshly prepared on the day of use, either from a mixed stock solution containing all the electrolytes except the calcium chloride or from individual solutions of the electrolytes. All stock solutions were stored at 4°C , and the mixed stock solutions were freshly prepared about once a week. The composition is given in Table 3.

The osmotic pressure given in Table 3 was measured using an Osmette S semi-automatic osmometer whose use will be described in Chapter VI. It is not possible to predict from theory the osmotic pressure of the final medium, since the activity coefficients of each electrolyte are substantially less than unity in a solution of ionic strength 154 mM . Further, the extended Debye-Hückel relationship for calculating approximate activity coefficients is not valid at ionic strengths over about 100 mM ; also, such a simple relation cannot take into account the heterogeneous nature of all the interacting ions.

The electrolytes, unless otherwise stated, are AnalaR reagents supplied by British Drug Houses Ltd., Poole, Dorset, and are dissolved in water which had been distilled twice, the second distillation being in a glass still.

Before use, each perfusate is equilibrated with 5% CO_2 in O_2 at 41°C for at least one hour per litre of perfusate. The pH of the normal perfusate is 7.6 and Figure 6 shows the time course of equilibration of one litre with the CO_2 .

This medium was originally selected because many previous authors have used it for intestinal incubations and perfusions with apparent success (e.g. Fisher & Parsons, 1949), and its use has been sustained since the preparation has been shown to function satisfactorily for

the purposes of the present work. No thorough attempt has been made to vary the composition with a view to maximising absorption rates or viability as this has not been necessary in the present investigations. However, there are two reasons why it might become desirable to modify the perfusate composition: (i) in the preparation being described, the rate of attainment of a steady-state in the secretion is proportional to the rate of water absorption (this will be amplified in Chapter VI); hence, maximum rate of water absorption might be advantageous for some purposes. (ii) instances will be mentioned where a medium which can be buffered without carbon dioxide might be useful. A phosphate-based perfusate might prove suitable. Although Smyth & Taylor (1957) found lower rates of water secretion in their preparation with phosphate buffers than with bicarbonate buffers, Professor Fisher and Mrs. F. O'Brien have made preliminary experiments which suggest that in fact the converse may be true for this type of preparation (personal communication).

For experiments on sodium depletion, most or all of the sodium salts are replaced by the corresponding salts of choline. These were supplied by the Sigma London Chemical Co. Ltd., but were not of analytical grade purity. The sodium concentration in typical 'sodium-free' (nominally) perfusates was found by atomic absorption spectrophotometry to be 0.04-0.5 mEq/litre.

Setting up the intestine

The anaesthetised animal is placed on its back on an operating table which stands within about 25 cm from the perfusion apparatus. Its head and neck are placed in a mask consisting of a short length of open-ended glass tubing which holds in position a pad of ether-moistened cotton wool. Light wires are tied round each limb to secure the animal to the table.

The caecum and most of the small intestine are exteriorised through a mid-line incision, and the duodeno-jejunal flexure is located as the highest part of intestine which can be withdrawn from the peritoneal cavity without undue traction on the Ligament of Treitz. At this flexure the duodenum is tied off, the intestine divided caudal to the ligature, and a glass cannula tied into the jejunum. Nylon wool has been used for the ligatures since this material is less likely to cut into the tissue than is conventional surgical linen or silk. The segment of intestine to be used (generally approximately 40 cm caudal to the duodeno-jejunal flexure) is rinsed out with warm oxygenated bicarbonate saline so as to displace solid debris into the lower ileum and caecum. Then the caudal end of the required segment is tied off, the intestine divided oral to the ligature, and the outflow glass cannula tied in. The organ chamber bung carrying the connections for the cannulae is lifted over to the animal, the cannulae are attached to the perfusion unit, and perfusion with the segmented flow of oxygenated warm perfusate is begun. Only now, once the supply of oxygen through the intestinal lumen has been established, can the animal's blood supply be dispensed with. The mesentery is clamped and cut distal to the clamp. The segment of intestine, now attached to the perfusion unit, is rinsed in a dish of warm saline and most of the mesentery gently pulled away. The intestine falls naturally into a series of coils through which a length of nylon wool is passed. The intestine is lifted into the organ chamber, where it is supported by the nylon wool sling.

The animal is now killed by opening the chest and cardiac puncture. The total time elapsing between opening the abdomen and placing the segment of intestine in the organ chamber is about 10-15 minutes.

The experiment was abandoned if the animal died before the segmented flow of perfusate through the lumen had been established.

Calculation of Absorption Rates etc.

Determination of volumes and concentrations enable the rates of absorption, secretion, and utilisation of solutes such as glucose to be estimated as follows.

The volumes of all collected samples of liquid have been determined by weighing and assuming the density to be 1 g/ml. Since the fluid secreted across to the serosal surface of the intestine is collected and weighed, the rate of water secretion is directly known. This was equated to the rate of water absorption since the water content of the intestine remains constant (see Table 13). If the glucose concentration in this secretion is estimated, then an estimate of the rate of glucose secretion is possible.

The rate of glucose absorption from the lumen is estimated by measuring the concentrations of glucose in the fluids entering and issuing from the lumen, provided that allowance is made for the water absorbed from the lumen. Thus, the amount of a solute absorbed from the lumen during a collection period is given by:-

$$(W_e + W_s) \cdot C_p - W_e \cdot C_e$$

where W_e is the weight of effluent from the lumen collected in a given time,

W_s is the weight of secretion collected during the same time,

C_p is the solute concentration in the perfusate entering the lumen,

and C_e is the solute concentration in the luminal effluent.

The rate of glucose utilisation is taken to be the difference between the rate of absorption from the lumen and the rate of secretion onto the serosal surface.

All rates have been expressed in terms of unit length^{*} of intestine (cm) and unit time (hour). The units, $\text{mg}\cdot\text{cm}^{-1}\cdot\text{hr}^{-1}$ etc, have been written as $\text{mg}/\text{cm}/\text{hr}$ etc for convenience. The approximate relation that 1 cm of intestine has a dry weight of 13 mg and a wet weight of 76 mg (Table 13) may be used to compare the results of these experiments with others expressed in terms of unit weight of intestine.

All these rates are net rates; they will be less than values for uni-directional flux rates of efflux from the lumen.

An error of 1% in the estimation of glucose concentration in either the lumen perfusate (C_p) or effluent (C_e) will result in an error of approximately 4.5% in the estimation of the rate of glucose absorption from the lumen under the conditions of the experiments described later. However this error will be somewhat less (at ca 1%) if both C_p and C_e are subject to the same amount of error. It is clearly essential that estimations of glucose concentration must be of the highest accuracy attainable if reliable measurements of absorption rates are to be gained.

Since the rate of glucose utilisation by the intestine is indirectly calculated as a fairly small difference between two larger numbers, this is the least accurate of the estimated rates. For instance, an underestimate of 1% in glucose concentrations in both secretion and lumen effluent could result in an overestimate of glucose utilisation rate by about 8.5%.

The rate of absorption is defined as the net rate of disappearance from the lumen. The rate of secretion is defined as the net rate of appearance at the outer wall of the intestine.

* The length of the segment was measured according to Fisher & Parsons (1949), namely by laying the segment without tension along a ruler.

CHAPTER III

THE ESTIMATION OF GLUCOSE

Introduction

As indicated in the previous Chapter, reliable calculation of the rate of glucose absorption requires accurate estimation of the glucose concentrations in the lumen effluent and in the perfusate, as well as of the rate of water absorption. If a 40 cm segment of jejunum and upper ileum is perfused with 5 mg/ml glucose at a rate of 4 ml/minute, the intestine will absorb about 2 mg of glucose and 0.12 ml of water per minute, and so the glucose concentration in the lumen effluent will be about 4.68 mg/ml. If an error of 1% is made in the estimation of the concentration in this effluent, then the resultant estimate of the rate of glucose absorption will be some 4.5% in error. Even larger errors will be incurred in the estimation of the rate of glucose utilisation. Clearly, the greatest possible accuracy in the estimation of glucose is desirable.

An existing enzymatic method which is precise, specific, and rapid has been modified to afford greater precision and convenience. It depends on the oxidation of β -D-glucose by atmospheric oxygen, catalysed by β -D-glucose oxidase (E.C. 1.1.3.4). D-glucono-5-lactone and hydrogen peroxide are formed, and in the presence of peroxidase (E.C. 1.11.1.7) the latter product will oxidise a chromogen such as o-tolidine (Middleton & Griffiths, 1957; Marks, 1959), o-dianisidine (Huggett & Nixon, 1957), or gum guaiacum resin (Hill & Cowart, 1966). The glucose oxidase enzyme is fairly specific towards β -D-glucose (Dixon & Webb, 1964), a valuable characteristic not shared by chemical methods.

The carcinogenic properties of o-tolidine and o-dianisidine make these agents undesirable. O'Brien (1969) and Fisher & O'Brien

(1971) have successfully adapted Hill & Cowart's (1966) method to the Technicon AutoAnalyzer system (Technicon Instruments Co., Chertsey, Surrey) in such a way as to combine rapid analysis (20 samples per hour) with excellent reproducibility (the standard deviation of an estimate in the range 5 to 15 μg glucose/ml is stated to be ± 0.06 $\mu\text{g}/\text{ml}$).

O'Brien's method requires that the samples presented to the AutoAnalyzer system be in the concentration range 5 to 15 $\mu\text{g}/\text{ml}$. As the samples encountered in the present investigations generally contained glucose in the concentration range 0.5 to 15 mg/ml , modifications were developed so as to render dilution of the samples unnecessary.

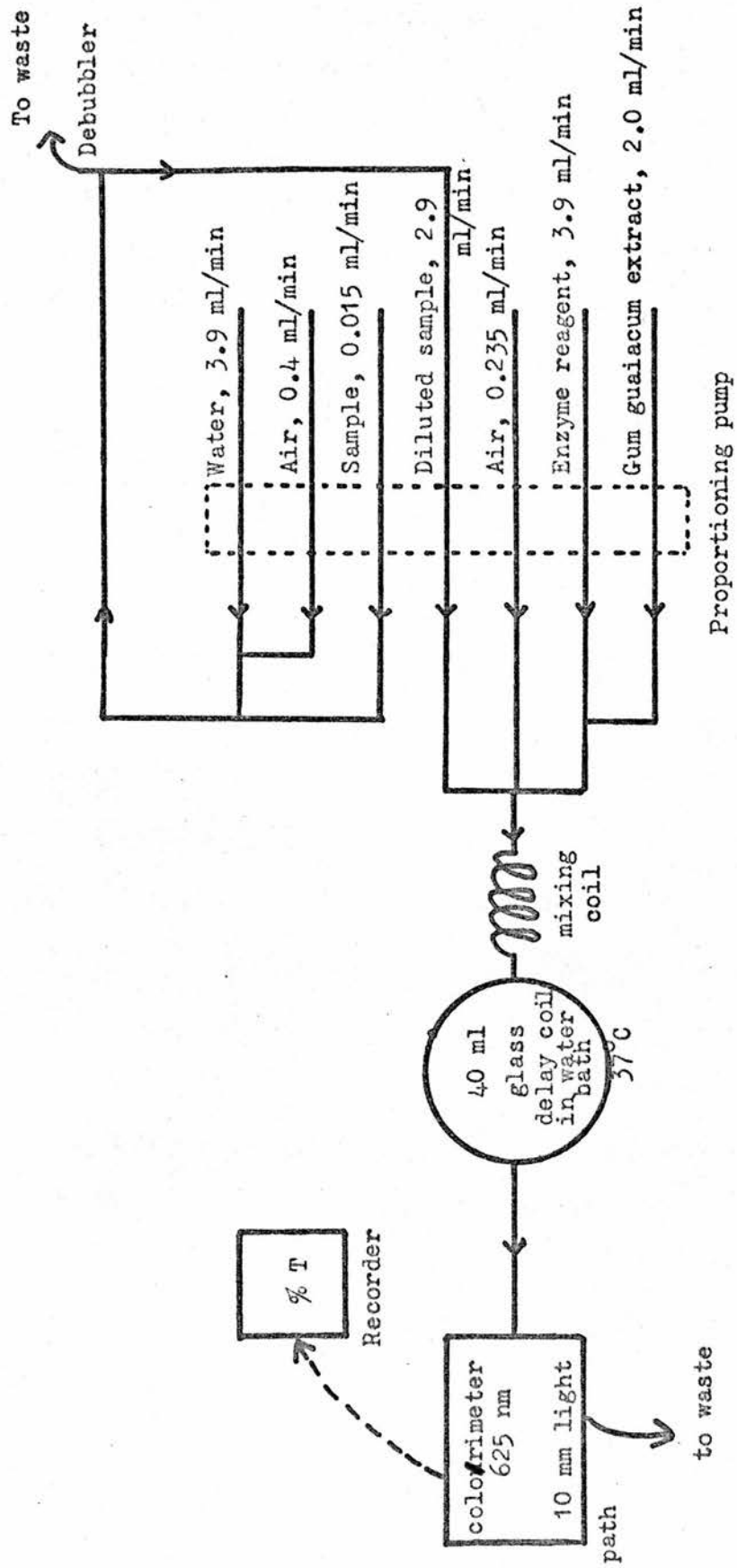
The AutoAnalyzer

The Technicon AutoAnalyzer is a continuous flow analytical system, built up from commercially produced modules (sampler, proportioning pump, mixing coils, heating bath, flow-cell colorimeter, and chart recorder). Sample and reagents are continuously aspirated in predetermined proportions by a peristaltic pump. They converge and are propelled through heating baths etc. into a colorimeter cuvette. Mixing is achieved by segmenting the continuous stream with air bubbles, which also serve to minimise cross-interactions between one sample and the next. The operation and use of the system has been amply described by its inventor Skeggs (1957; 1965), and in the Theses of Gilbert (1963), O'Brien (1969), and Nimmo (1970). The role which the air bubbles play in the mixing of the reaction mixture was evidently not appreciated by Skeggs (1957; 1965), and will be discussed later in another context (see Chapter V).

Such an automated system affords high precision, since each sample and standard solution receives identical treatment, and is

FIGURE 7

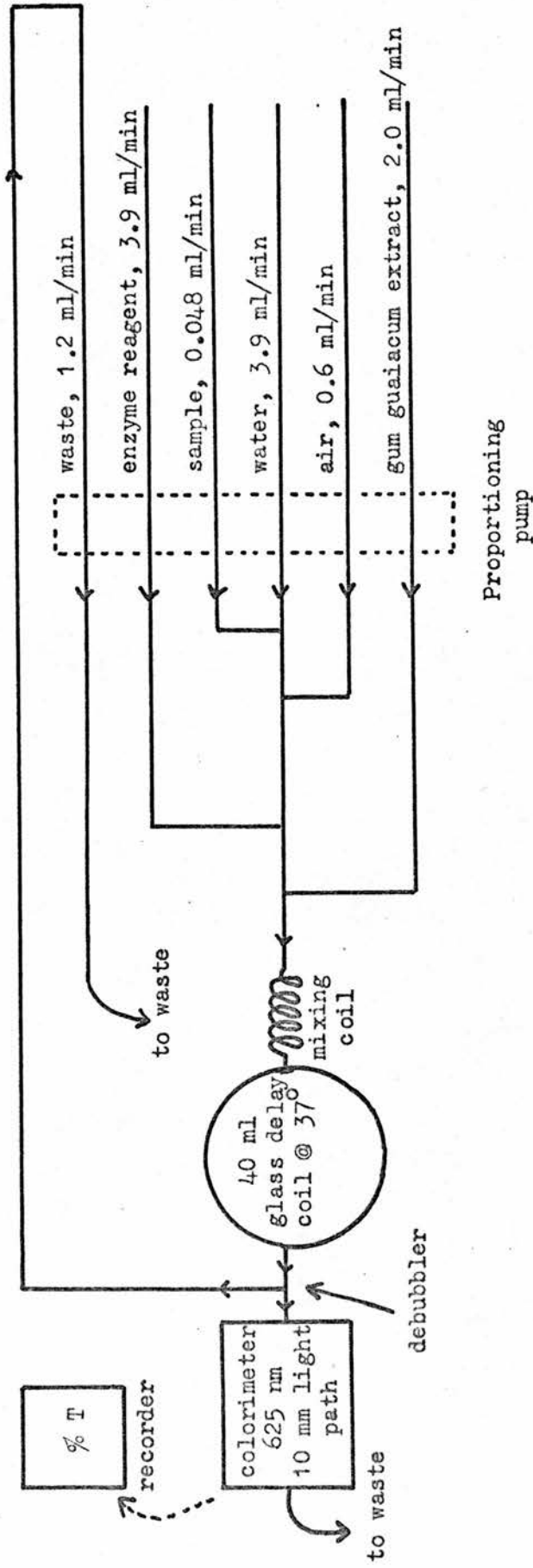
Auto-Analyzer system for glucose analysis



Sampling rate: 20/hour

FIGURE 8

Second AutoAnalyzer system for glucose analysis



Sampling rate: 40/hour

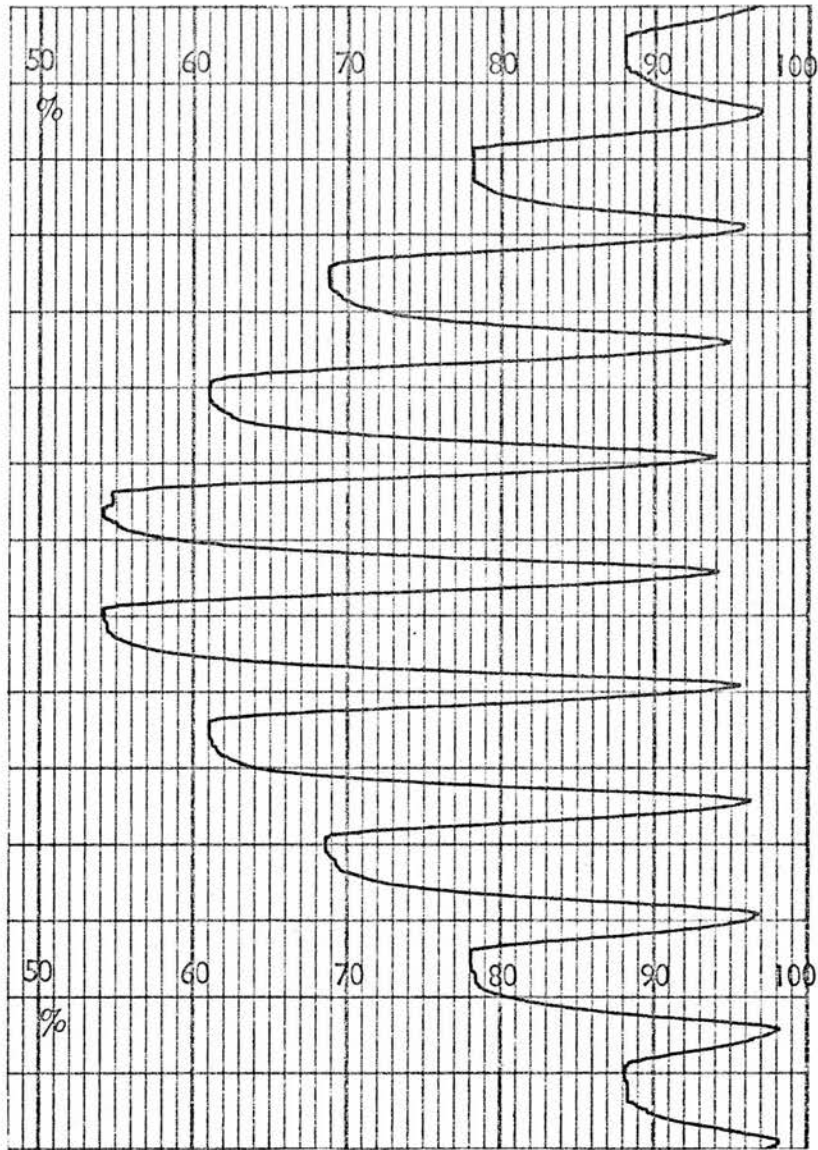


FIGURE 9

A typical AutoAnalyzer trace for glucose estimation

Solutions of the following glucose concentrations were aspirated: 0.25, 0.50, 0.75, 1.00, 1.25, 1.25, 1.00, 0.75, 0.50, 0.25 mg/ml. The chart is calibrated in units of %-transmittance.

Sampling rate: 40/hr.

unaffected by the variation in the proficiency of the operator which is bound to occur when large numbers of samples are processed manually. The coloured oxidation product of gum guaiacum extract, whose composition is apparently unknown, is unstable; thus any manual procedure would require very precise timing.

The arrangement of the system as used for glucose estimation for most of the work described in this thesis is shown in Figure 7. This is different from the system described by O'Brien (1969) in that the sample is automatically diluted some 240-fold before it mixes with the reagents. Also, the concentrations of enzymes in the reagents described below are much lower than those in O'Brien's reagent. Consequently, samples containing much higher concentrations of glucose can be presented to this system than to O'Brien's.

Towards the end of the investigations reported in this thesis, a further AutoAnalyzer pump and Sampler became available. When these were installed the system already described was slightly modified so that the rate of sampling could be increased from 20 per hour to 40 per hour. This more modern system is shown in Figure 8. Instead of a Technicon colorimeter with flow-cell, a Coleman Junior II (Perkin Elmer Ltd.) grating spectrophotometer was used. Its cuvette holder was modified by Mr. W. Tait in the Department's Workshops so as to receive a Fisons flow-through cuvette (supplied for use with a Vitatron colorimeter, by Fisons Ltd.). The output from this spectrophotometer was taken to a Servoscribe recorder (supplied by Smiths Industries Ltd.), the input of which was shunted by a 100 μ F electrolytic capacitor.

Typical recorder traces for glucose standard solutions run on the second AutoAnalyzer system are shown in Figure 9.

At regular intervals a warm solution of 'Decon 75' (B.D.H. Ltd.) was aspirated to clean the apparatus.

Reagents

The materials are as follows:-

- (i) Gum guaiacum resin (supplied by Brome & Schimmer Ltd.,
7 Leather Market, London).
- (ii) Molar sodium acetate buffer, pH 5.6
 - Sodium acetate, trihydrate 123.8 g
 - Acetic acid, glacial 5.2 ml
 - Distilled water to 1 litre.
- (iii) 'Triton-X-100' (supplied by British Drug Houses Ltd.)
This is a non-ionic detergent, a polymer of
p-isooctylpolyoxyethylene phenol. It renders the
alcoholic extract of gum guaiacum resin miscible with
the aqueous buffer, and also improves the bubble pattern
in the AutoAnalyzer system.
- (iv) 'Fermcozyme 635 AM' (supplied by Hughes & Hughes Ltd.,
Brentwood, Essex). This contains an impure preparation
of glucose oxidase (approx. 750 U/ml) from the fungus
Aspergillus niger.
- (v) Horse-radish peroxidase (supplied by Hughes & Hughes Ltd.).
- (vi) Stock gum guaiacum extract.

Finely powdered gum guaiacum resin (0.75 g) is extracted with 95% ethyl alcohol (12.5 ml) for at least 3 hours at room temperature. The extract is filtered into a mixture of sodium acetate buffer (600 ml) and Triton-X-100 (20 ml), and the whole diluted to 1.5 litres with distilled water. Throughout this investigation a single batch of gum guaiacum resin has been used, and the troubles experienced by O'Brien (1969) and Nimmo (1970) in connection with ageing of the reagent have not been observed. For use, 2 volumes of this stock solution are diluted with 1 volume of distilled water.

Enzyme reagents

The composition of the reagent depends on the concentration range of glucose in which measurements are to be made. The final reagent contains stock gum guaiacum extract (670 ml), distilled water (330 ml) and Fermcozyme and peroxidase as below.

	<u>Concentration range of glucose mg/ml</u>		
	<u>0.25-1.25</u>	<u>1.0-5.0</u>	<u>6.0-14.0</u>
Fermcozyme (ml)	6	1.5	0.75
Peroxidase (mg)	6	1.5	0.75

Standard solutions of glucose

These were prepared from AnalaR α -D-glucose (British Drug Houses Ltd.). When stock solutions were prepared, the glucose was heated with water at 90° - 100° C for about ten minutes, then cooled, so that the glucose would be at mutarotational equilibrium. This is essential since the glucose oxidase is specific to the beta anomer. Four, or occasionally 3, standards were used in each concentration range. Their concentrations bracketed those of the unknowns.

Calibration of system

In order to achieve maximum accuracy, each estimate is routinely duplicated. So as to reduce potential error due to any slight drift in the baseline caused by, for example, warming up of the reagent, these duplicate analyses are conducted in the following sequence:-

s1, s2, s3, s4, u1, u2, u6, u6, u2, u1, s4, s3, s2, s1

where s1 s4 are standard solutions,

and u1 u6 are unknown samples.

Thus, the mean time at which a pair of estimations is made is the same for all standards and unknowns, viz. the mid-point in the palindromic series.

Extinction @ 625 nm

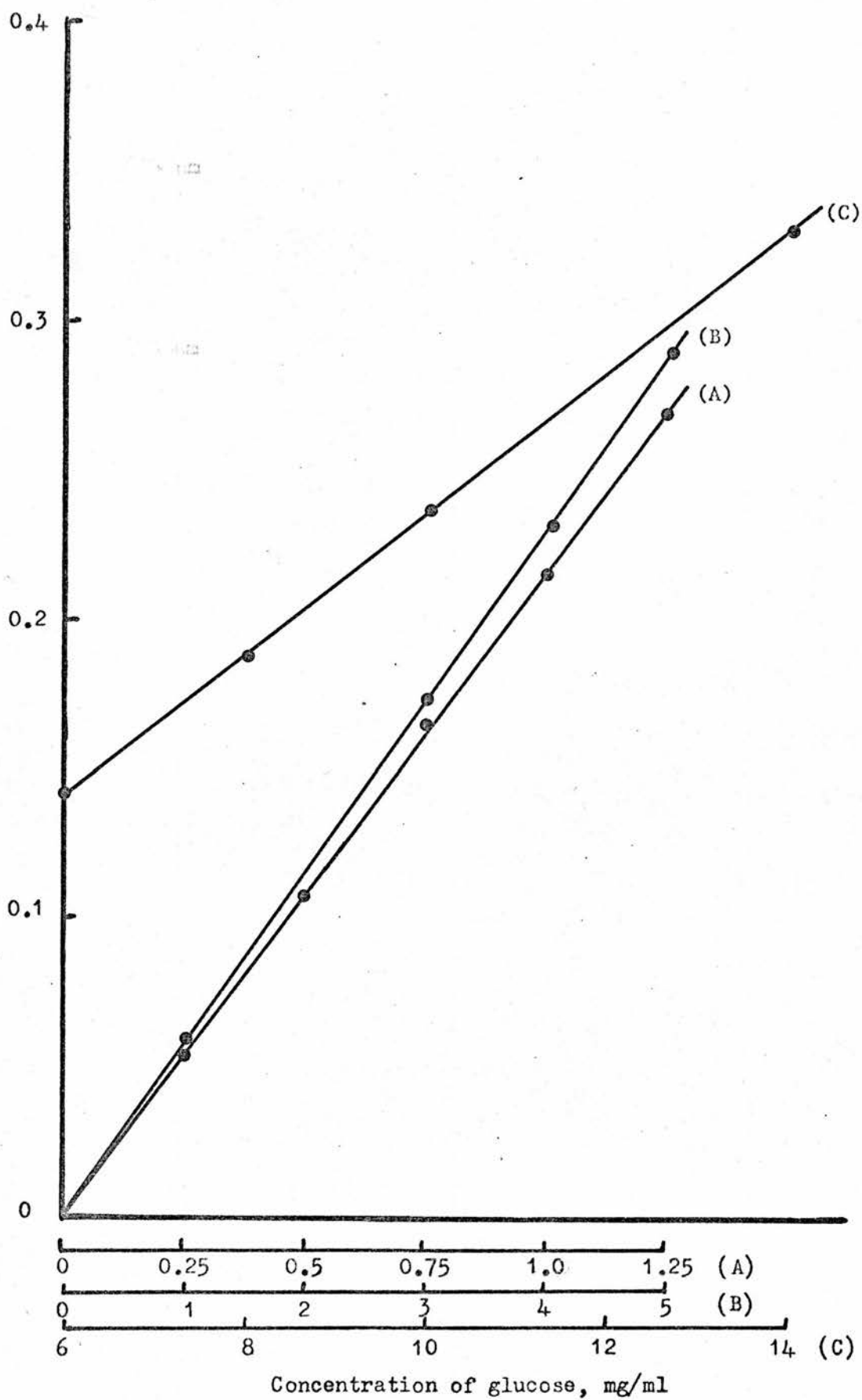


FIGURE 10

Glucose estimation - conformity to Beer's Law

The recorder is linear and its chart paper is calibrated in percentage transmittance; hence a reading could be linearly interpolated to 0.1% transmittance with confidence. The arithmetic mean of the extinctions corresponding to each pair of transmittance values is calculated, and a first-order regression of concentration on extinction computed. The fit of this line to the data for each standard solution is then assessed by inspection. If it appears satisfactory, then the concentrations of each unknown sample are calculated. This calculation is executed on a programmable calculating machine (Olivetti Programma P101, British Olivetti Ltd., Edinburgh) using a program written by Dr. I. A. Nimmo. In converting a value of percentage transmittance into one of extinction, an approximation given by Zucker (1965) is used to evaluate a common logarithm. Nimmo (1970) has shown that the error introduced by this approximation does not exceed 0.15%, provided that the transmittance falls between 40% and 100%. He also showed that the regression of concentration on extinction rather than of extinction on concentration is justifiable, even though extinction is the dependent variate, since the correlation coefficient between the two variates is very close to unity.

Conformity to Beer's Law

Figure 10 shows that the relations between concentration and extinction are linear over the respective concentration ranges covered by each reagent.

On only two occasions in almost 2 years was any serious deviation from Beer's Law noted. No explanation was found, but the relationship between concentration (C) and extinction (E) could then be adequately described by a relationship of the form:

TABLE 4

The precision of an estimate of glucose concentration

Apparent concentration of glucose, mg/ml		
	A	B
	5.9994	13.9697
	5.9612	13.9923
	6.0121	13.9698
	5.9866	14.0147
	5.9820	14.0147
		13.9698
	5.9941	
	6.0062	
	5.9823	
Mean	5.9905	13.9885
S.E.M.	± 0.0057	± 0.0090
S.D.	± 0.0161	± 0.0221
Coefficient of variation	± 0.27%	± 0.16%

Solutions containing 6.0 mg/ml (A) and 14.0 mg/ml (B) glucose were repeatedly estimated on three or two days respectively.

TABLE 5

Interference of raffinose in the estimation of glucose

Apparent concentration of glucose, mg/ml	
A	B
1.0088	1.0158
1.0019	1.0031
1.0158	1.0097
Mean \pm S.E. 1.0088 \pm 0.0040	1.0095 \pm 0.0037
Difference \pm S.D.	0.0007 \pm 0.0054
	$t_4 = 0.1288$
	N.S.

Solution A contained glucose (1 mg/ml).

Solution B contained glucose (1 mg/ml) plus raffinose (10 mg/ml).

$$\log E = a + b \cdot \log C$$

where a & b are the coefficients in a first-order regression.

Nimmo (1970) also reported similar occurrences.

Accuracy of an estimation

The standard deviation of an estimate of glucose concentration was derived by repeatedly estimating a single solution containing a known concentration of glucose. The results, given in Table 4, show that the coefficient of variation of an estimate in the range 5-15 mg/ml is in the region of 0.2%. This coefficient of variation is less than half that recorded by O'Brien (1969) for the unmodified AutoAnalyzer system.

That no significant interaction exists between successive samples was confirmed by repeatedly estimating a solution containing 14 mg/ml glucose, and then repeating the process but with sample cups filled with water interspersed between each sample. No significant difference was detected.

Interference of raffinose

In some experiments which will be described in Chapter IX raffinose is present as a marker of water absorption. The possibility that this sugar might interfere with the estimation of glucose was therefore tested.

The apparent concentrations of standard glucose solutions with and without added raffinose were estimated. The results (Table 5) show that, at a glucose concentration of 1 mg/ml, raffinose (10 mg/ml) has no measurable effect on the estimation of glucose. This ratio of raffinose to glucose is as high as any likely to be encountered.

TABLE 6

Effect of deproteinisation on the
estimation of glucose in secretion

Apparent concentration of glucose, mg/ml

A	B
1.3836	1.3795
1.3736	1.3802
1.3836	1.3802
1.3637	1.3802
1.3637	1.3802
1.3736	1.3802

Mean \pm S.E. 1.3736 \pm 0.0036 1.3801 \pm 0

Difference \pm S.D. 0.00645 \pm 0.00363

$t_{10} = 1.778$

$P > 0.1$

Solution A was deproteinised. $ZnSO_4$ & NaOH had been added.

Solution B was not deproteinised. Water had been added.

The deproteinisation of a sample

Intestinal secretion contains protein; therefore the possibility that the protein might interfere with the estimation of glucose was tested.

A commercial dialyser unit (Technicon Instruments Co. Ltd.) could have been added to the AutoAnalyzer system. However, as such an addition is likely to increase the variance of an estimation, this possibility was rejected in favour of chemical deproteinisation.

A sample of intestinal secretion was divided into twelve aliquots, each of 1 ml. Six were treated with 10% (w/v) zinc sulphate (0.2 ml) followed by 2% (w/v) sodium hydroxide to a phenolphthalein end-point. These mixtures and the 6 untreated aliquots were made up to 10 ml with distilled water, shaken, and centrifuged. Duplicate estimates were made of the glucose concentration in each supernatant.

The results (Table 6) show that the $ZnSO_4/NaOH$ deproteinisation treatment of Dawson, Elliott, Elliott & Jones (1959, page 622) does not affect the glucose estimation of intestinal secretion. Nimmo (1970) has confirmed that these protein precipitation reagents do not interfere with the estimation of glucose in a protein-free solution.

Therefore no material removable by $ZnSO_4/NaOH$ interferes with the estimation of glucose in intestinal secretion. Hence, samples were invariably presented to the AutoAnalyzer without prior deproteinisation.

Analytical recovery of glucose

Asp, Koldovsky & Hoskova (1967) found that mucosal homogenates of rat small intestine contained an inhibitor of the glucose-oxidase/peroxidase system. Therefore to establish whether any constituent of intestinal secretion interferes with the estimation of glucose,

TABLE 7

Analytical recovery of glucose added to intestinal secretion

Sample	Volume (ml) of added glucose solution (10.320 mg/ml) in a final volume of 10.0 ml	Expected glucose concentration (mg/ml)	Apparent glucose concentration (mg/ml)	Recovery (%)
A	0	-	1.3348	-
B	0	-	1.3447	-
C	1.0	2.3718	2.3965	101.0
D	1.0	2.3718	2.3545	99.3
E	2.0	3.4038	3.3887	99.6
F	2.0	3.4038	3.3776	99.2
G	3.0	4.4358	4.3785	98.7
H	3.0	4.4358	4.3785	98.7

Mean recovery \pm S.E.M. 99.42%
 \pm 0.35%

$t_5 = (100 - 99.42)/0.35$
 $= 1.677$
 $P > 0.1$

Therefore, the mean recovery of added glucose was not significantly different from 100.0%.

known amounts of glucose were added to samples of intestinal secretion, and the recoveries of glucose measured.

A sample of secretion was divided into eight aliquots, A-H, each of 1 ml. To each of these was added an aliquot of a glucose solution (approx. 10 mg/ml) as shown in Table 7, and distilled water to a final volume of 10 ml. The glucose concentrations in each were then estimated, and the results are shown in Table 7. The mean recovery \pm S.E.M. of the added glucose is 99.42% \pm 0.35%. As this does not differ significantly from 100% ($P > 0.1$ by Student's t-test) the concentration of glucose was routinely estimated in untreated secretion.

CHAPTER IV

THE ESTIMATION OF CATIONS

Introduction

Although chemical methods exist for the estimation of sodium and potassium (e.g. see Vogel, 1961), the technique of flame photometry (atomic emission spectrophotometry) has been adopted almost universally by many authors in the last twenty years. The following reasons may account for the wide use of this technique:-

- (i) small, inexpensive, simple, and reliable instruments are commercially available.
- (ii) sample preparation is minimal. The analyte simply has to be in solution at an appropriate dilution, and is aspirated into the flame for several seconds. A galvanometer reading is noted, and referred to a calibration graph prepared for standard solutions.
- (iii) versatility. Modern instruments can simultaneously estimate sodium and potassium; older instruments only required a simple change of filter when estimations were changed, and
- (iv) precision. Spectral interferences are small, and chemical interferences are claimed to be non-existent.

A more modern spectrophotometric method is that of Atomic Absorption Spectrophotometry. This technique, developed by Dr. Walsh at the C.S.I.R.O. laboratories, offers major advantages over atomic emission photometry. (Walsh, 1955; Reynolds & Aldous, 1970; Dawson & Broughton, 1968.)

In this Chapter the principle of this technique is described. This is followed by a description of the particular model of instrument which was selected and purchased. Finally, some typical calibration curves are given.

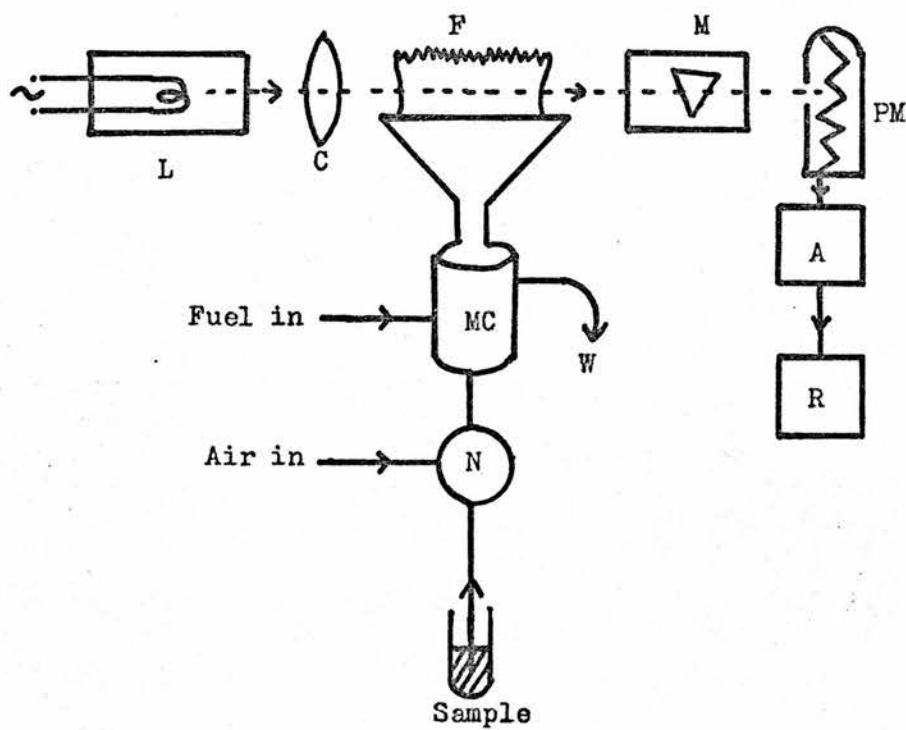


FIGURE 11

Diagrammatic representation of an atomic absorption spectrophotometer

Atomic Absorption Spectrophotometry

A solution containing metal ions is aspirated into a flame where the metal is atomised as in emission photometry. However, in this case the intensity of the emitted radiation is not measured. Instead, radiation at the wavelength of a resonance line of the element in question is generated and is passed through the flame. Atoms of the metal, in their ground state in the flame, are raised to an excited state by the incident radiation. Consequently, some of the incident radiation is absorbed. The intensity of the unabsorbed radiation which has passed through the flame is measured. The fractional transmittance of radiation can then be calculated; this is related to the number of atoms in the flame.

Figure 11 shows diagrammatically the main components of an atomic absorption spectrophotometer. An energy source (L), typically a hollow cathode lamp, whose spectrum is predominantly that of the element to be determined, directs radiation via a collimator (C) into the flame (F). This flame normally burns acetylene in air at about $2,300^{\circ}\text{C}$. Other flame systems, e.g. acetylene in nitrous oxide, may be employed particularly for refractory elements (such as barium, boron, and silicon) which are scarcely atomised at the lower temperature of the air-acetylene flame. A nebulizer (N) aspirates the test solution, disperses it into fine droplets, and directs them over vanes in a mixing chamber (MC) where they mix with air and fuel. Large droplets, which may comprise up to 90% of the sample aspirated, fall to waste (W), while a uniform fog passes to the burner. The radiation emergent from the flame is focussed onto a monochromator (M) which allows energy only at the selected wavelength to fall onto a photomultiplier tube (PM). The electrical output from this detector is amplified (A), and taken to a potentiometric recorder (R).

So that the radiation emitted in the flame as the excited atoms fall back to their ground-state is not recorded (the NET absorption is very small) the radiation from the hollow cathode is modulated at, say, 350 hertz by heating the cathode with an alternating current. The amplifier, A, contains a tuned circuit so that only the A.C. energy from the lamp is accepted.

Atomic absorption spectrophotometry affords three major advantages over emission photometry. (i) absence of spectral interferences, (ii) greater versatility, (iii) higher sensitivity and precision.

The virtual absence of spectral interferences stems from the fact that resonance spectral lines are very sharp, with a half-bandwidth of only some 0.05 \AA . Hence, with rare exceptions, spectral interferences are unknown (Reynolds & Aldous, 1970). Conversely emission spectral bands are much broader, and unless exceptionally high resolution monochromators are used spectral interferences can become significant. These arise from any partial overlap of the spectral bands.

Provided the chosen instrument has a suitable monochromator and a photomultiplier tube which is sensitive over a wide wavelength range (e.g. E.M.I. HTV R136 tube) the basic instrument is capable of analysis of a very wide range of elements from arsenic to zirconium. The only additional accessory required is a hollow cathode source for each element. Emission photometry is restricted to the alkali metals and only a few other elements.

The majority of atoms in the flame are in their ground state. Only about 10% are thermally raised to their excited state. These atoms are the ones which are estimated by emission techniques. The remaining 90% of the atoms, those in the ground-state, are

43

potentially capable of absorbing energy from the hollow cathode lamp. Hence, absorption techniques are much more sensitive than emission techniques; furthermore a small variation in the total number of atoms in the flame such as might be caused by minor fluctuations in the air pressure will cause a much greater relative error when the latter techniques are adopted.

The choice of an instrument

Thus on the grounds that atomic absorption spectrophotometry would potentially afford maximum precision in the estimation of all cations likely to be encountered in this investigation, a number of commercial instruments were considered. Finally, the EEL Model 240 Atomic Absorption Spectrophotometer (Evans Electroselenium Ltd., Halstead, Essex) was selected. The main considerations leading to this choice were (i) stability, (ii) safety, (iii) situation of burner, and low gas flow rate, and (iv) integrated output.

The instrument chiefly owes its stability to two features: the Czerny-Turner grating monochromator which is characteristically more stable than a prism monochromator, and the water-cooled 'chimney' which effectively isolates the burner compartment from the rest of the instrument.

Noteworthy safety features incorporated in the EEL 240 include:

- (a) suction applied to the mixing-chamber drain. This overcomes the danger of liquid building up in the mixing-chamber which could cause a reduction in gas velocity, and hence an air/acetylene explosion.
- (b) a thin PTFE diaphragm at the base of the burner. In the event of an explosion, this membrane will rupture rather than the heavy burner or mixing chamber being blown up.
- (c) the burner controls are all situated on the front panel of the instrument, and it is never necessary to put one's hand near the flame.
- (d) the fuel

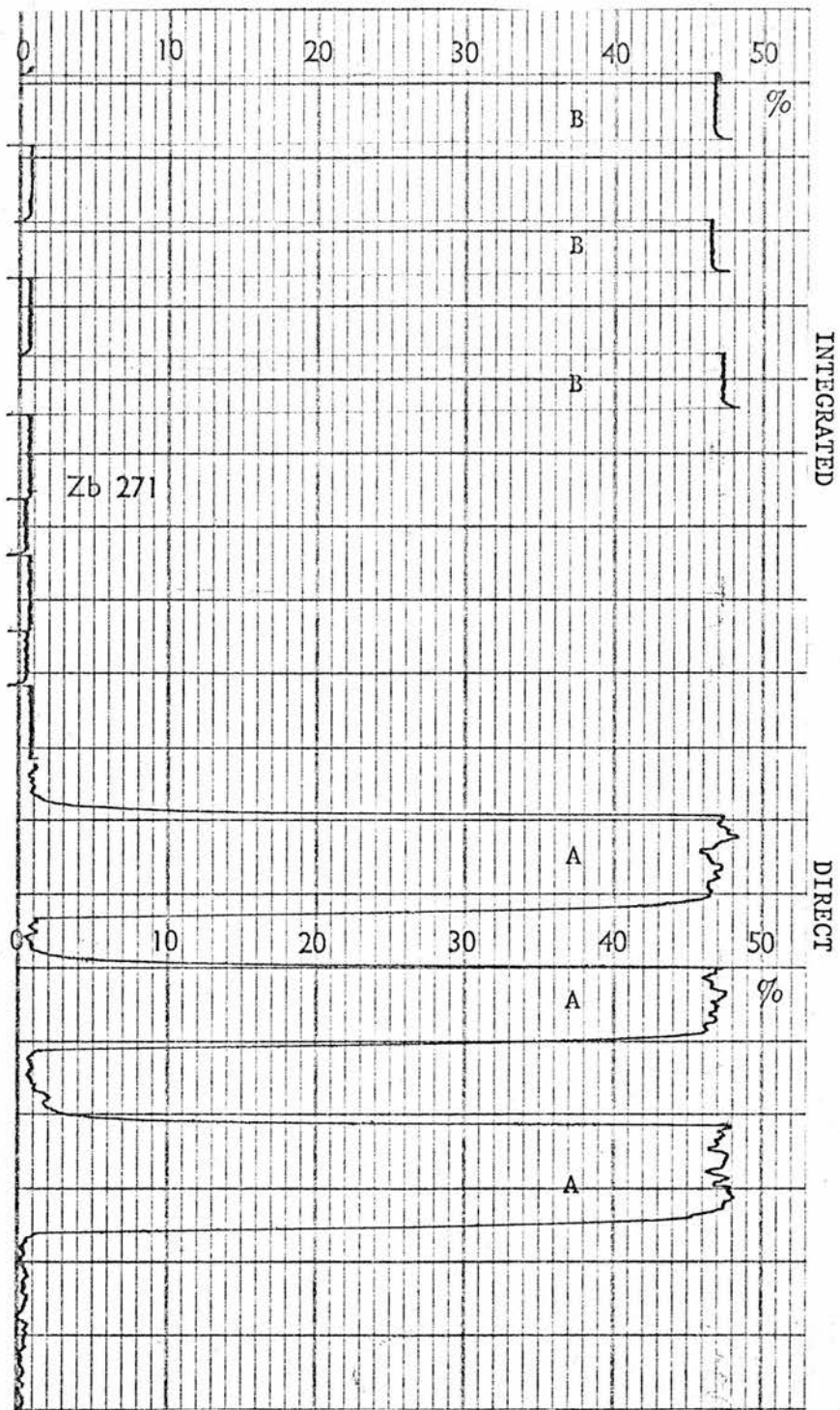


FIGURE 12

Output from the EEL 240 atomic absorption spectrophotometer

A solution containing potassium (0.04 mEq/litre) was repeatedly aspirated to the instrument in (A) direct output mode, and (B) integrated output mode. Distilled water was aspirated between samples. The chart is calibrated in units of %-absorbance. Chart speed: 3 cm/minute.

supply is equipped with a toggle valve which permits a very rapid shut-down.

In some instruments the burner is fully exposed. Especially when a high gas velocity is used, there is a severe tendency for atmospheric 'pollution' to be drawn into the flame. This is minimised in the EEL 240, with a resultant low level of 'flame noise'.

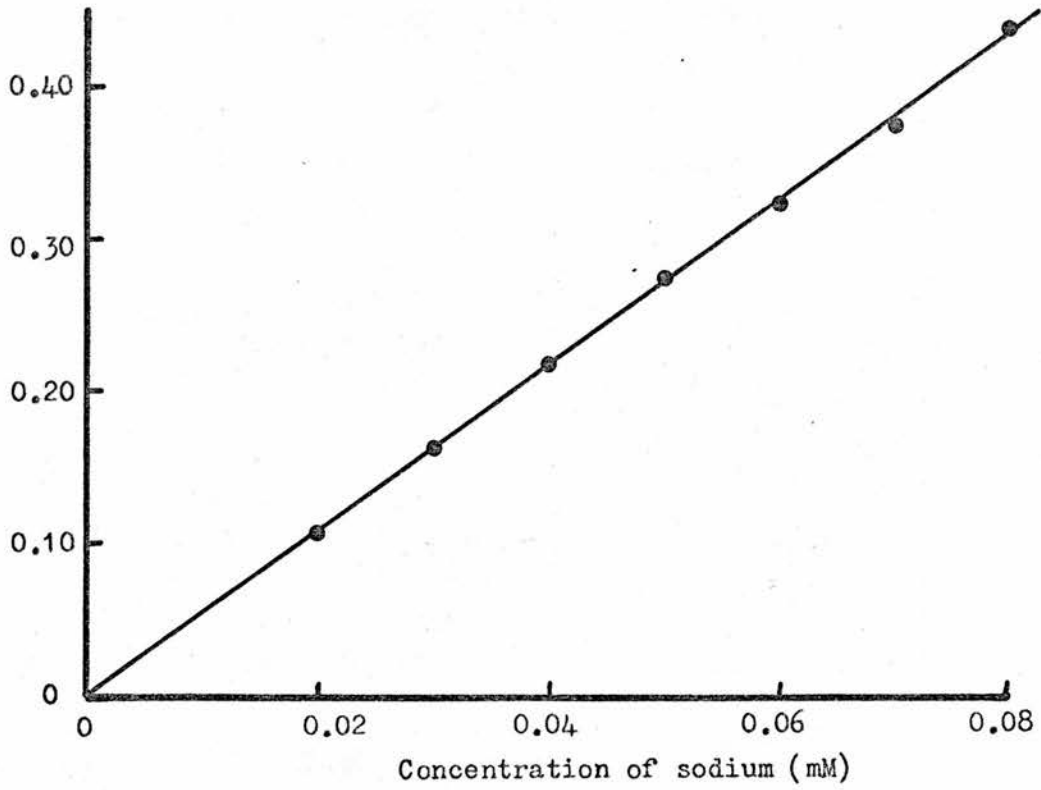
The EEL 240 is the only British atomic absorption spectrophotometer which is equipped with a built-in output integrator. This offers a major gain in precision. The output from all flame spectrophotometers contains a significant noise level which arises from practical considerations. The chief of these is slight variability in the rate of nebulisation. Damping the output signal by placing a capacitative load across the recorder merely increases the time-constant of the recorder, and is unsatisfactory. Instead, the output from the amplifier may be allowed to charge a capacitor for a fixed time. The input to the capacitor is disconnected, and the energy stored is measured by connecting a potentiometric recorder across the capacitor. This integration gives a true value of the mean signal, and it is equivalent to measuring the area under the noisy peak which would otherwise have been recorded. The EEL 240 can be operated in either direct or integrated mode, and Figure 12 reproduces a typical output obtained in each of these modes.

Calibration of instrument

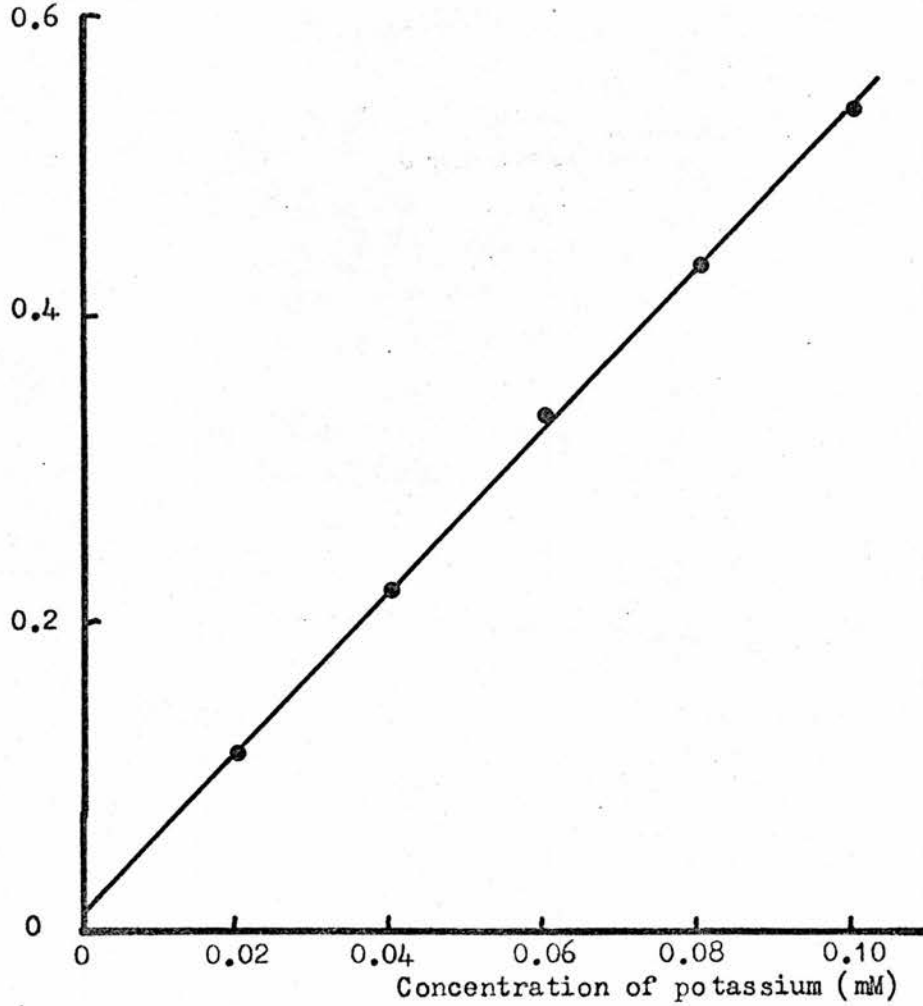
The results have been calculated from pairs of values of percentage transmittance in the same way as that described for glucose estimation on the AutoAnalyzer (Chapter III).

Figures 13, 14, 15 and 16 show typical calibration curves of extinction against concentration for sodium, potassium, calcium and magnesium respectively. In each case straight lines appear to fit

Extinction

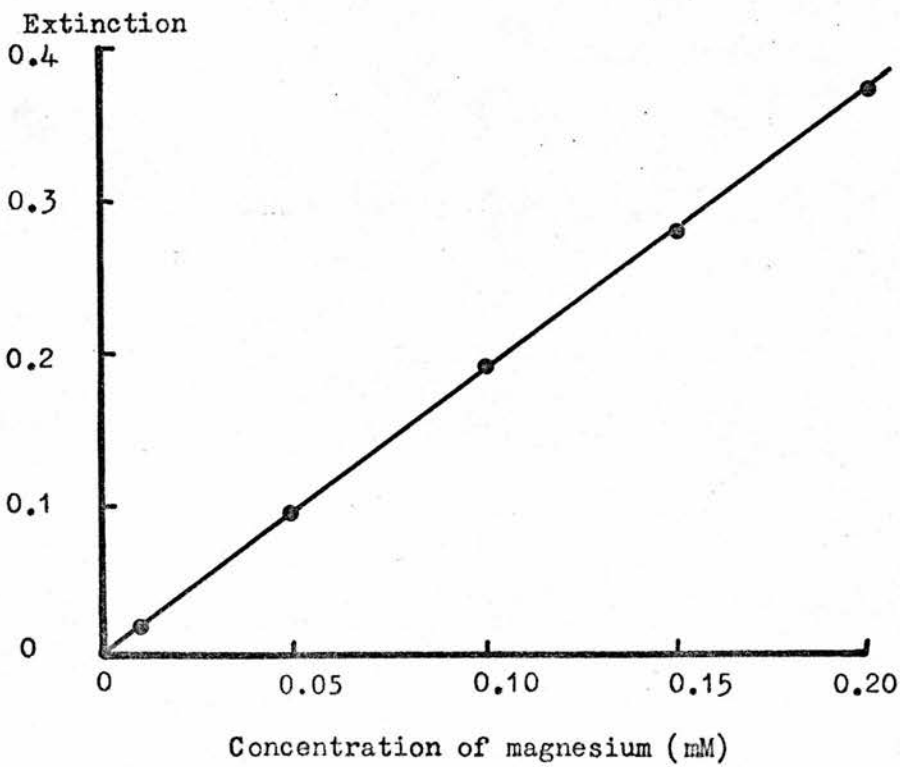
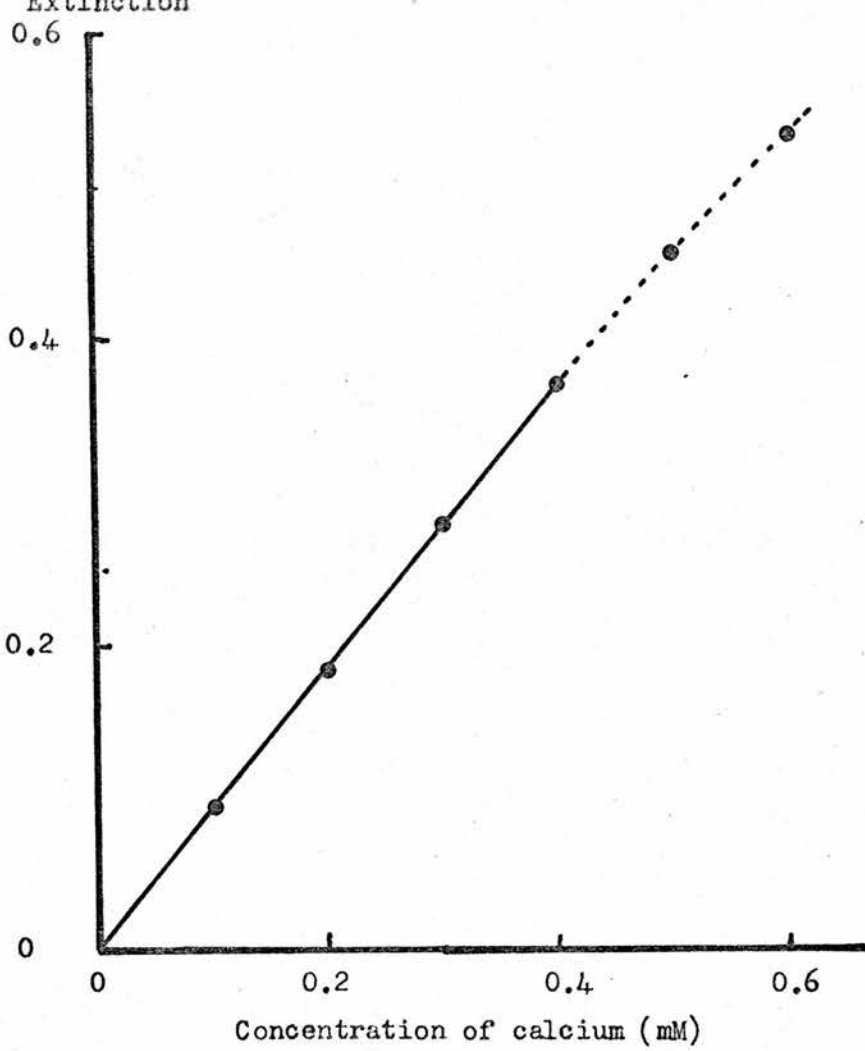


Extinction



FIGURES 13 & 14

Typical calibration curves for sodium and potassium estimation by atomic absorption spectrophotometry



FIGURES 15 & 16

Typical calibration curves for calcium and magnesium estimation by atomic absorption spectrophotometry

the data well, and the intercepts are not significantly different from zero. Therefore Beer's Law is obeyed in each of these estimations.

NOTE:

Although it was stated above that the EEL 240 instrument is a particularly stable model, severe drift with time (up to 20% of full scale deflection per hour) has been experienced. This drift occurred even when no flame was alight, and it was associated with the monochromator since the baseline could be reset simply by re-adjustment of the wavelength 'tuning' control. The problem seemed to be associated with only some of the production instruments rather than with the design, as instruments were seen which did not have this fault. A great deal of time and effort was spent in attempts to find and cure the cause of the problem. Eventually the manufacturers discovered a metallic stress in the monochromator drive unit. The replacement instrument appears to function satisfactorily, but further trials are necessary to confirm that drift is absent as the original instrument functioned well at times.

CHAPTER V

CHARACTERISTICS OF THE PREPARATION

CHAPTER V CHARACTERISTICS OF THE PREPARATION

Introduction

Before any results from a new experimental preparation can be interpreted, it is essential that one be familiar with the characteristics of the material and with the potential limitations. The work in this Chapter has been undertaken to measure the stability and viability of this intestinal preparation, and to compare them with the corresponding characteristics of other preparations. This work also provided the experimental data which are fundamental to the design of the investigations described in subsequent Chapters.

Stability of the preparation

The rates of water and of glucose absorption usually became stable within the first five or ten minutes after the segment of intestine has been placed in the organ chamber, and remained so over the next 100 minutes. In the subsequent half-hour, some falling off was often observed; therefore, experiments generally did not last longer than 90 or 100 minutes.

Over the first five minutes, the secretion was often cloudy, and apparently contaminated with blood which presumably had been washed out from the torn vasculature. This early secretion was normally discarded. On the few occasions on which an experiment was prolonged the concentration of glucose in the secretion was appreciably greater than that in the lumen, even after perfusion for five hours. At the end of this time, however, there was a noticeable amount of phenol red in the secretion, although its concentration was clearly much less than that in the lumen (see below).

In a series of experiments, measurements of the rates of water and glucose absorption were made over four successive 10-minute

TABLE 8

Stability of water absorption

Rates of water absorption, $\mu\text{l}/\text{cm}/\text{hr}$

PERIODS

Time after setting up intestine

	10-20 min	20-30 min	30-40 min	40-50 min
	70	80	87	89
	139	106	128	113
	192	209	197	185
	156	137	144	142
	202	185	206	201
<u>ANIMALS</u>	90	118	128	135
	149	134	139	153
	112	129	147	161
	118	104	126	154
	94	150	160	161
	128	94	122	186
	214	200	197	201
	243	231	223	217

Analysis of Variance

	Degrees of freedom	Mean square	Variance ratio (F)	
Periods	3	773.07	2.35	$0.1 > P > 0.05$
Animals	12	7045.94	21.45	$P < 0.005$
Residual	35	328.44		

TABLE 9

Stability of glucose absorption

Rates of glucose absorption, mg/cm/hr

PERIODS

Time after setting up intestine

	10-20 min	20-30 min	30-40 min	40-50 min
	1.5	1.5	1.4	1.4
	3.1	2.9	3.1	3.0
	4.2	4.0	4.0	3.1
	3.4	3.3	3.1	3.1
	5.1	4.4	5.7	5.6
	1.6	2.0	2.0	2.1
	3.6	3.6	3.6	3.6
	4.6	4.0	4.4	4.5
	4.3	4.4	3.9	4.0

Analysis of Variance

	Degrees of freedom	Mean square	Variance ratio (F)	
Periods	3	0.0433	0.4491	N.S.
Animals	8	5.6241	58.34	P < 0.005
Residual	24	0.0964		

periods following an initial 10-minute pre-period during which no collections were made. The results are shown in Tables 8 and 9 respectively, together with analyses of variance. The variance ratio tests demonstrate that there is no significant instability in either water or glucose absorption over this period of time, and that there is dramatically large inter-animal variation. A further analysis of variance showed that the variability was as great within days as between days (water absorption: $F = 1.181$, $n_1 = 1$, $n_2 = 3$. Glucose absorption: $F = 3.090$, $n_1 = 3$, $n_2 = 1$). Therefore environmental factors such as the weather are unlikely to be relevant as causes of experimental variability.

However, during the first hour or so the rate of glucose secretion onto the serosal surface of the intestine was not constant, but gradually increased. The reason for this lag will be detailed in Chapter VI in which a theoretical analysis and experiments which characterise this lag in secretion will be presented.

Rates of absorption etc. of glucose and of water

It has been known for many years that the rate of absorption of solutes from the intestinal lumen depends on their intra-luminal concentration. The relationship between absorption and luminal concentration for many solutes, including glucose, has been found to be saturable and formally similar to the kinetics derived by Michaelis & Menten (1913) for an enzyme catalysed reaction (e.g. Fisher & Parsons, 1953; Crane, Forstner & Eicholz, 1965). This finding has been one of the pieces of evidence in favour of carrier-mediated transport across the mucosa. It would be useful to compare the Michaelis-Menten parameters for this preparation with those obtained by earlier authors, especially since Crane et al (1965) have interpreted their values of K in terms of the sodium-

TABLE 10

Summary of rates of absorption of water & glucose,
also of mean rates of secretion & utilisation of glucose

Glucose concentration in perfusate	Mean \pm S.E. (number of intestines)			
	Rate of water absorption	Rate of glucose absorption	Rate of glucose secretion	Rate of glucose utilisation
mg/ml	μ l/cm/hr	mg/cm/hr	mg/cm/hr	mg/cm/hr
5.0	168.8 \pm 4.4 (57)	3.17 \pm 0.10 (57)	2.10 \pm 0.07 (52)	1.19 \pm 0.07 (52)
2.8	174.3 \pm 11.7 (9)	2.35 \pm 0.23 (9)		
2.0	179.4 \pm 14.0 (9)	1.88 \pm 0.23 (9)		
1.5	130.2 \pm 3.7 (17)	1.71 \pm 0.11 (14)	1.04 \pm 0.09 (17)	0.82 \pm 0.12 (13)

Note: These rates refer to 40 cm segments of jejunum and upper ileum.

1/rate of glucose absorption, cm.hr.mg⁻¹

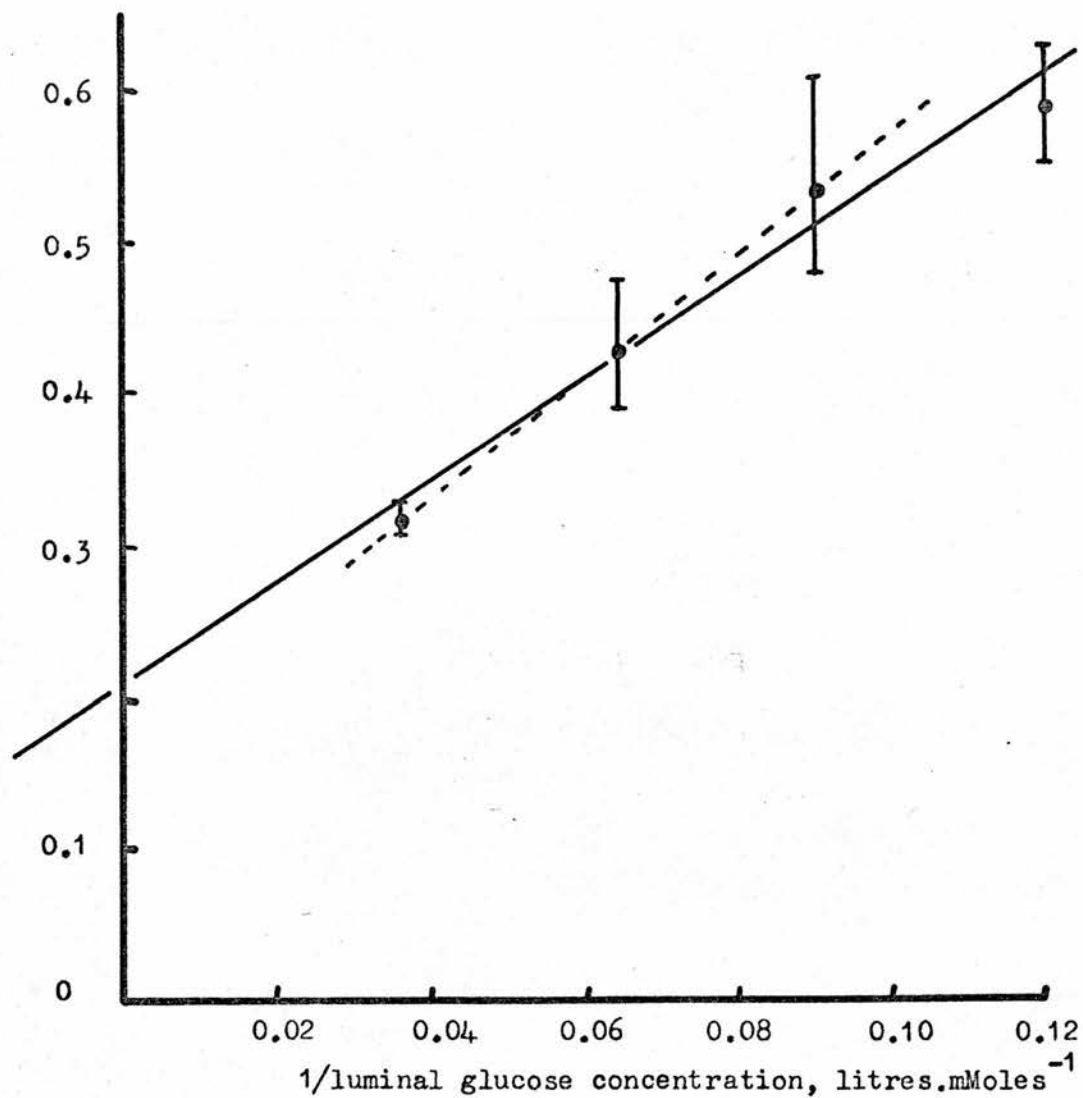


FIGURE 17

Lineweaver-Burk plot for glucose absorption

The continuous line was calculated by the method of least squares. The broken line has been drawn through the lower 3 points.

dependent affinity of a carrier system for glucose. Also, the V term gives an estimate of the maximum rate of glucose absorption from the lumen, assuming that the apparently hyperbolic relationship still holds at high glucose concentrations. Some of the difficulties of interpreting the K values will be discussed at the end of this Chapter.

Table 10 shows a summary of mean rates of absorption of glucose and of water, also of mean rates of secretion and utilisation of glucose at the steady-state, corresponding to several luminal concentrations of glucose. It must be stressed that these rates are mean values expressed in terms of unit length of intestine for segments of jejunum and upper ileum of about 4.0 cm length. It is now widely accepted that the rate per unit length depends on the length of the segment and the region from which it was taken.

Figure 17 shows these data plotted on an unweighted double reciprocal plot according to Lineweaver & Burk (1934). The error bars for $1/v$ have been drawn by calculating $1/(v + S.E.)$ and $1/(v - S.E.)$ where S.E. is the standard error of the mean absorption rate given in Table 10. The poor fit appears to be attributable to the last data point, as the broken line shows.

Estimates were made of the parameters K (the concentration for half-saturation), and V (the apparent maximum rate of absorption) in the Michaelis-Menten type of relation:-

$$v = \frac{V \cdot S}{K + S} \quad \text{where } v \text{ is the rate of absorption,}$$

and S is the luminal glucose concentration.

The method was that of Wilkinson (1961) which fits a rectangular hyperbola directly to the data by an iterative process. Initial estimates of the parameters are first obtained from the first-order regression of S/v on S with the data weighted by (S^2/v^4) . This calculation was executed on the Olivetti Programma P101.

TABLE 11

Estimates of the Michaelis-Menten parameters relating glucose absorption rate to luminal glucose concentration

These were calculated from the data given in Table 10 using the method of Wilkinson (1961)

$$v = \frac{V \cdot S}{K + S} \quad \text{where } v \text{ is rate of glucose absorption from lumen,}$$

S is glucose concentration in lumen.

	K(mM)	V(mg/cm/hr)
Taking S as concn in fluid entering lumen (S_{in})	19 ± 3.3	5.30 ± 0.488
Taking S as concn in fluid leaving lumen (S_{out})	14 ± 1.3	4.80 ± 0.225

As Sladen (1968) has pointed out, a rigorous treatment was not possible, since the glucose concentration (S) changes down the length of the intestine as glucose and water are abstracted. Therefore each calculation was made twice, first taking S as the concentration of glucose in the perfusate pumped into the intestinal lumen (S_{in}), and then taking S as the mean concentration in the lumen effluent (S_{out}). The results are in Table 11, together with estimates for the standard deviations of K and V . However, as in any calculation involving non-linear statistics, these standard deviations are not necessarily reliable.

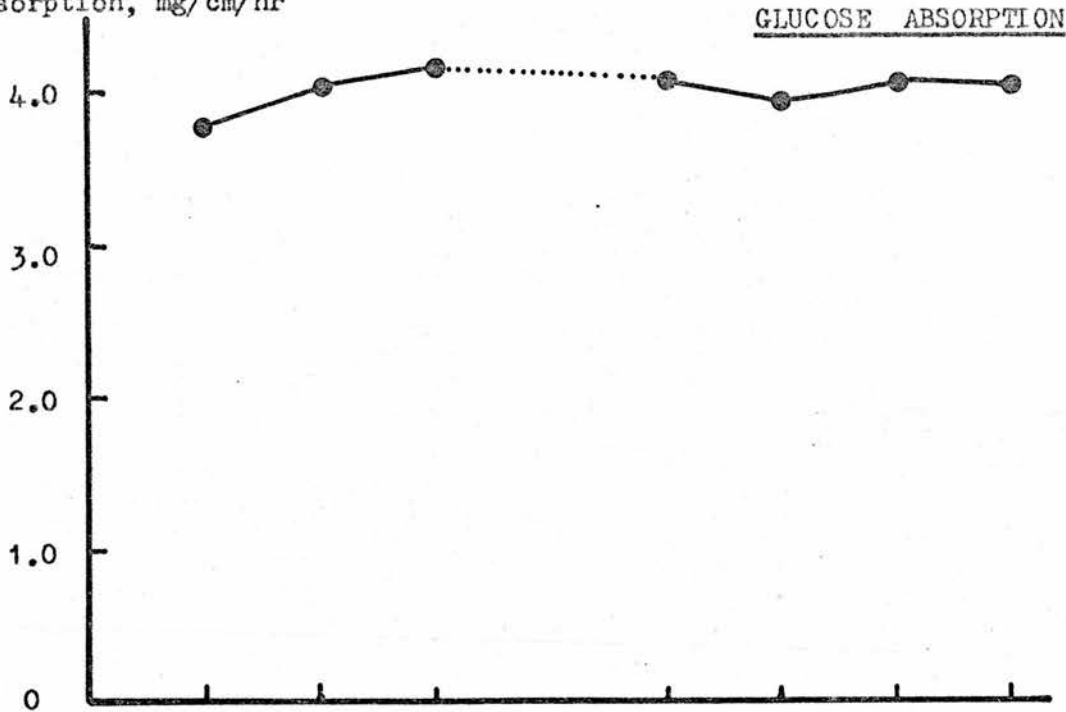
Clearly, the K and V values are slightly affected by whether S_{in} or S_{out} values were used in the calculation. The real values of K and V will be intermediate between those given by S_{in} and S_{out} . The estimated standard deviations are relatively small when one considers the poorness of fit to the double reciprocal plot in Figure 17.

Effect of doubling the magnesium concentration in the perfusate

The concentration in the perfusion media has routinely been one quarter of that in Krebs & Henseleit (1932) medium, instead of one half as is generally used in allowance for binding to plasma proteins (Greene & Power, 1931). To test whether this discrepancy has affected the properties of this preparation two experiments were made in which a normal perfusate was replaced by one which contained twice the concentration of magnesium.

The intestinal segments were set up and perfused as normal for fifteen minutes, then 3 collections over consecutive five minute periods were made of lumen effluent and of secretion. The perfusate was replaced by the high-Mg one and after a five minute pre-period four further collections were made. The results are shown in

Rate of glucose
absorption, mg/cm/hr



Rate of water
absorption, μ l/cm/hr

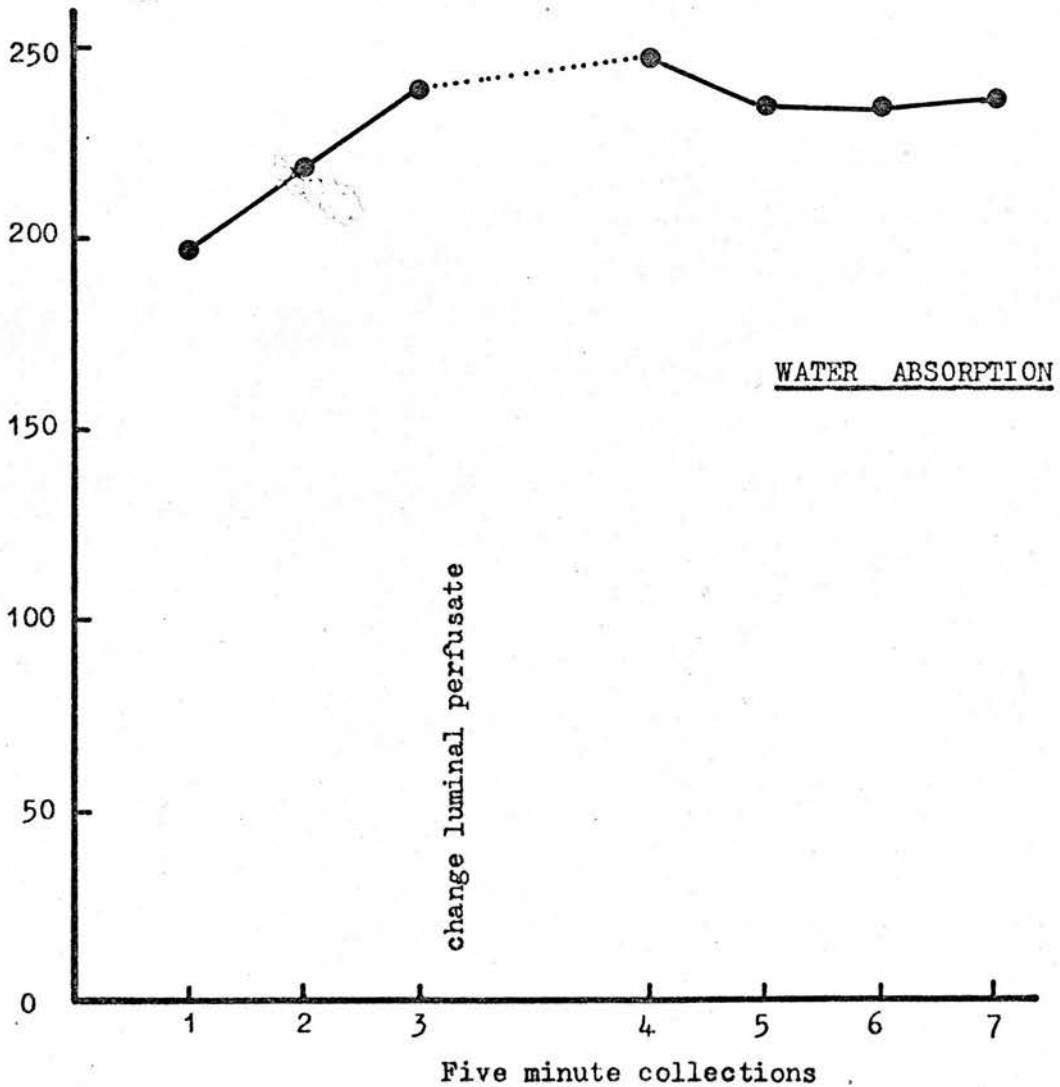


FIGURE 18

Effect of doubling the luminal magnesium concentration (Two experiments)

Figure 18 and no effect on glucose or water absorption can be attributed to the change in luminal magnesium concentration.

In one single experiment it appeared that complete removal of the magnesium from the perfusate did not affect glucose or water absorption. A similar result was obtained by J. J. Pratt (personal communication), and it is worth extending this observation, especially in view of the requirement for magnesium by certain enzymes notably the Na-K-stimulated ATPases.

Effect of pancreatin on glucose absorption

The possibility was considered that these absorption rates in the intestine in vitro may be low compared with the rates in the intact animal.*

When a segment of the intestine of an anaesthetised animal was perfused in situ through its lumen, the rates of absorption were indistinguishable from those in the present investigation (M. L. G. Gardner, unpublished results). The mean rates (\pm S.E.) from the preparation in vivo perfused with glucose (5 mg/ml) were:-

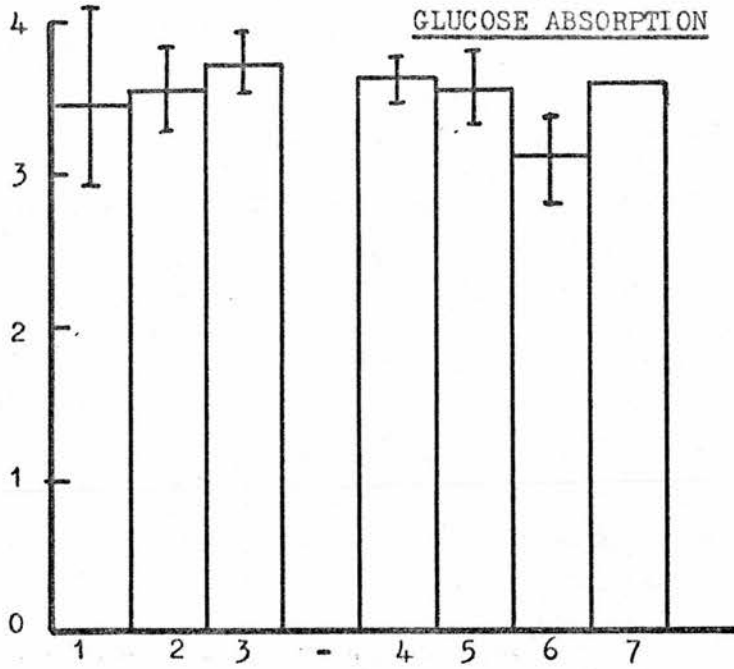
glucose absorption	3.10 ± 0.145 (23) mg/cm/hr
water absorption	174 ± 13.0 (23) μ l/cm/hr

Therefore any discrepancy between the absorption rates in the perfused preparation and those in the intact animal can be attributed neither to the removal of the segment of intestine from the animal nor to the absence of blood flow. In the experiments in vivo, the lower duodenum was ligated oral to the point of cannulation. The possibility therefore exists that tying off the lower duodenum excludes from the lumen of the aboral segment some agent which stimulates intestinal absorption. Biliary, duodenal, gastric or pancreatic secretions might contain this agent.

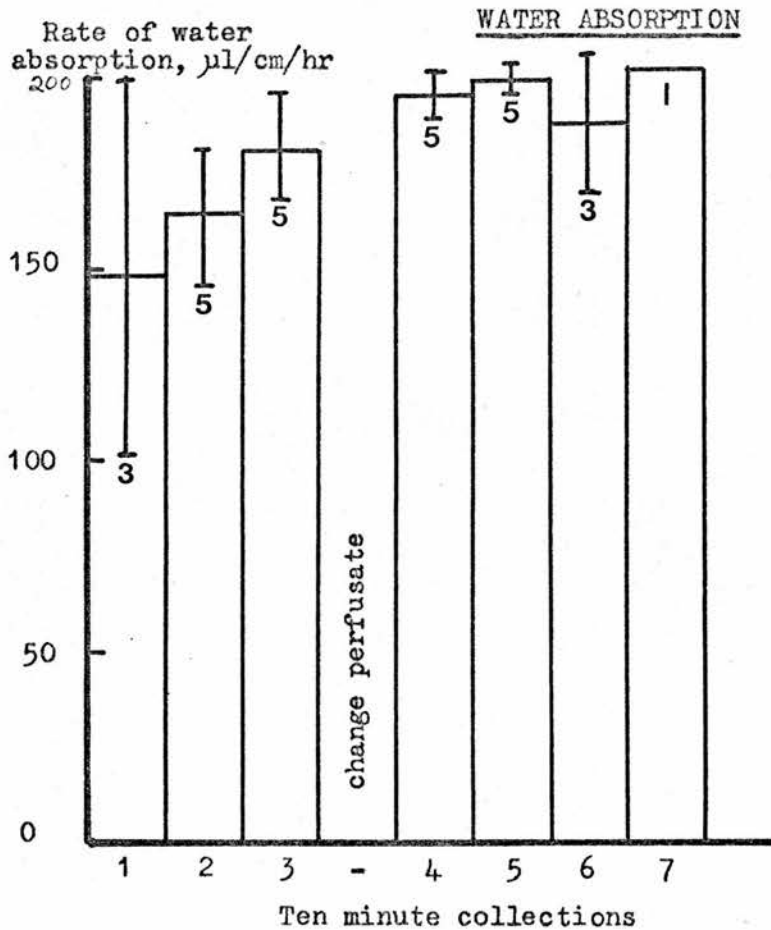
* Fisher & Parsons (1950, p.292) quote data from Westenbrink to the effect that the rate of absorption of glucose from a 50% solution is about 4 mg/cm/hr in the intact rat.

FIGURE 19

Rate of glucose absorption, mg/cm/hr



Rate of water absorption, $\mu\text{l/cm/hr}$



Effect of crude pancreatin on glucose and water absorption

A normal perfusate was replaced by one containing pancreatin (5 mg/ml). Vertical bars show S.E.M. Number of experiments shown in water absorption blocks.

Although close attention has not been directed to maximising rates of glucose absorption, a series of experiments was made to test whether pancreatic juice might affect the absorption of glucose.

Five segments of intestine were set up for perfusion. Control measurements were made over three consecutive periods of ten minutes each. Then the perfusate was replaced by one to which crude pancreatin (5 mg/ml: supplied by British Drug Houses Ltd.) had been added. Further measurements were made over 10 minute periods, and the results are shown in Figure 19. Clearly there has been no significant effect attributable to the pancreatin.

Potential interference by phenol red

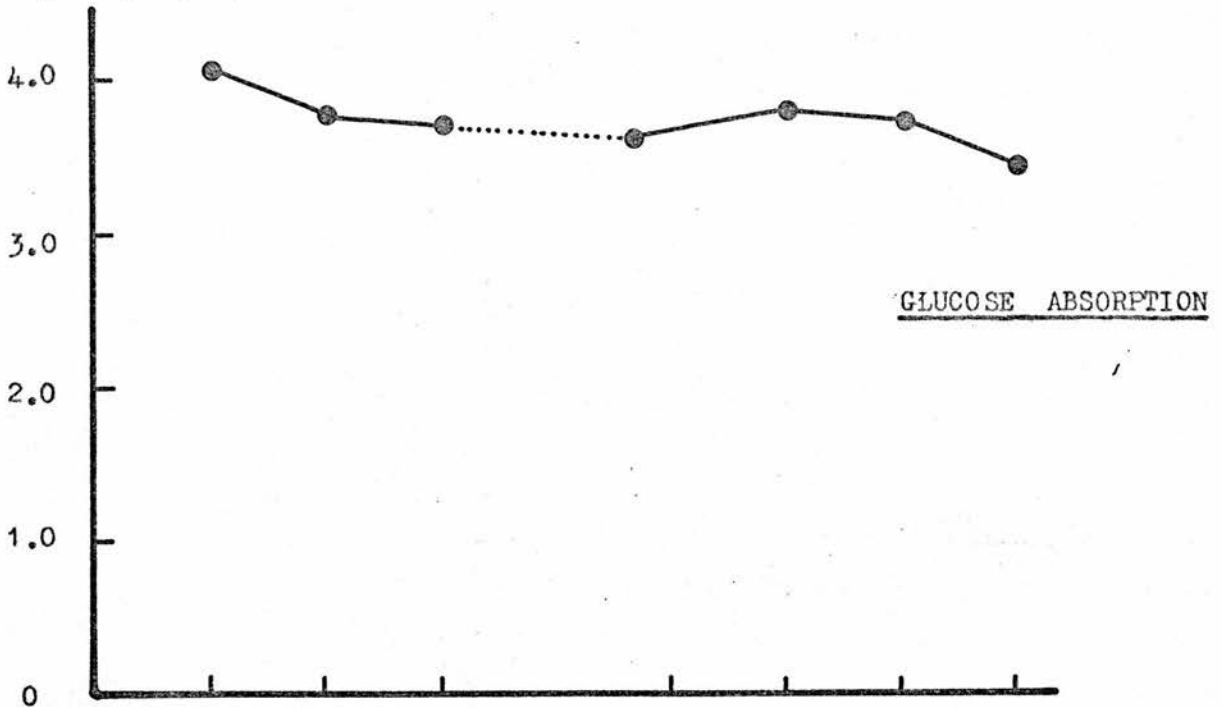
A number of authors have included phenol red (sulphonphenolphthalein) in luminal perfusates, either as a so-called 'unabsorbable marker' for measurement of water absorption (e.g. Summers & Schedl, 1968) or as an indicator of a leak across the mucosa (e.g. Fisher & Parsons, 1949). In all the present work it has been routinely included at a concentration of 50 $\mu\text{g}/\text{ml}$ as an indicator of leaks.

To test whether this presence of phenol red in the lumen might affect the properties of the preparation two experiments were made. An intestine was set up and perfused with a medium which contained no phenol red. After a 15 minute pre-period, three consecutive collections each over five minutes were made of lumen effluent and secretion. The perfusate was then replaced by a normal one containing phenol red (50 $\mu\text{g}/\text{ml}$). After a five minute pre-period, four further collections were made.

Figure 20 shows the mean rates of water and glucose absorption from these two intestines. No effect of phenol red on either glucose or water absorption can be detected. This contrasts with



Rate of glucose absorption, mg/cm/hr



Rate of water absorption, μ l/cm/hr

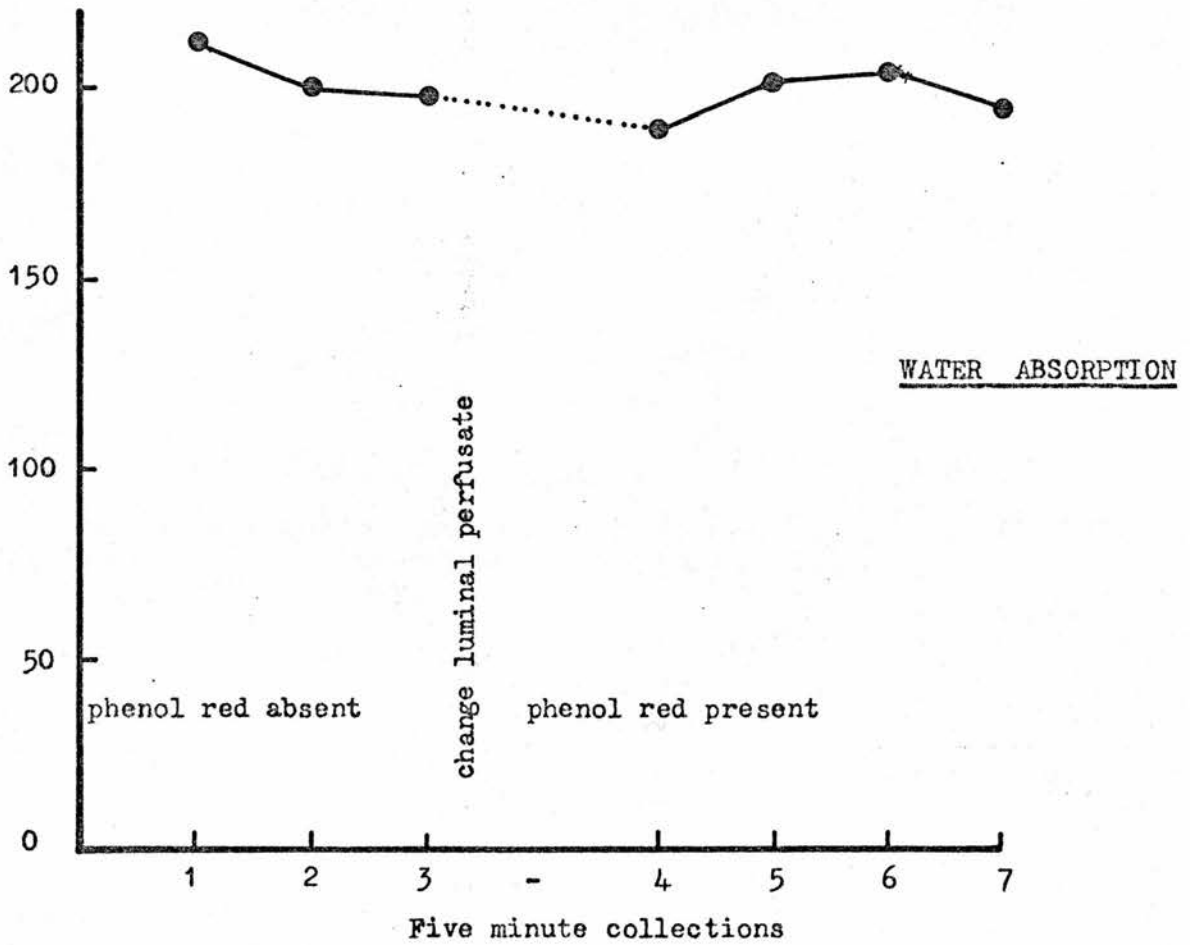


FIGURE 20

Absorption of glucose and water in the presence and absence of phenol red (50
(mean of two experiments)

the known effects of the un-sulphonated compound, phenolphthalein, (Hart & McCall, 1967; Adamic & Bihler, 1967; Hand, Sanford & Smyth, 1966).

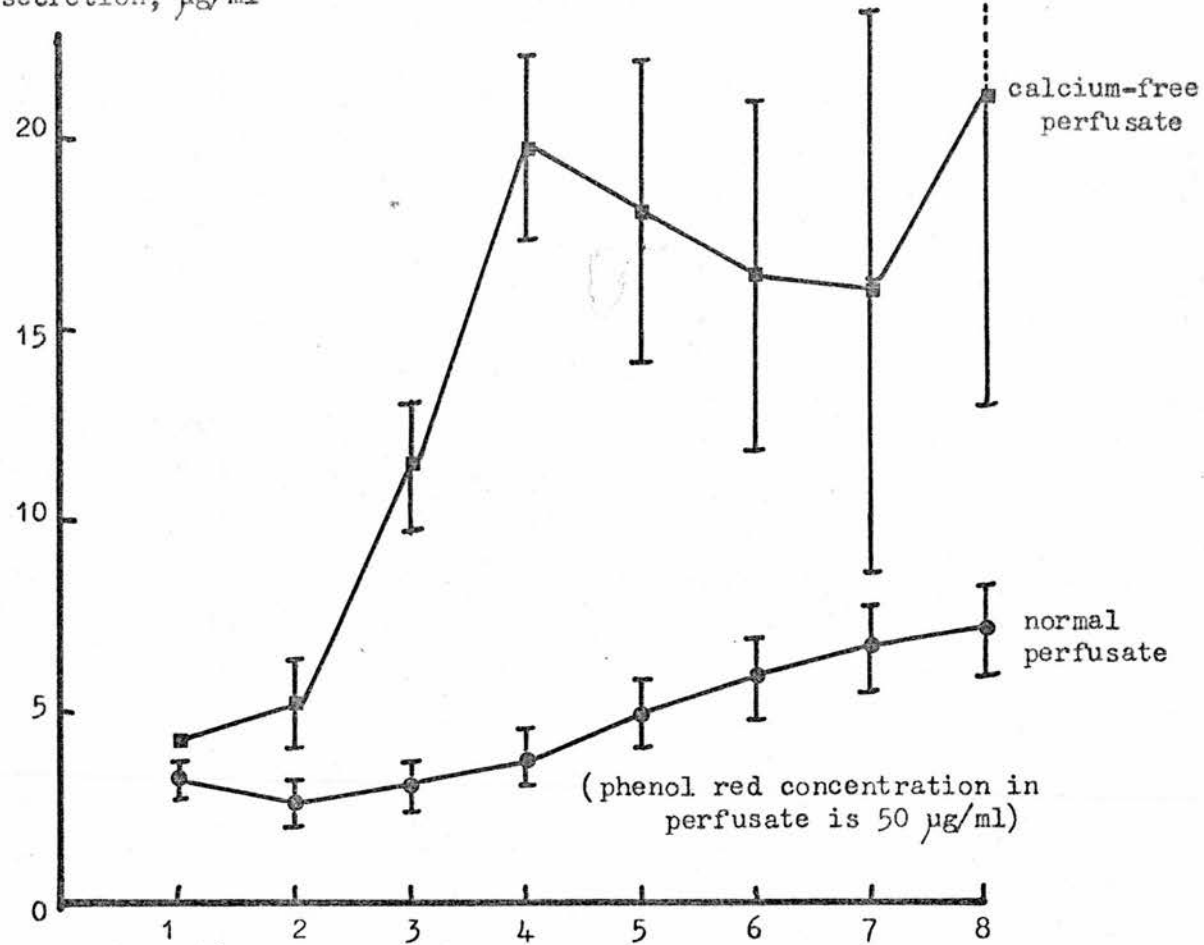
Effect of a calcium-free perfusate on water absorption

Tidball and his co-workers have suggested that calcium and magnesium ions may play a role in regulating the permeability of the intestine to water and to phenol red (Tidball, 1964; Tidball, Cassidy, Hantsoo & Thomas, 1966). From electron micrographs they further proposed that changes at the 'tight junctions' between cells might be involved (Cassidy, Goldner & Tidball, 1965).

It has already been stated that the concentration of phenol red in the secretion is much less than that in the lumen, and it has been suggested that the appearance of phenol red in the secretion may reflect, at least in part, a 'leak' across the mucosa. Certainly if the secretion in the first few minutes of any experiment contains a noticeable amount of phenol red then it is probable that the intestine has been damaged in the course of preparation: the regions at each cannula are particularly susceptible to such damage. However the reason for the gradual increase in the rate of appearance of phenol red in the secretion is not known; this may reflect a gradual deterioration in the viability of the intestine. It will be shown below that this concentration of phenol red is very low relative to the luminal concentration ($50 \mu\text{g/ml}$) during the first hour or two of perfusion.

Therefore experiments were made on the rate of water secretion and the appearance of phenol red in the secretion over a period of two hours when the perfusate was normal, and when it contained no calcium. Additionally, single experiments were made with a glucose-free and sodium-free perfusate respectively, but no phenol red

Concentration of phenol red in secretion, $\mu\text{g/ml}$



Rate of water absorption, $\mu\text{l/cm/hr}$

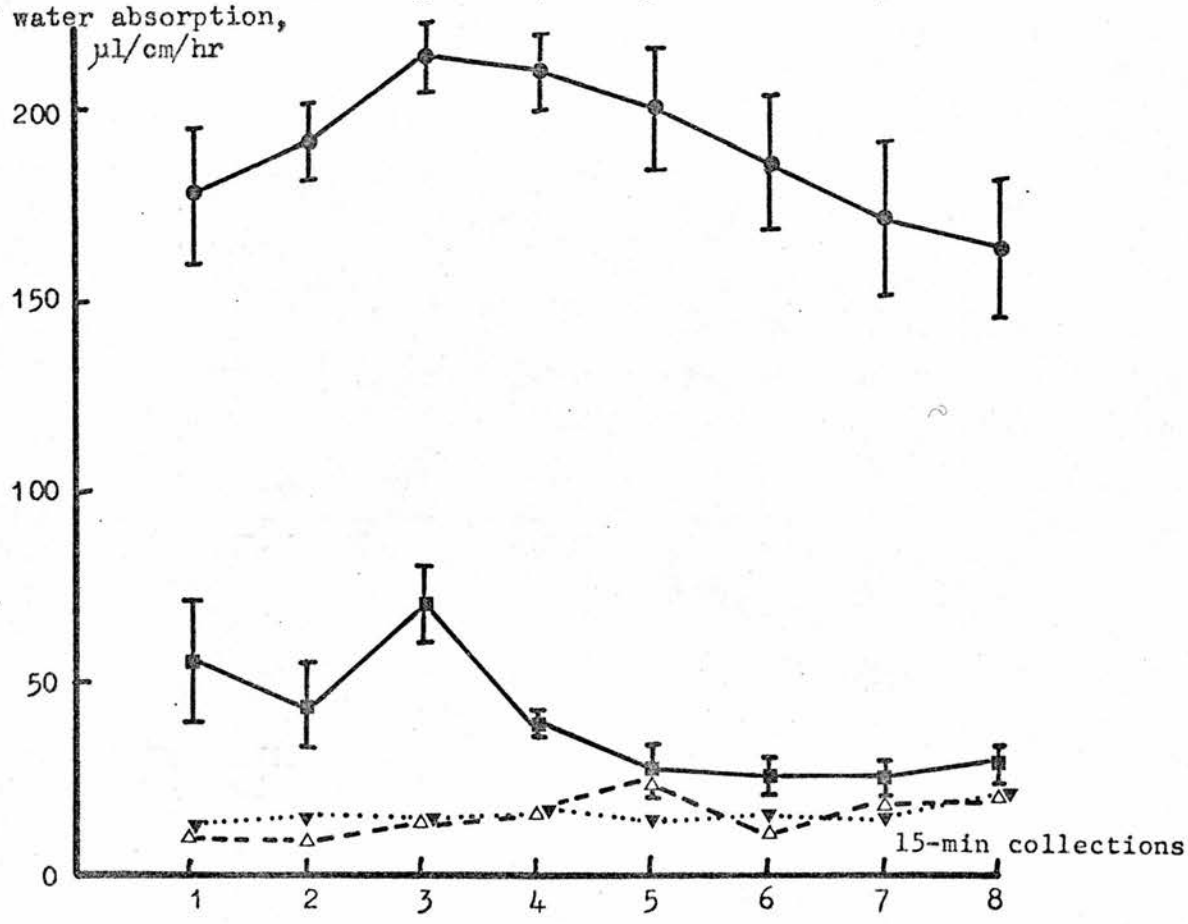


FIGURE 21

Water absorption rate and phenol red concentration in secretion when the perfusate was normal (4 expts \odot), and when it contained no calcium (3 expts \blacksquare), no glucose (2 or no sodium (1 expt \blacktriangledown) (vertical bars show S.E.M.)

estimations were made owing to the small volumes of secretion. In the latter case the sodium salts were iso-osmotically replaced by the corresponding salts of choline.

Phenol red was estimated by diluting the secretion with 0.1 N sodium hydroxide and measuring the extinction at 560 nm. A calibration curve was constructed by measuring the extinction of standard solutions containing phenol red. No allowance was made for protein error or other analytical interferences, since J. J. Pratt (personal communication) has suggested that under these conditions any interferences are likely to be trivial. However these results should be regarded as only approximate, but they give qualitatively useful information.

Figure 21 shows the results. Although the rate of water secretion was not as stable as normal during the four control experiments it was clearly greater than during the calcium-free perfusion (3 experiments). Further, the concentration of phenol red in the secretion, even although it progressively increased during the two hours was much less in the control experiments than when the calcium was absent. Thus, the actual rate of phenol red secretion was similar in the two types of experiments. When the perfusate lacked either sodium or glucose the rates of water secretion were, as in the absence of the calcium, only about 10% of the 'normal' rates.

These results are consistent with the notion that the 'normal' water transported consists of two components: one which has leaked through pores or imperfections in the mucosa and a second which depends on metabolic energy. The phenol red might move via the former, but not the latter, route. However it will be difficult to test this idea. Fisher & Parsons (1949) and Smyth & Taylor (1957) both noted that the rate of water absorption was almost

independent of the intra-luminal distension pressure. Therefore it might be useful to examine whether distension pressure affects the rate of water absorption in the absence of calcium, sodium or glucose. Experiments with other supposedly unabsorbed markers would also be interesting.

To facilitate identification of the two ends of a perfused segment once it has been removed from the perfusion apparatus, the two cannulae have been labelled recently. It has been noted occasionally that at the end of a perfusion experiment the lower (ileal) part of the segment is more heavily stained red than the upper part. The significance of this is yet unknown; it would be interesting to compare rates of phenol red secretion from jejunal and ileal segments.

Properties of a Segmented Flow

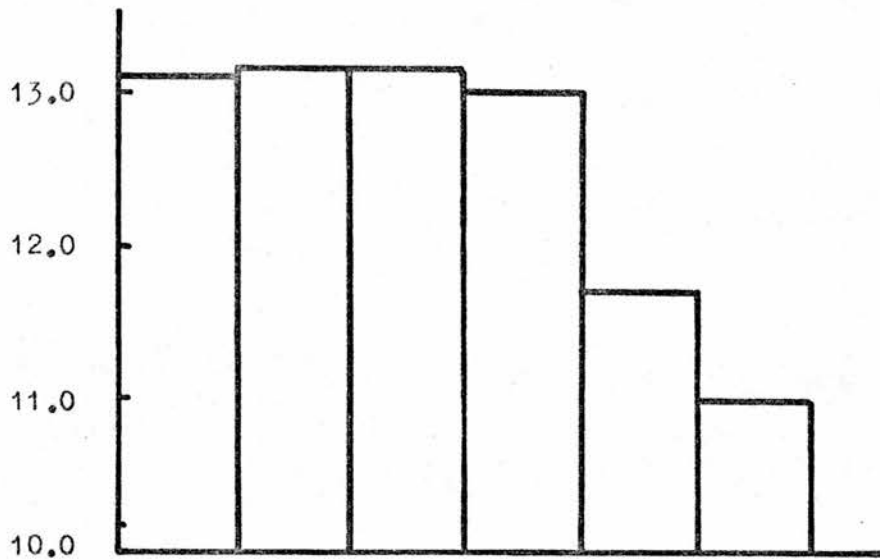
The use of a segmented flow through the lumen - i.e. a sequence of slugs of perfusate separated by bubbles of 95% oxygen (Figure 2) - was introduced as a means of supplying adequate oxygen to the tissue even at low rates of lumen perfusion. However, additional advantages accrue and these will now be discussed.

Replacement of segmented flow by all-liquid flow

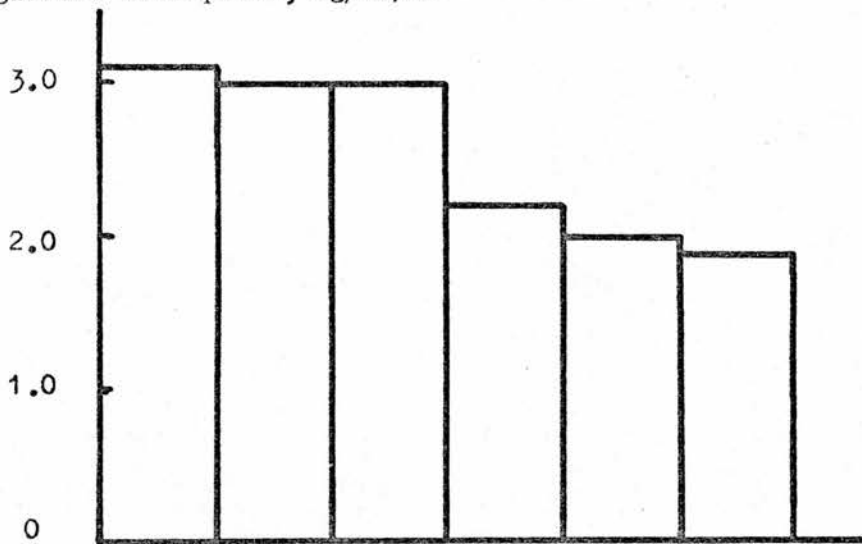
Experiments were made in order to examine the role played by the bubbles of oxygen in the segmented flow, and to test the assertion that the rate of luminal perfusion has to be in excess of at least 8 ml/minute if adequate oxygen is to be supplied from solution.

Segments of intestine were perfused with a normal perfusate containing glucose (5 mg/ml). After a 50-minute pre-period, three serial collections each lasting 10 minutes were made of lumen effluent and of secretion. The oxygen supply to the pump (pump B in Figure 4) was then disconnected and replaced by a second tube

Glucose concentration in secretion, mg/ml



Rate of glucose absorption, mg/cm/hr



Rate of water absorption, μ l/cm/hr

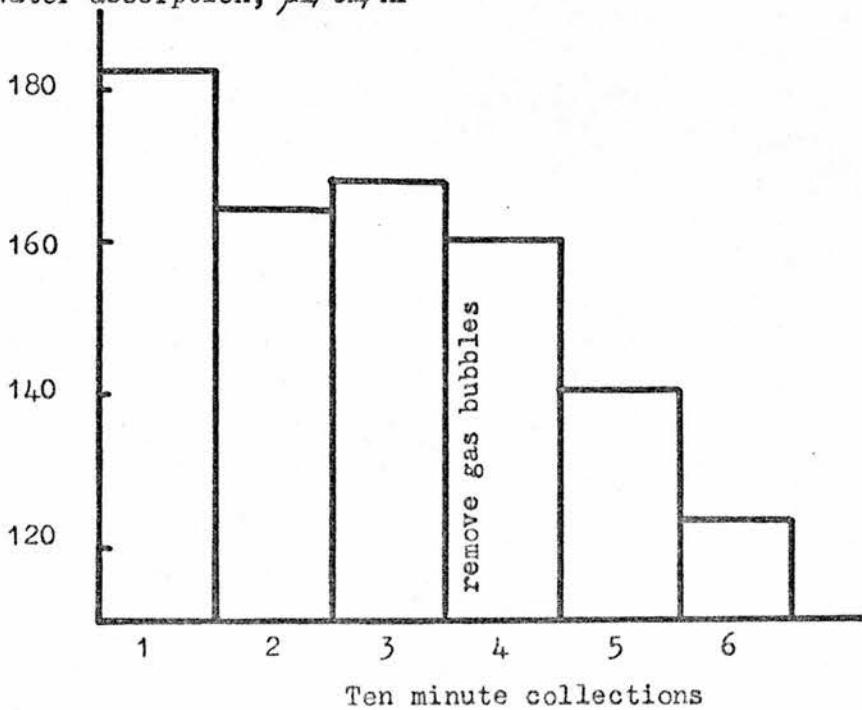


FIGURE 22

Effect of replacement of segmented flow by all-liquid flow

withdrawing perfusate from the reservoir. Three further collections were made.

The results, shown in Figure 22, demonstrate clearly that the removal of the segmented flow rapidly produces a marked deterioration in the rates of water and glucose absorption.

The stirring effect of a segmented flow

At low rates of flow, a continuous stream of liquid exhibits laminar flow. However, when such a stream is segmented with bubbles of gas, the flow may become turbulent. Indeed, the success of the Technicon AutoAnalyzer system must depend on this phenomenon to ensure complete mixing of the reaction mixtures as much as for the prevention of interaction between successive samples.

When a segmented flow is pumped through the intestinal lumen, one would expect that the perfusate would be effectively stirred, so that the solute concentration at the mucosal surface where absorption occurs would be little less than that in the axial stream of perfusate. The following experiment demonstrates the stirring effect of a segmented flow - Figure 23 and Plates 1-4.

Whole blood is sucked up into a clean glass vertical tube of about 3 mm internal diameter, and is allowed to drain out leaving a thin, but visible, film of erythrocytes adhering to the glass surface. The bottom of the tube is wiped clean, and an isotonic saline (so as not to haemolyse the cells) is gently sucked up into the tube. When the saline is allowed to drain slowly from the tube a red 'spike' of erythrocytes appears in the axis of the saline column, and this axial spike moves downwards faster than does the saline/air interface (Figure 23A, Plate 1). The effect is as though the meniscus were scraping the cells from the walls of the tube and passing them into the fast moving axial stream.

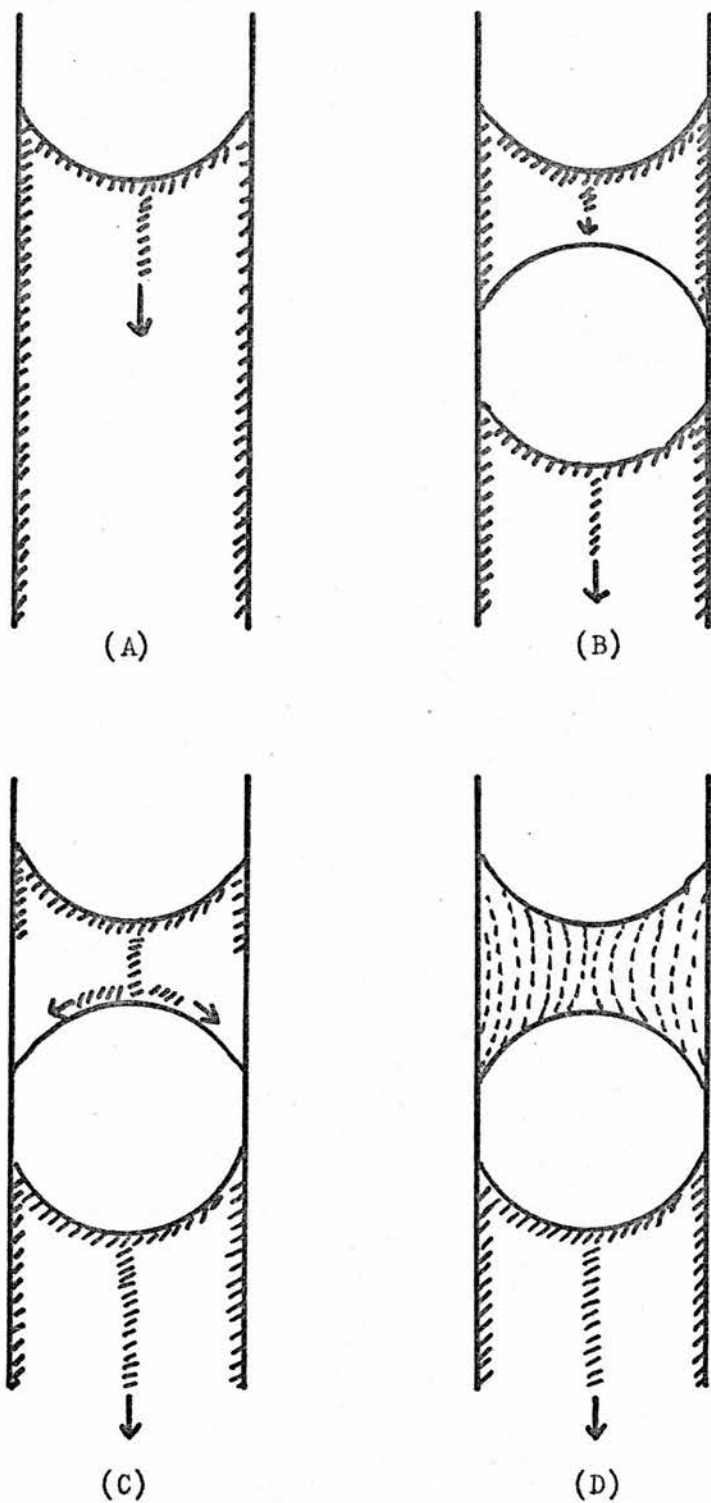
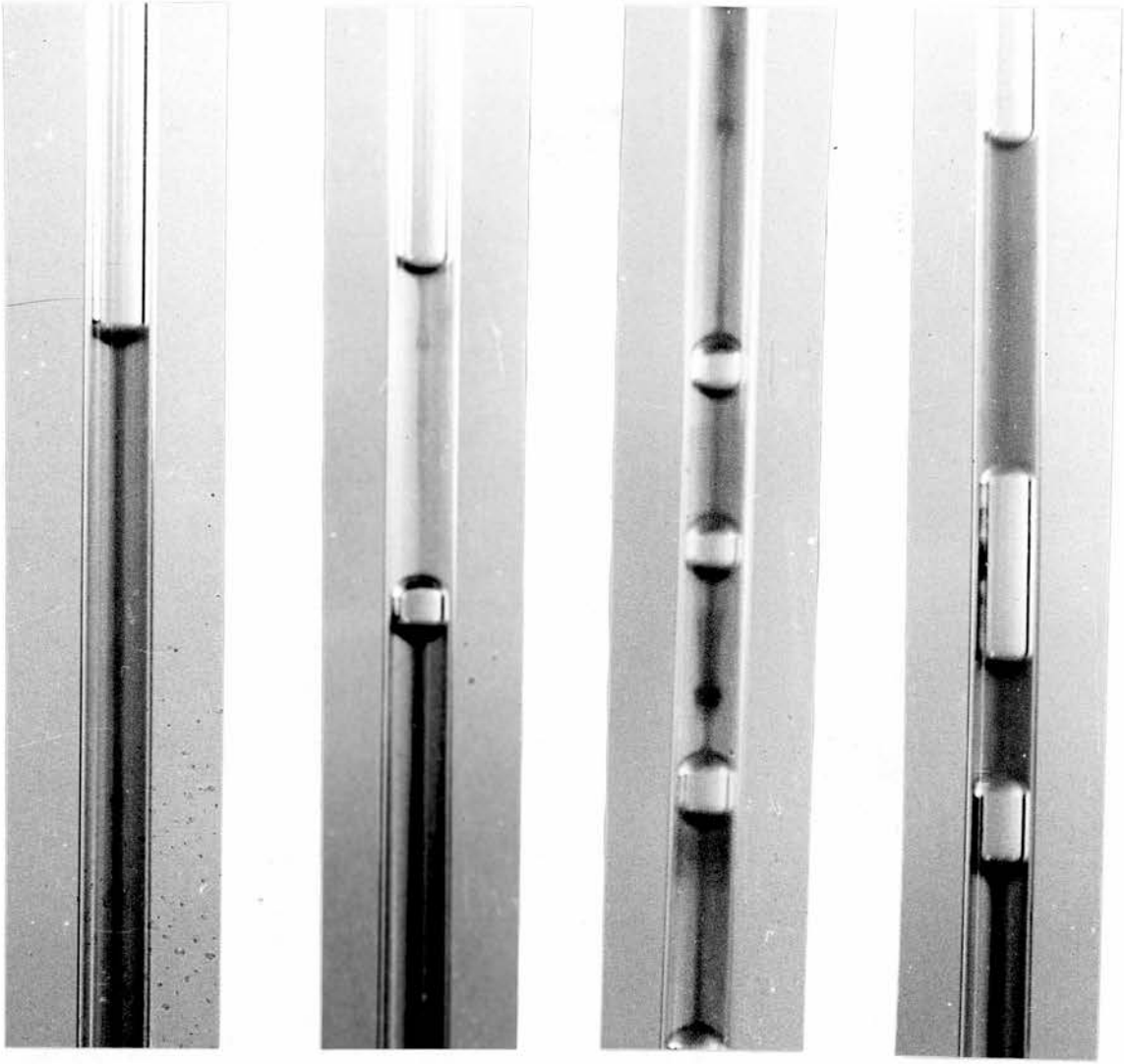


FIGURE 23

A hydrodynamic model which illustrates the stirring effect of a segmented flow



1

2

3

4

PLATES 1 - 4

An experiment which illustrates the stirring effect of
a segmented flow
(see text)

The experiment is repeated, but with an air bubble introduced through a fine needle or tube into the saline column some 1-2 cm from the top. In this instance, the red axial spike which develops at the uppermost saline/air meniscus is dispersed to the walls of the tube and recirculated once it reaches the meniscus at the lower end of that slug of liquid (Figure 23 B & C, Plates 2 & 3). At all but very low rates of flow, the effect is that the upper slug of liquid above the air bubble rapidly comes to contain an apparently uniform suspension of erythrocytes (Figure 23D, Plate 4) - that is, it behaves as though it were well stirred. However, the saline below the bubble remains clear except for the red axial spike; it is apparently unstirred.

Thus, the hydrodynamic effect of the insertion of gas bubbles into the stream of perfusate should be two-fold:-

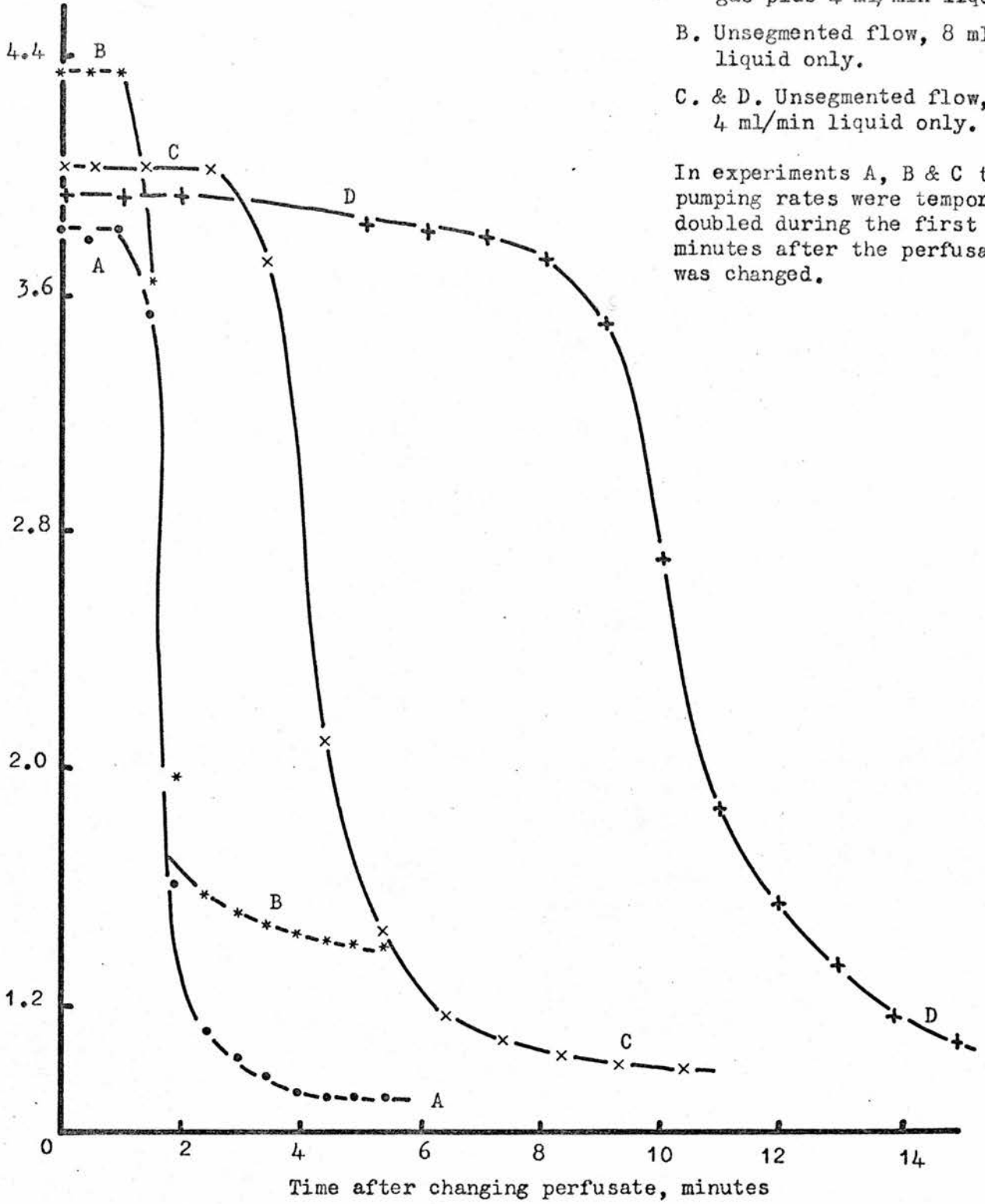
- (i) to maintain a nearly uniform concentration across the radius of the tube, and
- (ii) to sweep off the boundary layer more efficiently than does laminar flow.

Similar phenomena have been claimed to operate in 'bolus flow' through capillary blood vessels (Prothero & Burton, 1961). These authors suggested that slugs of plasma separated by single erythrocytes in narrow capillaries will be subjected to stirring effects.

The rate of attainment of a steady-state in the lumen

One of the valuable features of this intestinal preparation is that it allows one to compare the effects of two or more treatments, such as changes in the composition of the lumen perfusate, in one and the same segment of intestine. Thus each experimental intestine can serve as its own control; this is particularly advantageous in view of the large inter-experiment variance which was manifest in Tables 8 and 9. However, it is highly desirable that, when the

Glucose concentration in luminal effluent, mg/ml



A. Segmented flow, 4 ml/min gas plus 4 ml/min liquid.
 B. Unsegmented flow, 8 ml/min liquid only.
 C. & D. Unsegmented flow, 4 ml/min liquid only.
 In experiments A, B & C the pumping rates were temporarily doubled during the first two minutes after the perfusate was changed.

FIGURE 24

The time-course of a change in luminal conditions

At zero time the luminal perfusate which contained 5 mg/ml glucose was replaced by one containing 1.5 mg/ml glucose. The concentration of glucose in the luminal effluent was estimated at intervals of 0.5 or 1 minute.

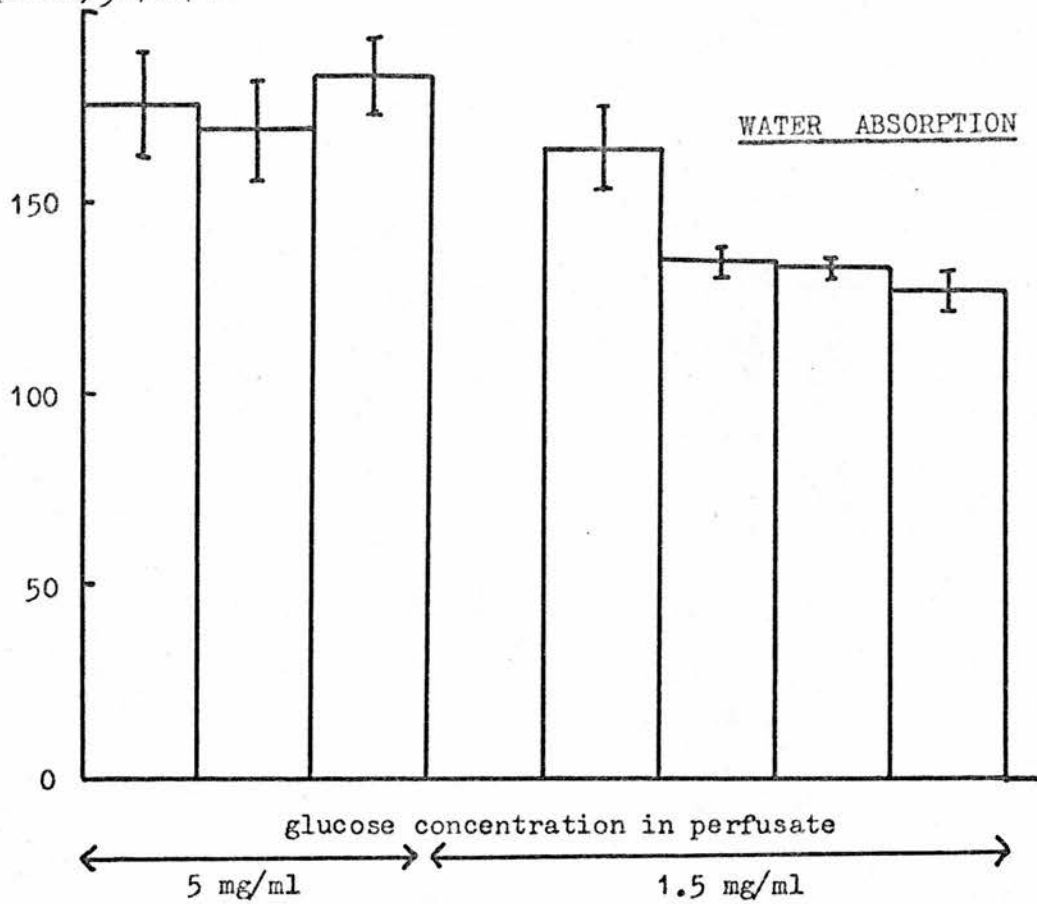
composition of the luminal perfusate is changed, the new steady-state in the lumen be achieved in as short an interval as possible. The experiments described below were made to study the time course of the attainment of this new steady-state when the concentration of glucose in the perfusate is suddenly changed.

A polypropylene 3-way stopcock (type K-75, made by Pharmaseal Laboratories, Glendale, California) selects the reservoir from which perfusate is to be pumped. When the perfusate is to be changed, the tap is operated and the rate of pumping is temporarily doubled to 8 ml/minute for the first two minutes after the changeover.

After a segment of intestine had been perfused for about 30 minutes, a collection of lumen effluent was made. The perfusate containing 5 mg/ml glucose was replaced by one which contained 1.5 mg/ml glucose, and collections were made of lumen effluent at half minute intervals. The glucose concentration in the effluent was estimated, and the time-course of its change is shown in Figure 24A. The perfusion rate was 4 ml/minute liquid, and 4 ml/minute gas. The experiment was then repeated, but with an unsegmented flow of liquid only at 8 ml/minute (Figure 24B) and at 4 ml/minute (Figure 24C). Finally, the experiment was repeated with the unsegmented flow at 4 ml/minute, but this time the normal routine of temporarily increasing the pump speed during the first two minutes after the perfusate changeover was not followed (Figure 24D).

Under the conditions normally employed - i.e. segmented flow of 4 ml/minute liquid plus 4 ml/minute gas and temporarily doubled - the change in the lumen concentration was complete within less than five minutes (Figure 24A). With the unsegmented flow of 4 ml/minute, the change was not complete in 10 minutes, although when the perfusion rate was 8 ml/minute the time was again less than five minutes.

Rate of water
absorption, $\mu\text{l}/\text{cm}/\text{hr}$



Rate of glucose
absorption, $\text{mg}/\text{cm}/\text{hr}$

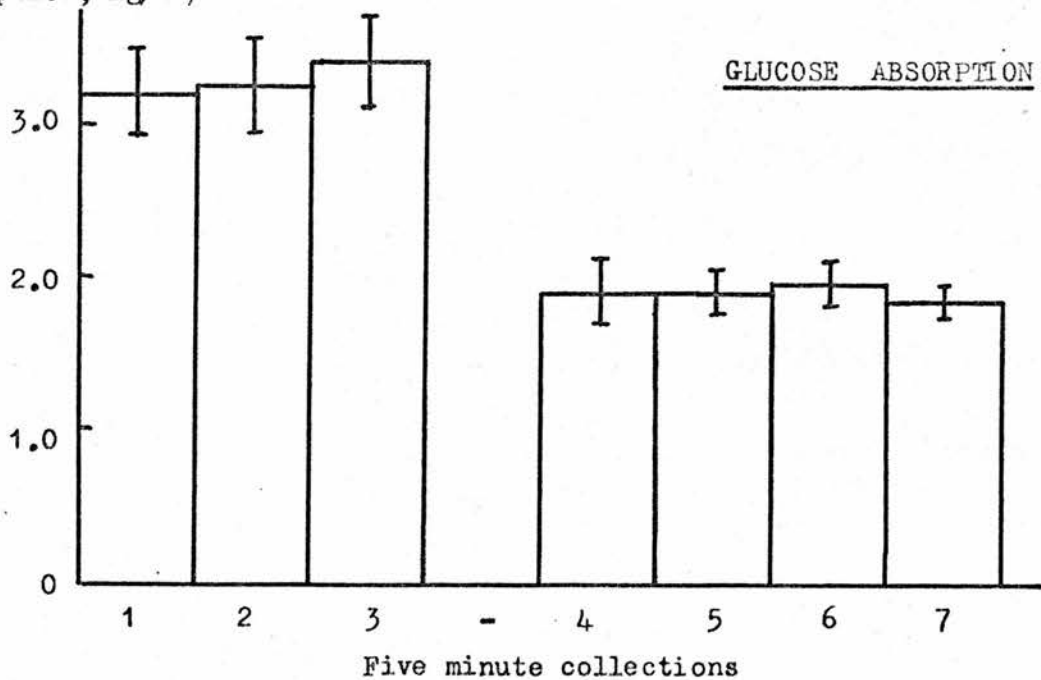


FIGURE 25

Absorption of glucose and water when the concentration of glucose in the perfusate is abruptly changed from 5 mg/ml to 1.5 mg/ml. (Mean of 8 experiments. The vertical bars indicate the S.E.M.)

Therefore the inclusion of the oxygen bubbles in the perfusate, by doubling the linear flow rate through the intestinal lumen, favours a rapid change in conditions. This would otherwise only be possible at increased rates of liquid flow which would obviously be at the expense of accuracy in measurement of absorption rate. Additionally, the procedure of temporarily doubling the perfusion rate for two minutes at the time of changing the perfusates is clearly worthwhile - compare Figures 24C and D.

These results further indicate that not only do the hydrodynamic properties of the system favour a rapid change in the lumen steady-state, but also that any change in the rate of absorption of glucose from the lumen when the luminal concentration of glucose is changed must itself be rapid. Consequently, measurements were made of the rates of absorption of glucose immediately before and five minutes after a change in lumen glucose concentration. Figure 25 represents the mean of results from eight such experiments where the two perfusates contained 5 mg/ml and 1.5 mg/ml glucose respectively; this clearly confirms that the rate of glucose absorption does indeed change, and reaches its new steady-state within about 5 minutes of a change in luminal glucose concentration. The corresponding change in water absorption is also quite rapid, but not so fast as the change in glucose absorption.

However, the corresponding change in the measured rate of glucose secretion onto the serosal surface of the intestine is less rapid when the luminal glucose concentration is changed: instead there is a lag of somewhat more than an hour in the attainment of the new steady-state. This will presently be discussed in some detail (see Chapter VI).

Effect of hypoxia during the setting-up of the intestine

In the discussion of several types of preparation of surviving intestine, the need has been stressed for continuous oxygenation of the tissue even while the organ is being removed from the animal. Consequently, preparations such as everted sacs or tissue accumulation slices which make this impossible have been adjudged as unsuitable for reliable studies on absorption and metabolism.

Fisher & Parsons (1949) suggested that one of the prime reasons for the singular success of their preparation lay in the stringent precautions which they adopted to ensure that the intestine was never deprived, even momentarily, of oxygen. They never interrupted the blood supply to the experimental segment until after the fast flow of oxygen-saturated perfusate had been established through the lumen. However, they gave no quantitative evidence to prove that this procedure was obligatory.

Robinson used a tissue accumulation technique to study amino-acid accumulation in the rat. He found that if the tissue was removed from a rat which had been dead for 15 minutes the accumulation of L-proline was only some 25% of that when the tissue was removed from an ether anaesthetised animal (Robinson, 1966; Robinson, Jequier, Felber & Mirkovitch, 1965). He also found that partial occlusion of the arcadian vessels to an experimental segment for 5 minutes caused a significant decrease in the subsequent accumulation of L-phenylalanine. However, in this case the controls may not be valid since adjacent segments (location unspecified) were used. These experiments point to the necessity for continuous oxygenation of the tissue.

TABLE 12

The effect of temporary hypoxia while setting up the intestine

	Mean \pm S.E. (number of intestines)			
	Rate of water absorption	Rate of glucose absorption	Apparent rate of glucose secretion*	Apparent rate of glucose utilisation*
	$\mu\text{l/cm/hr}$	mg/cm/hr	mg/cm/hr	mg/cm/hr
Control	168.8 \pm 4.4 (57)	3.17 \pm 0.10 (57)	2.10 \pm 0.07 (52)	1.19 \pm 0.07 (51)
Hypoxic	135.0 \pm 17.0 (8)	2.80 \pm 0.24 (8)	1.20 \pm 0.21 (8)	1.50 \pm 0.15 (8)
Difference \pm S.D.	33.75 \pm 13.2	0.37 \pm 0.29	0.90 \pm 0.21	0.31 \pm 0.19
t	2.545	1.281	4.316	1.602
P	<0.02	>0.2	<0.001	>0.1
Ratio Hypoxic/control	0.80	0.88	0.57	1.26

* The estimates of secretion and utilisation are not strictly valid, since the secretion was not at a steady-state.

In the current work, the precautions of Fisher & Parsons have been rigorously applied, and if an animal died before the segmented flow of perfusate had been established then that experiment was abandoned. The following series of experiments tested the consequences of neglecting these precautions.

In eight special experiments, the intestine was prepared for perfusion in the normal manner until after the lumen had been rinsed with the oxygenated saline. Then the mesenteric vasculature was clamped off, and the cannulae were attached to the perfusion apparatus but with no perfusate flowing. The segment was dissected from the animal and the serosal surface was washed with saline. Five minutes after the blood supply had been occluded perfusion was commenced. Five or eight collections over 5 minute periods were taken of the lumen effluent and secretion, and estimates were made of the rates of glucose and water absorption, and also of the apparent rates of glucose secretion and utilisation. The mean rates for each intestine were compared by Student's t-test with the mean rates for control intestines set up in the customary manner in which the recommendations of Fisher & Parsons were followed. Note that because of the large inter-experimental variability (see Tables 8 and 9) more experiments are necessary where the experimental design prevents control and experimental measurements being made on the same segment of intestine.

The results (Table 12) demonstrate that even temporary hypoxia for 5 minutes during the setting up has a significantly detrimental effect on the absorbing performance of rat small intestine. However, the effect is not large.

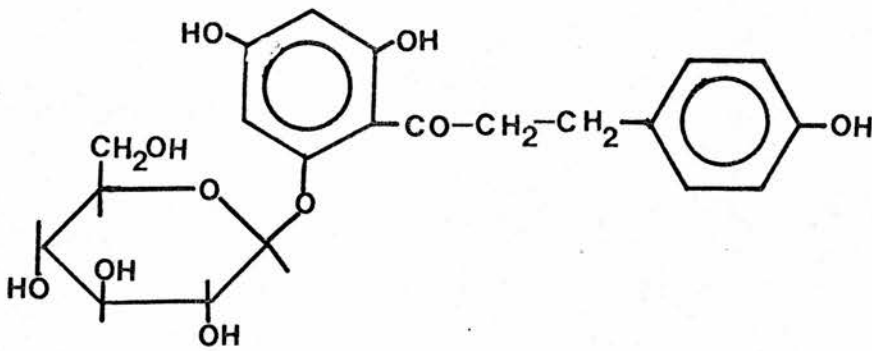
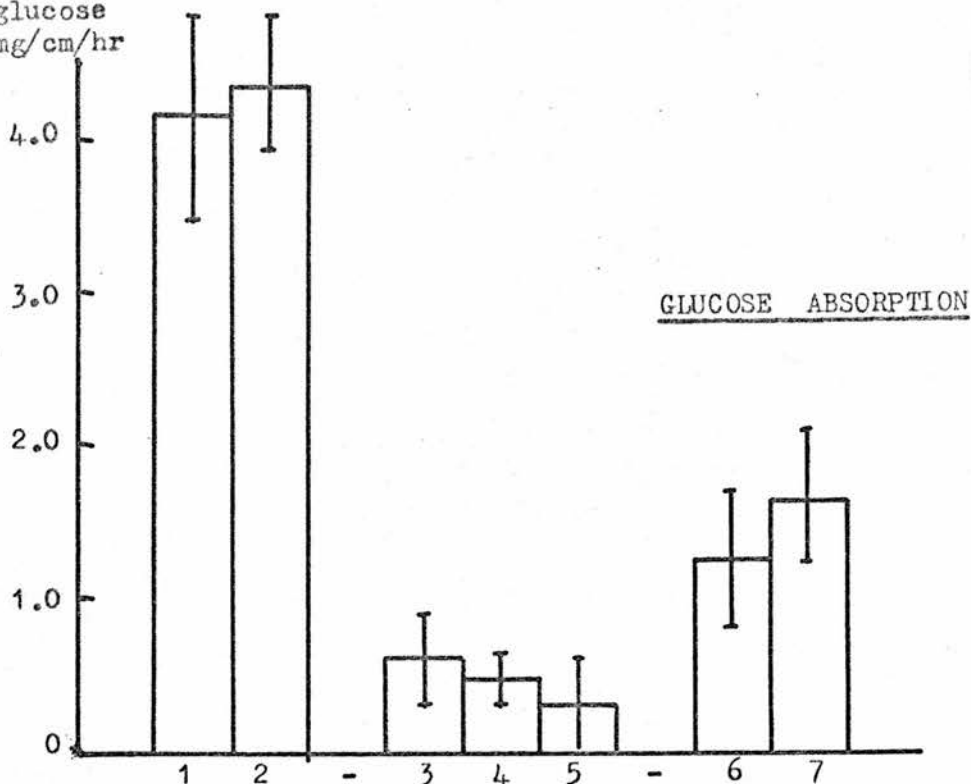


FIGURE 26

Phloridzin

Rate of glucose
absorption, mg/cm/hr



Rate of water
absorption, μ l/cm/hr

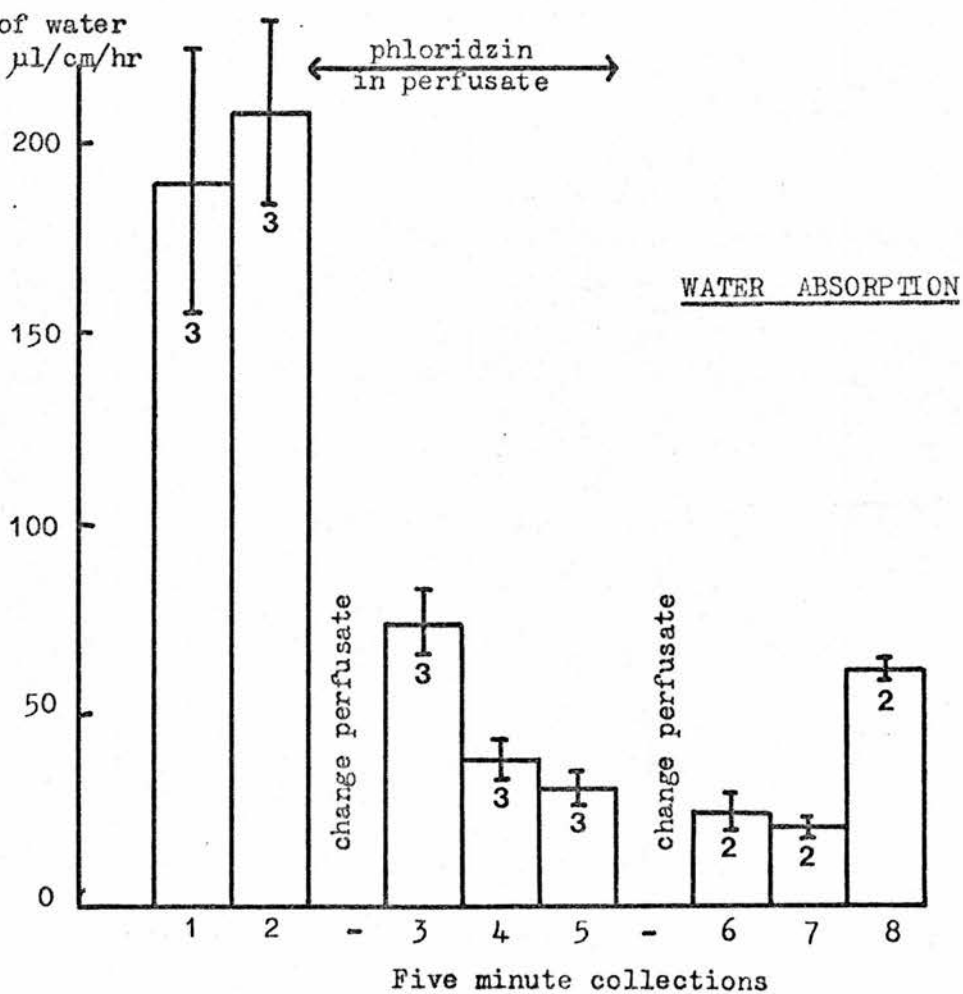


FIGURE 27

Effect of luminal phloridzin (1 mg/ml)

The vertical bars show the S.E.M. The number of experiments is shown within the blocks for water absorption.

Effect of phloridzin on glucose and water absorption

Phloridzin, a glucoside whose formula is shown in Figure 26, is known to be a rapid and potent inhibitor of glucose resorption by the kidney and of absorption by the intestine (see review by Lotspeich, 1961). Although its mode of action is still not understood, it has been suggested that the inhibitor competes with glucose for a binding site on a carrier in the luminal membrane (Alvarado & Crane, 1962).

Therefore a short series of experiments was designed to test the sensitivity of this new intestinal preparation to luminal phloridzin, and to establish whether any inhibition was immediate.

Three segments of intestine were perfused with a normal perfusate containing glucose (5 mg/ml; 28 mM). After two control collections each over five minute periods had been made the perfusate was replaced by one which was identical except that it contained phloridzin (1 mg/ml; 2.1 mM - supplied by British Drug Houses Ltd.). After a five minute pre-period, 3 further collections were made; then (in 2 of the experiments) the original phloridzin-free perfusion was re-established and further measurements were made.

Figure 27 shows the means of the results. It is clear that under these conditions the effect of luminal phloridzin (2.1 mM) is to inhibit rapidly both water and glucose absorption almost entirely. This effect is not ^{instantaneously complete} and is at least partly reversible. Glucose absorption seems to be affected more rapidly than water absorption is by this inhibitor.

The water content, and wet and dry weights of intestine

It is desirable to know whether perfusion affects the water content of the intestinal segments, especially since the estimate of absorption rates requires the assumption that the rate of water

TABLE 13

The water content, and wet & dry weights of segments of intestine
(upper 40 cm segments of jejunum & ileum)

	Means \pm S.E. (number of intestines)		
	Wet weight mg/cm	Dry weight mg/cm	Water content μ l/cm
Control			48.2 \pm 1.5 (7)
1 hour perfusion with normal perfusate			53.9 \pm 2.6 (4)
2 hours perfusion with normal perfusate			52.5 \pm 2.9 (2)
1 hour perfusion with normal perfusate followed by 1 hour with low Na (5mM) perfusate	64.5 \pm 0.2 (2)	13.8 \pm 0.4 (2)	50.7 \pm 0.6 (2)
Miscellaneous perfused intestines	76.4 \pm 2.9 (16)	12.9 \pm 0.4 (16)	57.7 \pm 2.0 (29)

absorption is equal to the rate of water secretion. Fisher & Parsons (1953) found that the water content of perfused segments of intestine was much higher than that of control segments which had not been perfused. Accumulation of water in the intestinal wall during absorption is implicit in the observations of Diamond & Torney (1966). These authors suggested on the basis of electron micrographs that the lateral inter-cellular spaces become grossly distended during fluid absorption. Parsons (1967) has discussed these observations.

The lengths of segments of intestine which had been perfused were measured, their serosal surfaces were lightly blotted on filter paper, and a gentle stream of compressed air was passed through the lumen so as to blow out surplus surface moisture. The segments were then dried at 75°C to constant weight on aluminium foil.

Control segments were treated similarly. The lumen was rinsed with warm oxygenated saline to displace all solid debris, and compressed air was gently passed through the lumen while the intestine was in situ in the anaesthetised animal. The segments of intestine were excised, blotted, and dried to constant weight.

The means of the results are given in Table 13. The values for the perfused segments are not significantly different from those for the controls. This conclusion contrasts sharply with that of Fisher & Parsons (1953). The present water content data for perfused segments are remarkably similar to the corresponding data of Fisher & Parsons ($51.25 \pm 2.14 \mu\text{l}/\text{cm}$); this indicates that the results reported here have not been influenced by experimental artifact such as inadequate removal of surface liquid. However the water content of the control segments in the present work is about twice the value found by Fisher & Parsons (1953) for their controls. Other measurements in this laboratory agree with the data in Table 13

(Mrs. F. O'Brien, personal communication), while other values from the Oxford laboratory substantiate those of Fisher & Parsons (McHardy & Parsons, 1957). There is no obvious explanation for these two discrepancies: 1 - the difference in water content of the control segments of intestine; and 2 - the difference in the swelling of the organ during perfusion. Differences between the animals or between their diets may be relevant. Steiner, Bourges, Freedman & Gray (1968) obtained values for the water content of control segments (about 59 mg/cm) which agree well with those in the present work. They noted that after total starvation for six days the water content fell to about 28 mg/cm. Whether or not the liquid diet which Fisher & Parsons used overnight prior to experiment could affect the water content is not known. At the moment these differences are inexplicable.

DISCUSSION

In general, the objectives of the new preparation have been well achieved. Experimental tests on the new preparation have established that its properties offer advantages over most other preparations, and that it has potential value for subsequent investigations. Some of the respective advantages and disadvantages are discussed below.

Three major advantages accrue from the use of the segmented flow of oxygen bubbles separated by slugs of perfusate through the intestinal lumen. Firstly and chiefly, it is possible to supply much more oxygen to the mucosal cells while maintaining a low rate of perfusion than would be possible if all the oxygen were in solution in the perfusate. Secondly, the stirring effect of the segmented flow should tend to eliminate a radial concentration gradient from the axial flow to the peripheral site of absorption. Unstirred layers at the mucosal boundary should be minimised, although it is most unlikely that they be eliminated. For example, Green & Otori (1970) made direct measurements of the unstirred layer of fluid surrounding the posterior surface of a rabbit cornea, and estimated that vigorous stirring could reduce the thickness of this layer from some 350 μm to about 65 μm . A detailed study on unstirred layers around frog skin preparations was made by Dainty & House (1966) who suggested that diffusion across unstirred layers might be the rate-limiting step in some membrane transport phenomena. Certainly, the lack of stirring of the luminal contents in some types of preparations - e.g. Thiry-Vella loops - may partly account for relatively low apparent rates of solute absorption. Thirdly, the additional fluid flow contributed by the oxygen through the

lumen helps to achieve a rapid change in luminal conditions - see Figure 24.

Removal of the oxygen bubbles from the segmented flow caused an immediate deterioration in the absorptive activity of the intestine even though the mucosal cells were presented with more glucose in unit time - Figure 22. This confirms the value of the segmented flow technique, and suggests that, whatever the reason may be, this preparation is more active than that of Gilman & Koelle (1960a,b). These authors modified Smyth & Taylor's (1957) preparation by replacing the rapidly recirculated luminal perfusion by slow single transit perfusion. They claimed that their flow rate of 5 to 10 ml/min could supply adequate oxygen to the intestine. However, it is not proven whether the fall in activity subsequent to removal of the oxygen bubbles (Figure 22) can be wholly attributed to incomplete oxygenation of the tissue, or at least in part to the removal of the stirring effect already described. Early experiments in vivo (M. L. G. Gardner, unpublished work) suggested that even when the tissue receives its oxygen via the intact mesenteric blood supply, a slight diminution in absorption rates occurred when segmented flow was replaced by all-liquid flow. Confirmation of this, and assessment of the relative gains from the improved oxygenation and from the stirring effect requires experiments in which the 5% CO₂ in O₂ can be replaced by 5% CO₂ in N₂. Alternatively and preferably, experiments might be made with a phosphate-buffered perfusate (which dispenses with the need for 5% CO₂), segmented with either oxygen or nitrogen bubbles.

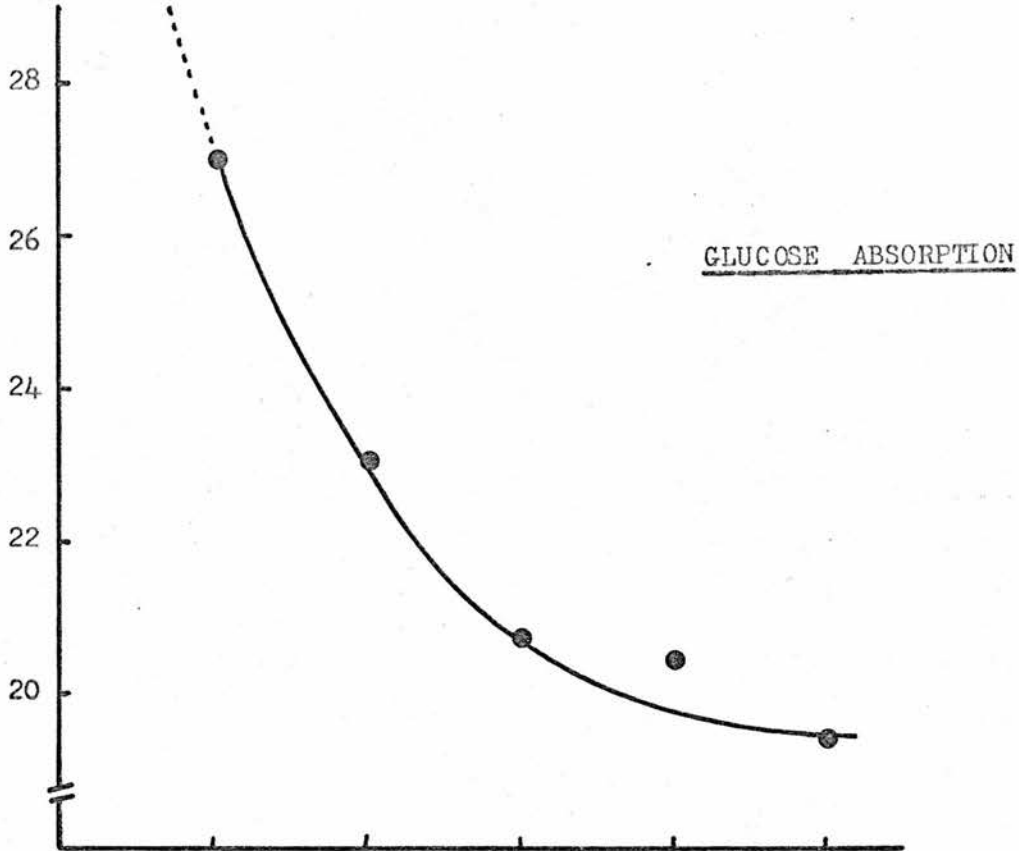
The excellent stability of the preparation over about two hours coupled with the ability to change conditions rapidly in the fluid bathing the mucosal surface, makes possible comparisons between two

or more treatments in the same segment of intestine. In view of the large variability between intestines (see analyses of variance in Tables 8 and 9), it is especially valuable that control and experimental measurements can now be made in one and the same intestinal segment.

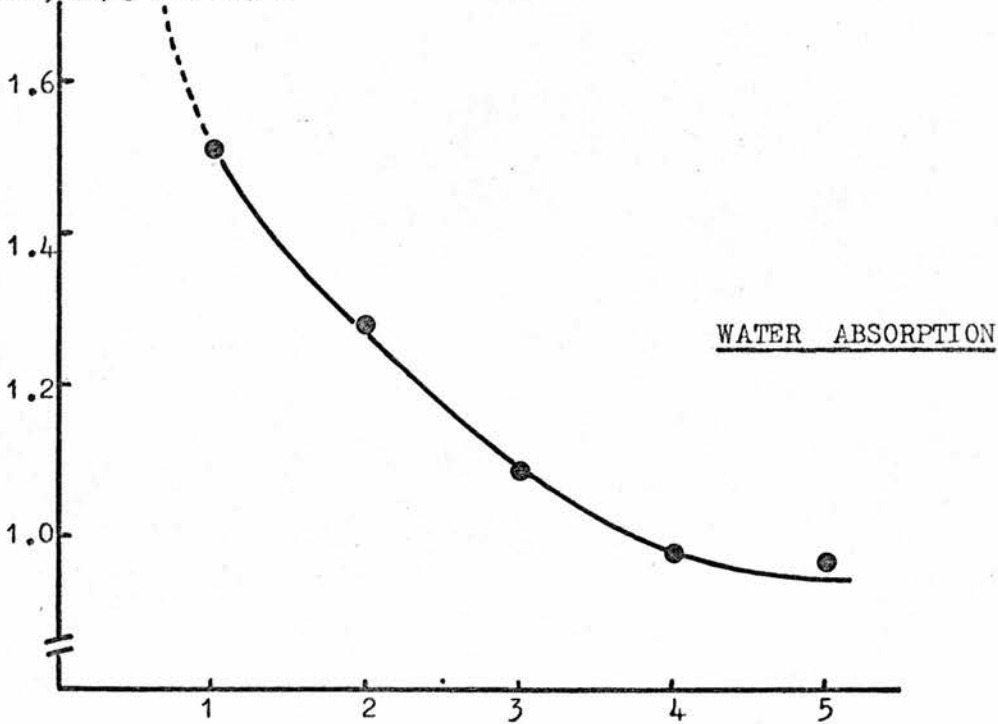
The extent to which this large inter-experimental variability is due to differences between the animals themselves is not clear; others have reported substantial variance which they have attributed to the animals. It has already been remarked that the variance between days is no greater than that within days; therefore climatic conditions are unlikely to be relevant as causes of animal variability. Experiments to compare variation within and between litters of animals would be valuable, since genetic factors may be involved. Evidence into the causes of the large inter-animal variability would be useful, since significant comparisons might be made with fewer animals if variance from this source could be reduced. For instance, Beveridge (1961, p.20) states that as much information on butterfat yield was obtained from a single pair of identical twin cows as from two groups each of 55 individuals!

In setting up most non-perfused types of intestinal preparation in vitro it is rarely possible to avoid temporary anoxia. With perfused preparations, perfusion and hence supply of oxygen can be established before the blood supply is interrupted. This precaution was stressed by Fisher & Parsons (1949), but since then Smyth (1963) has suggested that these precautions were not necessarily obligatory. He based his argument on experiments by Barry, Matthews & Smyth (1961) who removed segments of rat intestine, and at intervals of time set up everted sacs absorbing glucose and water. These authors used experiments of a Latin Square design to separate

Rate of glucose absorption
mg/mg dry wt/hr



Rate of water absorption, ml/g wet wt/hr



Order of preparing the sacs

FIGURE 28

Data of Barry, Matthews & Smyth (1961) - their Table 3 - plotted to show progressive decrease in absorption with delay in setting up everted sac preparations

(The sacs were set up at approximately six minute intervals.)

TABLE 14

Rates of absorption by other intestinal preparations

Preparation	Source of data	Rate of water absorption $\mu\text{l/cm/hr}$	Rate of glucose absorption mg/cm/hr	Segment of intestine
Everted sac	Barry, Matthews & Smyth (1961) Tables 4 & 5	78	1.9	Upper 40 cm
Everted sac	Newey, Sanford, Smyth & Williams (1963) Table 1	139	3.1	Middle 5th
This preparation with hypoxia	Table 12	135	2.8	Upper 40 cm
This preparation	Table 10	169	3.2	Upper 40 cm

variance due to the different regions of intestine from which the segments were taken and due to the order in which the sacs were set up. Figure 28 shows the rates of water and glucose absorption reported by Barry et al (1961) (their Table 3) corresponding to the whole jejunum and ileum set up at various intervals post mortem. Clearly there is some falling off in absorptive activity as this interval increases, although as Barry et al point out this decrease is not statistically significant. The slopes of the lines in Figure 28 are high at 6 minutes post mortem, and so it is reasonable to suggest that the broken lines might represent the true time-courses of absorption in the period immediately post-mortem.

The results already described in Table 12 indeed confirm that a period of as little as five minutes of temporary anoxia can reduce the absorption rates of both glucose and water. However, this reduction is only of the order of 20%, and cannot readily account for the very low activity in the sacs of Barry et al. Their results, together with those of Newey, Sanford, Smyth & Williams (1963), have been converted into the same units as used in this work by assuming that the wet weight of small intestine is about 70 mg/cm (see Table 13), and are summarised in Table 14. The differences between the results of Barry et al and those of Newey et al are large, and cannot be accounted for even when allowance is made for the slightly different regions of intestine involved. Recent experiments by Professor Fisher and Mrs. F. O'Brien using the present preparation have shown that further prolonged anoxia, over about 15 minutes, can further depress the subsequent absorptive activity but not to the level recorded by Barry et al. While the magnitude of the damage caused by five minutes anoxia is only about 20%, the mechanism of this damage is not at all understood. Thus, the damage

might reflect a specific effect on only part of the metabolic and transport machinery, rather than a general slowing down of all processes. It is for this reason that distrust has been placed in other preparations whose integrity may be open to question.

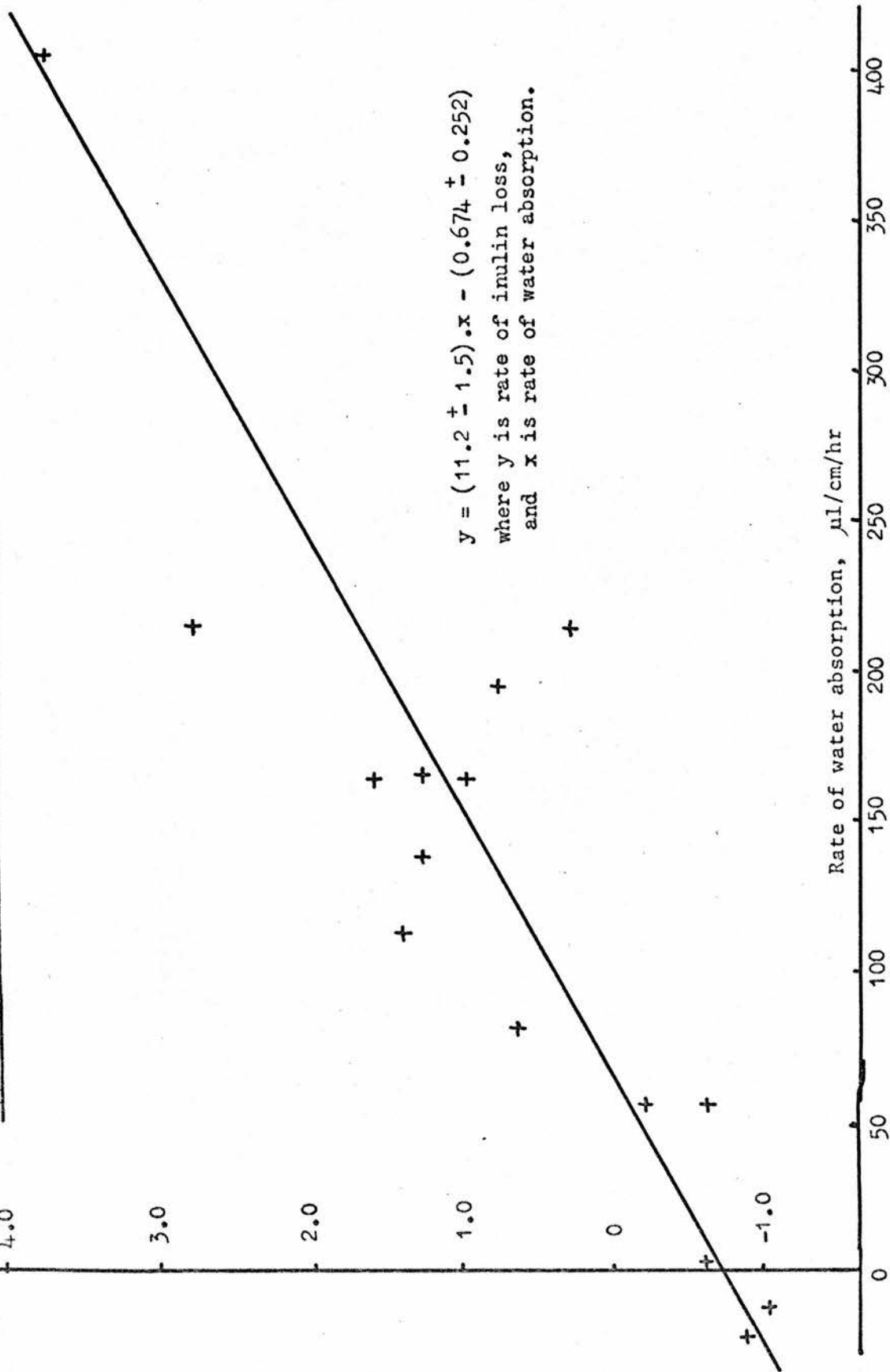
This preparation is ideally suited to the measurement of water absorption, since the fluid secreted on to the serosal surface can be collected and directly weighed. Indeed, it was specifically for a study of water transport that Smyth & Taylor (1957) removed the outer (serosal) fluid from the Fisher & Parsons (1949) type of preparation. The direct measurement of water absorption is decidedly preferable to relying on an 'unabsorbable marker' for three reasons: (i) The assumption that any marker really is totally unabsorbed or unchanged must be rigorously tested. (ii) Use of a marker requires measurement of two concentrations and a volume in the determination of water net flux. On the other hand, this direct method requires only a single volume measurement. When the marker method is used, the estimate of water transport will be subject to the same order of errors as already has been calculated for glucose absorption. In turn, the estimate of glucose absorption rate will become less reliable. (iii) The assumption that the marker itself does not interfere with solute or water movement must be justified; this is difficult in many preparations.

Phenol red, which is sometimes used in the lumen perfusate as a marker (Summers & Schedl, 1968), can be observed to appear in secretion, although only in relatively small concentrations - see Figure 21. That the error introduced by such 'absorption' is trivial must be demonstrated before the use of such a marker is permissible. McLeod, French, Good & Wright (1968) found a small, but significant, appearance of phenol red in bile and urine from human

FIGURE 29

Rate of loss of inulin from
lumen, mg/cm/hr

Apparent absorption of inulin from a perfusate containing 10 mg/ml inulin



subjects who had ingested phenol red; this suggests that the appearance of phenol red in secretion of an *in vitro* preparation may not necessarily be an artifact caused by damage. Kunze & Vogt (1967) claimed that phenol red could be absorbed actively, since they observed saturation kinetics.

Smyth & Taylor (1957) reported that inulin could appear in secretion from their preparation at about 14% of the concentration at which it was present in the luminal perfusate. However, Schanker, Tocco, Brodie & Hogben (1959) observed excellent recoveries of tracer inulin solutions slowly perfused through the lumen *in vivo* in the rat. Inulin is quite labile (e.g. see Bell, 1952), and it is possible that the material which Smyth & Taylor found in their secretion was not in fact inulin, but a smaller fructosan produced by partial hydrolysis of inulin. The analytical method which these authors used (that of Bacon & Bell, 1948) is not specific to inulin.

This explanation, however, seems unlikely in the light of some early work with the present preparation. Small amounts of apparently unchanged inulin can cross the mucosa of this preparation since inulin was detected in the intestinal secretion by paper chromatography (M. L. G. Gardner, unpublished work). In this early work, the quantitative recovery of infused inulin which Schanker et al reported could not be confirmed, and it appeared that the apparent loss of fructosan from the intestinal lumen might be proportional to the amount of water transported - see Figure 29. A further suspicion was that luminal inulin (10 mg/ml) interfered with glucose absorption, but this finding was not pursued and so remains unconfirmed.

Access to both the luminal perfusate and the secretion permits simultaneous estimates of sugar absorption, secretion, and hence

metabolism. Because it is difficult to measure metabolic loss of sugar in many other preparations, many authors have studied the absorption of non-metabolisable sugars such as galactose or 3-deoxy-glucose. However, under these conditions the intestine is provided with no exogenous nutrient and so studies extending over more than a few minutes may not be reliable. Furthermore, the present preparation allows the simultaneous investigation of two distinct, though related, subjects - namely, transport and metabolism. Gellhorn & Northrup (1933) studied the effect of certain hormones on the rate of appearance of glucose in a vascular perfusate in their elegant preparation of frog intestine. Because insulin, thyroxine and adrenalin caused an increase in the rate of appearance of the sugar in the vascular perfusate they concluded that these hormones had stimulated the absorption of glucose. However, the possibilities that glucose utilisation had been inhibited or that release of endogenous sugar had been stimulated were neglected.

It is hard to compare the rates of absorption of glucose and water by this and other preparations, especially on account of the gradients of absorptive activity which exist down the length of the small intestine. For example, Fisher & Parsons (1950) found that the rate of glucose absorption steadily increased in a linear fashion from upper jejunum to terminal ileum. Wilson & Wiseman (1954b) reported a similar gradient in the respiration of small intestine. However, Barry, Matthews & Smyth (1961) recorded that absorption of both water and glucose was most rapid about the middle of the small intestine; they, unlike Fisher & Parsons, also found substantial water absorption in terminal ileum. Furthermore, differences between animals used in different laboratories make comparisons between preparations used by different authors meaningless.

Nevertheless, the absorption rates shown by the present preparation are substantially higher than the values reported by many other authors. A few such values are collected together in Table 14.

Female rats have been used throughout this investigation. Fisher (1955), Deuel, Hallman, Murray & Samuels (1937) and Althausen & Stockholm (1938) have pointed out that absorption rates in male animals are some 10% lower. This use of females may in part contribute to the variability between animals. No attempt has been made to correlate absorption rates with the state of oestrus as Crocker (1971) has done. She noted substantial cyclical changes in water and sodium absorption rates in phase with the rats' oestrus cycle in experiments with everted sacs.

The values obtained for the parameters K and V in the Michaelis-Menten relationship between the rate of glucose absorption and the glucose concentration in the perfusate are in reasonable agreement with previous literature values. However, it must be stressed that any interpretation of the physical significance of these parameters can only be speculative at the present time. There is no evidence that the half-saturation concentration, K , is related to the affinity of any carrier system for its substrate, although some authors (e.g. Crane, Forstner & Eicholz, 1965) have extrapolated from enzyme kinetics to this conclusion. Nevertheless, whatever their physical significance may be, the Michaelis-Menten parameters do usefully characterise the relation between absorption rate and luminal concentration. Indeed the substrate concentration in the immediate proximity of the glucose carrier system may be different from the bulk concentration in the luminal fluid since it is now apparent that the rate-limiting step in glucose transport is located within the luminal membrane rather than on its luminal surface (Parsons &

Pritchard, 1968, 1971). The dependence of the K value on the luminal concentration of sodium as shown by Crane et al, 1965 and others for rat and hamster small intestine is most striking, but unfortunately cannot yet be interpreted. A remarkable fact which reinforces the desirability for some such interpretation is that the value of K_m , but not of V_{max} , for sucrase hydrolytic activity is Na-dependent (Semenza, 1970). On the other hand, Goldner, Schultz & Curran (1969) found that the value of K for 3-methylglucose absorption from rabbit ileum was independent of the mucosal sodium concentration, but the value of V was Na-dependent. Furthermore, Kolinska & Semenza (1967) have reported for sucrase in the rabbit that the V_{max} , but not K_m , was Na-dependent. This striking coincidence of the sodium dependence of sugar transport and disaccharidase activity merits further attention. The relationship between these two processes is not at all clear: Parsons & Pritchard (1971) have now demonstrated that the two processes are not due to a single system by studying competition between free glucose and disaccharides in the lumen for hydrolysis and transport; it is possible that a single 'multi-enzyme' complex exists with separate sub-units for hydrolysis and transport. This complex might be subject to conformational change by sodium ions in some way.

The new preparation is not without its disadvantages. Some of these will now be discussed. The arrangement currently used to maintain the intra-luminal distension pressure allows this pressure to fluctuate between about 30 and 40 cm water, depending on the ratio of gas to liquid in the fluid head in the lumen outflow tube from the intestine. This means that the conditions of the preparation are not constant; however, both Fisher & Parsons (1949) and Smyth & Taylor (1957) have shown that changes in this hydrostatic

pressure across the intestinal wall cause trivial changes in the rate of water transport. Future modifications to the apparatus will include the provision of a Starling resistance in the luminal outflow tube to control the distension pressure.

Until further work has been done on the kinetics of the appearance of phenol red in the secretion it is not possible to determine whether the slow rate of phenol red appearance is due to an artifact in the preparation. Evidence has already been mentioned which indicates that this may be a perfectly physiological process (McLeod et al, 1968).

At the present time it is not possible to make measurements of transmural potential difference or of short-circuit current in this preparation. In view of the difficulties attendant on interpreting such measurements this is perhaps no great loss (e.g. Barry, Smyth & Wright, 1965).

The use of segmented flow through the lumen is restricted to the intestines from small animals, since the surface tension between perfusate and gas is not great enough to maintain the integrity of the oxygen bubbles in an intestine of internal diameter larger than about one centimetre when distended. This could be a disadvantage in, for example, specific studies which can only be made in larger animals such as the effects of bacterial toxins which affect cattle.

The delay in attainment of the steady-state in the secretion can be regarded as a disadvantage; however, in this work this lag has been characterised in detail and has been used to obtain further information. This will be the subject of the next two Chapters.

The major disadvantage inherent in this preparation is the inability to control directly the composition of the intestinal

tissue fluid although this composition can be readily determined (see next Chapter). This disadvantage will be further discussed in the light of future experimental results, and means of overcoming it will be described later.

On the whole, the advantages outlined above far outweigh the disadvantages of this preparation. The advantages of this new preparation over all, or almost all, others make it potentially valuable for a wide variety of studies on intestinal absorption. Although special apparatus is required, it is sufficiently simple and easily manipulated to be of wide-spread application.

CHAPTER VI

INTESTINAL SECRETION

THE KINETICS OF APPEARANCE OF SOLUTES IN THE SECRETIONIntroduction

It has already been stated that the rate of secretion of glucose onto the serosal surface of the intestinal segment does not reach a steady-state within at least the first hour of perfusion, although the rate of glucose absorption from the lumen becomes steady within four or five minutes from the setting-up of the preparation. The reason for this lag should be clear from Figure 3. Although this Figure is not drawn to scale, it emphasises that the extracellular water occupies a relatively large bulk of the intestine. (The measurements of water content and dry weight of the tissue already presented in Table 13 show that the total water content accounts for about 75% of the gross wet weight of the tissue.) Thus, any material secreted by the mucosal cells into the sub-epithelial space will be diluted by this extracellular water - the tissue fluid. If the secretion collected is a sample of this extracellular fluid, then the concentration of a solute in the secretion will only gradually approach that of the fluid secreted from the mucosal cells.

In this Chapter, the kinetics of such a one-compartment system are first derived. Then methods for fitting the model to experimental data are discussed and tests are described which establish whether the theoretical kinetics are consistent with those observed in practice.

Theoretical kinetics

On the basis of this single compartment model which is depicted in Figure 30 the kinetics of appearance of a solute in the intestinal secretion can be predicted.

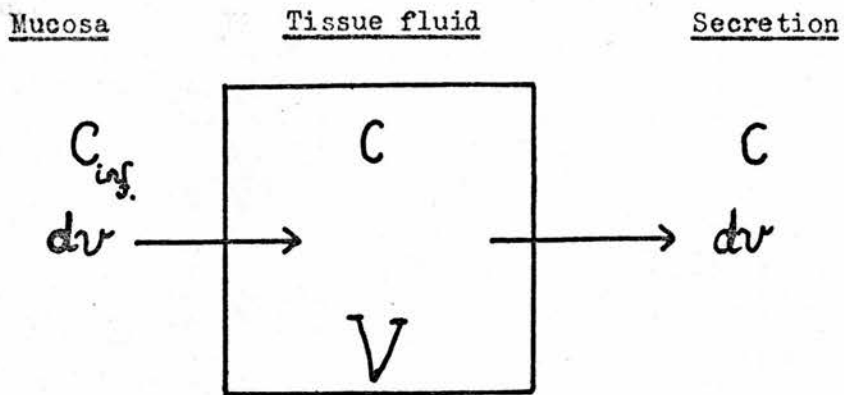


FIGURE 30

A one compartment system

dv = increment in volume of water added to tissue fluid by mucosa,

V = volume of tissue fluid,

C_{inf} = solute concentration in fluid added to tissue fluid by the mucosa,

C = solute concentration in tissue fluid and secretion.

Consider the fluid secreted from the mucosal cell into the sub-epithelial space (Figure 30). If the volume, V , of this extracellular space remains constant, then for every increment, dv , of volume which enters this space from the mucosa, an equal volume, dv , must be displaced and collected as secretion.

Let the concentration of a solute in the extracellular space be C , and let the concentration of that solute in the fluid entering this space from the mucosa be C_{inf} (assumed constant). Assume that the fluid secreted from the cell admixes uniformly with the extracellular fluid and that, at any time, the secretion collected is a fair sample of this extracellular fluid.

Then, when the increment of volume, dv , enters this space, the change in concentration of the solute in the extracellular fluid, and hence in the secretion, dC , is:-

$$dC = (C_{inf} \cdot dv - C \cdot dv) \cdot 1/V$$

or:-

$$\frac{dC}{(C_{inf} - C)} = \frac{dv}{V}$$

Integration yields:-

$$- \log_e (C_{inf} - C) = v/V + K$$

where K is a constant of integration.

Let the concentration of the solute in the extracellular fluid, and hence in the secretion, be C_0 when $v = 0$, i.e. when no fluid has been secreted through the compartment.

Then $K = -\log_e (C_{inf} - C_0)$

So, $-\log_e (C_{inf} - C) = v/V - \log_e (C_{inf} - C_0)$

Rearranging, we obtain:-

$$\log_e \frac{(C_{inf} - C)}{(C_{inf} - C_0)} = -v/V$$

Hence, $\frac{(C_{inf} - C)}{(C_{inf} - C_0)} = \exp(-v/V)$

or, $C = C_{inf} + (C_0 - C_{inf}) \cdot \exp(-v/V) \dots\dots\dots I$

Hence one can predict that the concentration of a solute in the secretion will change exponentially with respect to v , the total (cumulative) volume secreted, provided that the assumptions made are valid.

Furthermore, if a function of the form of equation I can be fitted to the experimental data, then the constants C_{inf} , C_o , and V can be estimated.

Note that when v is infinite, $C = C_{inf}$ - i.e. the concentration of solute in the secretion becomes equal to that in the fluid secreted by the mucosal cells. Also, note that the rate constant of the exponential function is $1/V$, i.e. it is the reciprocal of the volume of the extracellular fluid with which the material from the epithelial cells admixes.

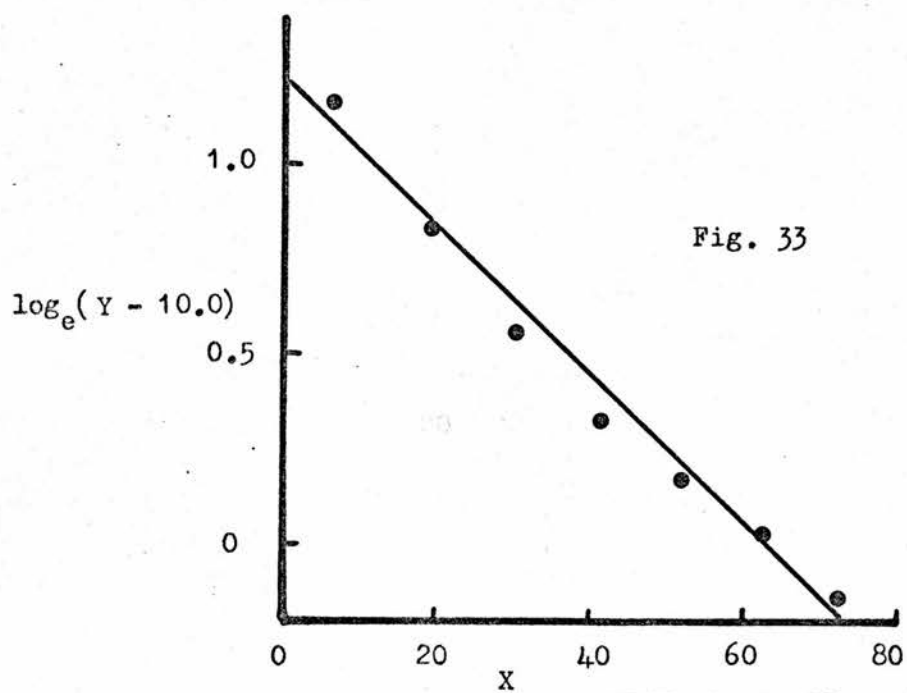
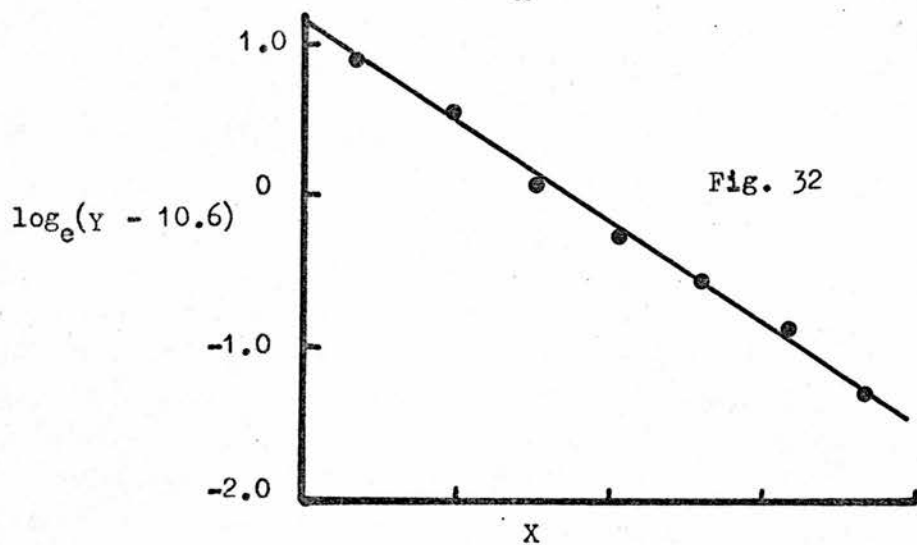
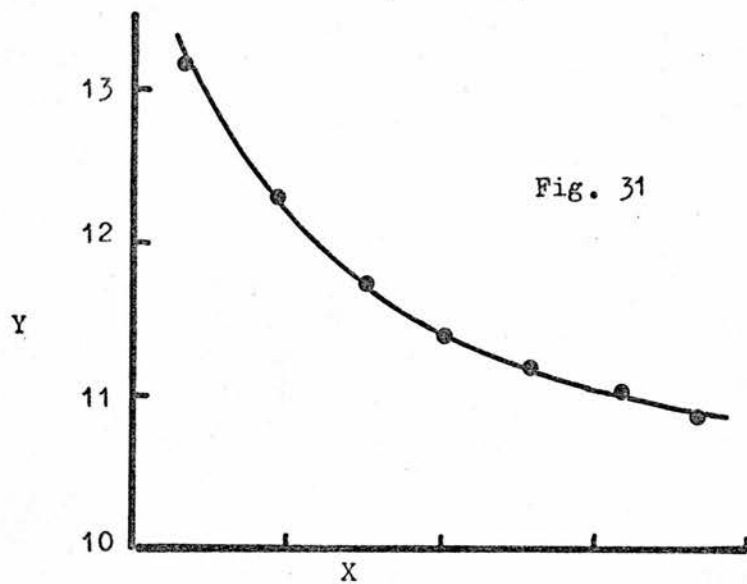
Methods for fitting exponential functions to data

It has been shown above that the time course of appearance of a solute in intestinal secretion might be expected to follow an exponential function of the form:-

$$C = C_{inf} + (C_o - C_{inf}) \cdot \exp(-v/V)$$

In order to test whether this theoretically derived function does indeed describe the experimentally observed kinetics, and if so, to evaluate the constants C_{inf} , C_o , and V it is necessary to fit the exponential function to the observed data.

Given a function of the form $y = A \cdot \exp(-kx)$ where A and k are constants, then a semi-logarithmic plot of $\log_e y$ against x will be linear, yielding estimates of A from the intercept on the ordinate, and k from the slope. However, if the function is of the form $y = B + A \exp(-v/V)$, where B is an additional constant, then the plot will not be linear. The plot of $\log_e (y - B)$ against



FIGURES 31, 32 & 33

Arithmetic and semi-log plots for an exponential with non-zero asymptot

x is linear, but clearly is only possible when the value of B is known. B can sometimes be evaluated approximately by inspection, but as the following instance illustrates severe error in the estimation of k can attend such an empirical procedure. Experimental data known to be fitted reasonably well by an exponential function of the form $y = B + A \exp(-kx)$ are plotted in Figure 31.

Inspection suggested that the value of B , the asymptote, probably lies between 10.0 and 11.0. Subtracting $B = 10.6$ from each experimental value of y , and plotting $\log_e (y - B)$ against x gave the line in Figure 32. A similar plot, but with $B = 10.0$, is shown in Figure 33. If these two lines are assumed to be straight, as the semi-logarithmic plot should be, then the rate constants estimated from the slopes of the first-order regressions are 0.0333 (Figure 32) and 0.0194 (Figure 33) respectively. In fact, a more appropriate method, such as will be duly described, for estimating B and k gave the optimum parameters as $B = 10.572$, and $k = 0.0330$, and showed that these data are excellently described by the single exponential function. An extension of this graphical method is to calculate the first-order regression of $\log_e (y - B)$ on x for a series of values of B . The sum of squares of residuals about the regression line is calculated, and the value of B which gives the minimum sum of squares is taken to be the 'best-fit' value.

However this whole calculation took from 2 to 5 hours for execution on the Olivetti Programma P101 for an exponential with only six data points. It was also run on an I.B.M. 360/50 computer. A serious disadvantage of this type of method is that taking logarithms introduces unequal weighting into the raw data. Further unequal weighting is introduced when a constant, B , is subtracted from the dependent variable, y , since the nearer that y is to the

asymptote, B , the relatively larger will be the variation in $(y - B)$ caused by variation in y . If the true value of y is very close to B , then experimental error may make $(y - B)$ negative. In this case, $\log_e (y - B)$ is insoluble and the data point can only be ignored. This is unacceptable, and so use of this method was discontinued.

The graphical method of Guggenheim (1926) is applicable to problems of this sort where the asymptote, B , is not known. Values of $\log_e (y_n - y_{n+1})$ are plotted against x_n , where y_n and y_{n+1} are successive values of y . However, the method is only valid for data measured at equal intervals of x . Moreover, even with large numbers of accurate data points, the accuracy of the estimated parameters is poor. Cleland (1970) has suggested a modification so that the analysis can be extended to measurements at unequal intervals in x , but he stressed that both his method and the original one of Guggenheim are hardly capable of acceptable accuracy.

Similar inadequacies in graphical methods for exponential curve fitting have also been emphasised by Wagner & Metzler (1967), who estimated rate constants for the absorption and elimination of a drug by a human subject. Both graphical analysis and digital computer methods were used but substantially different results were obtained by the two methods. Because the values for the standard deviations of the parameters were much smaller when the computer method was used, they concluded that it was the better method. Perhaps a better criterion would have been the sums of squares of the residuals about the fitted curves or the standard deviation of the residuals, since estimates of parameter standard deviations are only approximate in non-linear statistics. Atkins (1969, page 104)

comments: "Apart from the simple case of fitting a regression line to a single exponential, the use of graphical analysis has become obsolescent. Its main use nowadays ought to be restricted to the estimation of initial values of parameters for subsequent use in one of the curve fitting procedures". Therefore more elaborate methods for curve-fitting were explored.

The 'partial sums method' of Cornell (1962) and several similar procedures are only applicable to data at equal intervals of x . Furthermore, at least ten data points are generally considered to be necessary. The independent variable in the case for which the fit is currently required is v , the cumulated volume of fluid secreted by the intestine (equation I, page 76). Although it would have been possible to modify the apparatus so that the fraction collector was actuated by a drop counter mechanism, this would have been objectionable in practice since all other measurements are expressed on a time basis. For example, glucose absorption rate is in mg glucose per cm length per hour.

Therefore a powerful curve-fitting procedure capable of fitting small numbers of data points at unequal intervals of v with high precision was sought. These requirements were met by an elaborate digital computer program written by Dr. G. L. Atkins of the Department of Biochemistry to whom I am most grateful for all his help. This program is based on the rapid descent methods of Fletcher & Powell (1963) and Davidon (1959) for minimising a function (Atkins, 1971a). In this case the function is the sum of squares of residuals about a curve. Atkins' program is highly versatile in that it can be adapted to analyse numerous different functions. Uses have hitherto included Michaelis-Menten kinetics (Atkins, 1971b), two- and three-exponential functions (Apps, 1971), sets of linear and non-linear

first-order differential equations (Atkins, 1969 and 1971c respectively), sine curves, sigmoid curves, power functions, and minimising a sum of squares of areas (Atkins, 1971b).

For this work the program has been written in IMP(AA) and punched on cards. (IMP(AA) is an advanced computer language derived from ALGOL via Atlas Autocode.) It has been stored on magnetic disc, and has been run on the I.B.M. 360/50 and occasionally the I.C.L. 4-75 computers at the Edinburgh Regional Computing Centre.

An alternative curve-fitting procedure based on a Taylor expansion was used for a short time. The program was written by Dr. I. A. Nimmo in BASIC and was run on-line on a G.E.C. GE-235 computer operated by G.E.I.S. Computer Time Sharing Service.

Represent $y = B + A \cdot \exp(-v/V)$

by $y = f(V)$

Let r_0 be a provisional estimate of V ,

so $y = f(r_0 + V - r_0)$

$$= f(r_0 + d) \text{ where } d = V - r_0$$

This can be expanded in an infinite Taylor series (see Snedecor & Cochran, 1967 - page 467):

$$f(r_0 + d) = f(r_0) + d \cdot f'(r_0) + \frac{d^2}{2!} \cdot f''(r_0) + \dots$$

$$\approx f(r_0) + d \cdot f'(r_0) \text{ if terms containing}$$

the square or higher powers of d are ignored.

Now, $f(r_0) = B + A \cdot \exp(-v/r_0)$

So, $f'(r_0) = -A \cdot (v/r_0) \cdot \exp(-v/r_0)$

Let $X_1 = 1$, $X_2 = \exp(-v/r_0)$, and $X_3 = -(v/r_0) \cdot \exp(-v/r_0)$

Then, $f(V) \approx B \cdot X_1 + A \cdot X_2 + d \cdot A \cdot X_3$

If one then calculates the multiple linear regression of y on X_1 ,

X_2, X_3 i.e. $y = b_1 \cdot X_1 + b_2 \cdot X_2 + b_3 \cdot X_3$

then b_1 is a first estimate of B

b_2 is a first estimate of A

and b_3 is a first estimate of A.d

$$\text{So, } d = (V - r_0) \cong b_3/b_2$$

Hence, r_1 , a first estimate of V, is given by $r_0 + b_3/b_2$

A second iteration with r_1 in place of r_0 gives better estimates of B, A, and V. Further iterations may be made, until $d = 0$, or until 'best' estimates of B, A, and V have been derived.

The method proved to be extremely convenient, since the program was stored on magnetic drums at the computer and the data were typed in at an on-line teletypewriter terminal near to the laboratory. Print-out was complete within one or two minutes of entry of a single data set. However, administrative complications precluded the extensive use of this system, and detailed evaluation was never completed. The parameters and sums of squares of residuals were calculated for four sets of data using both Atkins' method and this Taylor expansion procedure. The values obtained by each method were almost identical. The former method has the merit that it can be adapted very readily to more complex functions such as sums of 2 or more exponential terms (used in Chapter X), hyperbola etc. and that each data point can be weighted easily if required. The Taylor expansion method could probably be programmed for calculation on a desk top computer with adequate storage facility such as the Wang Model 700b. Atkins' method invariably gave smaller estimates for the standard deviations of the parameters than did the Taylor expansion method. However, as already stated, the superiority of a program cannot be judged on this criterion.

Testing Atkins' computer program

To establish whether Dr. Atkins' method gives unbiased estimates

TABLE 15

Best-fit parameters for exponential functions with simulated error

$$C = B + A \cdot \exp(-v/V)$$

At each level of data error six exponential functions were simulated with:

$$B = 12.0$$

$$A = -6.0$$

$$V = 30.0$$

(six data points from $v = 20.0$ to $v = 120.0$ in each)

Simulated data error	FITTED PARAMETERS		
	Mean \pm S.E.M. (& actual % error)		
	A	B	V
0	-6.000 (0)	12.000 (0)	30.000 (0)
0.5%	-5.944 \pm 0.113 (0.9)	12.025 \pm 0.079 (0.2)	30.524 \pm 1.309 (1.7)
1%	-6.227 \pm 0.524 (3.8)	12.009 \pm 0.069 (0.1)	29.257 \pm 1.799 (2.5)
2%	-6.556 \pm 1.045 (9.3)	12.068 \pm 0.321 (0.6)	30.611 \pm 9.145 (2.0)
5%	-7.796 \pm 3.683 (31.6)	12.254 \pm 1.109 (2.1)	40.254 \pm 32.929 (34.2)
10%	-7.576 \pm 2.474 (26.3)	13.965 \pm 2.670 (16.4)	65.785 \pm 59.704 (119.3)

of the parameters in the exponential function, and to test whether the method can be reliably used with only, say, six data points the following test was performed.

Values for $C = B + A \cdot \exp(-v/V)$ were calculated for $v = 20, 40, 60, \dots, 120$, with the constants $B = 12.0$

$$A = -6.0$$

$$\text{and } V = 30.0$$

These values for the constants were selected after preliminary experiments had indicated that they were approximately equal to the respective parameters for the appearance of glucose in intestinal secretion.

Experimental error was then simulated by multiplying each 'true' value of C by a pseudo-random number taken from a series whose mean was unity and whose standard deviation was 0.005, 0.01, 0.02, 0.05, or 0.10. These pseudo-random numbers were generated on a G.E.C. KDF9 computer by Dr. I. A. Nimmo using the sub-routine 'RANDOM K' from the Edinburgh Regional Computing Centre Library. Hence, simulated time courses with 'experimental error' of 0.5%, 1%, 2%, 5%, and 10% respectively were obtained - six time courses at each level of simulated error. The exponential function was then fitted to the simulated data using Atkins' program. Table 15 shows the mean value for each parameter computed at each level of 'experimental error'.

Clearly, even with only the six data points, this program can give reliable estimates for all three parameters B , A , and V . In no case was the estimated parameter significantly different from the actual value of 12.0, -6.0, or 30.0 respectively. Myhill (1967) adopted a similar approach in evaluating a method for fitting a sum of two exponentials to data. However his problem was more complex than the present one, and so it is not surprising that he found that for a data error of 1% the parameter error could be as high as 14%. At 10% data error the parameter error could be 86%.

% of results falling within $\pm N$ standard deviations from actual value

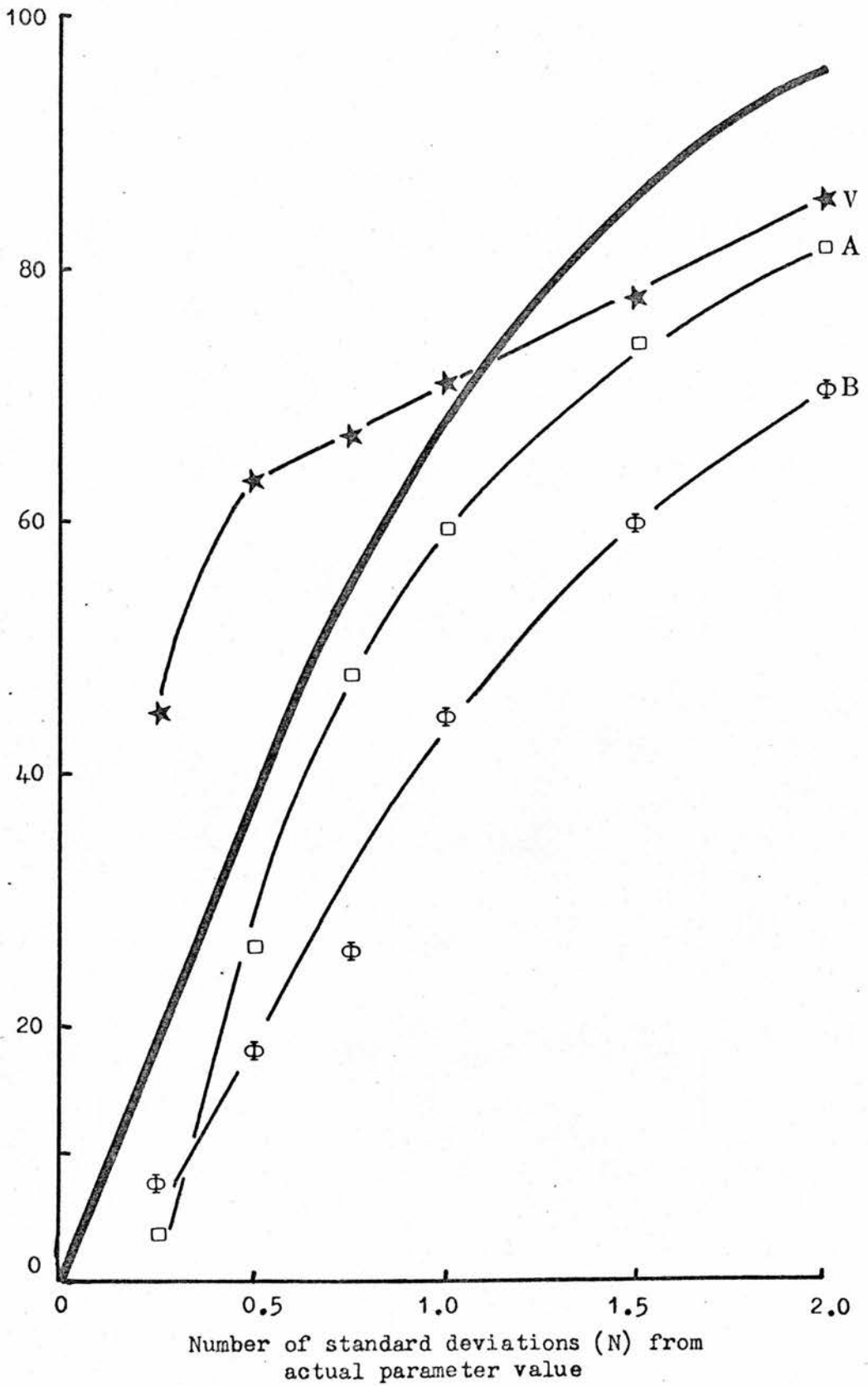


FIGURE 34

A test of the validity of estimates of parameter standard deviation given by Atkins' computer program (see text)

The output from Atkins' program includes estimates of the standard deviations of the computed parameters. In non-linear statistics such standard deviations are usually not reliable. The validity of Atkins' standard deviations was roughly tested as follows. For each of the 27 simulated exponentials (Table 15) the actual parameter error was calculated as a fraction of the calculated standard deviation (S.D.) of the parameter. The results are plotted in Figure 34. The heavy line through the origin is the theoretical curve for a normal distribution, and is plotted from Table A3 of Snedecor & Cochran (1967, p.548). Thus, 95% of results should fall within 2 S.D.'s of the true value, 67% should fall within 1 S.D., and 39% should fall within 0.5 S.D., etc. However, all the points for A and B fall below this theoretical curve (Figure 34); this suggests that the computed S.D.'s for the parameters A and B are underestimates. Nevertheless, this discrepancy is not so marked in the points for V, the reciprocal of the rate constant.

The computed 'standard deviations' have been ignored throughout this work since they seem to be unreliable.

Testing goodness of fit

The goodness of fit of any particular model to sets of data cannot be assessed easily, and any such judgement is largely subjective. For instance, the closeness of the observed points to the theoretical line predicted by a model may be crudely estimated by visual inspection, especially if the chosen model can be represented by a linear relation. This procedure is no more useful than the Matthews test for significance (sic-Matthews, 1966)!

The following method has been used to try and reduce the subjectivity of such a test. A number of fitted exponentials were tabulated together, and the mean residual (\pm S.E.M.) of the first data

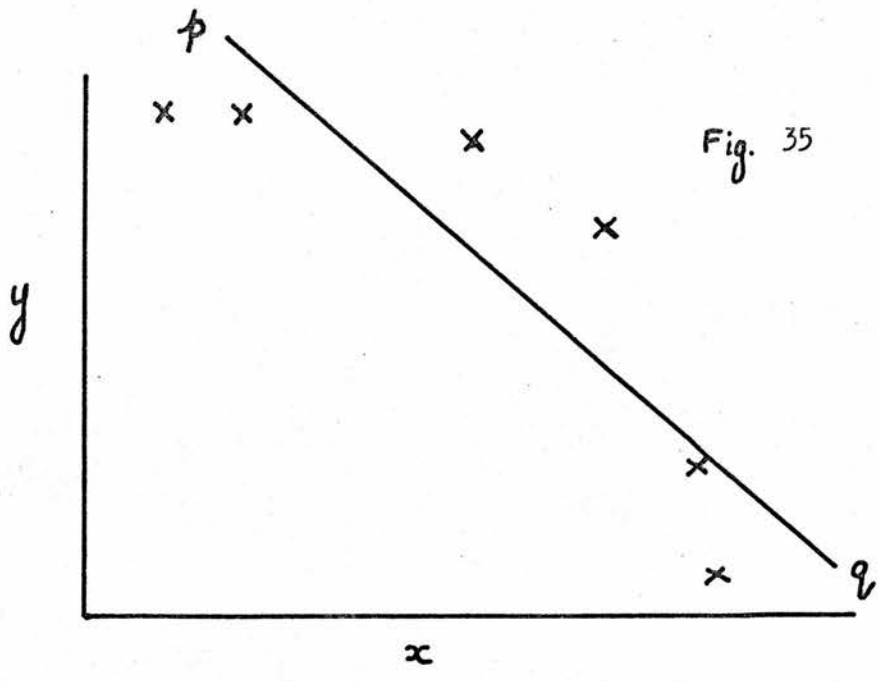


Fig. 35

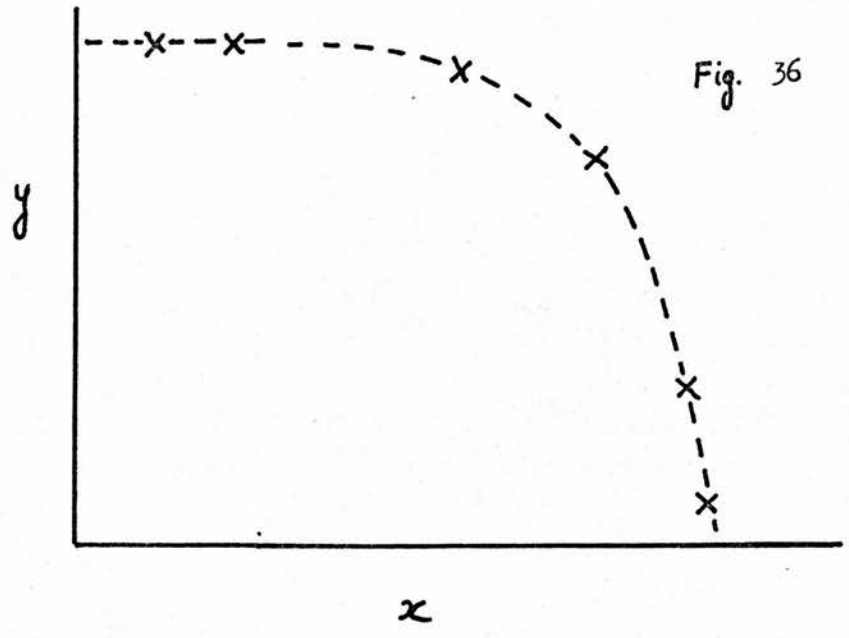


Fig. 36

FIGURES 35 & 36

Testing goodness of fit

If the line pq is drawn through the points in Fig. 35 the residuals of the first two and last two points are negative. If the line were a 'good fit' then the residuals would not differ significantly from zero (see text).

point (i.e. for the lowest value of the independent variable) was calculated. The mean residual was then tested for significant deviation from zero by a t-test. The mean residuals of each subsequent data point were similarly treated. If the mean residual for each set of data points was not significantly different from zero it has been assumed that the exponential model fairly represents the experimental data. However, an instance will later be discussed which shows that only limited confidence can be placed in this still subjective test (Chapter X). Ideally the method requires that the independent variables are the same in successive experiments. In practice they are only approximately the same.

A simple illustration will clarify the use of this test. Suppose that a model predicts a linear relation between y and x , and that the experimental data from a particular experiment are as shown in Figure 35. In fitting the model to the data, the line pq is obtained by a first-order regression. The residuals - i.e. $(y - bx - a)$ where b and a are the first-order regression coefficients - are negative for the first two and the last two points. They are positive for the intermediate points. If subsequent experiments yield similar data, so that the mean residuals for the first two and last two points are significantly less than zero, then clearly a curvilinear model such as that represented in Figure 36 would be a better representation of the experimental data.

Cumulative volume of secretion

The cumulative volume of secretion, the term v in the exponent of equation I, has been taken as the sum of the volumes of secretion in the previous collections plus half the volume of the present collection. Thus it represents the total volume of secretion collected up to (approximately) the mid-point of the present collection.

Glucose concentration
in secretion, mg/ml

Total nitrogen concentration
in secretion, $\mu\text{g/ml}$

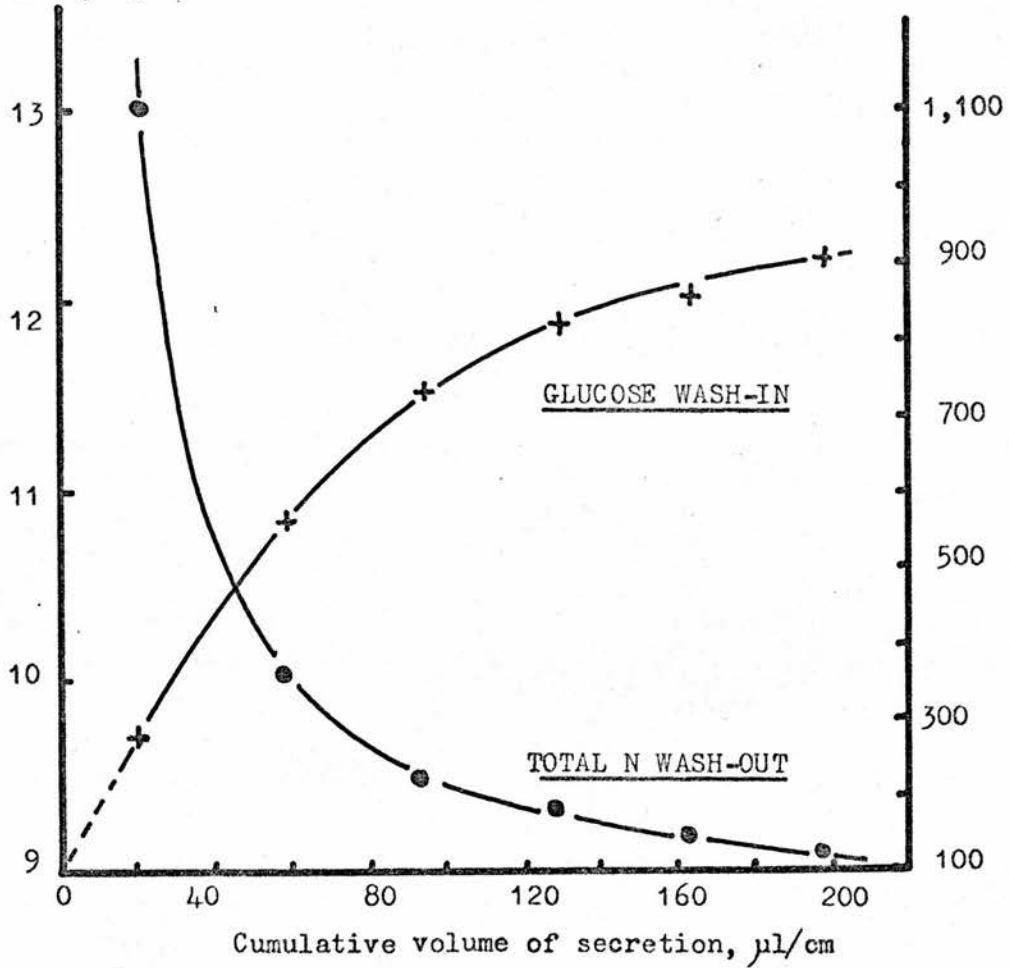


FIGURE 37

Simultaneous wash-in of glucose and wash-out of endogenous nitrogenous material into intestinal secretion

The approximation is made that the mean concentration of a sample of secretion is equal to the concentration at the mid-point of that collection. If the concentration changes exponentially so that the concentration C at time t is given by:-

$$C = A.e^{-kt}$$

then for a period of length $2a$ centred on t , the mean concentration over the period will be:-

$$\begin{aligned} \frac{1}{2a} \int A.e^{-kt} .dt &= \frac{A}{2ak} .(e^{ak} - e^{-ak}) .e^{-kt} \\ &= \frac{(A}{ak} . \sinh ak) .e^{-kt} \end{aligned}$$

Therefore the mean concentration over the whole period differs from the true concentration at the mid-point of the period by the factor:-

$$(\sinh ak)/ak$$

This factor is negligibly different from unity when ak is small.

When the period length ($2a$) is 10 minutes, $a = 5$ minutes. In the present work k is about $1/30 \text{ min}^{-1}$, so $ak = 0.167$ and $(\sinh ak)/ak = 1.0046$ which is trivially different from unity. Therefore negligible error is introduced by the assumption that the mean concentration of a sample of secretion is the same as that at the mid-point of the collection.

Wash-in of glucose into secretion

A typical time-course, or strictly a volume-course, for the appearance of glucose in the secretion during the first hour or so of an experiment is shown in Figure 37. The luminal concentration of glucose was 5 mg/ml. Clearly, the steady-state concentration of glucose had not yet been reached by the time that 200 μ l of secretion per cm length of intestine has passed across to the serosal surface. This corresponds to over one hour on a time basis, since the mean rate

TABLE 16

Best-fit parameters for the time-courses of
appearance of solutes in secretion

$$C = B + A \cdot \exp(-v/V)$$

where C is the concentration of solute in secretion,

and v is the cumulated volume of fluid secreted.

A, B, and V are constants whose significance is discussed in the text.

	Means \pm S.E.M.			Number of experiments
	A mg/ml	B mg/ml	V μ l/cm	
Protein wash-out	13.8 \pm 2.0	0.475 \pm 0.067	29.1 \pm 1.7	9
Total-N wash-out	1.46 \pm 0.15	0.101 \pm 0.012	29.6 \pm 2.4	15
Glucose wash-in	-5.81 \pm 0.47	12.7 \pm 0.3	29.8 \pm 2.0	27
Glucose wash-out	5.50 \pm 0.62	8.08 \pm 0.56	34.9 \pm 2.5	19
Overall mean			V = 30.86 \pm 1.21 μ l/cm	70

$\text{Log}_e(C - B)$

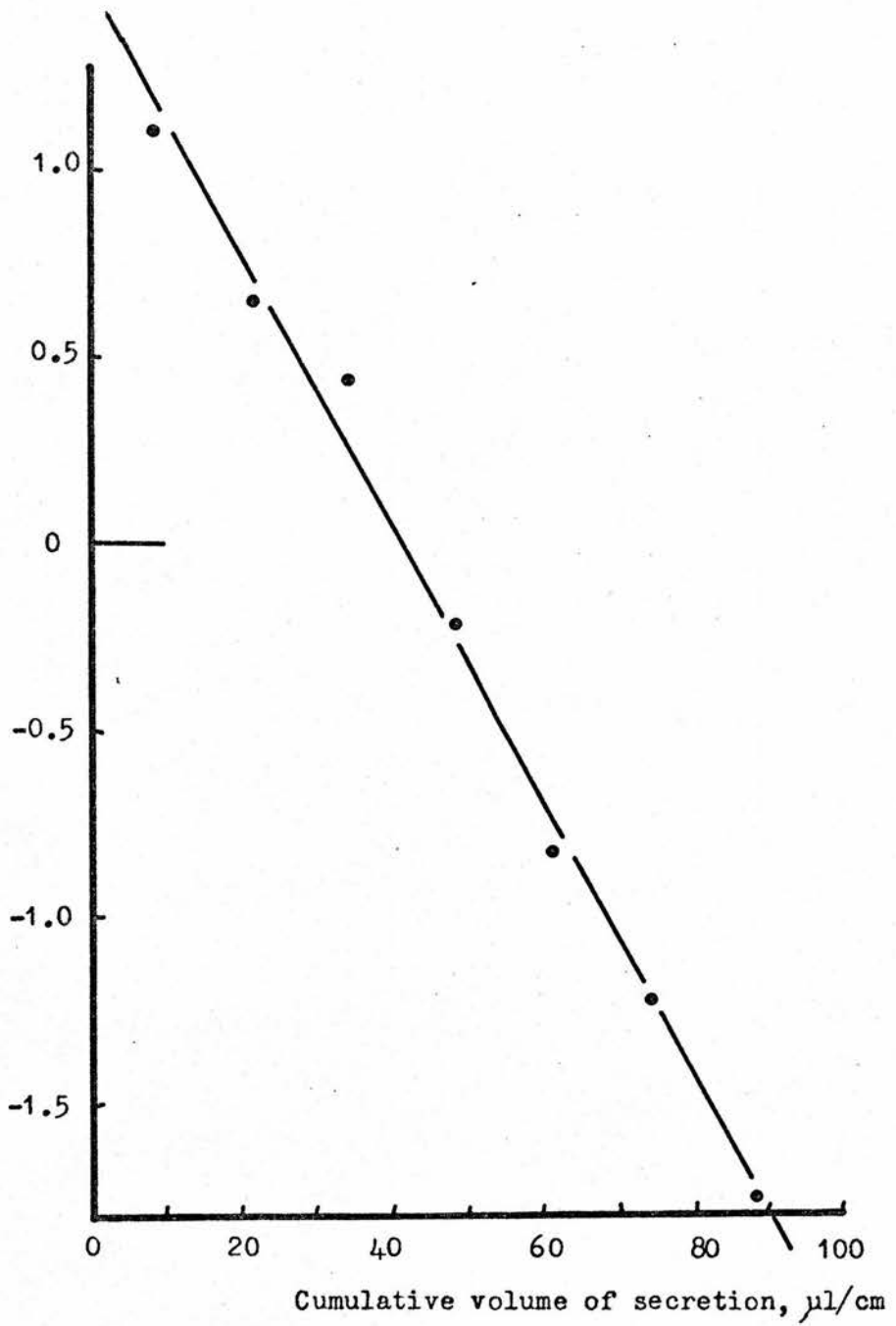


FIGURE 38

Semi-log plot for glucose wash-in to intestinal secretion

TABLE 17

Tests of the goodness of fit of single exponential models to kinetics of appearance of solutes in secretion

Data point	Glucose Wash-in					(7)	(8)
	(1)	(2)	(3)	(4)	(5)		
Mean residual	-0.0491	0.0913	-0.0711	0.0423	-0.0127	-0.0069	0.0958
S.E.M.	0.0184	0.0368	0.0362	0.0266	0.0377	0.0409	0.0417
D of F	29	29	29	29	26	22	7
t	2.665	2.481	1.965	1.590	0.337	0.170	2.302
P	<0.02	<0.02	<0.05	>0.1	NS	NS	<0.05
Data point	Protein Wash-out					(7)	(8)
	(1)	(2)	(3)	(4)	(5)		
Mean residual	-0.0441	0.1326	-0.0873	0.0049	0.0024	0.0269	-0.0471
S.E.M.	0.0326	0.0849	0.0510	0.0387	0.0274	0.0412	0.0467
D of F	9	9	9	9	9	9	7
t	1.354	1.562	1.711	0.127	0.087	0.652	1.010
P	>0.2	>0.2	>0.2	NS	NS	NS	>0.3
Data point	Nitrogen Wash-out					(7)	(8)
	(1)	(2)	(3)	(4)	(5)		
Mean residual	1.929	4.719	16.276	1.552	0.6707	-9.602	8.445
S.E.M.	7.233	13.979	17.40	10.684	10.783	2.969	6.632
D of F	14	14	14	14	14	12	8
t	0.267	0.338	0.935	0.145	0.062	3.234	1.273
P	NS	NS	NS	NS	NS	<0.01	>0.2
Data point	Glucose Wash-out					(7)	(8)
	(1)	(2)	(3)	(4)	(5)		
Mean residual	-0.027	0.0916	-0.0448	-0.0420	-0.0148	0.0870	-0.0274
S.E.M.	0.021	0.0433	0.0395	0.0475	0.0452	0.0513	0.0459
D of F	20	20	20	20	20	20	13
t	1.260	2.119	1.133	0.883	0.327	1.694	0.596
P	>0.2	<0.05	>0.2	NS	NS	>0.1	NS

of water secretion is 169 $\mu\text{l}/\text{cm}/\text{hr}$ (Table 10).

The model:-

$$C = B + A \cdot \exp(-v/V)$$

was fitted by Atkins' method to data for the kinetics of appearance of glucose in the secretion during the first hour or so of perfusion with perfusate containing glucose (5 mg/ml). Mean values for the parameters are given in Table 16. The mean value (\pm S.E.) calculated for B, the asymptote, is 12.7 ± 0.3 (30) mg glucose per ml; this is the concentration in the secretion which would be measured at the steady-state, and corresponds to that in the fluid secreted by the mucosal cells. Thus, the glucose is transported actively from the lumen against a 2.5-fold net concentration gradient.

Figure 38 shows a semi-logarithmic plot of data from a typical experiment. The straight line appears to be a good fit to the data of $\log_e(y - B)$ against v in this instance. However, Table 17 gives the results of applying the test described above to the residuals for glucose wash-in in some 30 experiments. This test implies that the fit is not very good, since the residuals are significantly different from zero for several of the data points. The residuals of the 2nd and last points are positive and those of the 1st and 3rd points are negative. Therefore a simple convex or concave is no better a fit than the straight line.

Wash-out of glucose into secretion

If the perfusate is suddenly replaced by one which contains a different concentration of glucose, then the new steady-state in the secretion is slowly attained in an exponential fashion. In contrast, as has been shown in Figure 25, the rate of glucose absorption from the lumen reaches its new steady-state almost instantaneously.

Figure 39 shows the change of glucose concentration in the secretion

Glucose concentration in
intestinal secretion, mg / ml

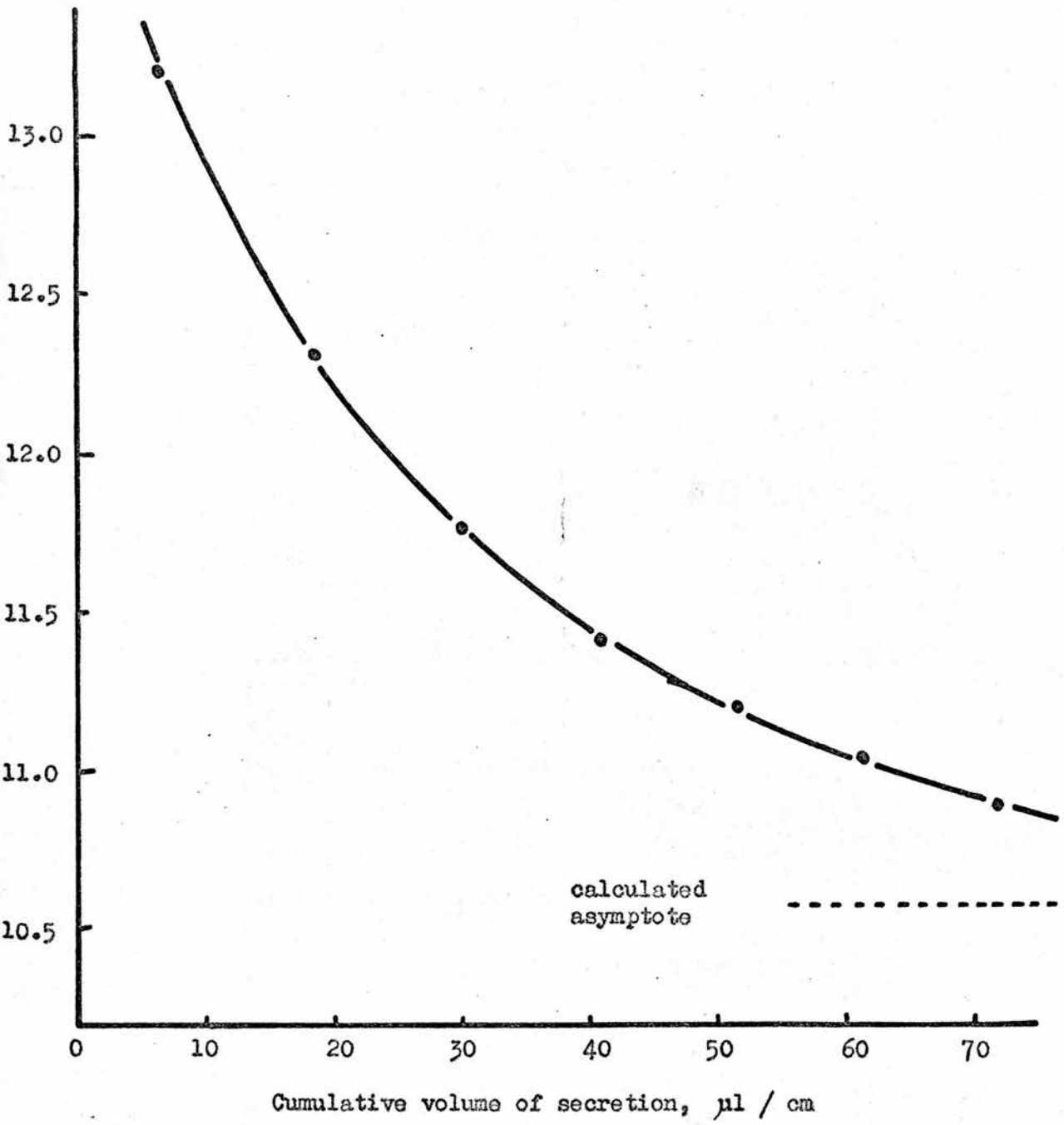


Figure 39

WASH-OUT OF GLUCOSE INTO INTESTINAL SECRETION, when the luminal
glucose concentration is abruptly reduced

$\text{Log}_e(C - B)$

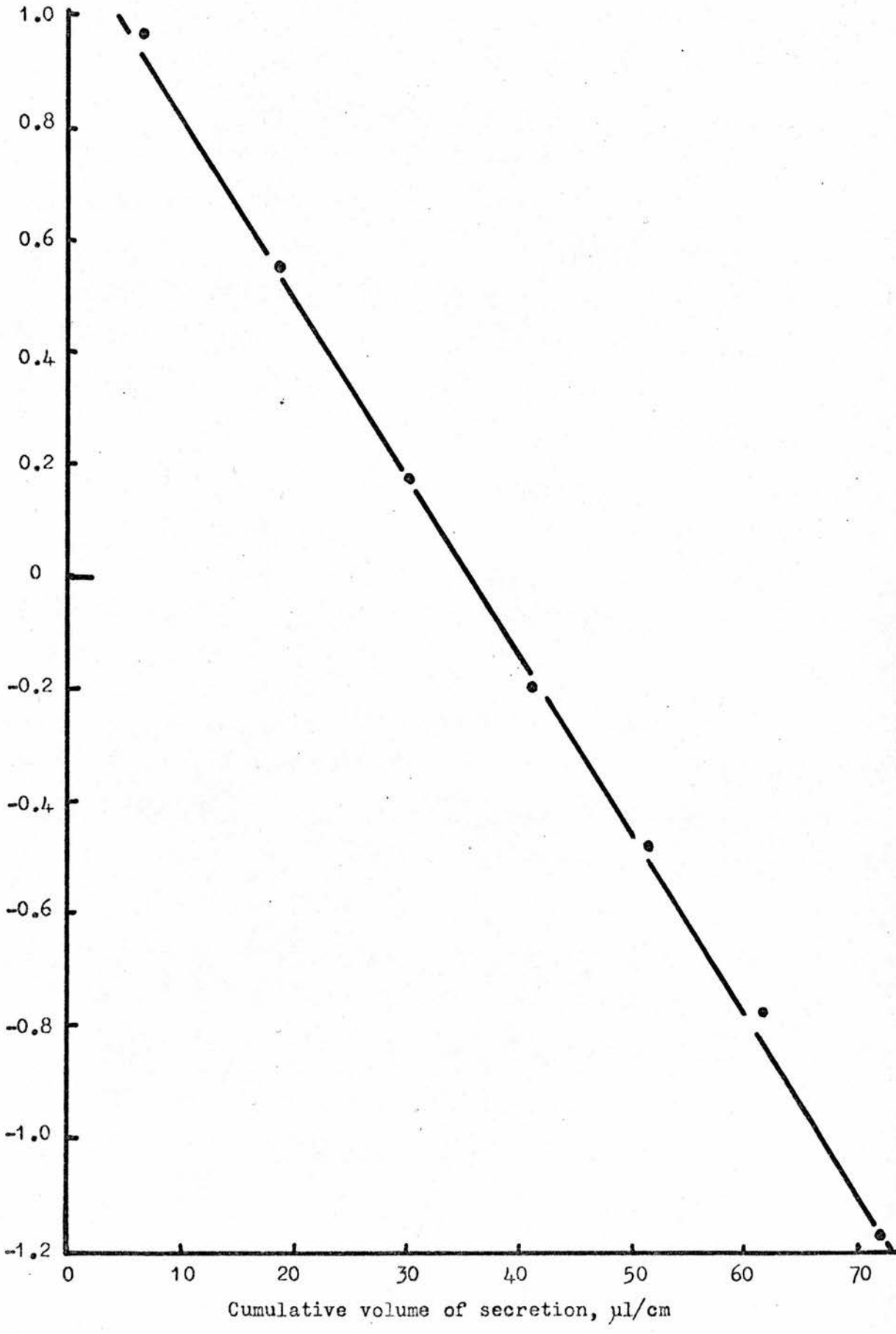


FIGURE 40

Semi-log plot for glucose wash-out into intestinal secretion

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during a typical experiment in which a perfusate containing 5 mg/ml glucose was abruptly replaced by one containing 1.5 mg/ml glucose. Table 16 shows the mean exponential parameters computed for nineteen such experiments. The steady-state concentration in the secretion (B) is 7.97 ± 0.55 (19) mg/ml, corresponding to a luminal concentration of 1.5 mg/ml. In this case, the sugar has been transported actively from the lumen against a 5-fold net concentration gradient.

Figure 40 shows the fit of the single exponential model to the data for a single representative experiment. The data are plotted in the semi-logarithmic form $\log_e(y - B)$ against v , and the straight line appears to be a good fit. The test of residuals in Table 17 suggests that the data are generally well described by the model. All save one of the residuals are not significantly different from zero.

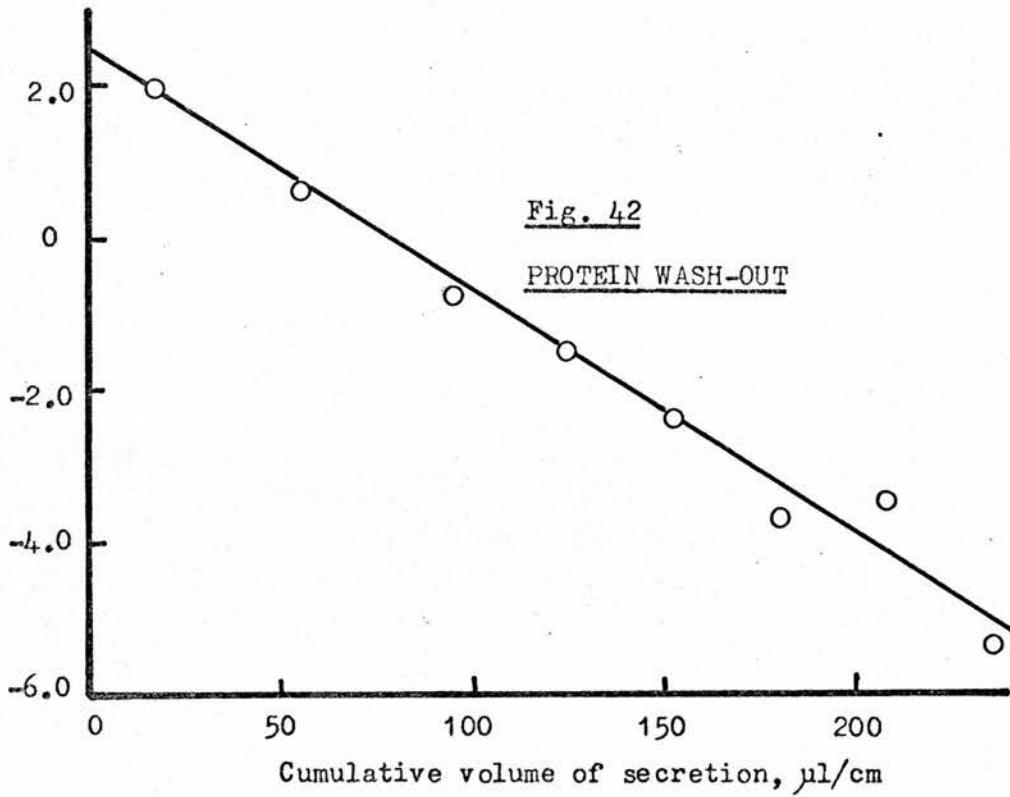
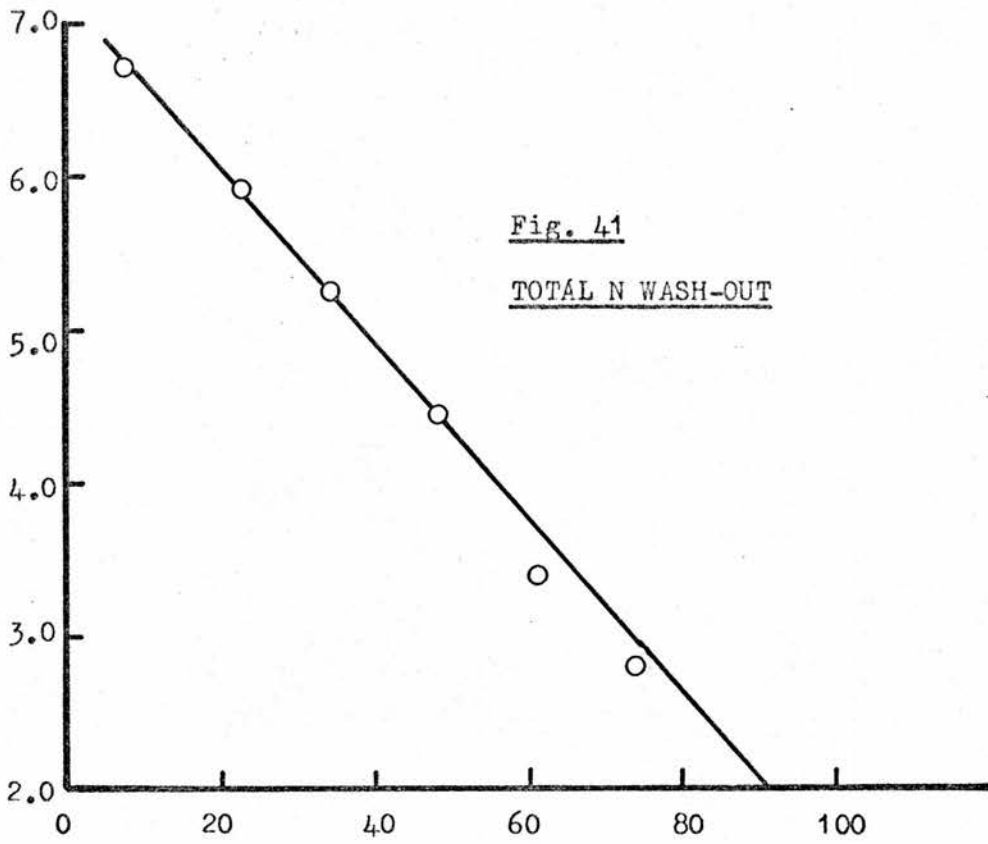
A paired t-test showed no significant difference between the reciprocals of the rate constants for glucose wash-in and wash-out when both these were determined on the same segments of intestine ($t = 0.293$, 4 degrees of freedom).

Wash-out of endogenous nitrogenous material into secretion

Intestinal lymph, and therefore intestinal tissue fluid, contains a high concentration of protein (Yoffey & Courtice, 1956; Barrowman & Roberts, 1967). This can be expected to be washed out into the secretion by the fluid which is transported from the lumen to the serosal surface. If the theoretical assumptions already made were valid then it could be predicted that this wash-out of protein should have the same rate constant as has the wash-in of glucose into secretion.

Total nitrogen in secretion was measured by a modification of Bolleter, Bushman & Tidwell's (1961) method. Secretion was digested

$\text{Log}_e(C - B)$



FIGURES 41 & 42

Semi-log plots for total nitrogen and protein wash-out into intestinal secretion

in micro-Kjeldahl tubes with concentrated sulphuric and perchloric acids with hydrogen peroxide. The resulting ammonium sulphate was estimated by a phenol-hypochlorite reaction adapted to AutoAnalyzer use by Mr. J. J. Pratt (unpublished work).

Typical time-courses for the simultaneous wash-out of pre-formed total nitrogen and wash-in of glucose into secretion are shown in Figure 37. Best-fit parameters in the exponential function were computed for a number of experiments in which the total nitrogen concentrations in secretion were estimated, and are summarised in Table 16.

Similar computations were also made using data for protein (including peptide) concentration in secretion, and the parameters are also shown in Table 16. I have to thank Professor Fisher, also Mr. J. J. Pratt, who made these protein measurements by a biuret method (Stevens, 1958) and who allowed me to use them. Some of the raw data used in computing the nitrogen wash-out exponentials had also been kindly provided by J. J. Pratt.

Figures 41 and 42 show the fit of the single exponential model to the nitrogen and protein wash-out data respectively from representative experiments. The data are plotted in the semi-logarithmic form $\log_e(y - B)$ against v , and the straight lines appear to be reasonable fits. This impression is supported by the test of residuals, shown in Table 17, where the residuals are seen to be insignificantly different from zero for both cases.

THE COMPOSITION OF THE INTESTINAL SECRETION

Introduction

It has already been shown that intestinal secretion contains relatively high concentrations of glucose, and that the composition of the secretion changes exponentially towards a steady-state. It has also been confirmed that the tissue metabolises some of the glucose which it absorbs, but it is not known what the end-product of glucose utilisation is. So as to obtain an estimate of the rate of anaerobic glycolysis measurements have been made of lactate concentration in secretion. Also, as a foundation for future studies on the relationship of ions to the absorption of sugars and water, some measurements on the major inorganic ions present in secretion were made. These are briefly reported, together with details of the analytical methods used, in this section.

Methods

(i) CATIONS

Inorganic cations were estimated by Atomic Absorption Spectrophotometry, using an EEL Model 240 instrument (Evans Electro Selenium Ltd., Halstead, Essex). These methods have been described in Chapter IV. In the early stages of this work a Unicam SP 90 Series 1 Atomic Absorption Spectrophotometer was used, and I am grateful to Dr. H. J. Cruft for allowing me to borrow this instrument from the Biochemistry Honours Class.

(ii) LACTATE

Lactate was estimated by the method of Lundholm, Mohme-Lundholm & Vamos (1963) with minor modifications. L-(+)-lactate is oxidised to pyruvate, in the presence of lactate dehydrogenase (E.C. 1.1.1.27) and NAD. The amount of reduced NAD (NADH) is determined by measuring the extinction at 340 nm. In order to drive the reaction

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to completion, the reaction mixture is buffered at pH 9.0 and the pyruvate which is formed is trapped by hydrazine. Hydrazine, a carbonyl reagent, reacts with the keto-acid to form its hydrazone.

The modifications were mainly those described by O'Brien (1969), who reported a variable increase in the extinction of the reaction mixture at 340nm when the mixture was poured or shaken. This potential source of error was confirmed, but its cause remains unknown. It did not appear to be attributable to atmospheric oxygen, since the trouble was no more serious when the reaction mixtures were incubated under oxygen. For this reason, O'Brien conducted the incubations in the actual cuvettes in which the extinctions were to be measured. A further modification was here introduced, in that the enzyme and co-enzyme were dissolved in the glycine-hydrazine buffer immediately before use. Then the mixed reagents could be added to the sample with a single pipetting operation, rather than the three used by O'Brien. Furthermore, lactate is present in intestinal secretion at such a high concentration that very small aliquots of the order of 20 μ l are required. With these and the pre-mixed reagent no stirring or agitation was found to be necessary before incubation.

The reagent

Glycine (4.67 g), sodium hydroxide (0.4 g), and hydrazine hydrate (1.94 ml) are dissolved, made up to 100 ml with distilled water, and the pH is adjusted with NaOH to 9.18 at 20°C. This corresponds to pH 9.0 at 25°C, the temperature at which the incubations are conducted. This medium is 0.63 M with respect to glycine, and 0.4 M with respect to hydrazine.

Immediately before the reagent is used, NAD (80 mg of grade II material supplied by the Boehringer Corporation Ltd., London) and

L-(+)-lactate dehydrogenase from rabbit muscle (0.1 ml of the 10 mg/ml suspension supplied by Boehringer Ltd.) are added and the mixture inverted once.

Method

To the sample (20 μ l) containing lactate in a Spectronic 20 cuvette (Bausch & Lomb Ltd.) was added reagent (4.0 ml). After the tubes, sealed with 'Parafilm', had been incubated at 25°C for one hour the extinctions at 340 nm were measured in a Coleman II Junior Spectrophotometer (Perkin Elmer Co. Ltd.). Blanks containing water in lieu of lactate, and lactate standards were also set up.

Lactate standards

The concentrations of lactate in the unknown solutions were calculated from the molar extinction coefficient of NADH (see below) but standard 10 mM lactate solutions were always set up with the unknowns to check the reliability of the assay.

The lactate standard was supplied gratis by Boehringer Ltd. as a 1.0 M solution. An aliquot (0.1 ml) was heated at 95°-100°C with about 1-2 ml of distilled water for about 20 minutes to decompose lactones. After cooling, the solution was diluted to 10.0 ml with glycine-hydrazine buffer. There is little tendency for cyclisation to lactones to occur at this high pH, 9.0.

Calculation of lactate concentration

The molar extinction coefficient of NADH was assumed to be $6.22 \times 10^6 \text{ cm}^2 \cdot \text{mole}^{-1}$ (Kornberg, 1957).

The effective light path of the cylindrical Spectronic 20 cuvettes used in the Coleman spectrophotometer was estimated by measuring the extinctions at 560 nm of a series of phenol red solutions in both the Coleman Junior II spectrophotometer with

Extinction in Unicam SP500
with 10 mm cells

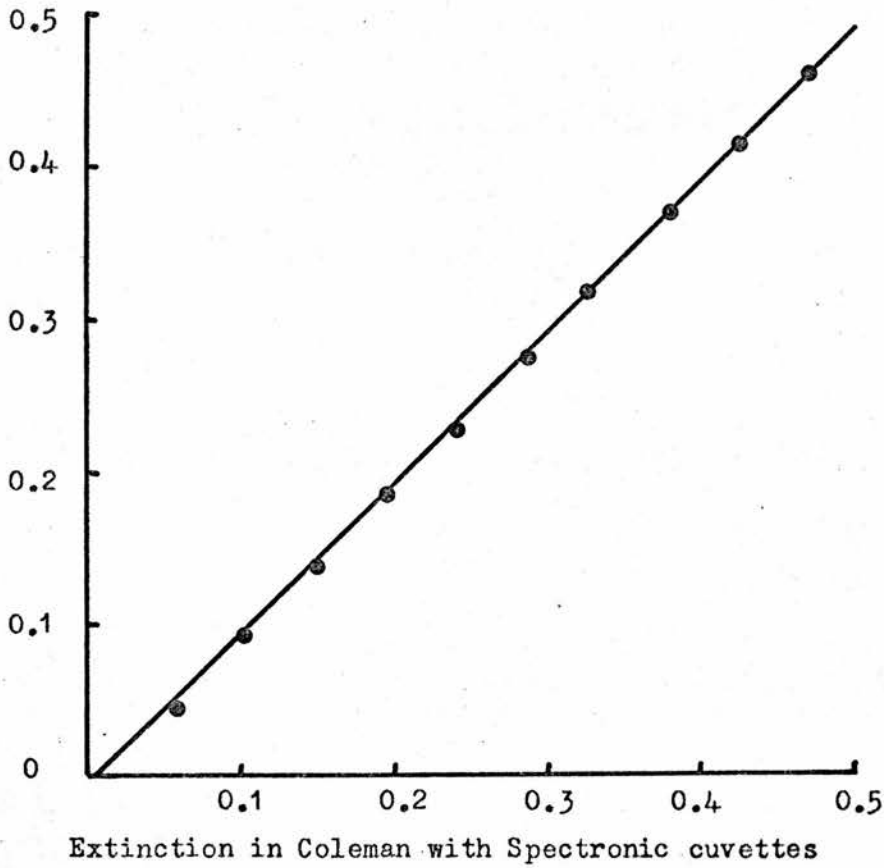


FIGURE 43

Determination of apparent light path length of Spectronic 20 cuvettes
when used in Coleman Junior II spectrophotometer

The extinctions of a series of phenol red solutions were measured in this instrument and in a Unicam SP500 with 10 mm path length cells.

Extinction @ 340 nm

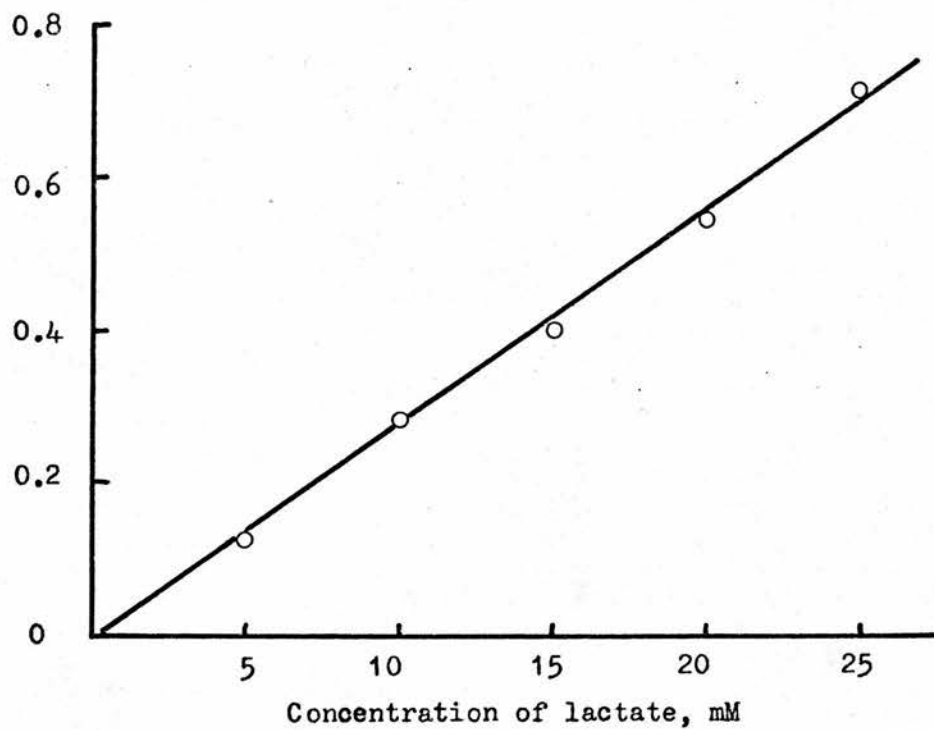
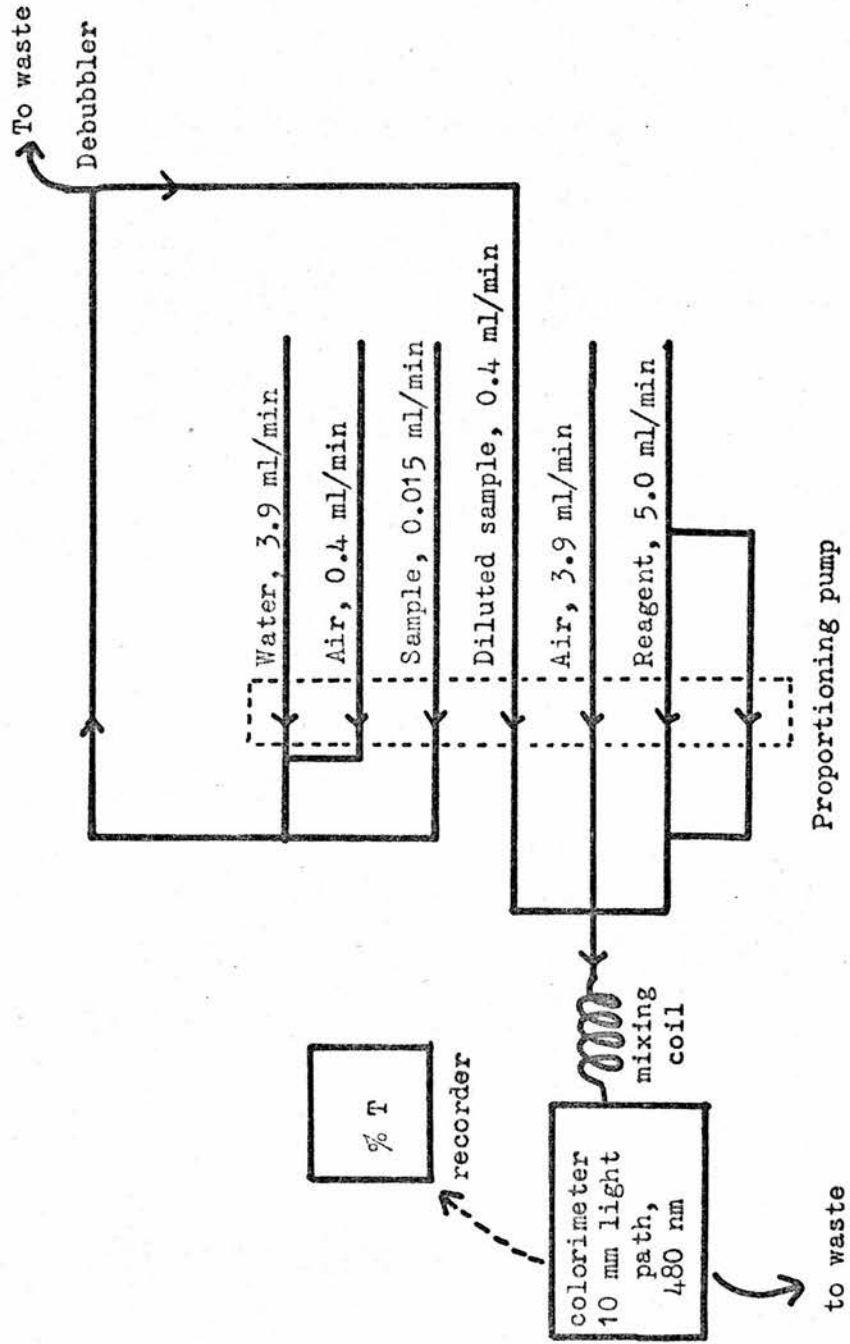


FIGURE 44

Conformity to Beer's Law for lactate estimation

FIGURE 45

AutoAnalyzer system for chloride estimation



Sampling rate: 20/hour

Spectronic 20 cuvettes, and in a Pye-Unicam SP500 spectrophotometer with Pye-Unicam 10.0 mm cuvettes. Figure 43 shows the relationship between the extinctions measured in each instrument. The slope of the line, calculated by the first-order regression, indicates that the effective light-path of a Spectronic cuvette when used in the Coleman spectrophotometer is 9.97 ± 0.057 mm.

Accuracy of an estimate of lactate concentration

Five estimates were made of lactate concentration in a standard solution containing 10.0 mM lactate. The mean (\pm S.E.) of the measured concentrations was 10.077 ± 0.057 mM. This is not significantly different from 10.0 mM. The standard deviation of a single estimate was 0.129 mM - i.e. the coefficient of variation was 1.3%.

Figure 44 shows the conformity to Beer's Law. The calculated slope of the first-order regression is 0.02854 ± 0.00088 litre.mMole⁻¹ which is not significantly different from the theoretical value of 0.03085 litre.mMole⁻¹ calculated from the molar extinction coefficient of NADH and the estimated path length of the spectrophotometer cuvette.

(iii) CHLORIDE

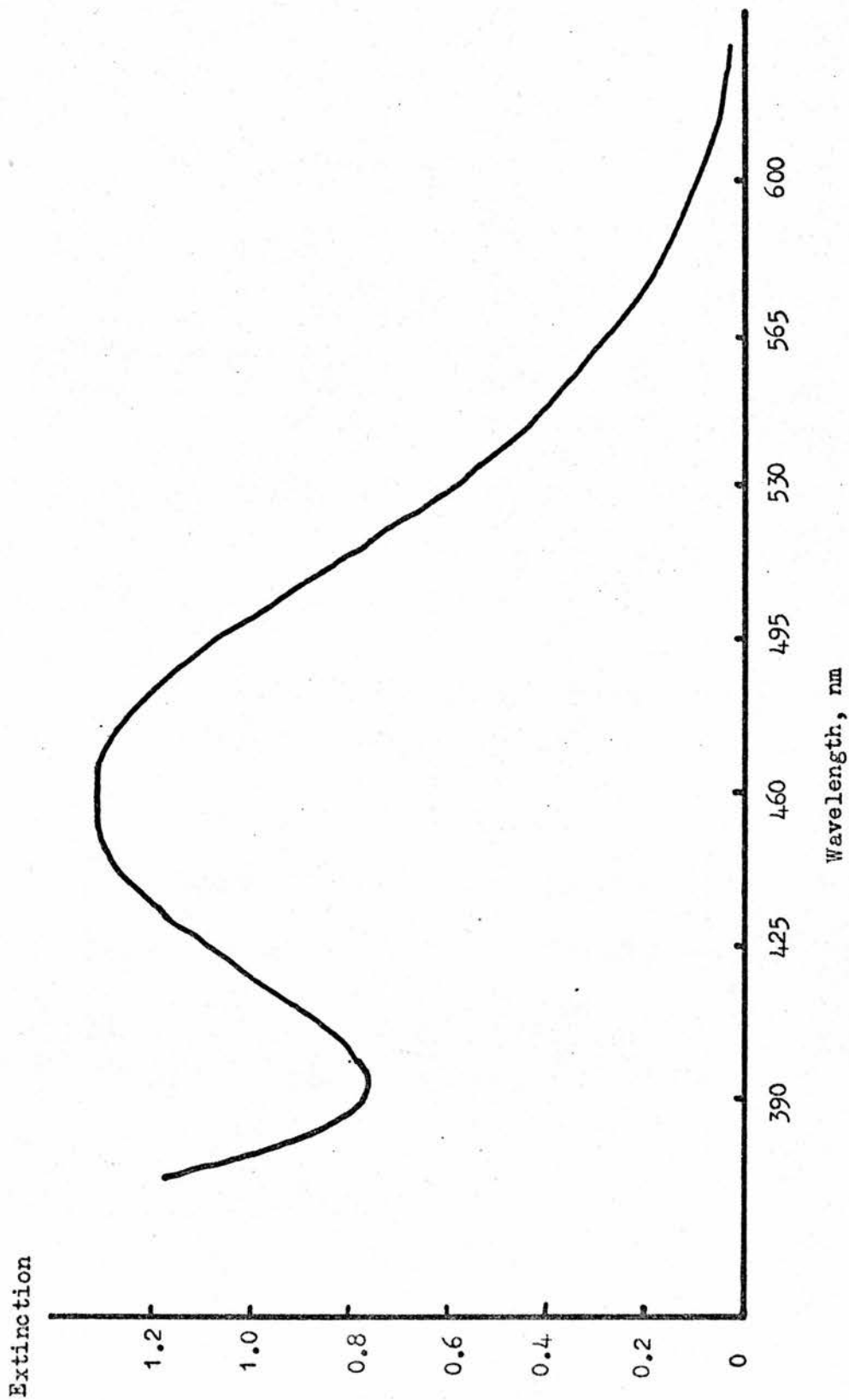
The method was that of Zall, Fisher & Garner (1956) as modified for the AutoAnalyzer by Skeggs (1958). This method depends on the displacement of thiocyanate ions from a solution of mercuric thiocyanate by chloride ions. The displaced thiocyanate is estimated colorimetrically as complexes of the form $\text{Fe}(\text{CNS})_n$ in the presence of ferric nitrate.

The AutoAnalyzer system

This is shown in Figure 45. The rate at which air is pumped was found to be critical, but also to depend on the actual glass 'cactus' junction piece where the reagent, air, and sample streams converge. More or less air gave noisy traces on the recorder chart.

FIGURE 46

The absorption spectrum of the complex $\text{Fe}(\text{CNS})_n$



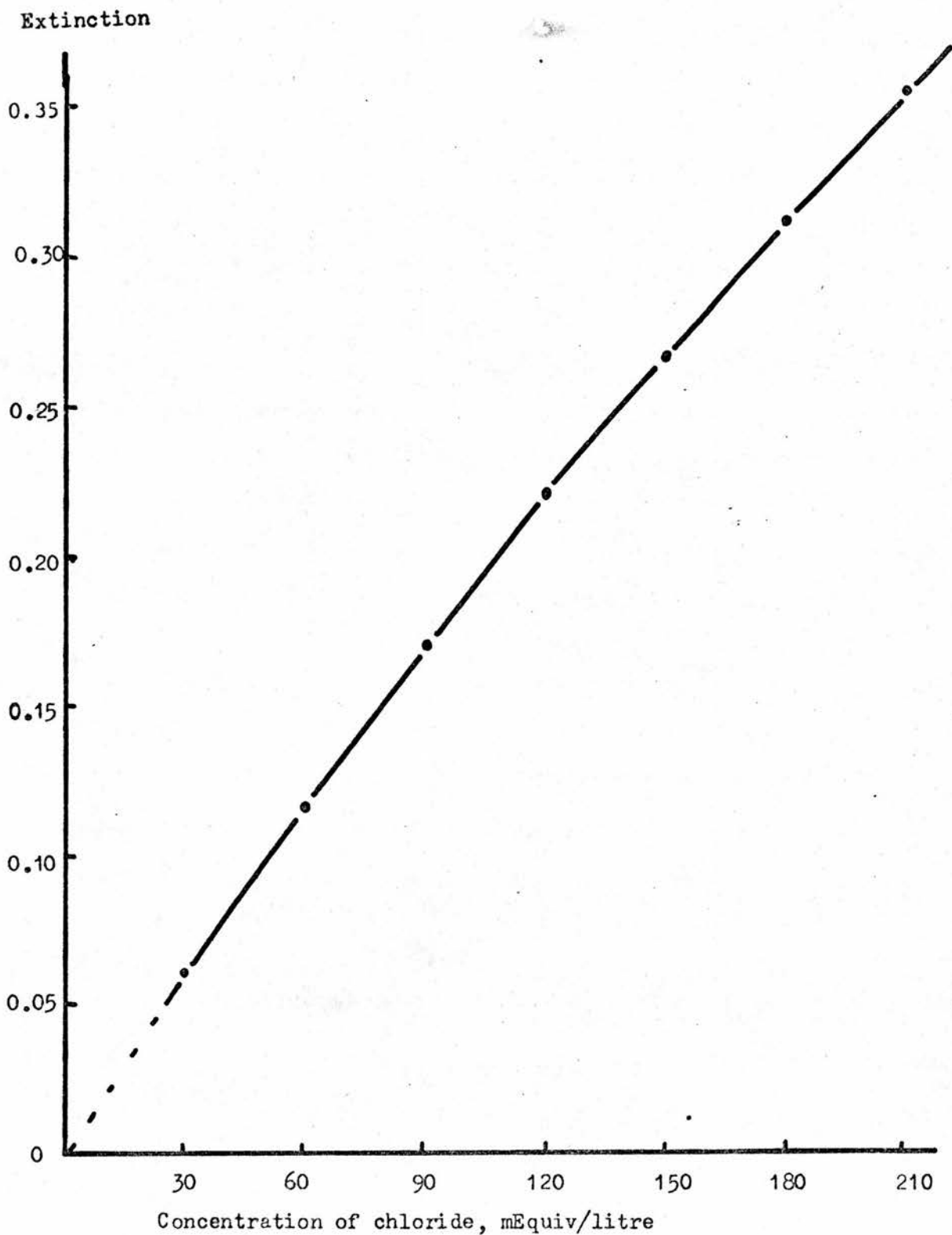


FIGURE 47

Estimation of chloride: a typical calibration curve

Increasing the reaction time by insertion of a 40 ml glass delay coil in series with, and immediately before, the colorimeter had no effect on the extinction.

Reagent

Ferric nitrate (202 g) in concentrated nitric acid (32 ml) is diluted to 1 litre with distilled water. To this is added a saturated solution of mercuric thiocyanate in water (110 ml). The mercuric nitrate additive recommended by Skeggs (1958) was found to be unnecessary.

The absorption spectrum of the coloured product

This spectrum was determined from 390 to 630 nm in a Unicam SP 800 ultra-violet recording spectrophotometer, and is shown in Figure 46. The absorption maximum occurs at approximately 460 nm. As a 460 nm interference filter was not available for the AutoAnalyzer colorimeter, measurements were made at 480 nm, at which wavelength the extinction is about 90% of that at 460 nm.

Calibration of the system

Sodium chloride standard solutions of 30.0, 60.0, 90.0, 210.0 mM were aspirated to the AutoAnalyzer. Figure 47, a plot of extinction against concentration, shows that Beer's Law is not obeyed. However, a second-order function of the form

$$C = a + b.E + c.E^2 \quad \text{where } C \text{ is concentration}$$

and E is extinction,

was found to represent the data closely. Table 18 shows the goodness of fit to the quadratic function, together with an analysis of variance. This regression calculation was executed on the Olivetti P101 calculator, using a program written by Dr. I. A. Nimmo according to Moore & Edwards (1965).

TABLE 18

Fitted values in a typical calibration for chloride estimation

The experimental data were fitted to the quadratic function:

$$C = a + b \cdot E + c \cdot E^2$$

with $a = 0.000813$

$b = 0.4641$

$c = 0.3549$

where C is concentration (μ Eqv/litre)
and E is extinction

Actual Concentration mEqv/litre	Apparent Concentration mEqv/litre
30.0	30.19
60.0	59.59
90.0	90.03
120.0	120.34
150.0	149.91
180.0	179.90
210.0	210.03

Analysis of variance

	Mean square	Degrees of freedom	Variance ratio
Due to linear regression	0.02514	1	
Due to adding quadratic term	0.00006109	1	582.9
Total due to quad- ratic regression	0.01260	2	
Residual	0.0000001048	4	

Precision of an estimate

A standard solution of sodium chloride (90.0 mM) was analysed six times. The mean (\pm S.E.) measured concentration was 89.93 ± 0.3865 mM; this is not significantly different from 90.0 mM. The standard deviation of a single estimate was 0.95 mM - i.e. the coefficient of variation was 1.1%.

(iv) DETERMINATION OF OSMOLARITY

The osmolality of secretion was measured using an 'Osmette S Automatic Osmometer' (supplied by Precision Systems, 44 Rumford Avenue, Waltham, Mass. 02154, U.S.A.). This instrument semi-automatically measures the freezing point of the sample, and from the depression of freezing point below that of pure solvent gives the total osmolality of the solution

Operation of instrument

Samples (2.0 ml or 0.2 ml) are placed in a sample tube, which is then lowered into a sample well in the instrument. Into the sample dip a stirrer, whose amplitude can be preset, and a thermistor - i.e. a semiconductor whose resistance changes markedly with small changes in its environmental temperature. This thermistor is one arm in a Wheatstone Bridge network, the output of which is measured by a sensitive galvanometer. A pump inside the instrument circulates an anti-freeze solution, refrigerated by a solid state cooling device, around the sample tube. The falling temperature of the stirred sample can be followed on the meter. In the absence of nuclei on which ice crystals can form, the solution supercools - i.e. the temperature of the liquid falls below the freezing point. When a certain degree of supercooling has occurred, freezing is initiated by seeding, by vigorously agitating the sample. The meter immediately registers a sudden rise in temperature as the

TABLE 19

The composition of intestinal secretion

	Mean \pm S.E.M. (number of experiments)	
	SECRETION mM	PERFUSATE mM
Sodium	109 \pm 4.0(5)	143.4
Potassium	4.3 \pm 0.11(5)	5.9
Calcium	*0.51 \pm 0.024(3)	1.27
Magnesium	0.192 \pm 0.0186(3)	0.30
Chloride	57.0 \pm 0.17(3)	125.8
Glucose	70.0 \pm 1.74(5)	27.8
Lactate	26.9 \pm 1.03(3)	-
Actual osmolarity (mOsm/litre)	317 \pm 2.7 (4)	318 \pm 1.9 (4)

* Note: No allowance was made for possible interference by phosphate in the estimation of calcium. Phosphate can suppress the atomic absorption of calcium, and so this value may be slightly too low.

latent heat of fusion is emitted, and the temperature settles out around the freezing point of the solution. The final temperature will not be exactly the freezing point, since only a small fraction of the sample is allowed to freeze and so the degree of supercooling will affect the plateau temperature. The temperature meter is calibrated in units of milliosmoles per kilogram of water for aqueous solutions, and in practice the instrument is calibrated before use, with solutions of known osmolarity. These standard solutions were supplied by the manufacturers of the instrument.

Results

Samples of secretion were collected from 40 cm segments of upper small intestine which had already been perfused for 70 minutes with the normal perfusate containing glucose (5 mg/ml). The results are summarised in Table 19 where the composition of the luminal perfusate is also repeated for convenience.

Note that the measured osmolarity of the secretion is not significantly different from that of the perfusate. However, the calculated osmotic pressure of the secretion, assuming unit activities and that there is bicarbonate or some other monovalent anion to balance the cations, is substantially less than the measured value. Part of this discrepancy may be caused by the fact that the various measurements were made on different samples of secretion. Further, there may be some constituent unaccounted for.

In several experiments, the steady-state rate of sodium secretion and the rate of glucose absorption from the lumen have been determined simultaneously. The rate of sodium absorption from the lumen cannot be measured directly, since the difference between the sodium concentrations in perfusate and lumen effluent is very small (less than 1% of the perfusate concentration). However, at the steady-

state the rate of sodium secretion should equal the rate of absorption from the lumen. The mean ratio (\pm S.E.M.):-

(rate of glucose absorption)/(rate of Na secretion)

was 0.87 ± 0.04 (15) mMoles/mEq. This is significantly less than unity ($P < 0.01$).

DISCUSSION

The kinetic analysis of the appearance of solutes in the secretion clearly demonstrates that a steady-state can be achieved only slowly: more than 200 μ l of secretion per cm length of intestine must be collected before the steady-state in the secretion is reached. In this preparation, the mean rate of water absorption was 169 μ l/cm/hr (Table 10), when the luminal glucose concentration was 5 mg/ml. Hence, estimates of glucose secretion and utilisation rates made within the first hour or so from the setting-up of the preparation are of little value - they will be too low and too high respectively. This restriction probably applies to other intestinal preparations including that of Fisher & Parsons (1949) and everted sacs etc., but probably excludes preparations of stripped sheets of mucosa or isolated cells.

In their Figure 6, Smyth & Taylor (1957) show the concentrations of glucose in their secretions corresponding to several luminal glucose concentrations. With 5 mg/ml glucose in the lumen, the concentration of glucose is about 7 mg/ml; this contrasts with the steady-state value of about 13 mg/ml found in this work. However, this discrepancy is not as great as it appears at first sight. Smyth & Taylor's sample of secretion seems to have been a pooled sample, collected over the whole of the first hour of perfusion; therefore, their data are mean values, rather than steady-state

values or even instantaneous values. It is clear from Figure 37 that if an estimate of glucose in secretion is made before some 200 μl of secretion per cm of intestine has been collected and discarded, the concentration will be less than the steady-state value which corresponds to the composition of the fluid leaving the mucosal cell. As already shown, the concentration of glucose in the secretion is given by:-

$$C = C_{\text{inf}} + (C_0 - C_{\text{inf}}) \cdot \exp(-v/V)$$

Let p be the rate of water secretion,

and t be the time from commencement of perfusion.

Then, $v = p \cdot t$

Assume that the glucose concentration in the tissue fluid, and hence in the secretion, at zero time (C_0) is zero.

So, $C = C_{\text{inf}} - C_{\text{inf}} \cdot \exp(-pt/V)$

If a mean value (\bar{C}) of concentration is measured over a period of time T ,

$$\text{then, } \bar{C} = \frac{1}{T} \int_0^T (C_{\text{inf}} - C_{\text{inf}} \cdot \exp(-pt/V)) \cdot dt$$

$$= C_{\text{inf}} - \int_0^T \left[C_{\text{inf}} \cdot \frac{V}{p} \cdot \exp(-pt/V) \right]$$

Taking $V = 30.9 \mu\text{l/cm}$ (Table 16),

and $p = 100 \mu\text{l/cm/hr}$,

and $T = 1 \text{ hr}$

$$\begin{aligned} \text{Then, } \bar{C} &= C_{\text{inf}} \cdot (1 + 0.309 \exp(-100/30.9) - 0.309 \exp(-0)) \\ &= 0.7 \times C_{\text{inf}} \end{aligned}$$

So in Smyth & Taylor's work,

$$C_{\text{inf}} = \bar{C}/0.7 = 7.0/0.7 = 10.0 \text{ mg/ml}$$

Thus, the expected steady-state concentration is 10 mg/ml in Smyth & Taylor's experiments.

The estimates of initial concentration (C_0) obtained from the glucose wash-in and total nitrogen (or protein) wash-out exponentials are not equal to the actual concentrations of solutes present in the extracellular fluid in vivo, since secretion is not collected during the first few minutes after the isolated intestine has been set up.

Protein wash-out into the intestinal lymph of intact rats was recorded by Barrowman & Roberts (1967) when the animals drank water or saline. Although these authors did not characterise the kinetics of wash-out, their data appear consistent with the results of this work. Barrowman & Roberts estimated the protein concentration in normal intestinal lymph as nearly 30 mg/ml; this seems in accord with the present estimate of $C_0 = 14.3$ mg/ml made shortly after wash-out had started (Table 16).

Note that C_{inf} for protein wash-out (0.475 ± 0.067 (9) mg/ml) and for total nitrogen wash-out (0.101 ± 0.012 (15) mg/ml) are both significantly greater than zero. This indicates that the intestine, in a steady-state, continues to lose protein material. The cause could either be a steady disintegration of the tissue or 'normal' protein synthesis. Although there is continual turnover of the mucosa the former possibility is unlikely, since the preparation is known to be stable with respect to the active absorption of glucose for quite a long time. The second possibility seems likely, since the intestine is well known to be active in protein synthesis. However, the source of amino acid precursors is not known and it might be premature to suggest that this steady 'secretion' of protein is relevant to the subject of intestinal absorption of dietary protein. The problem has been in the hands of another worker in Professor Fisher's laboratory.

The mean steady-state concentration of glucose in the secretion

corresponding to a luminal concentration of 5 mg/ml was 12.7 ± 0.3 (27) mg/ml when estimated from the glucose wash-in kinetics. This value is not significantly different from 13.6 ± 0.45 (19) mg/ml which was obtained as C_0 from the glucose wash-out data ($P > 0.05$).

This ability of the preparation to transport glucose against such an adverse concentration gradient confirms qualitatively the viability of the preparation. Indeed, with 1.5 mg/ml glucose in the lumen, the concentration ratio is even greater since the steady-state concentration in the secretion is then 7.97 ± 0.55 (19) mg glucose per ml.

Such high concentrations of glucose in the tissue fluid constitute highly unphysiological conditions, corresponding to very severe hyperglycaemia. The normal blood glucose concentration in the rat is about 0.6 mg/ml (Spector, 1956, p.53). Consequently, it is possible that the unusual conditions obtaining in the tissue fluid may alter the metabolism of the mucosa. For instance, the high rate of anaerobic glycolysis which is indicated by the relatively large amounts of lactate in the secretion (Table 19) might be related to this condition, rather than to oxygen lack.

The reciprocals (V) of the rate constants determined for protein wash-out (29.1 ± 1.7 (9) $\mu\text{l}/\text{cm}$), for nitrogen wash-out (29.6 ± 2.4 (15) $\mu\text{l}/\text{cm}$), for glucose wash-in (29.8 ± 2.0 (30) $\mu\text{l}/\text{cm}$), and for glucose wash-out (34.9 ± 2.5 (19) $\mu\text{l}/\text{cm}$) are not significantly different: indeed, they are in remarkable agreement with each other. This, and the apparent goodness of fit of the exponential model, supports the belief that the secretion which is collected is a fair sample of the tissue fluid bathing the serosal pole of the mucosal cells. The kinetics of appearance of solutes are those of a single compartment system. While it can be foolish to equate a mathematical

compartment to a specific anatomical compartment such as the sub-mucosal extracellular space, it may be pointed out that the apparent volume of this calculated compartment (30.9 ± 1.21 (73) $\mu\text{l}/\text{cm}$) is a reasonable figure for the extracellular water of the intestine.

(The total water content is 50-60 $\mu\text{l}/\text{cm}$ - Table 13.)

The good agreement between the predicted and observed kinetics supports the validity of the assumptions which were made in the theoretical derivation. For example, it illustrates the constancy of the solute concentration in the fluid secreted from the serosal pole of the epithelial cells. This is consistent with the good stability of the preparation. However, in the kinetic analysis no allowance was made for glucose etc. refluxing into the mucosal cells and lumen from the tissue fluid. Wilson (1962, page 84) gives the ratio of mucosal to serosal/serosal to mucosal flux rates of glucose as about 20:1. Therefore neglect of the refluxing glucose in the kinetic analysis could lead to an underestimate of the apparent compartment size by about 5%. (The theoretical section of the following Chapter shows how the exponential rate constant can be a function of both the compartment size and the ratio of the unidirectional flux rates. If the reflux from the tissue fluid is at a rate independent of the solute concentration in the tissue fluid, then the rate constant will be unaffected by this back flux. If, however, the rate of reflux is proportional to the tissue fluid concentration then the rate constant will be larger than the reciprocal of the tissue fluid volume. If the rate of reflux is a non-linear function of the tissue fluid concentration, then the data will not fit to a single exponential.) No error would be incurred in the computation of the asymptotic concentration at the steady-state.

The good agreement between the rate constants for glucose and protein wash-out is quite consistent with the absence of back flux.

Superficially, the approximately 1:1 ratio of glucose to sodium absorption is in agreement with the Crane (1962) hypothesis that sodium and glucose enter the mucosal cell on a common carrier. These rates are NET rates, and it is probable that the unidirectional rate of glucose efflux from the lumen is only slightly greater than the net rate. The unidirectional rate of sodium efflux almost certainly is much greater than the net flux rate (see Chapter VIII). Therefore the ratio of unidirectional flux rates of glucose and sodium may be much less than 1:1.

CHAPTER VII

THE ABSORPTION OF DEUTERIUM OXIDE

BY RAT SMALL INTESTINE

CHAPTER VII THE ABSORPTION OF DEUTERIUM OXIDE
BY RAT SMALL INTESTINE

Introduction

Deuterium oxide ('heavy water') and tritium oxide have been used frequently as tracers for water in measurements of unidirectional flux rates across the mucosa. The validity of their use depends on the assumption that they behave as perfect tracers: i.e. that deuterium oxide, tritium oxide, and water are absorbed at equal rates by the intestine, and that the presence of tracer in no way modifies the function of the tissue. Many authors have tacitly made this assumption, but without any experimental justification. This may be because a test of the validity is difficult, or even impossible, to carry out with most preparations of intestine. Many authors have therefore relied on the fact that the diffusion rates of the tracer and of water might be expected to differ only trivially, since the difference in their masses is small. As will be discussed later, this assumption is not necessarily justifiable, whether or not water absorption occurs by a passive diffusion process.

As will become clear below, this type of preparation of isolated intestine is well suited to a direct comparison of the rates of absorption of water and of tracer. By collecting the intestinal secretion one can determine the composition of the sub-epithelial tissue fluid while water and marker are being absorbed from the lumen. The kinetic analysis which has already been developed in Chapter VI to represent the time-course of the appearance of solutes, such as glucose, in secretion can be extended to the unidirectional movements across the epithelium of substances such as deuterium oxide. Simple measurements of the appearance of the tracer may thus afford approximate values for the mucosal to serosal

and serosal to mucosal unidirectional flux rates, in addition to testing the suitability of the tracer.

The experiments described in this Chapter were all performed with the collaboration of Dr. R. J. Bywater from the Department of Veterinary Pharmacology, Royal (Dick) School of Veterinary Studies, University of Edinburgh. The work was started because he had been using deuterium oxide as a tracer in Thiry-Vella loops in calves and he wished to justify the crucial assumptions.

Theoretical kinetics

Consider the time-course of transport of deuterated water from the lumen of the intestine, across the epithelium and sub-epithelial space, and through the torn lymphatics and blood vessels on to the serosal surface.

Suppose that deuterium oxide and water may be differentially absorbed, so that the concentration of tracer in the fluid entering the mucosal cells from the lumen may be different from the concentration in the lumen; similarly, that the concentration of tracer in the fluid re-fluxing from the extracellular fluid back into the lumen may not be equal to the concentration in the extracellular fluid.

Let:- P be the luminal concentration of tracer,

C be the concentration of tracer in the extracellular fluid, and hence in the secretion,

i be the rate of unidirectional flux of water out of the lumen,

e be the rate of unidirectional flux of water back into the lumen,

s be the net rate of water secretion onto the serosal surface,

V be the volume (supposed constant) of sub-epithelial extracellular fluid,

r be the ratio of mucosal-serosal water flux rate to the net flux rate; i.e. $r = i/s$,

$$a = \frac{\text{tracer concentration in fluid leaving lumen}}{\text{tracer concentration in lumen}}$$

$$\text{and } b = \frac{\text{tracer concentration in fluid refluxing into lumen}}{\text{tracer concentration in extracellular fluid}}$$

Since V is constant, $i = e + s$

but, $r = i/s$

$$\text{so, } e = (r - 1) \cdot s$$

When infinitesimal changes di, de, and ds occur in i, e, and s respectively, then the corresponding change, dC, in C is given by:-

$$V \cdot dC = a \cdot P \cdot di - b \cdot C \cdot de - C \cdot ds$$

$$\text{So, } \frac{dC}{\frac{a \cdot P \cdot r}{b \cdot (r - 1) + 1} - C} = \frac{b \cdot (r - 1) + 1}{V} \cdot ds \dots\dots (i)$$

On integration, this yields the exponential function,

$$C = B + A \cdot \exp(-kv) \dots\dots\dots (ii)$$

where $B = \frac{a \cdot P \cdot r}{b \cdot (r - 1) + 1} \dots\dots\dots (iii)$

$$A = C_0 - B \text{ where } C_0 \text{ is the value of } C \text{ when } v = 0$$

$$\text{and the rate constant, } k = \frac{b \cdot (r - 1) + 1}{V} \dots\dots\dots (iv)$$

and, v is the cumulative total volume of secretion collected.

Note that the asymptote, B, is the value of C at the steady-state - i.e. when v is infinite. If there were no isotope effect, then

$$a = b = 1$$

and so $B = P$ - i.e. the concentration of marker in the secretion at the steady-state would equal the concentration in the lumen. Equation (ii) is identical in form to that already derived in Chapter VI to represent the kinetics of appearance of glucose in secretion. The values of B and of k (equations (iii) and (iv)) have however been modified by terms in r to take into account the

back-flux of fluid into the lumen, and in a and b to allow for possible isotope effects.

The problem of estimating the constants B, A, and k in equation (ii) given experimental data for C (deuterium oxide concentration in secretion) and v (cumulated volume of secretion collected) has already been discussed in Chapter VI. It was there concluded that the digital computer program of Atkins (1971a) was the most suitable solution to this problem. A method for assessing the goodness of fit of a theoretical model to experimental results was also described in Chapter VI, and is used here.

If the isotope effects for flux out of and back into the lumen are equal, then $a = b$. Equations (iii) and (iv) can then be solved for b and for r.

$$\text{Hence, } a = b = 1 - V \cdot k \cdot (1 - B/P) \dots\dots\dots (v)$$

$$\text{and, } r = \frac{B \cdot (1 - b)}{P \cdot b \cdot (1 - B/P)} \dots\dots\dots (vi)$$

If however, there is an isotope effect for transport of tracer out of the lumen, but not for the passage back into the lumen, then $a \neq 1$ but $b = 1$. In this case equations (iii) and (iv) give:-

$$a = B/P \dots\dots\dots (vii)$$

$$\text{and, } r = V \cdot k \dots\dots\dots (viii)$$

In the other extreme case, with no isotope effect for the efflux out of the lumen but with one for the flux back into the lumen $a = 1$, but $b \neq 1$. Equations (iii) and (iv) now yield:-

$$b = \frac{(k \cdot V) - 1}{(k \cdot V \cdot B/P) - 1} \dots\dots\dots (ix)$$

$$\text{and, } r = k \cdot V \cdot B/P \dots\dots\dots (x)$$

In each of these cases r can give the two unidirectional flux rates i and e, since

$$r/(r - 1) = i/e \dots\dots\dots (xi)$$

and the net rate of secretion is

$$s = i - e$$

Methods

A segment of intestine was perfused as already described, except that the length of segment taken was about 80 cm. The perfusate was a normal one containing glucose (5 mg/ml, 28 mM). Approximately five minutes after the segment had been placed in the organ chamber and had begun to secrete fluid onto its serosal surface, the perfusate was replaced by one identical except that it contained deuterium oxide (supplied by Koch-Light Ltd., Colnbrook, Buckinghamshire) - approximately 1 ml/100 ml perfusate. After a five minute pre-period to allow the conditions in the lumen to reach their new steady-state (see page 56) collections of secretion were made at two minute intervals.

These samples were weighed, diluted with distilled water (0.5 ml) and covered with Parafilm. They were centrifuged and the deuterium oxide concentration in them was estimated in duplicate by measuring the extinction at $3.98 \mu\text{m}$ in a Perkin-Elmer Model 257 double beam infra-red spectrophotometer (Turner, Neely & Hardy, 1960). Calcium fluoride cells ('Unipak' cells with 0.1 mm path length made by Ross Scientific Co., Hornchurch, Essex) were used. The reference cell contained distilled water, and to minimise thermal drift, this cell was removed from the spectrophotometer when a reading was not actually being taken. The reference water was replaced by water at room temperature immediately before a reading was made. The concentration of tracer in the perfusate was also estimated as a mean of about six measurements for each experiment. We are grateful to Professor G. S. Boyd for allowing us to use his spectrophotometer.

The concentrations of these samples were calculated from the first order regression of extinction on concentrations for standard solutions containing 0.2, 0.4, 0.6, and 0.8% w/v deuterium oxide

Extinction

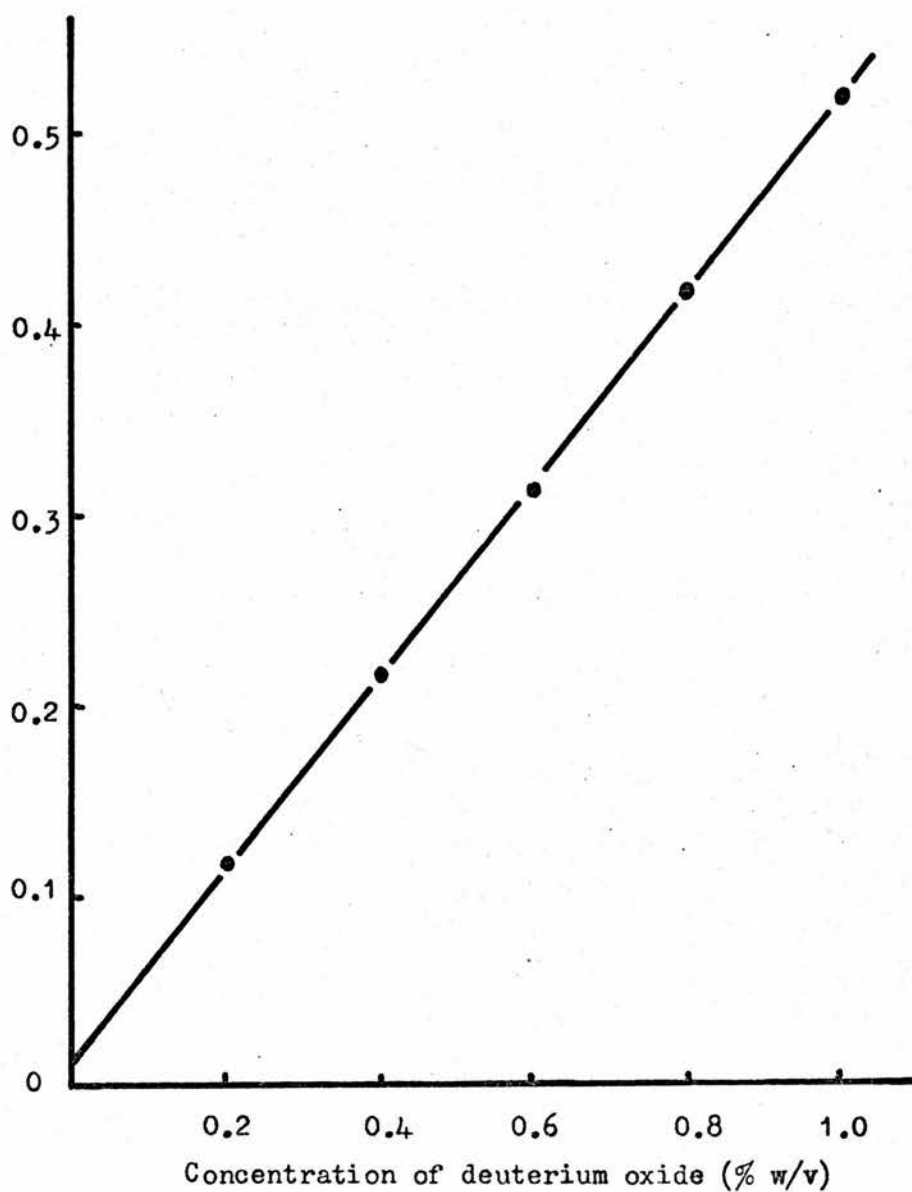


FIGURE 48

Estimation of deuterium oxide; a typical calibration curve

TABLE 20

Appearance of deuterium oxide in intestinal secretion

B, A, and k are the best-fit parameters in the function:

$$C = B + A \cdot \exp(-k \cdot v)$$

C is the deuterium oxide concentration in secretion,
 v is the cumulative volume of secretion,
 P is the perfusate concentration of deuterium oxide,
 B is the asymptotic concentration of deuterium oxide
 computed for the steady state in secretion.

Best-fit parameters

B(%)	A(%)	k(cm/ μ l)	P(%)	P-B	B/P
1.0450	-0.7632	0.11604	1.085	0.0400	0.9631
1.1265	-0.6161	0.18285	1.165	0.0385	0.9669
1.1630	-0.5723	0.16972	1.165	0.0020	0.9983
1.1620	-0.8403	0.31429	1.165	0.0030	0.9974
1.1269	-0.8137	0.29364	1.165	0.0381	0.9673
1.1188	-0.7129	0.16695	1.165	0.0462	0.9603
1.0523	-1.0364	0.09095	1.0916	0.0393	0.9640
1.0610	-0.8404	0.09941	1.0895	0.0285	0.9738
1.0828	-0.8454	0.10235	1.0896	0.0068	0.9938
1.0766	-0.9392	0.15684	1.0969	0.0203	0.9815
1.0482	-0.7498	0.07193	1.0755	0.0273	0.9746
			Mean	0.02636	0.97645
			S.E.M.	0.004852	0.004295

Each line refers to a separate experiment

Concentration of deuterium
oxide in secretion, % (w/v)

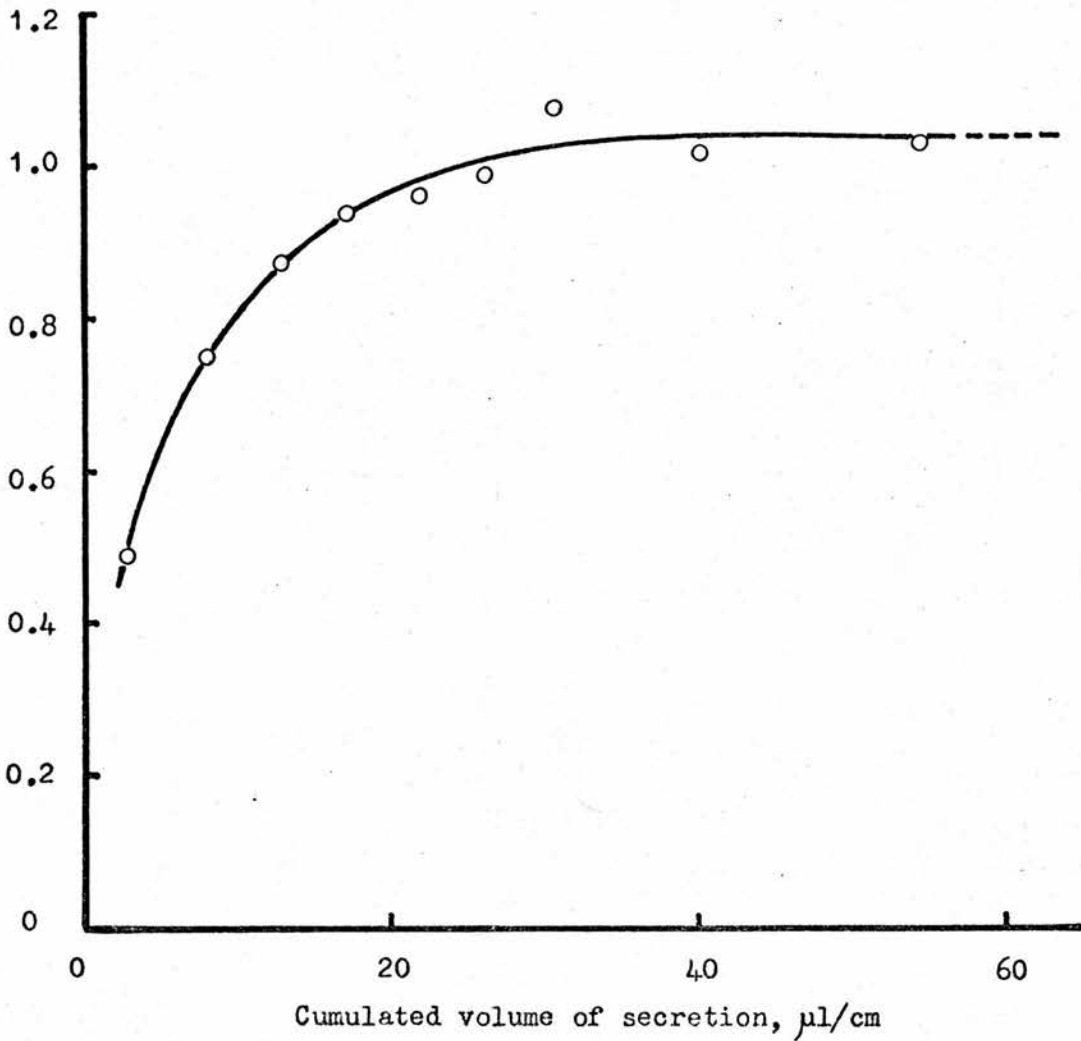
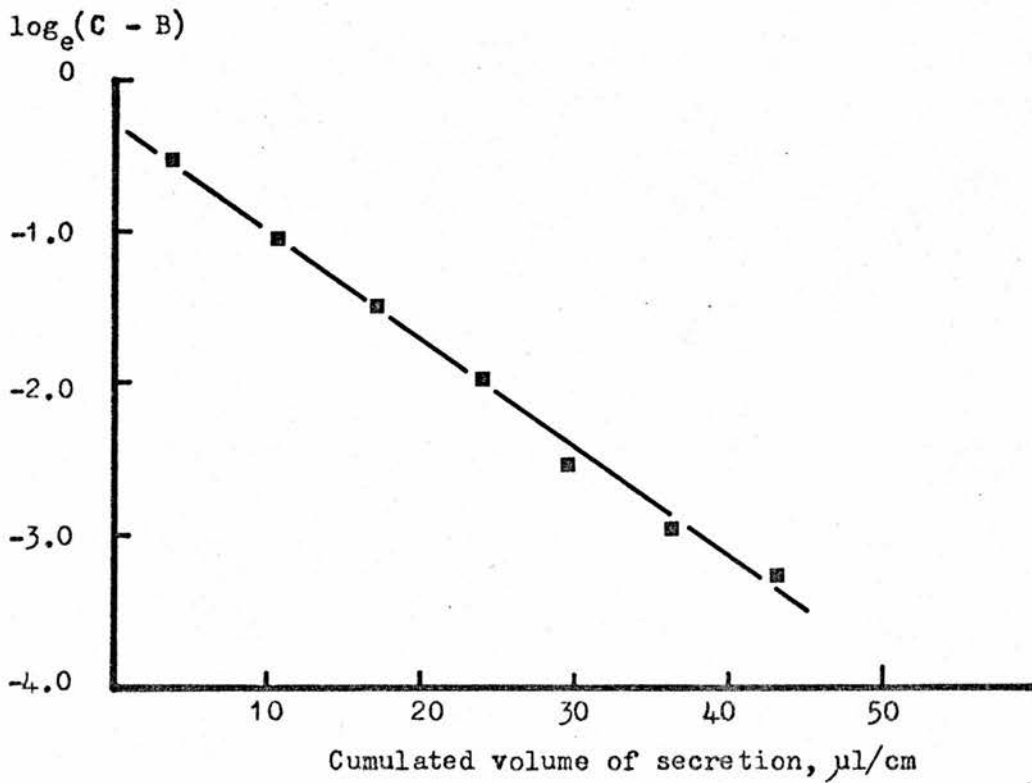
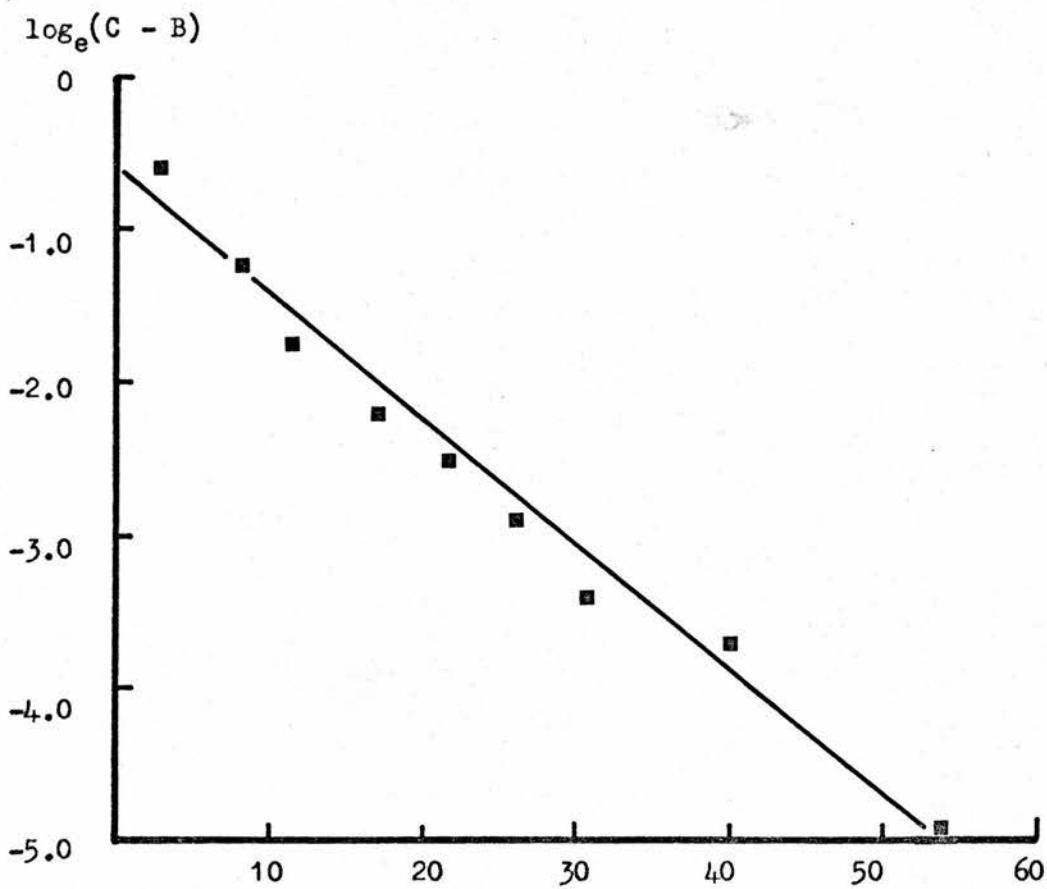


FIGURE 49

Wash-in of deuterium oxide into intestinal secretion

The luminal perfusate contained deuterium oxide (approx 1% v/v)
(one typical experiment).



FIGURES 50 & 51

Semi-logarithmic plots for deuterium oxide wash-in to secretion
(two typical experiments)

(C is the concentration of D_2O in the secretion; B is the steady-state asymptotic concentration of the tracer in secretion)

TABLE 21

Tests of the goodness of fit of the single exponential model to the kinetics of deuterium oxide secretion

<u>Data point</u>	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Mean residual	0.00220	-0.00780	-0.00475	0.00930	0.02788	0.01260	-0.00457	-0.00853	-0.01347
S.E.M.	0.00221	0.00655	0.00720	0.00544	0.02011	0.00947	0.00843	0.00743	0.00847
D of F	10	10	10	10	10	10	10	9	8
t	0.995	1.192	0.660	1.710	1.386	1.330	0.542	1.149	1.590
P	NS	>0.2	NS	>0.1	>0.1	>0.2	NS	>0.2	>0.2

respectively. Figure 48 shows the conformity to Beer's Law. The method of Atkins (1971a) was used to fit the function:-

$C = B + A \cdot \exp(-kv)$ (equation (ii) above) to the experimental results, where C is the concentration of deuterium oxide in the secretion when a cumulative volume of $v \mu\text{l}$ secretion per cm length of intestine has been collected.

Results

Best-fit values for the parameters B , A , and k are given in Table 20, together with the tracer concentration (P) in the lumen perfusate. Also shown are the differences ($P - B$) between the perfusate concentration (P) and the computed steady-state concentration (B) in the secretion, and the ratios, B/P , for each of 11 experiments each on a separate segment of intestine.

Student's t -tests show that the differences, $P - B$, are significantly ($P < 0.001$) greater than zero, and the ratios, B/P , are significantly ($P < 0.001$) less than unity. These differences suggest that there is an isotope effect: i.e. that deuterium oxide (HDO) is more slowly absorbed from the lumen than is water (H_2O).

The kinetics of appearance of tracer in secretion are shown in Figure 49 for a typical experiment. Figures 50 and 51 show semi-logarithmic plots of $\log_e(C - B)$ against v for two typical experiments. As would be predicted from the model, the straight lines appear to be a good fit to the experimental data. So as to reduce the subjectivity of such a judgement, the residuals were tested for randomness in the manner described in Chapter VI. The results of this test are given in Table 21, and as none of the residuals differ significantly from zero this has been taken as evidence of a good fit.

It is not possible to calculate the actual size of the isotope effect, since it is not yet known whether the isotope effect for the 2

TABLE 22

Deuterium oxide isotope effects, and ratio of unidirectional fluxes of water across the mucosa

a is the isotope effect for D₂O flux out of lumen

b is the isotope effect for D₂O flux into the lumen

$$\frac{r}{r-1} = \frac{i}{e} = \frac{\text{rate of water flux out of lumen}}{\text{rate of water flux into lumen}}$$

Values of a, b, and i/e were calculated for each of the three special cases:

(i) <u>if a = b</u>		(ii) <u>if a = 1</u>		(iii) <u>if b = 1</u>	
a = b	i/e	b	i/e	a	i/e
0.8677	1.336	1.735	1.671	0.9631	1.387
0.8130	1.175	1.330	1.286	0.9669	1.215
0.9911	1.233	1.311	1.309	0.9983	1.236
0.9747	1.112	1.133	1.130	0.9974	1.115
0.7033	1.087	1.186	1.147	0.9673	1.124
0.7952	1.191	1.389	1.334	0.9603	1.240
0.8988	1.497	2.429	2.342	0.9640	1.552
0.9195	1.444	2.036	1.983	0.9738	1.483
0.9804	1.453	1.882	1.870	0.9938	1.462
0.9103	1.237	1.386	1.360	0.9815	1.260
0.9435	1.772	6.382	6.208	0.9746	1.818
Mean 0.8907	1.322	2.018	1.967	0.9765	1.354
S.E.M. 0.0270	0.0617	0.453	0.440	0.0043	0.064

Each line refers to a separate experiment.

TABLE 23

'Simulated secretion' experiment to test recovery
of deuterium oxide from perfusion apparatus

Simultaneous collections were made of perfusate and of 'simulated secretion' which had dripped over a glass coil inside the organ chamber.

Collection	Deuterium oxide concentration, % (w/v)	
	'Simulated secretion'	Perfusate
1	1.0481	1.0706
2	1.0593	1.0735
3	1.0509	1.0764
4	1.0509	1.0593
5	1.0425	1.0792
6	1.0425	1.0792
Mean	1.0490	1.0730
S.E.M.	0.00257	0.00307

$$t_{10} = 5.995$$

$$P < 0.001$$

directions of flux are equal or not. However the three extreme cases for which equations (iii) and (iv) were solved above for a, b, and r can be examined. Table 22 shows these results. The value of V has been taken to be 30.9 $\mu\text{l}/\text{cm}$ - see Table 16.

The detection of this apparent isotope effect has depended solely on the measurement of a small difference in tracer concentrations in the steady-state secretion and in the perfusate. An alternative explanation of the observed difference might lie in an experimental artifact - e.g. the secretion collected might not be a fair sample of the fluid secreted by the mucosal cells. For instance, the 5% CO_2 in O_2 which passes through the organ chamber and over the serosal surface of the intestinal segment contains H_2O vapour but not HDO vapour. Therefore the possibility was considered that tracer from the secretion was being lost by exchange into the vapour from the liquid phase which was collected for analysis.

The apparatus was set up as for intestinal perfusion. However, the perfusate was not pumped into the lumen of a segment of intestine, but was slowly delivered (at ca 0.1-0.2 ml/min) onto the outer surface of an AutoAnalyzer mixing coil inside the organ chamber. After running over part of the surface of this glass coil, which simulated the serosal surface area of intestine, this fluid - the 'simulated secretion' - drained into the exit tube at the bottom of the organ chamber and was collected as secretion normally is. Simultaneous collections were made of perfusate pumped directly from the reservoir to a second collecting tube.

The results (Table 23) from such serial measurements prove that there is loss of deuterium oxide from the 'simulated secretion', presumably into the vapour phase. This loss almost exactly equals the observed difference, P - B, on which the foregoing recognition

TABLE 24

Appearance of deuterium oxide in intestinal secretion

In these experiments the gas passing over the serosal surface of the intestinal segment contained 1% deuterium oxide in water vapour.

B, A, & k are the best-fit parameters in the function:

$$C = B + A \cdot \exp(-k \cdot v)$$

C is the deuterium oxide concentration in secretion

v is the cumulative volume of secretion

P is the perfusate concentration of deuterium oxide

B is the asymptotic concentration of deuterium oxide computed for the steady state in secretion

B(%)	A(%)	k (cm/μl)	P(%)	P - B	B/P
1.10256	-1.13808	0.09344	1.0384	-0.06416	1.0618
1.04887	-1.37068	0.17720	1.0720	0.02313	0.9784
1.07778	-1.06099	0.09179	1.0586	-0.01918	1.0181
1.02827	-0.89421	0.11336	1.0682	0.03993	0.9626
1.02631	-0.96895	0.09790	1.0569	0.03059	0.9711
1.04492	-1.07388	0.10732	1.0566	0.01168	0.9889
1.05264	-0.95923	0.10921	1.0626	0.00996	0.9906
0.93082	-1.08863	0.20331	1.0744	0.14358	0.8664
1.16138	-1.19942	0.14595	1.0818	-0.07958	1.0736
1.10378	-1.11346	0.13497	1.0818	-0.02198	1.0203
Mean				0.007397	0.9932
S.E.M.				0.01963	0.01834

Each line refers to a separate experiment.

of an isotope effect rested. Therefore it seems likely that there may be no measurable isotope effect; the results already tentatively attributed to an isotope effect may be solely an experimental artifact.

Consequently, ten further experiments were made exactly as before but with one modification: the 5% CO₂ in O₂ which passes through the organ chamber was taken from the reservoir containing the deuterated perfusate. Hence this gas should be moist with 1% HDO in H₂O vapour, and no exchange between HDO in secretion and the vapour phase at the steady-state would be predicted. Table 24 summarises the kinetic parameters etc. from these additional experiments, and it can be seen that there is no significant difference between the concentrations of tracer in the steady-state secretion (B) and in the lumen perfusate (P).

The mean (\pm S.E.M.) ratio of the unidirectional fluxes, i/e, is 1.38 ± 0.040 (10) from equations (viii) and (xi) above.

DISCUSSION

The conclusion from these experiments is that, under the conditions of the present study, there is no measurable isotope effect in the absorption of deuterium oxide - i.e. that deuterium oxide and water cross the intestinal mucosa at the same rate. This finding validates the use of deuterium oxide as a tracer for water in intestinal studies.

Previous evidence in the literature is equivocal concerning the relative rates of diffusion and transport of water and deuterium and tritium oxides across biological membranes. Little of it relates to the intestine. Hevesy, Hofer & Krogh (1935) found that water crossed frog skin more rapidly than deuterium oxide did. Parpart (1935) and Brooks (1935) both noted slower haemolysis of

erythrocytes in deuterium oxide than in water. Similar observations on protozoa suggested that the rate of permeation of deuterium oxide was less than that of water (Kitching & Padfield, 1960). On the other hand the rate of permeation of deuterium oxide into sea urchin eggs (Lucke & Harvey, 1934) and into giant amoeba (Lovtrup & Pigon, 1951) could not be differentiated from that of water. Some of these investigations may have been invalidated by the use of relatively high concentrations of the isotope which are known to be toxic. In recent tracer work the maximum concentration of deuterium oxide which is customarily used is about 1%.

The rates of movement of deuterated and tritiated water have been compared in several tissues, and either have been found to be indistinguishable (Chinard & Enns, 1954; Takashina, Lazzara, Cromvich & Burch, 1962; King, 1969) or the differences seen have been attributed to experimental artifact (Elford, 1970). This has been taken as evidence for the validity of work using the isotopes of hydrogen as tracers of water across membranes (King, 1969). However this conclusion is not justifiable.

If specific transport mechanisms exist for water then a substitution of either deuterium or tritium for hydrogen atoms could equally well reduce the rate of fluid transport. Even if non-specific mechanisms are responsible for water transport it is not possible to predict a difference in the diffusion rates on the sole basis of molecular weight. For example, HTO and H_2O^{18} have virtually identical molecular weights. Yet Wang, Robinson & Edelman (1953) found that the self-diffusion coefficients of these two molecules differ by 14%. They suggested that this might be owing to a difference in the moments of inertia. A further possibility which does not appear to have been mooted is that these

molecules (and HDO etc.) aggregate into different sized polymers. Thus, water exists mainly as $(H_2O)_4$, but there seems to be no information available regarding the degree of polymerisation of HDO or HDO in H_2O etc. Hence the relative molecular weights might be very different from that predicted on the basis of 'mono-water' molecules. Therefore (i) one cannot predict the relative rates of diffusion simply from the molecular weight, (ii) nor can one deduce anything about the type of transport mechanisms involved from knowledge of the relative rates of transport, (iii) nor can one predict that the rate of, say, H_2O transport is the same as that of HDO and HTO simply because the rates for the last two tracers are equal.

The present kinetic analysis has also allowed estimation of the two unidirectional flux rates of water across the mucosa. The ratio of mucosal-serosal/serosal-mucosal fluxes was 1.38 ± 0.04 (10) and this compares favourably with existing estimates in the literature (e.g. 1.30 ± 0.09 (7) calculated from the results of Berger, Pecikyan & Kanzaki, 1970). Estimation of the ratio depends on several assumptions, the chief of which is that one knows the volume of the fluid with which the fluid secreted by the mucosal cells mixes - i.e. the compartment size. This was evaluated in Chapter VI, but itself rests on several assumptions. As already mentioned in Chapter VI, if glucose, protein etc. re-flux from the tissue fluid back into the lumen, then the estimate of compartment size may be too low (e.g. see wash-out of sodium into secretion, Chapter VIII). In turn, this error would result in overestimation of the ratio of mucosal-serosal/serosal-mucosal flux rates.

CHAPTER VIII

THE EFFECT OF A CHANGE IN LUMINAL SODIUM ION

CONCENTRATION ON GLUCOSE ABSORPTION

CHAPTER VIII THE EFFECT OF A CHANGE IN LUMINAL SODIUM ION
CONCENTRATION ON GLUCOSE ABSORPTION

Introduction

The earliest reports of an effect of sodium on the intestinal absorption of sugars are probably those of E. W. Reid (1900, 1902). However, perhaps because of the singular lack of a viable in vitro preparation, the matter was scarcely reconsidered for many years. Althausen, Anderson & Stockholm (1939) demonstrated that the reduction in glucose absorption subsequent to adrenalectomy could be avoided if the adrenalectomised animals were given sodium chloride in their drinking water. Then in 1958, Riklis & Quastel reported investigations into the effect of cations on intestinal absorption of sugars in the guinea pig. They concluded that the presence of sodium ions was essential for active absorption of glucose. Csaky & Thale (1960) confirmed that sodium was obligatory, and suggested that it had to be in the mucosal (luminal) medium.

Since then the importance of sodium ions for the active absorption of sugars has been confirmed and emphasised by many workers using a wide variety of species and methods. In what has now become a vast literature one conclusion is unanimous: that in the absence of sodium ions from the medium bathing the luminal surface of the epithelium, active transport of sugars is inhibited. However, there is no agreement as to the nature of the relationship between the sodium ion and the mechanism(s) for sugar absorption.

One theory, reviewed in the General Introduction, which has gained substantial acceptance, is that of R. K. Crane (Crane, Miller & Bihler, 1961; Crane, 1965). According to this view, sodium ions and sugar enter the mucosal cells bound to a common allosteric carrier, which has binding sites for both entities, at the luminal

membrane. The affinity of the carrier for glucose is modified by bound sodium ions. The sodium ion enters the cell by facilitated diffusion down its electrochemical gradient; consequently, the glucose may be carried into the cell up its electrochemical gradient. Thus, the active transport of sugar is directly dependent on the downhill transport of sodium ion into the cell. In turn, the latter requires that the intracellular electrochemical activity of sodium be kept low, below that in the lumen. Crane, Miller & Bihler (1964) suggested that low intracellular sodium concentration might be maintained by the active expulsion of sodium ions back into the lumen by a 'sodium pump'. Schultz & Zalusky (1964b) supported this scheme, but proposed that the sodium pump is situated at the serosal surface of the mucosal cell.

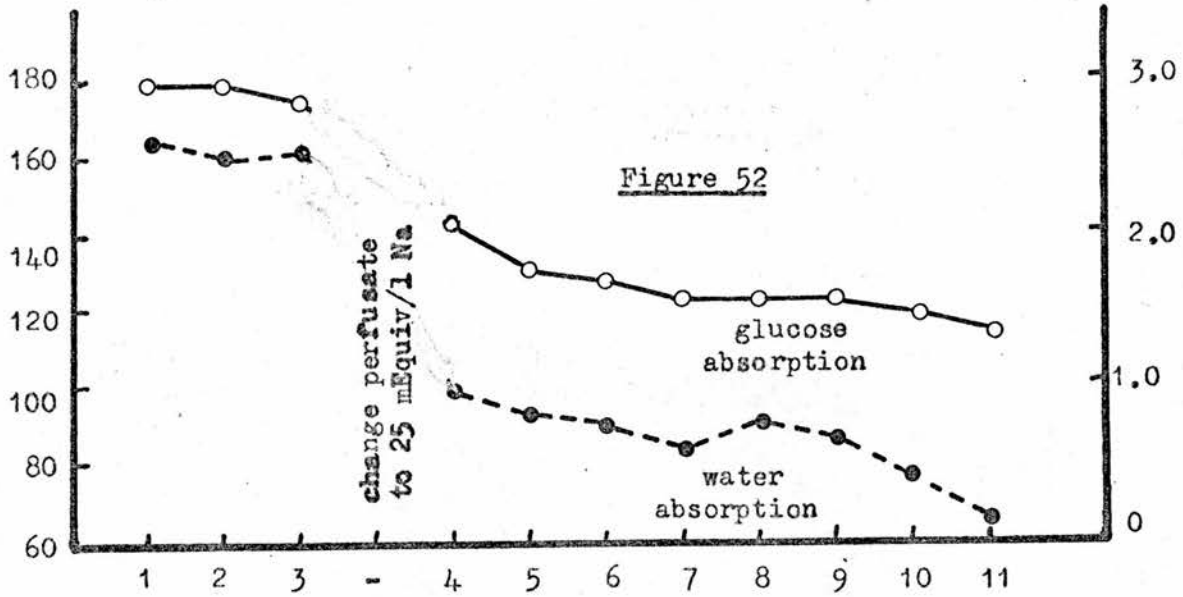
Crane (1967) has further proposed that his general model of 'gradient-coupled' transport might be widely applicable to many other instances of biological transport, and it is obviously desirable to test his hypothesis.

Stein (1967, page 183) has given, in a flattering review of "... the admirable model of Crane (1965) ...", a list of experimental implications predicted by the model. In addition to these tests, it is obvious that, according to Crane's scheme, the rate of glucose absorption would be expected to change immediately when the transmembrane sodium gradient is changed, e.g. by an abrupt alteration in the luminal sodium concentration. Further, if the direction of the gradient of sodium ion concentration were reversed then glucose absorption should cease or even become negative.

The preparation which has already been described is ideally suited to making such an experimental test. It has already been shown that the rate of glucose absorption from the lumen of this

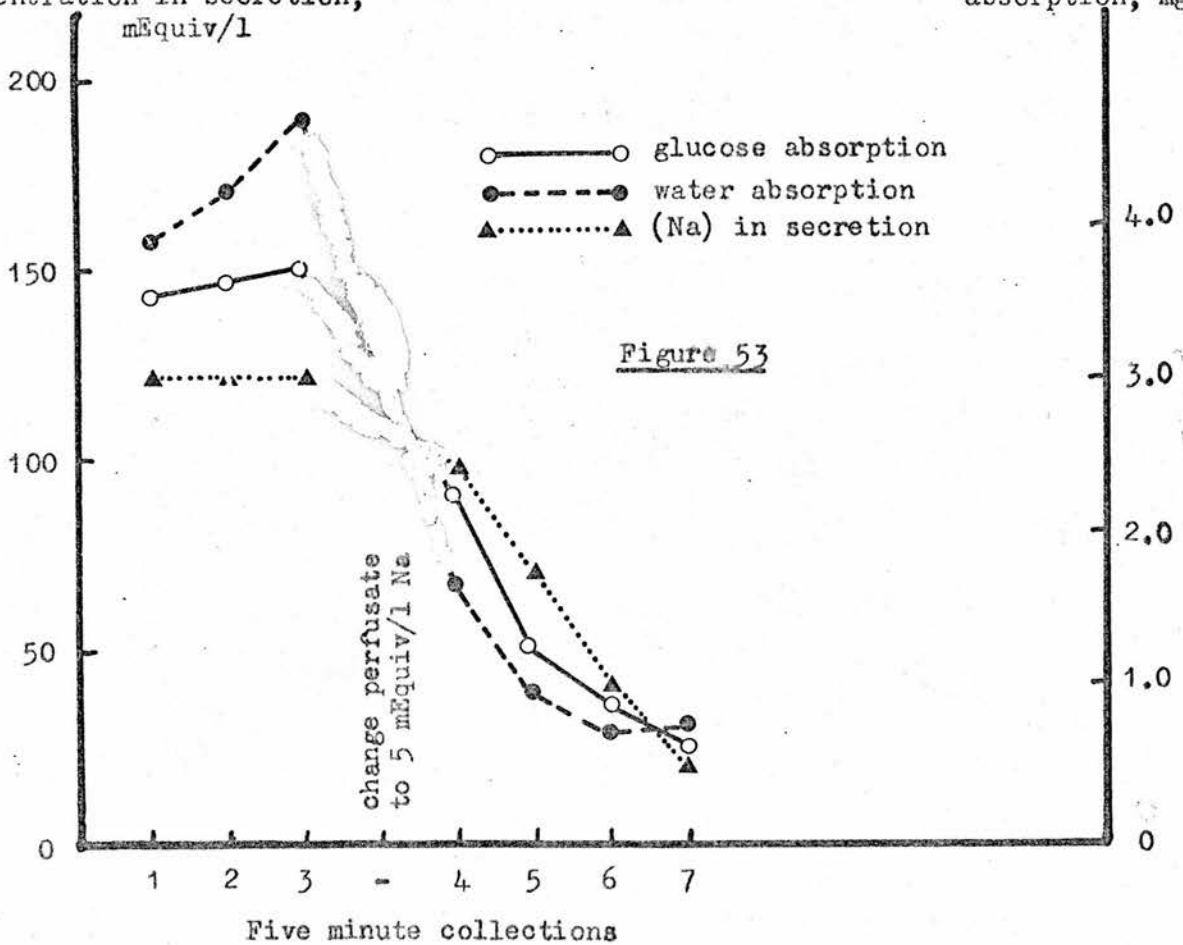
Rate of water absorption, $\mu\text{l}/\text{cm}/\text{hr}$

Rate of glucose absorption, $\text{mg}/\text{cm}/\text{hr}$



Rate of water absorption, $\mu\text{l}/\text{cm}/\text{hr}$ or sodium concentration in secretion, mEq/l

Rate of glucose absorption, $\text{mg}/\text{cm}/\text{hr}$



FIGURES 52 & 53

Effect of changing the sodium concentration in the perfusate from 144 mEq/litre to 25 mEq/litre or 5 mEq/litre, respectively

(Each point is the mean of 3 experiments)

preparation changes almost instantaneously when the luminal glucose concentration is changed (Chapter V - Figure 25).

Methods

Segments of intestine were perfused with the normal medium containing glucose (5 mg/ml, 28 mM). The sodium concentration of this perfusate is 144 mEq/litre. After 3 serial collections of lumen effluent and of secretion the luminal perfusate was replaced by one in which some or all of the sodium had been replaced by an osmotic equivalent of the corresponding salts of choline. In one experiment, mannitol was used to replace the sodium chloride.

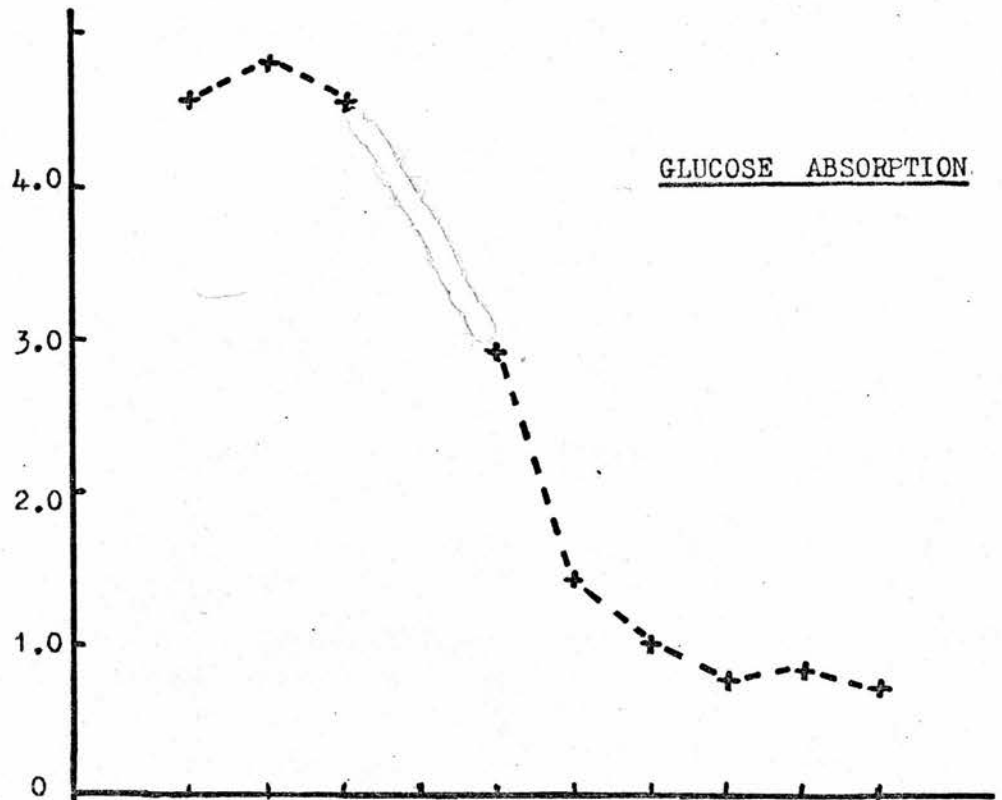
In some of the experiments the sodium concentrations in the secretions were estimated by atomic absorption spectrophotometry.

Results

The results are summarised in Figures 52, 53, 54 and 55. Figure 52 shows the mean rates of water and glucose absorption when the normal perfusate was replaced by one in which the sodium concentration was 25 mEq/litre, all the sodium chloride having been replaced by choline chloride. The rate of glucose absorption from the lumen fell to about 50% of its control value, but the new steady-state was not reached immediately after the perfusate change-over. This may not be obvious from the figure, but was confirmed by an analysis of variance.

Figure 53 shows the mean absorption rates for three experiments in which the second perfusate contained 5 mM sodium chloride; the remainder of the sodium chloride and all the sodium bicarbonate had been replaced by choline chloride and bicarbonate respectively. The rate of glucose absorption clearly falls only gradually once the sodium concentration in the perfusate has been reduced. Indeed, it

Rate of glucose
absorption, mg/cm/hr



Rate of water
absorption, μ l/cm/hr

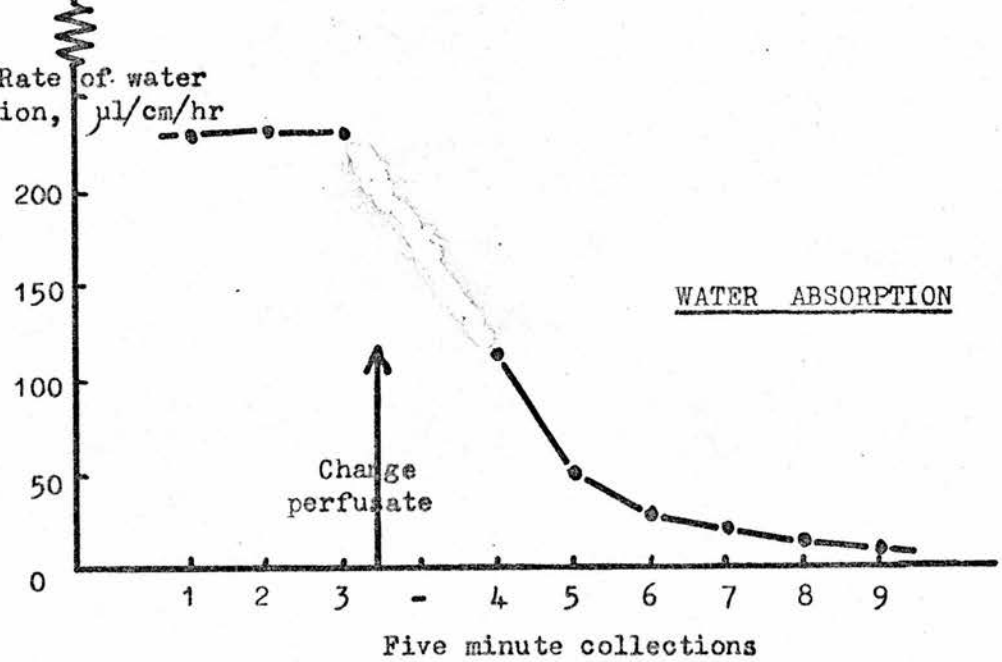
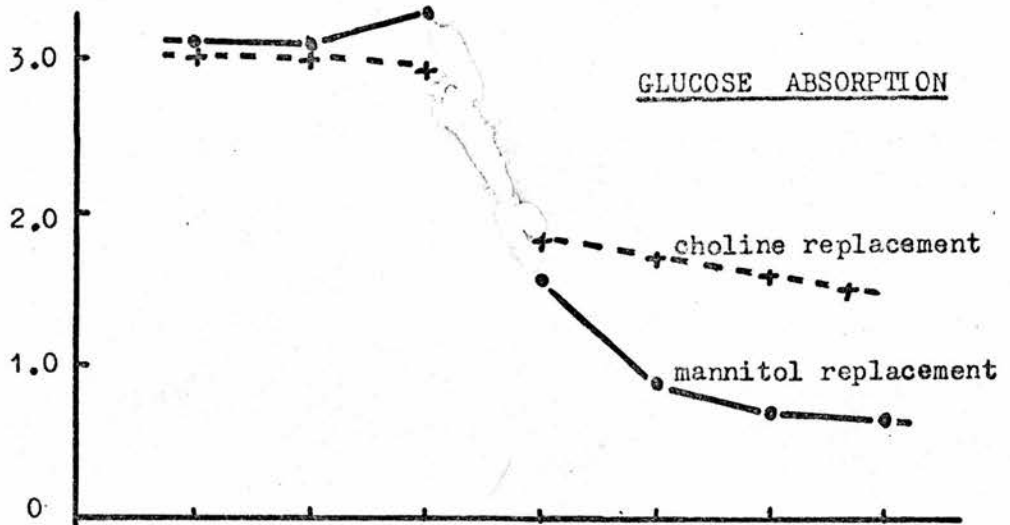


FIGURE 54

Effect of total replacement of luminal sodium by choline
(one experiment)

Rate of glucose
absorption, mg/cm/hr



Rate of water
absorption, μ l/cm/hr

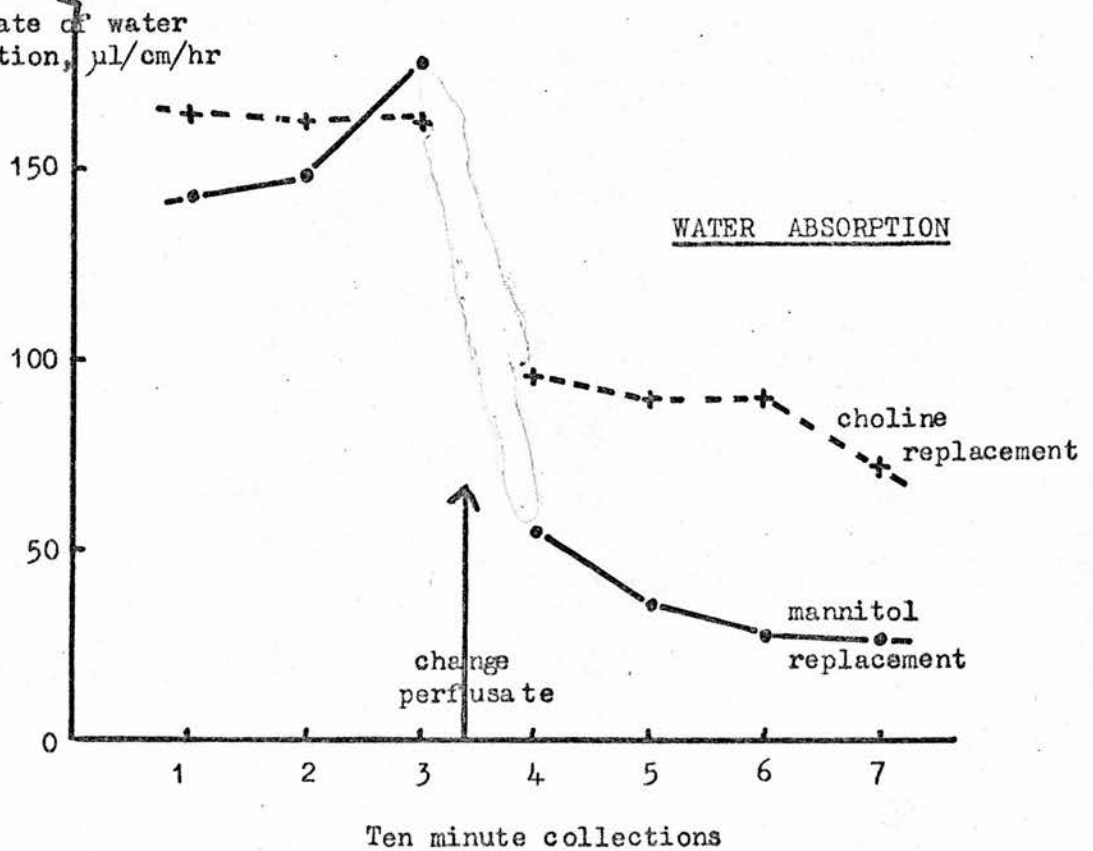


FIGURE 55

Effect of changing the sodium concentration in the perfusate from
144 mEq/litre to 25 mEq/litre

In 3 experiments the sodium was replaced by choline;
in one experiment it was replaced by mannitol.

falls no more rapidly than does the sodium concentration in the secretion (Figure 53). This finding contrasts with the results of changing the glucose concentration in the perfusate. In that case, the rate of glucose absorption reached its new steady-state within about 5 minutes from the perfusate change-over (Figure 25).

Figure 54 shows the results from a single experiment in which all the sodium was iso-osmotically replaced by choline. The diminution in glucose and water absorption was so marked that the experiment was not repeated, since it is difficult to make reliable estimations on very small volumes of secretion. Nevertheless, again it is seen that the reduction of glucose absorption is not immediate, but is gradual.

Figure 55 shows the results from a single experiment in which the second perfusate contained 25 mM sodium bicarbonate, but with mannitol replacing iso-osmotically the sodium chloride. The broken line shows the glucose absorption data of Figure 52 (choline chloride plus 25 mM NaHCO_3) also repeated here, and it is noteworthy that the reduction in glucose absorption in the presence of mannitol was more rapid than it was with choline chloride. This difference might be attributable to the chloride ion, since anionic effects on sugar absorption have also been reported. Alternatively, slower dilution of the intracellular sodium by mannitol than by the choline chloride might be at least partly responsible.

Figure 56 shows data for the sodium concentration in the secretion plotted against the cumulative volume of secretion when the luminal sodium was removed. Although sufficient data have not been collected to show whether a one compartment (single exponential) model is a good fit to the data for the sodium wash-out, it appears that this may be the case. The mean of the apparent rate constants

Na concentration in secretion, mEquiv/litre

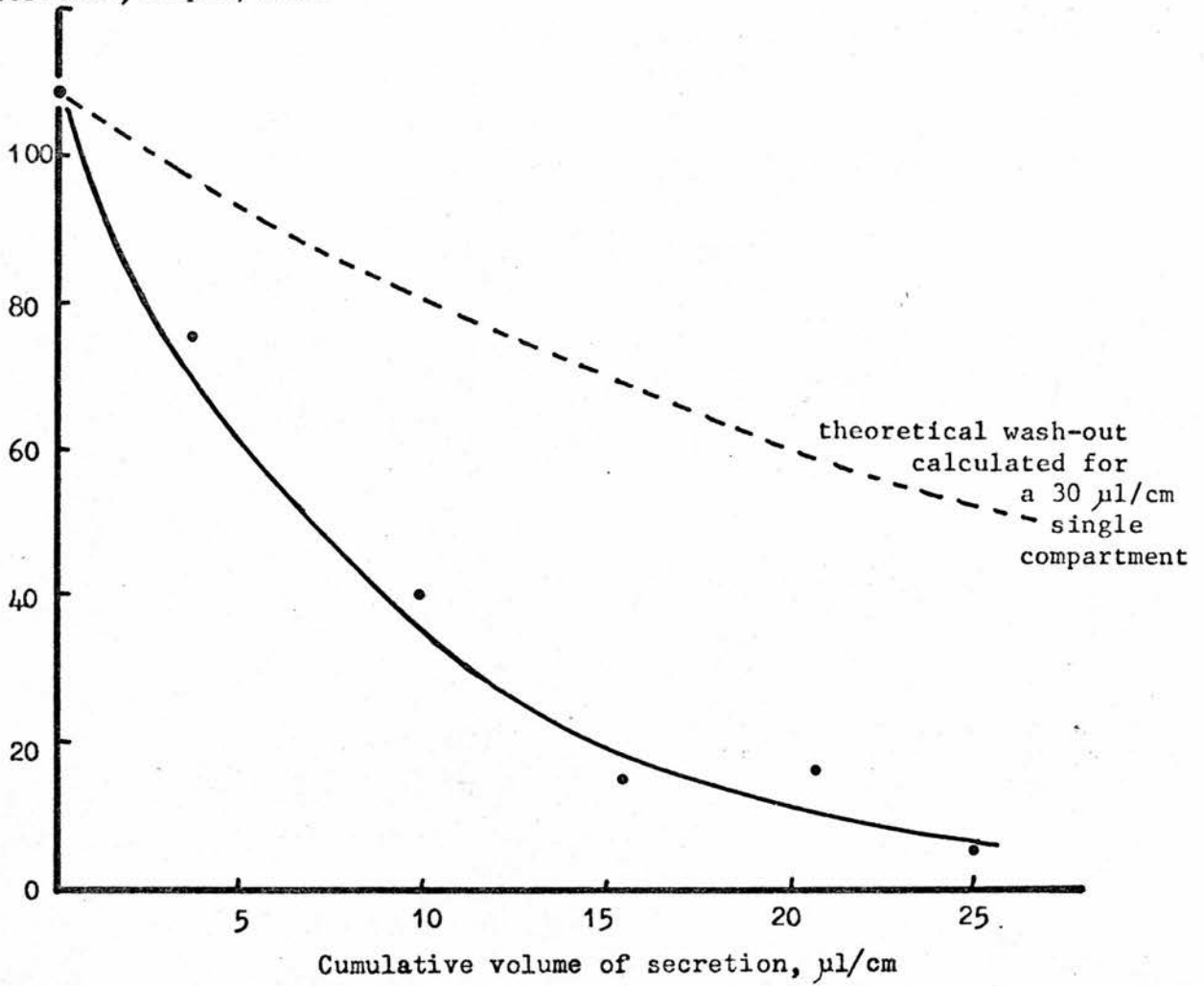


FIGURE 56

Sodium wash-out into secretion, following total removal of sodium from the luminal perfusate

(one experiment)

Rate of glucose
absorption, mg/cm/hr

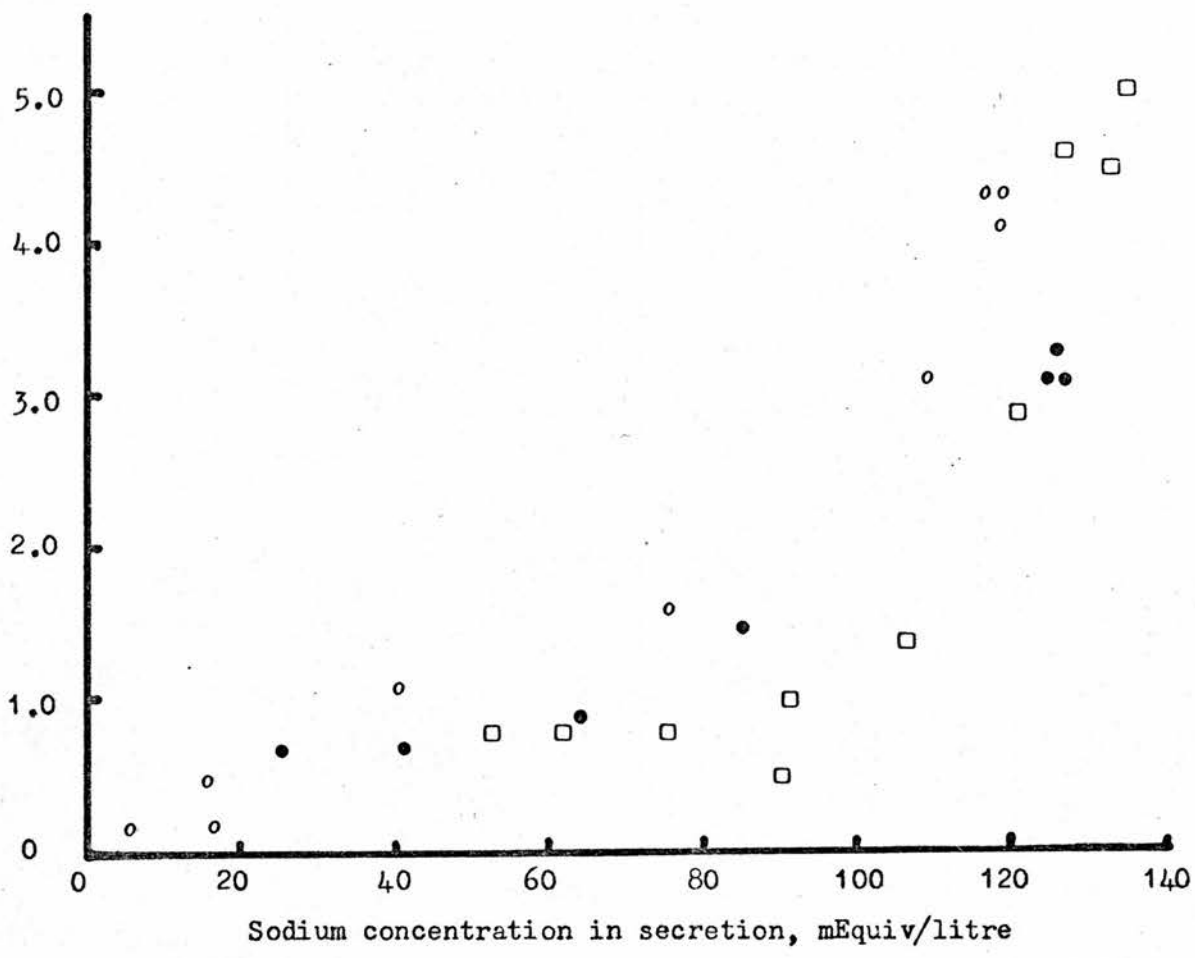


FIGURE 57

The relation between the rate of glucose absorption and the sodium concentration in the intestinal secretion, following the removal of the luminal sodium.

(Each type of symbol represents the results from a single experiment)

(\pm S.E.) for 4 such experiments was 0.0903 ± 0.0117 cm/ μ l; this is some three times that obtained for wash-out of glucose, total nitrogen, or protein (see Chapter VI). This difference could be explained in terms of the sodium leaving the tissue fluid in two directions (i) into the secretion, and (ii) refluxing back into the lumen. The ratio of the sodium flux rates appears to be approximately:

$$\frac{\text{mucosal to serosal flux}}{\text{serosal to mucosal flux}} = 1.56$$

This value is in reasonable accord with other values in the literature (e.g. 1.4 for rat ileum - Curran & Solomon, 1957). An alternative explanation for the relatively large rate constant for sodium wash-out is that a smaller compartment is accessible to sodium than to glucose, protein etc. There is no evidence in support of this, and the kinetics of sodium wash-out are similar to those of deuterium oxide wash-in, for which this alternative is not available.

The sodium ions which reflux from the tissue fluid into the lumen must augment the luminal sodium concentration. Hence the sodium concentration of the fluid in the lumen may not change as rapidly as might be expected from the rapid change-over of perfusate. Measurements of sodium concentration in the luminal effluent were not made at this stage in the investigation, largely owing to the instrumental problems mentioned on page 45. However appropriate measurements were made later, and will be presented in Chapter IX.

Figure 53 showed that the concentration of sodium in the secretion fell at about the same speed as did the rate of glucose absorption when the luminal perfusate was changed. Figure 57 shows results from three further experiments: clearly any relation between the tissue-fluid sodium concentration and the rate of glucose absorption is not simple.

DISCUSSION

The results shown in Figures 54 and 55 are remarkable. The prediction drawn from Crane's (1965) model as discussed in the Introduction to this Chapter has not been fulfilled.

To recapitulate: If the driving force for glucose absorption were provided by downhill facilitated diffusion of sodium ions from the intestinal lumen into the mucosal cells, then a sudden change in the luminal concentration of sodium ions would cause an almost instantaneous change in the rate of glucose absorption from the lumen. The experiments reported here have shown that, on the contrary, the absorption of glucose declined only gradually. When the sodium ion concentration in the perfusate was taken to zero so that there was presumably a negative gradient of sodium ion concentration between the lumen and the cell, there was no evidence of counter-transport of glucose as might have been expected and as Crane (1964) claimed to show.

The possibility that the sodium concentration in any unstirred layer of fluid in contact with the luminal membrane did not fall rapidly when the perfusate was changed can be rejected as unlikely subject to reservations discussed below; the rate of glucose absorption has already been shown to fall virtually instantaneously when the glucose concentration in the luminal perfusate was abruptly changed (Figure 25). No unstirred layer lag was found.

Similarly, the suggestion that the trans-membrane gradient of sodium ion concentration changed gradually because of a change in the intracellular sodium concentration secondary to the lowering of the luminal concentration can be discounted. The intracellular sodium will indeed probably change when the sodium is removed from the lumen, but it must surely decrease rather than increase.

Thus, a change in intracellular sodium concentration will tend to increase the concentration gradient into the cell, and so the glucose absorption rate would slowly increase after a sudden and immediate fall, according to Crane's hypothesis.

Consequently, it appears that the Crane model may be untenable. A 'gradient-coupled' mechanism may not account for the entry of glucose into the mucosal cells of the rat small intestine.

Between the date on which these experiments were completed and the time of writing, two other groups have arrived at a similar conclusion - one for glucose transport (Kimmich, 1970b) and the other for methionine transport (Newey, Rampone & Smyth, 1970). Again, the evidence was a lag in the response to sodium depletion. However, such a conclusion cannot be unequivocal at the present time for several reasons which will now be discussed.

Kimmich (1970b) used a preparation of isolated mucosal cells which had been scraped from the chicken intestine following an incubation with hyaluronidase. There are several major objections to Kimmich's work. Firstly, the integrity of the cells remains unproven. It cannot be denied that hypoxia, even for brief periods, can cause irreversible damage to intestinal tissue. The exact nature of the damage is unknown, and so it seems rash to assume that it merely slows down the metabolism and transport processes of the cells. The results in Chapter V stressed the dangers of hypoxia, as did the experiments of Robinson (1966) which have already been discussed. The defence that all steps subsequent to the 30 minute incubation with hyaluronidase were conducted at 0°C does not amount to justification. Kimmich has not calculated the Q_{O_2} of the cells to which he claims to ascribe metabolic viability. He suggests that the maintenance of a fairly constant respiration is evidence that

"... the metabolic and transport capabilities are the same after isolation as before ...". He then points out that his preparation has a steady lactate and carbon dioxide production for at least two hours, and claims that "... this represents a period of linear metabolic activity about three times longer than the best preparations reported elsewhere in the literature ...". However, he does give two different figures for the rate of carbon dioxide production from ^{14}C -glucose (Kimmich, 1970a, Tables I and III). Taking his higher figure of 315 nmoles CO_2 produced per mg of protein per hour, one can calculate a likely upper limit for the Q_{O_2} of his preparation. It is 7 $\mu\text{l O}_2$ per mg dry weight per hour. In fact, the actual value may be less than this figure, which was obtained on the assumption that the cell dry weight is all contributed by protein. (This estimate assumes that labelled glucose is the only substrate which is oxidatively metabolised.) Figures for the Q_{O_2} of viable chicken mucosal cells are not available; however a Q_{O_2} of 7 seems unreasonably low. It is desirable to attempt to measure the respiration of the intestine in the present perfused preparation.

Secondly, the active accumulation of galactose has not in fact been proved in Kimmich's experiments. The apparent accumulation which he observed in the sodium-free media could equally be attributed to efflux of water from the cells. Furthermore, the sodium-free media which he used contained mannitol rather than a chloride, in spite of the well documented effects of anions on sugar absorption. For instance, Csaky & Thale (1960) found that replacement of sodium chloride by sodium sulphate in the lumen led to a 60% inhibition of glucose absorption. Hence, it is plausible to suggest that the effects which Kimmich has reported might have been due to the removal of chloride ions rather than of sodium ions. It is possible

that chloride ions moved out of his cells into the mannitol media, and water may have been concurrently extruded. Mainly for these reasons, the work of Kimmich, while certainly suggesting that the Crane model is inadequate, cannot be accepted without reservations.

Newey, Rampone & Smyth (1970) studied the uptake of L-methionine by everted sacs of rat intestine following a 30 minute pre-incubation in either a sodium-containing or a sodium-free medium. These respective pre-incubations were supposed either to maintain normal or to lower the intracellular level of sodium ions. In the subsequent incubation the uptake of methionine was measured after 5, 30, 60, or 120 seconds. It seemed that the intracellular sodium concentration was more important than the direction of the sodium electrochemical gradient across the mucosal membrane during the actual incubation period. However, as Newey et al pointed out, there was no evidence that the composition of the bulk of the mucosal medium was the same as that of the fluid in actual contact with the cell membrane - e.g. there might be an unstirred layer, or sodium ions originating from the serosal fluid might be refluxing via a co-transport mechanism (see below). Nevertheless these experiments are at present more readily reconciled with Csaky's (1963) hypothesis than with the Crane (1965) mechanism.

It is well known that sodium ions can reflux from the tissue fluid back into the intestinal lumen (e.g. Schultz & Curran, 1968), and this was borne out by the relatively large rate constant for sodium wash-out into the secretion (Figure 56). It is possible that the refluxing sodium ions are expelled from the inside of the luminal membrane, perhaps by a sodium pump as envisaged initially by Crane, Miller & Bihler (1961), so that they can be captured more readily by the Crane allosteric carrier than they can pass into the

bulk of the luminal fluid. If this were true, there could conceivably be co-transport of glucose and sodium ions into the mucosal cells even although sodium were absent from the bulk of the luminal perfusate. Further, the present experiments have not established that the sodium concentration in the luminal effluent falls to zero within five minutes of replacing the perfusate by a sodium-free one. This will be further considered in Chapter IX. Similar objections can be raised against Kimmich's (1970b) and Newey et al's (1970) experiments.

The lag in the fall in glucose absorption rate as shown in this Chapter and in Kimmich's work (also the lag in methionine absorption in Newey et al's (1970) work) could therefore still be consistent with the Crane hypothesis. Further experiments are necessary to elucidate the problem.

The data of Figure 53 suggested that the glucose absorption rate might be related to the concentration of sodium in the tissue fluid. However, the more detailed data given in Figure 57 showed that the relationship was not a simple one. As the sodium concentration in the secretion fell from the normal level of about 120 mEq/litre to about 100 mEq/litre so the glucose absorption rate fell swiftly. But further reduction in the sodium concentration in the secretion down to some 5 mEq/litre was hardly accompanied by further fall in glucose absorption.

The intracellular sodium concentration must fall gradually after the removal of the luminal sodium in this isolated preparation. Therefore it is possible that it was the relatively slow change in the sodium concentration inside the mucosal cells which caused the gradual decrease in the glucose absorption rate when the luminal sodium was removed. This is essentially the view of Csaky (1963).

Regrettably, it is not possible to control directly the composition of the tissue fluid, nor that of the cellular water, in this preparation. Consequently, these possible relationships cannot be tested at the present time using this preparation.

CHAPTER IX

A NEW TECHNIQUE: A MODIFIED INTESTINAL PREPARATION

WITH ARTERIAL INFUSION

CHAPTER IX A NEW TECHNIQUE: A MODIFIED INTESTINAL
PREPARATION WITH ARTERIAL INFUSION

Introduction

One of the chief disadvantages associated with the isolated preparation which has been developed and used in the foregoing investigations is the inability to control the composition of the sub-epithelial tissue fluid. Indeed, when the intestine is absorbing glucose from the lumen, this tissue fluid becomes extremely hyperglycaemic and hyperlactaemic. And there is no firm justification for assuming that the cellular metabolism under these abnormal conditions is 'normal'.

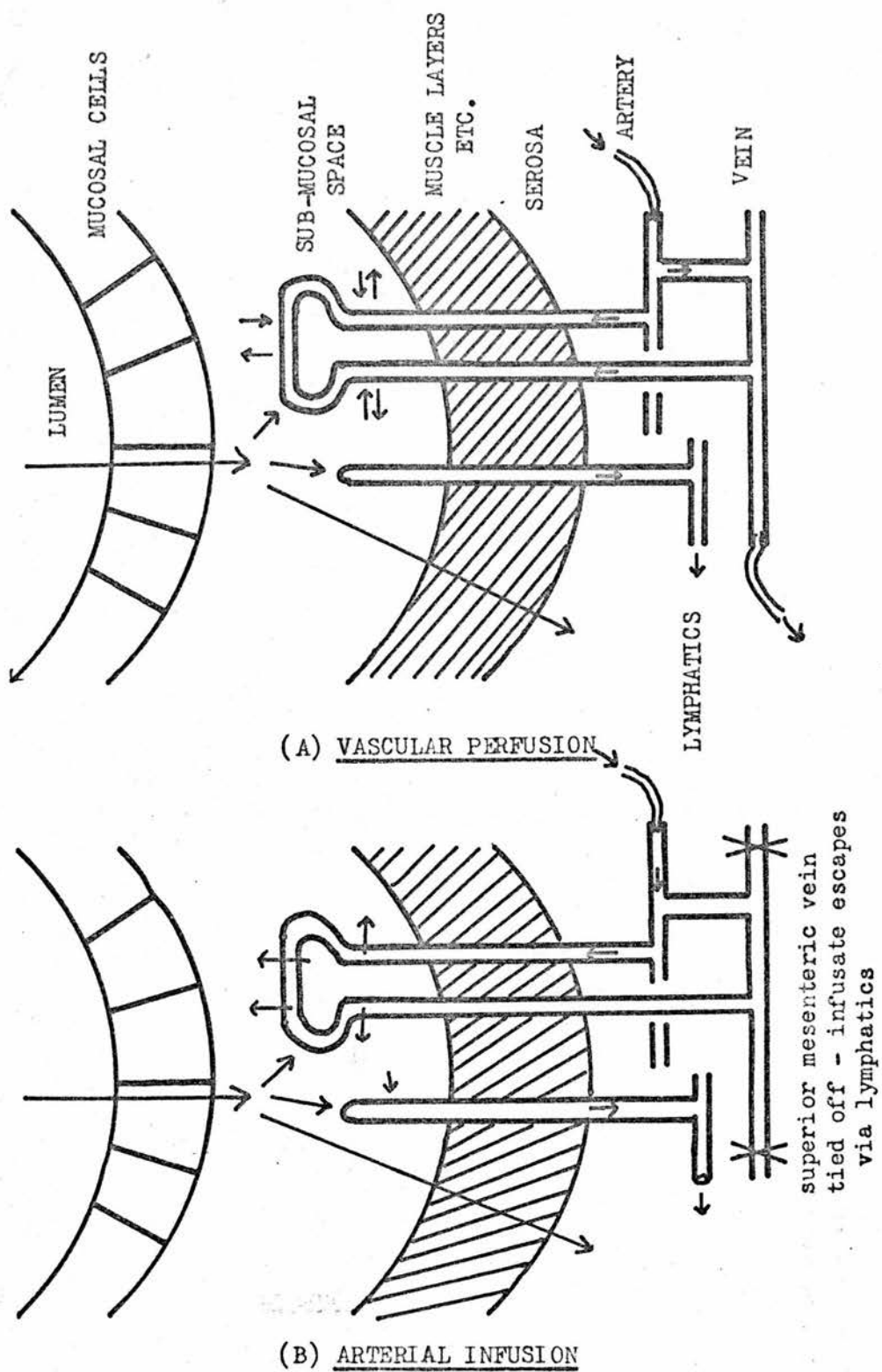
Some of the disadvantages of techniques in which the serosal surface of a perfused segment of intestine is bathed in a fluid, such as those of Fisher & Parsons (1949) and Wiseman (1953), have already been discussed. Furthermore, as Lifson & Parsons (1957) and Csaky & Hara (1965) have pointed out, there is little basis for the assumption that the composition of the sub-epithelial fluid in contact with the mucosal cells is that of the serosal medium. Indeed, Csaky & Hara (1965) estimated that the concentration of an inhibitor which they had put into the serosal compartment of a flux chamber was about an order of magnitude less in the tissue fluid than in the bulk of the medium. The serosal layers of connective tissue must constitute a formidable barrier to diffusion into the sub-epithelial space; additionally, the bulk flow of water outward towards the serosal surface opposes such net movement.

The second serious disadvantage inherent in the preparation, and indeed in many other preparations, is the long delay before a steady-state can be attained. Although conditions in the lumen can reach a steady-state rapidly, there is a lag of over an hour before

the steady-state is attained in the secretion. Therefore it is difficult to make valid measurements of secretion and utilisation rates, although under certain circumstances a study of the lag can provide valuable information - see Chapters VI and VII.

Techniques of vascular perfusion, while controlling the extracellular composition to some extent, introduce yet other variables. A major drawback is that the intestinal vasculature contains numerous arterio-venous shunts. Therefore the space which is accessible to the vascular perfusate is unpredictable, and is not necessarily constant even within one intestine. Parsons & Pritchard (1968) reviewed some thirty accounts of intestinal preparations with vascular perfusion, and concluded that "... the technique of perfusion of the intestinal vascular system appears to have been attended by singular ill-success throughout its history ...". This is particularly true of preparations of rat small intestine.

A new technique of 'arterial infusion' has been developed with the intention of overcoming these limitations. While the lumen of an isolated segment of intestine is perfused with a segmented flow in the manner already described, a carefully filtered saline medium (the infusate) is pumped into the cannulated superior mesenteric artery as for a vascular perfusion. However, the superior mesenteric vein is also ligated. In consequence the fluid, instead of perfusing an unknown fraction of the blood vessels, is forced across the capillary walls, through the extracellular space, and escapes via the lymphatics. The infused fluid thus exchanges rapidly with the sub-epithelial extracellular fluid. No attempt is made to supply oxygen to the tissue via the vascular system; the segmented stream of perfusate through the lumen has already been shown to satisfy, at least largely, the intestine's oxygen



(A) VASCULAR PERFUSION

(B) ARTERIAL INFUSION

superior mesenteric vein
tied off - infusate escapes
via lymphatics

FIGURE 58

A diagrammatic comparison between vascular perfusion (A) and arterial infusion (B)

requirements. The arterial infusate simply serves to control the composition of the fluid bathing the serosal pole of the epithelial cells, and to carry away the material which is secreted into the extracellular space by the cells. Figure 58 shows a diagrammatic comparison between vascular perfusion and arterial infusion.

Consequently, with direct access to the fluids bathing both sides of a single layer of epithelial cells and the ability to achieve rapidly a steady-state in both these fluids, many valuable experiments are now possible. Problems to which application of this new technique may prove particularly exciting include effects on intestinal absorption and metabolism of ions, drugs and hormones, resorption of metabolites from the extracellular space, the roles of active and osmotic processes in water absorption, and many others. In this Chapter the experimental details of the new preparation are described. Subsequent Chapters will be devoted to the evaluation of the preparation and the application to one of these problems - the interaction between sodium ions and the mechanism(s) for sugar absorption.

The apparatus

The only modifications to the normal perfusion apparatus which was described in Chapter II involve the addition of components for the delivery of an infusate into the superior mesenteric artery. This infusate must be warm, well filtered, and free from bubbles, and it must be pumped at a known but variable rate at a known pressure. These components are shown diagrammatically in Figure 59.

Warm oxygenated infusate, in equilibrium with 5% CO₂ in O₂, is pumped from a reservoir, R, via Q, a polypropylene 3-way tap (type K-75, made by Pharmaseal Laboratories, Glendale, California, 91201, U.S.A.). The peristaltic pump, P, is similar to the one which

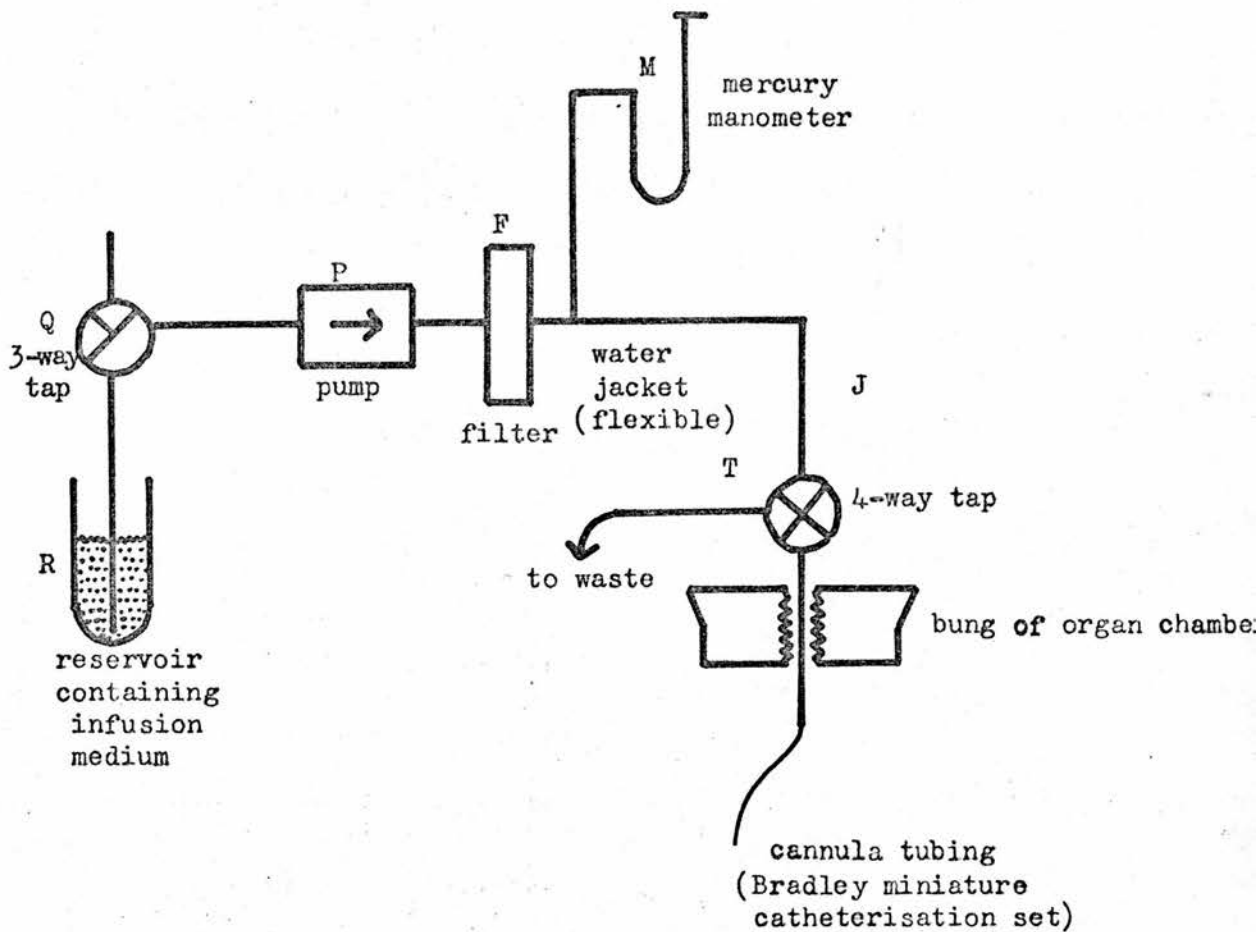


FIGURE 59

Additional components of the perfusion apparatus for arterial infusion.

The basic perfusion apparatus is as shown in Figure 4. The components shown are added to the apparatus so that a warm oxygenated and well filtered infusion can be delivered to the superior mesenteric artery.

impels the lumen perfusate. The infusate passes through a cellulose acetate filter which is 49 mm in diameter and of 0.3 μ m pore size (Millipore (U.K.) Ltd., Wembley, Middlesex). The filter is supported on a disk of hardened filter paper (Whatman No. 50) in a perspex holder, F, which was described by O'Brien (1969). This holder is designed to expose a large area of filter to the infusate with a minimum of dead volume, and it was built by Mr. W. Tait in the Workshops of the Department of Biochemistry. Recently, 0.45 μ m 'Acropor' membrane filters made of acrylonitrile co-polymer on a nylon support have been used (supplied by Gelman Hawksley Ltd., Lancing, Surrey). These filters are mechanically strong, and dispense with the need for a filter paper support.

The filtered infusate is taken within a flexible water-jacket, J, via a mercury manometer¹, M, with bubble trap to a 4-way tap, T (Baxter BR-62S stopcock, made by Baxter Laboratories Ltd., Thetford, Norfolk). This tap, which is made of polypropylene, is attached to the bung of the organ chamber so that one outlet passes through the bung into the chamber, while the inlet and the second outlet are immediately outside the organ chamber. A short length of tubing leads to waste from the latter outlet. To the outlet inside the organ chamber is attached five or six centimetres of hard polyethylene tubing (O.D. about 1 mm) by a Luer fitting. This is cut from a 'Bradley miniature catheterisation set' (made by Portex Ltd., Hythe, Kent) and serves as the arterial cannula. (I am grateful to Dr. M. George who suggested the use of a Bradley catheter.)

The vascular effluent drips from the intestine, presumably from the torn lymph vessels, and it is collected as secretion was from

¹ The manometer has been replaced recently by an aneroid dial gauge reading from 0-300 mm Hg, supplied for use with a sphygmomanometer ('Pressostabil', obtained through A. Young and Son Ltd., Forrest Road, Edinburgh).

the unmodified preparation. Recently, this effluent has been pumped through a filter and into the receiving tubes. The filter (49 mm diameter discs of Nos. 54 and 50 Whatman filter paper in series) removes particles which might otherwise obstruct the AutoAnalyzer sampling tube, and is more convenient than centrifugation of individual samples.

The arterial infusate

The normal medium is based on the modified Krebs & Henseleit (1932) bicarbonate buffer which was described on page 25 for the lumen perfusates. However, the infusate contains no phenol red, and raffinose (10 mg/ml, 16.8 mM) is substituted for the glucose. The raffinose serves as an extracellular marker from whose dilution the net rate of water secretion into the sub-epithelial space is calculated.

Changing the arterial infusate

The system was designed so that the infusion media could be changed rapidly, and so that no gas bubbles would enter the arterial cannula. Bubbles would cause embolism in the blood vessels, and so would progressively restrict the extent of infusion into the sub-epithelial space.

The 3-way tap (Q in Figure 59) fulfils two purposes: (i) Air in the tubing from the reservoir can be displaced into a syringe attached to one limb of this tap, and (ii) A rapid change-over of infusion media is possible, while air is denied access to the system. As this type of tap was noted to leak slightly after prolonged use it was inspected regularly.

The 4-way tap (T in Figure 59) permits the infusate to bypass the intestine. This is convenient when it is desired to replace an

infusate by a second one as rapidly as possible without subjecting the vasculature to an unduly high pressure. Therefore the following regime was adopted whenever the infusate was to be changed in the course of an experiment.

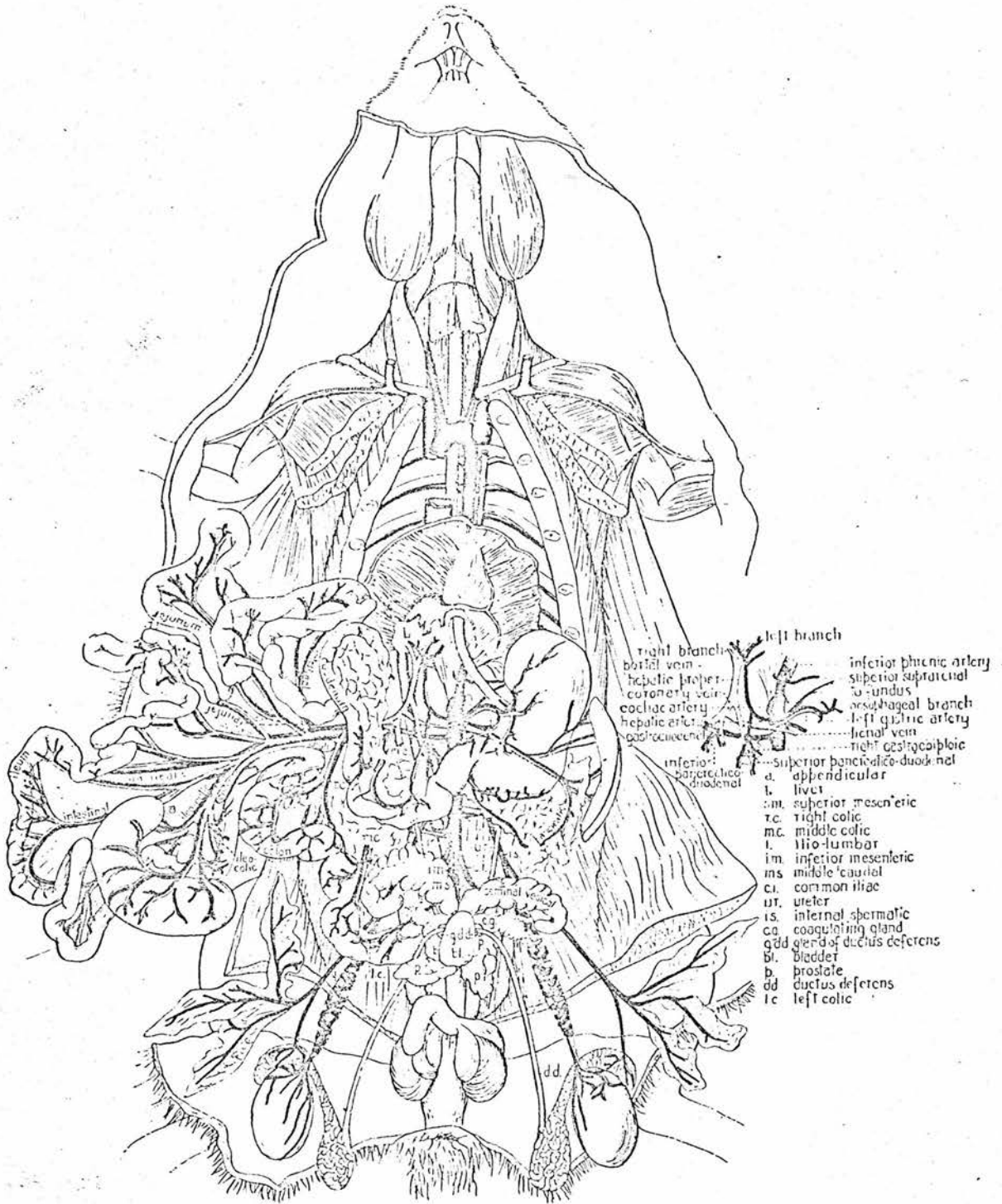
1. Reservoir selected by tap Q,
2. Tap T opened so as to allow infusate access to both intestine and drain,
3. Pump rate doubled for two minutes,
4. Finally, tap T closed to drain. The new infusate now enters only the arterial cannula.

Anaesthesia

As the surgical preparation of the intestine is lengthy, a long acting non-volatile anaesthetic was sought. Urethane, ethyl carbamate, was selected because it is very long acting, easily administered, and the dose is not critical. A disadvantage is that urethane is slightly carcinogenic (see e.g. British Empire Cancer Campaign, 1967; Boyland & Rhoden, 1949). However, Professor R. A. M. Case of the Royal Cancer Hospital, London, kindly advised that its use for this work was without significant hazard. Surgeons gloves were routinely worn when animals which had received urethane were handled.

The urethane was supplied by British Drug Houses Ltd., and the dose was 1 ml of a warm aqueous solution containing 0.7 g urethane (i.e. 0.7 g urethane plus 0.7 ml water) injected subcutaneously. For the injection, the animal was temporarily anaesthetised with ether. Surgical anaesthesia was usually complete within 60 to 90 minutes.

It was often noted on surgery that the mesenteric blood was abnormally venous, perhaps owing to respiratory depression under



Liver removed and viscera displaced to show the portal system of veins and the abdominal branches of the aorta.

FIGURE 60

The vascular anatomy of the rat

(reproduced from Greene, 1959)

anaesthesia. In view of the damaging effects of hypoxia which have already been recorded, the animals have recently been put in an oxygen tent immediately after administration of the anaesthetic. Supply of oxygen was continued throughout the operation, and the mesenteric blood generally appeared to be much more arterial than in the earlier work. Baldwin & Robinson (1939) reported that brief exposure to an oxygen-enriched atmosphere increased the rat's resistance to anoxia. However, results will be given below which indicate that the oxygen therapy had no effect on the subsequent absorption rates.

Setting up the intestine

The initial stages of the operation are similar to those in the setting-up of the unmodified preparation; however, especial care is needed to maintain the integrity of the vasculature, since the infusate must escape only through the permeable capillary walls and not through torn arteries and veins. The success of the preparation may be attributed partly to the use of crude diathermy to divide the arcadian blood vessels, and to arrest bleeding. For this a small electric soldering iron with thermostatic control ('Ceco miniature iron', made by Cardross Engineering Co. Ltd., Woodford Yards, Dumbarton) has been used. Figure 60 may be referred to for details of the vascular anatomy.

The anaesthetised animal is secured to the operating table, and oxygen administered through a simple mask. A mid-line T-incision is made into the abdomen, and the animal is covered with a plastic drape which prevents the fur from sticking to the intestine. The caecum, then ileum and jejunum are gently exteriorised and reflected towards the left hand side of the animal as seen from the posterior. The duodeno-jejunal flexure is identified as the highest part of

intestine which can be withdrawn without undue traction on the Ligament of Treitz. The arcadian blood vessels to the upper 1 or 2 cm of jejunum are cauterised and divided close to the intestine using the soldering iron. The collateral vessels may also be cauterised. The duodenum is tied off and cut free, and a glass cannula is tied into the jejunum. Warm oxygenated saline under low pressure is then pumped into the cannula to displace any solid debris from the small intestine into the caecum and colon. In a rat which has recently fed some gentle manipulation may be necessary to move the debris from the lower ileum. The ileo-colic artery and vein are now identified among the adipose tissue close to where the colon emerges from the caecum, and are divided between silk ligatures. The arcadian vessels to the terminal 1 or 2 cm of ileum are cauterised and divided, and the ileum is tied off and divided near to the ileo-caecal valve. The outflow cannula, directed orally, is tied into the lumen of the terminal ileum. After a further rinse with warm saline through the lumen, the segment of intestine in situ is attached to the perfusion apparatus and luminal perfusion is commenced. The flexible tubing and water-jacketing allows the organ chamber bung which carries the attachments for the cannulae to be clamped immediately above the operating table. Care is necessary so that the mesentery is not injured.

The small intestine and caecum are then reflected towards the right hand side of the animal, and the adipose tissue and omentum which bind the mesentery to the colon are parted with the hot soldering iron. When the right colonic vein is exposed it is cauterised. With the aid of an illuminated magnifier with a wide field of view (I.V.A. Engineering Co., Camberley, Surrey - kindly loaned by Dr. J. W. Minnis) the neighbouring superior mesenteric artery and

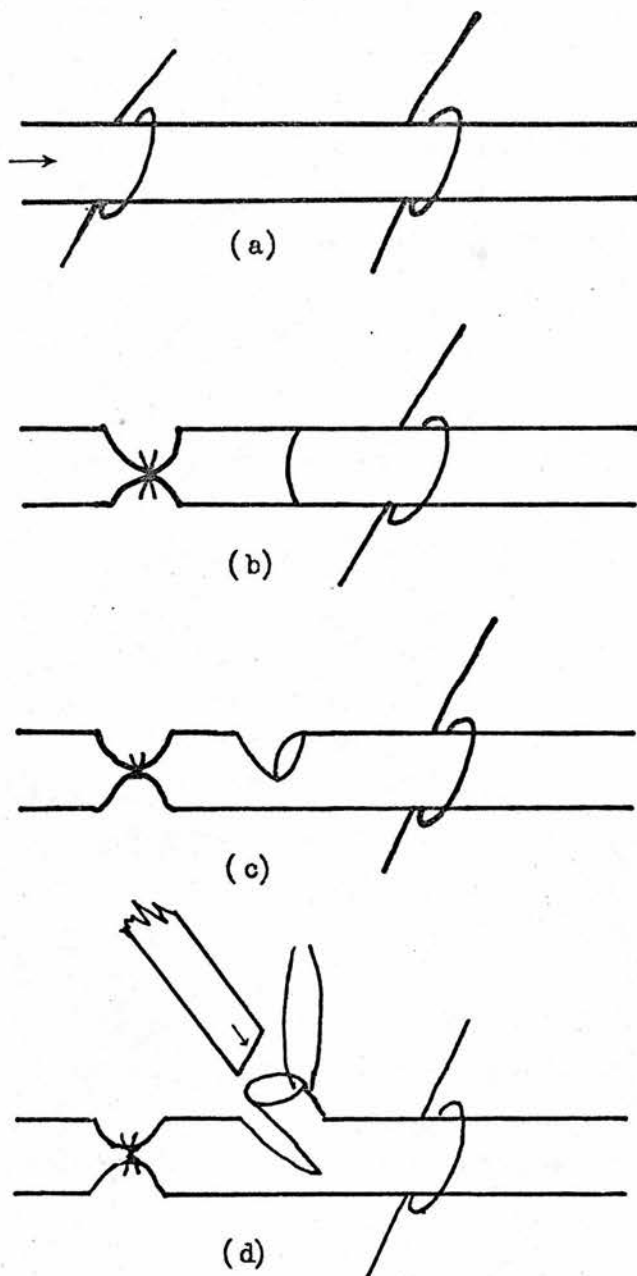


FIGURE 61

Cannulation of an artery

vein are exposed and cleared by blunt dissection. Two loosely tied silk ligatures are placed around the vein, and a small strip of white plastic sheet, about 2 mm thick, is placed below the artery but above the vein. This serves as a firm support for the artery during cannulation, to protect the vein from damage, and as a light background to aid vision. The artery is then cannulated.

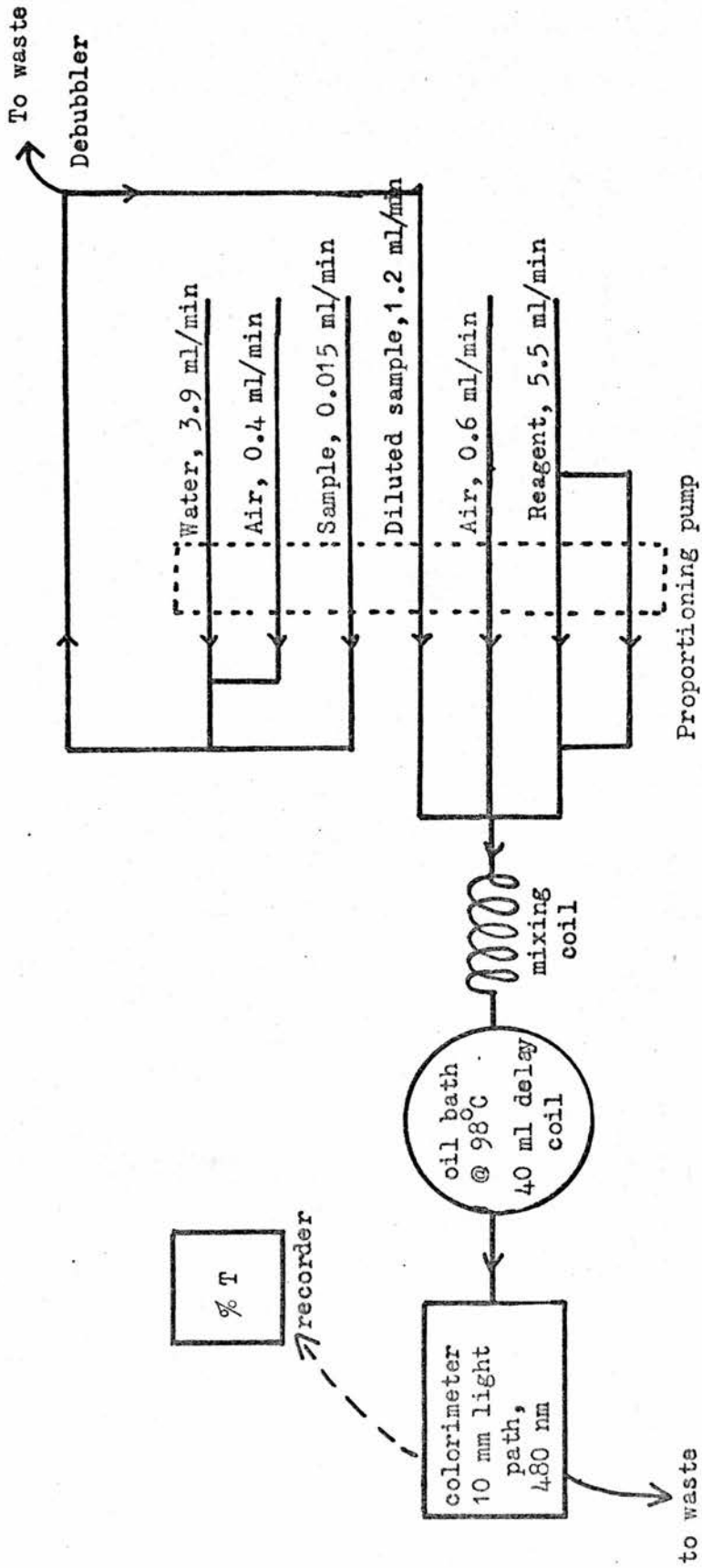
The superior mesenteric artery is only about 1 mm in outside diameter, and its cannulation requires some practice and a certain amount of luck. Occasionally groups of rats have been encountered with unusually small arteries which appear to go into spasm as soon as they are cut. In these cases failure is the regular outcome.

The infusion pump is started to deliver an almost imperceptibly slow drip from the arterial cannula tubing. Two loose ligatures of silk are placed about 6 mm apart around the artery (Figure 61a) and the proximal one is tightened. (Different coloured silk is used for the arterial and venous ligatures to aid identification.) An oblique transverse arteriotomy is made immediately distal to this tie through about half the depth of the artery with eye scissors (Figure 61 b & c). The distal cut wall is lifted with sharply pointed forceps ('Precista No. 4' made by Inox, Switzerland), and the cannula is pushed into the lumen of the vessel and the ligature drawn tight (Figure 61d).

The infusion rate is increased to about 3 ml/min. The manometer should now record a pressure of about 100-130 mm Hg if all is well. As soon as most of the blood has been displaced visibly from the mesenteric vessels the superior mesenteric vein is tied off and divided between the ligatures which have already been placed around the vessel. The artery wall proximal to the point of cannulation is cut, the whole small intestine is lifted carefully

FIGURE 62

AutoAnalyzer system for raffinose estimation



Sampling rate: 20/hour

into a nylon hairnet sling, and it is transferred to the organ chamber. Great care is taken so that the hairnet supports the weight of the organ and that the blood vessels are not torn.

The animal is then killed. The whole operation takes about 40 minutes on average, but extreme limits of 20 and 70 minutes have been recorded.

ESTIMATION OF RAFFINOSE*

Raffinose was estimated on the AutoAnalyzer by the fructosan method of Fisher & Gilbert (1970), fuller details of which have been given in the theses of Gilbert (1963) and Nimmo (1970). This method depends upon the colorimetric estimation of a red/yellow compound produced when fructosans react with ferric chloride and resorcinol in the presence of hot concentrated hydrochloric acid (Seliwanoff reaction).

The AutoAnalyzer system is shown in Figure 62. The only modification to the system which Nimmo (1970) described was that the sample was diluted some 240-fold with water by the AutoAnalyzer pump before it converged with the reagent stream. Consequently, samples containing 5 to 15 mg raffinose/ml can be estimated directly without prior treatment.

The reagent

A solution of resorcinol (1 g) and ferric chloride (0.05 g) in water (400 ml) is added to concentrated hydrochloric acid (600 ml). The acid was of commercial grade, supplied by Richard Smith Ltd., Glasgow. Consignments of acid from alternative suppliers have been found to be less concentrated; unless allowance is made for this, the less concentrated acid is unsatisfactory (I. A. Nimmo, personal communication).

* Raffinose is α -D-galactosyl-(1-6)- α -D-glucosyl-(1-2)- β -D-fructoside.

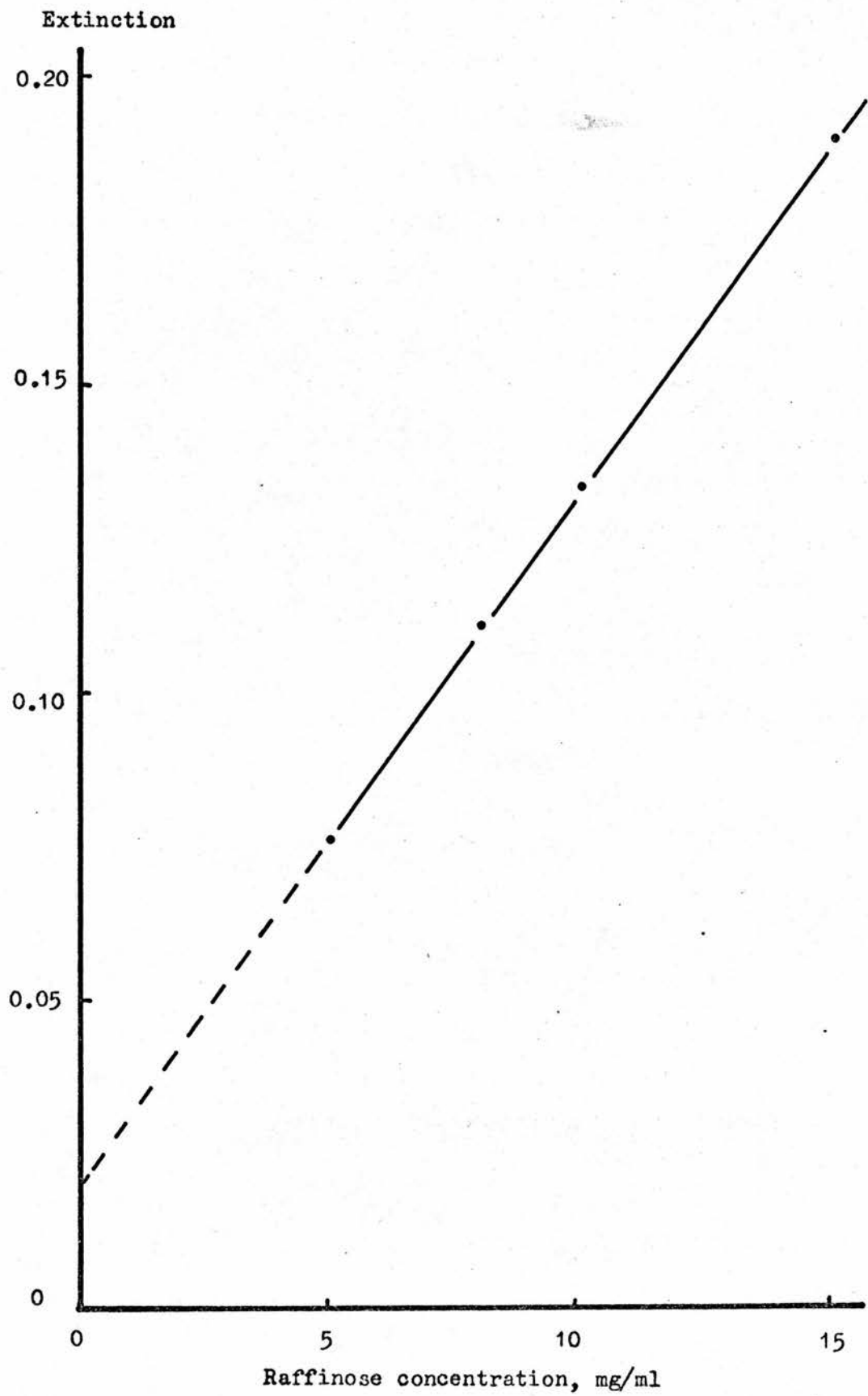


FIGURE 63

Estimation of raffinose: a typical calibration curve where Beer's Law was not obeyed

Extinction @ 480 nm

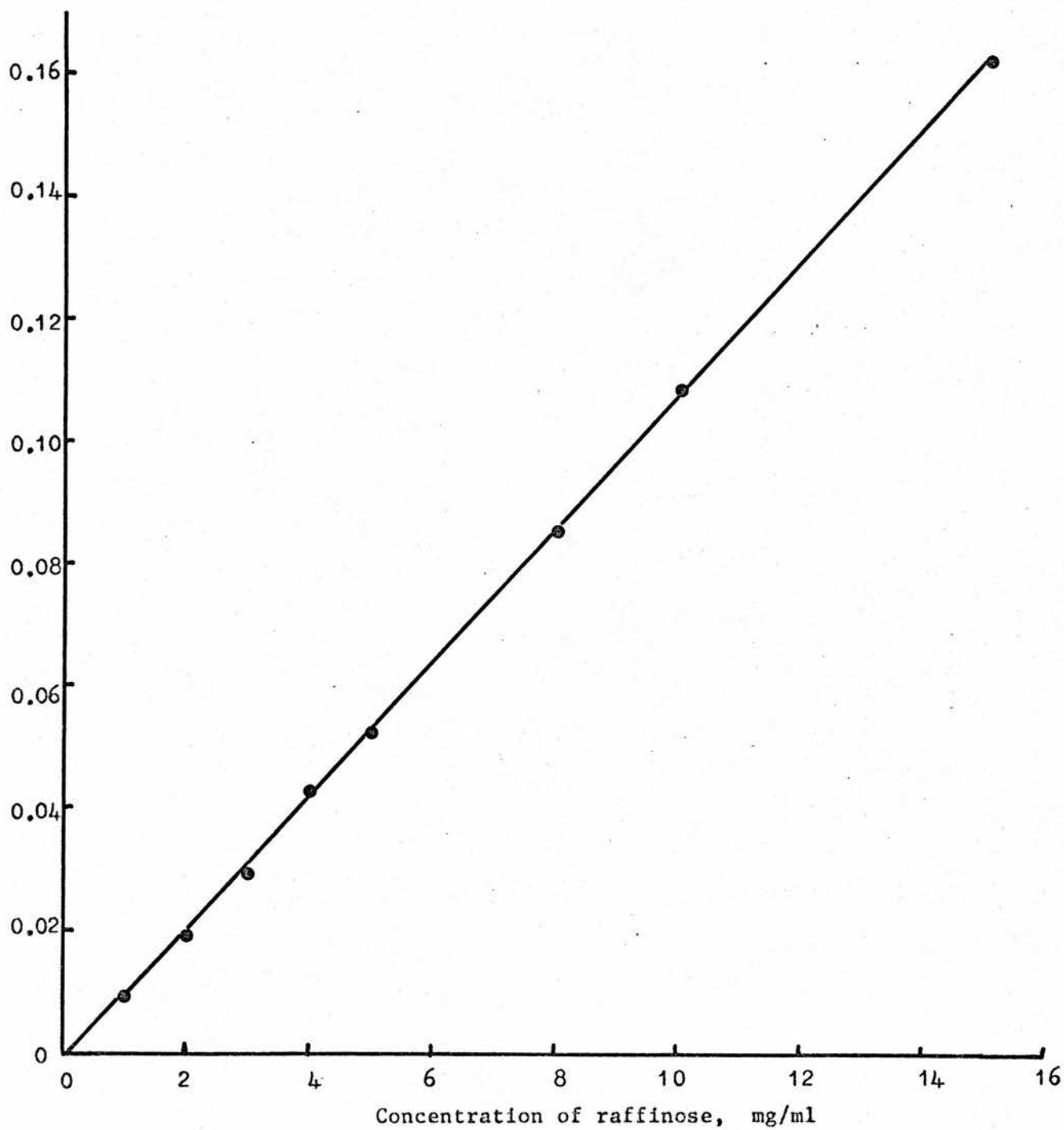


FIGURE 64

Estimation of raffinose: a typical calibration curve where Beer's Law was obeyed

Calibration of the system

The system was used in the same manner as that already elaborated in Chapter III for glucose estimation. Standard solutions containing 5, 8, 10, and 15 mg raffinose/ml respectively were aspirated immediately before and after each palindromic series of unknowns. Figure 63 shows a typical plot of extinction against concentration. Although Beer's Law is not obeyed, since the line does not pass through the origin, the deviation from linearity in the region between the standards is small. Therefore the first-order regression was used to fit the calibration curve; in practice the extinctions for the standard solutions seldom differed by as much as 1% from the values predicted by the regression. However, on a subsequent occasion a linear calibration curve through the origin for extinction against concentration of raffinose was obtained, and this is shown in Figure 64. A different batch of resorcinol was used in the reagent for this latter calibration, and may account for the discrepancy between the two occasions. Gilbert (1963) had already claimed that Beer's Law was obeyed for this estimation, but Nimmo (1970) clearly showed the converse.

The precision of an estimate

When a solution containing 10.0 mg raffinose/ml was repeatedly estimated 5 times, the mean apparent concentration (\pm S.E.) was 9.998 ± 0.01646 mg/ml - i.e. not significantly different from 10.0 mg/ml. The standard deviation was 0.037 mg/ml.

Interference of glucose

A solution containing raffinose (10 mg/ml) and a similar one to which glucose had been added to a final concentration of 2 mg/ml were estimated four times each. This represents the highest ratio of glucose to raffinose expected to be encountered in the present work.

TABLE 25

Interference of glucose in the estimation of raffinose

Apparent raffinose concentration, mg/ml

A	B
10.1133	10.1673
10.0054	10.1945
10.0593	10.1403
10.0862	10.1133
Mean \pm S.E.M. 10.0661 \pm 0.0230	10.1539 \pm 0.0175

$$t_6 = 3.039$$

$$0.05 > P > 0.02$$

Solution A contained raffinose (10 mg/ml).

Solution B contained raffinose (10 mg/ml) plus glucose (2 mg/ml).

The apparent concentrations of raffinose in the two solutions were not quite significantly different - see Table 25. Therefore potential interference by glucose was discounted.

The raffinose 'Blank' of vascular effluent

Gilbert (1963) and Nimmo (1970) found that rat heart perfusate and red blood cell haemolysates respectively gave a positive reaction with the reagent for raffinose estimation, as though these tissues contained fructosan material. So an intestine was perfused with arterial infusion in the normal manner, except that the arterial infusate contained no raffinose. Samples of the vascular effluent were estimated for raffinose, but none could be detected. The AutoAnalyzer manifold was temporarily modified so that the sample was not diluted before it converged with the reagent. Again no raffinose was detectable in the vascular effluent. Therefore the apparent raffinose 'blank' could not have been greater than about 1 $\mu\text{g}/\text{ml}$.

Calculation of absorption rates etc.

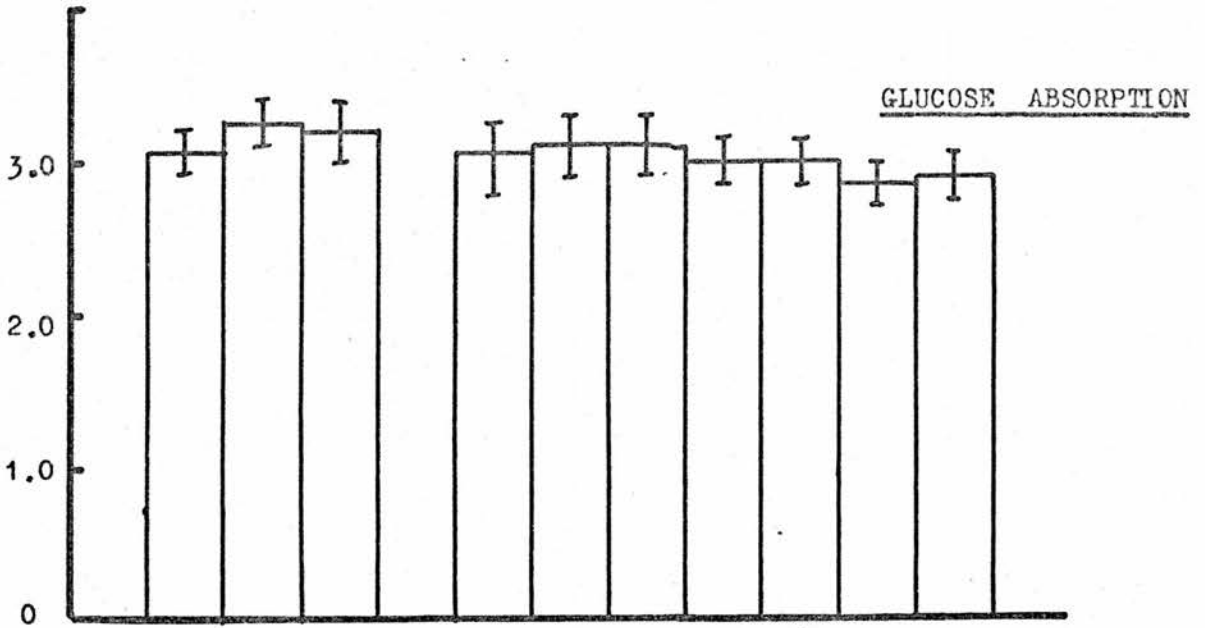
The calculations were made in a similar manner to that already described, with the exception that rates of water transport were determined from the dilution of the raffinose in the arterial infusate.

CHAPTER X

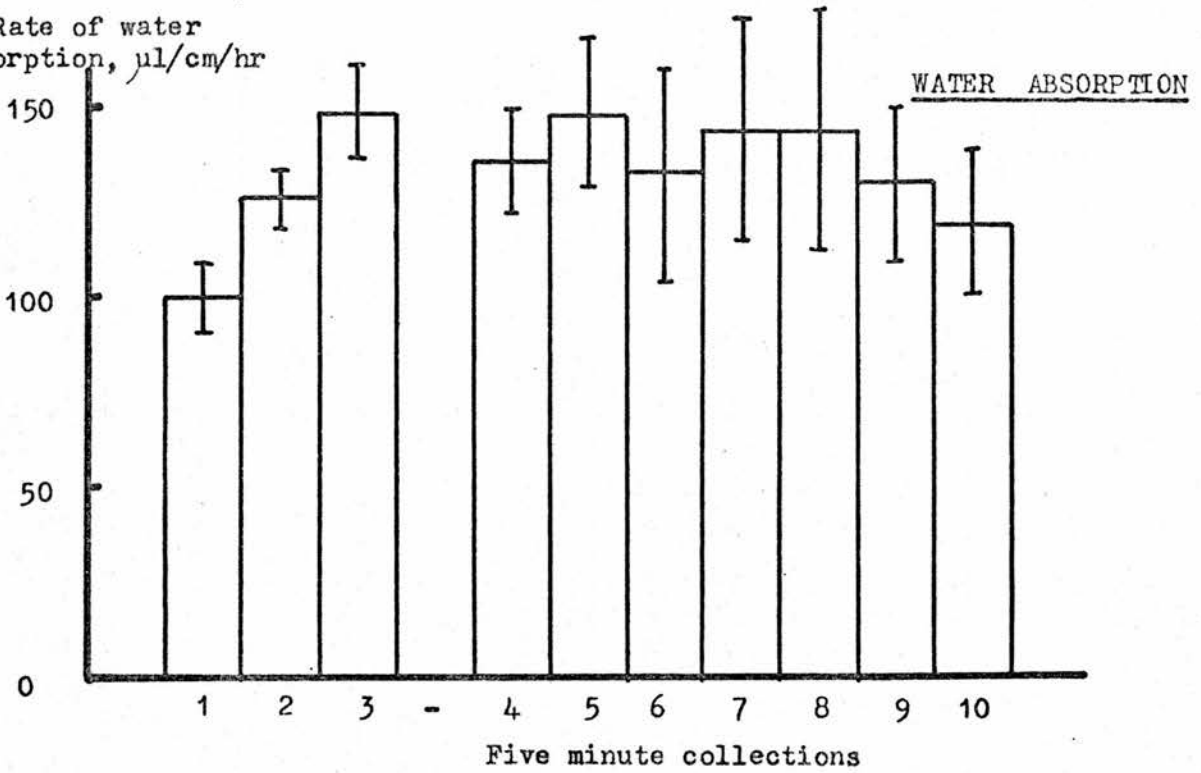
CHARACTERISTICS OF THE NEW PREPARATION

WITH ARTERIAL INFUSION

Rate of glucose
absorption, mg/cm/hr



Rate of water
absorption, μ l/cm/hr



Pressure, mm Hg

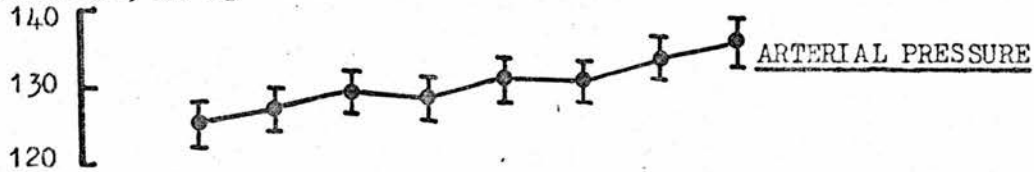


FIGURE 65

Stability of the new preparation with arterial infusion
(vertical bars show S.E.M.)

CHAPTER X CHARACTERISTICS OF THE NEW PREPARATION WITH
ARTERIAL INFUSION

Stability of the preparation

Figure 65 summarises the mean rates of glucose and water absorption from a luminal perfusate containing glucose (5 mg/ml, 28 mM) over the first hour after the intestine had been put in the organ chamber.

Clearly the stability of glucose absorption over the whole period is excellent, while that of water absorption is fairly good after some fifteen minutes have elapsed. This impression has been confirmed by analyses of variance.

Figure 65 also shows the pressure recorded by the manometer at the arterial cannula, and this too is remarkably stable over this period. This pressure is the sum of the pressures across the arterial cannula (about 60-80 mm) and across the vascular space.

The raffinose space

In many of the experiments a coloured dye was injected into the arterial cannula after the intestine had been removed from the perfusion apparatus as an indication of the space to which the infusate had had access. In some of the earliest experiments the dye (Evans Blue or Nile Blue dissolved in saline) was seen to escape from ruptured arcadian vessels. In subsequent work this has been rare. Generally the tissue rapidly became almost uniformly blue after small volumes of the dye had been injected. Down the length of the intestine the blue colour was generally fairly uniform, but the upper 2 to 4 cm and lower 1 to 3 cm were often not stained.

While the blue dye confirmed that the infusion medium was not leaking out from torn vessels to a noticeable extent it gave no information as to the actual space to which the infusate had access.

Raffinose concentration (mg/ml)
in vascular effluent

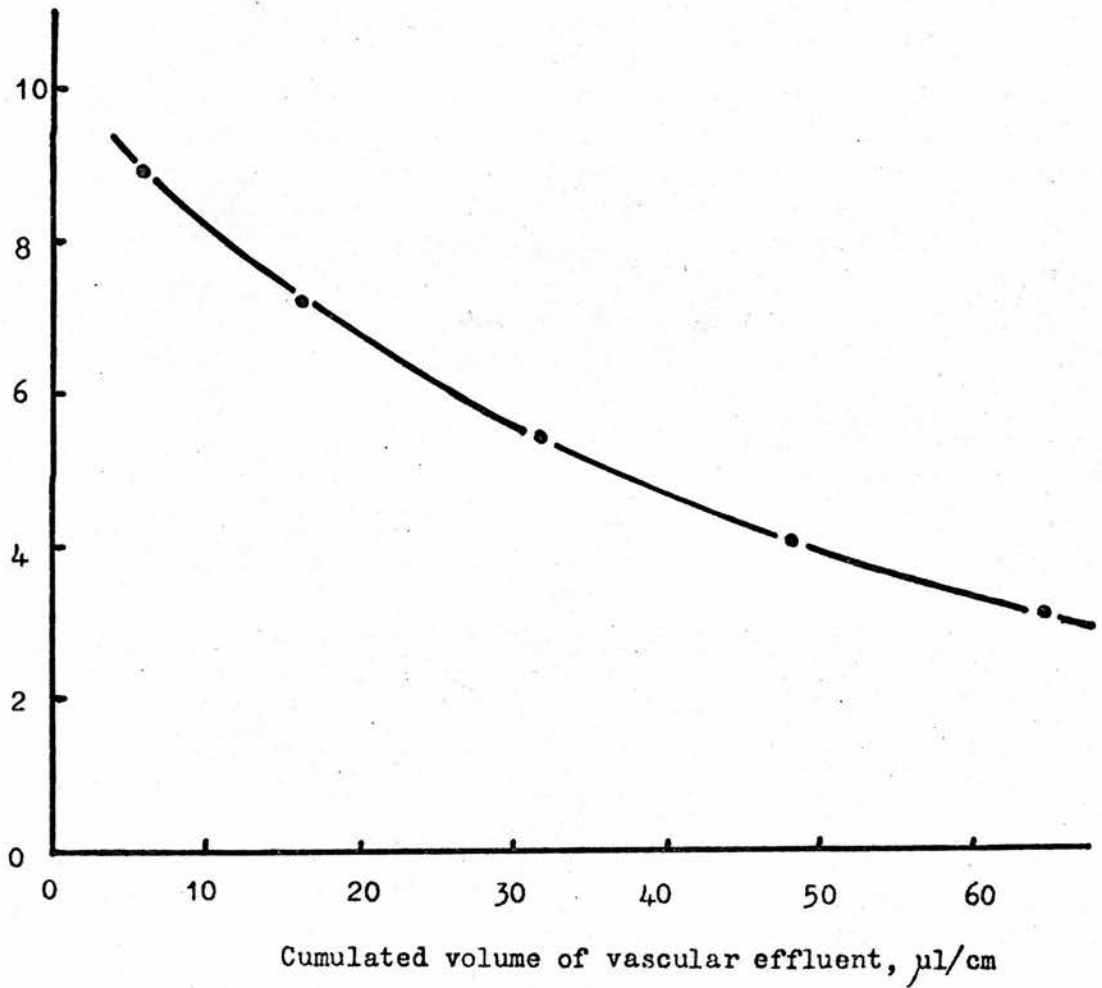


FIGURE 66

Raffinose wash-out in vascular effluent

$\text{Log}_e(\text{raffinose concentration})$

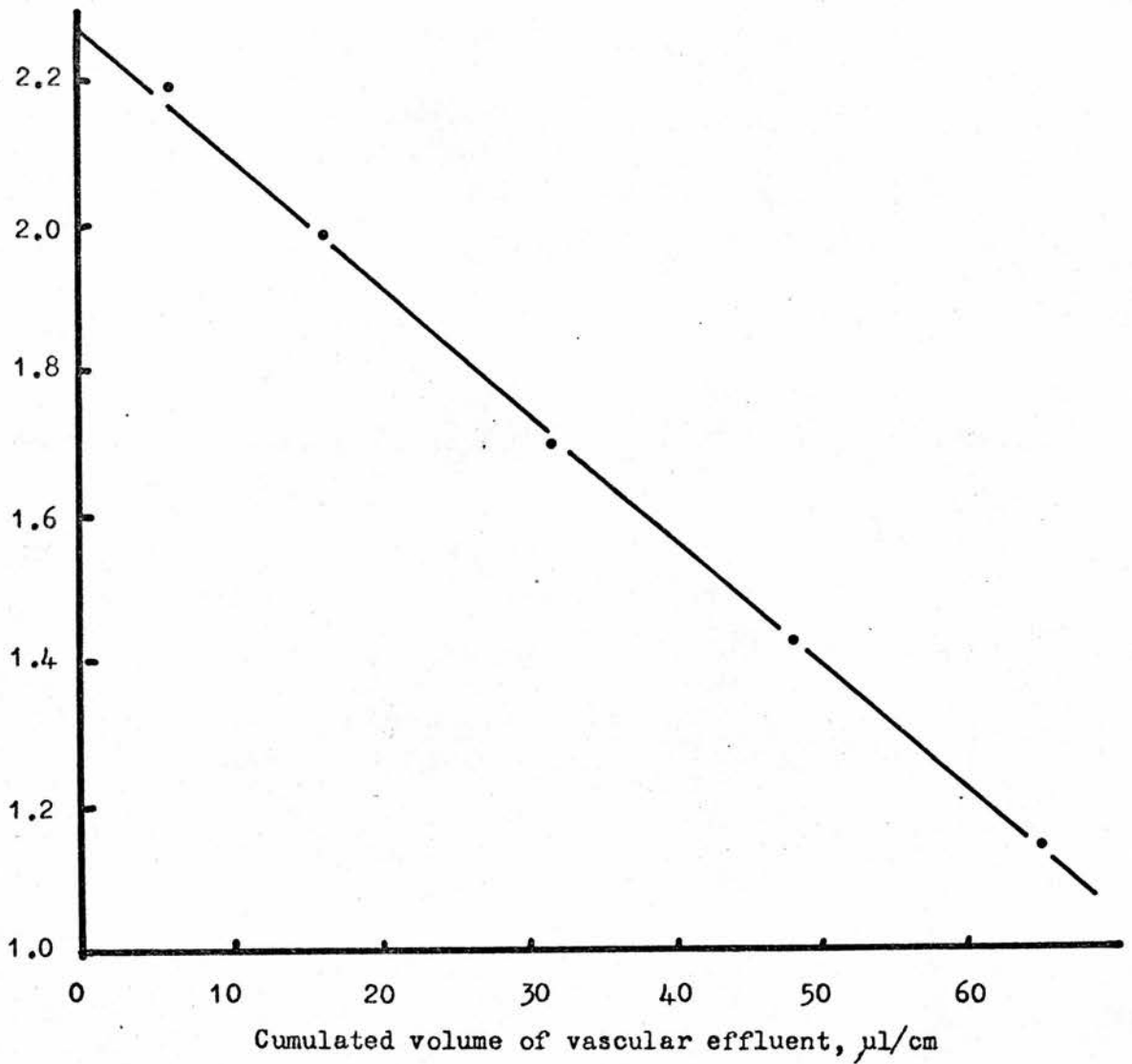


FIGURE 67

Semi-logarithmic plot for raffinose wash-out in vascular effluent

(single experiment - date of Fig. 66)

TABLE 26

Computed parameters for raffinose wash-out (single exponential model)

$$C = B + A \cdot e^{-v/V}$$

	B mg/ml	A mg/ml	V μl/cm
	1.210	8.854	42.248
	2.026	6.869	26.631
	0.567	5.188	53.333
	2.033	7.082	29.197
	1.841	6.468	24.079
	1.132	5.714	17.346
	1.097	7.938	43.271
	2.357	5.887	30.340
	2.071	6.163	34.662
Mean	1.593	6.685	33.456
S.E.M.	0.2011	0.3838	3.712

Each line refers to a separate segment of intestine.

Therefore measurements were made of the raffinose space by following the kinetics of raffinose wash-out in the vascular effluent when the raffinose concentration in the infusate was abruptly changed.

After infusion for about an hour with a normal infusion medium containing raffinose (10 mg/ml), this infusate was replaced by a similar one but which contained glucose (5 mg/ml or 3.6 mg/ml) and no raffinose. After the change-over, samples of the vascular effluent were collected at about 20 second intervals and were analysed for raffinose. The raffinose concentration in this effluent diminished in an apparently exponential manner (Figure 66) as predicted for a single compartment system (Atkins, 1969, page 25). This analysis is similar to the one used by Bleehen & Fisher (1954) to estimate the extracellular volume of perfused hearts, and it follows as a special case of the kinetics derived in Chapter VI to represent the appearance of solutes in intestinal secretion.

When the data for raffinose wash-out were plotted on a semi-logarithmic graph of \log_e (raffinose conc. in effluent) against the cumulated volume of effluent, reasonable straight lines were obtained (e.g. Figure 67). Therefore, an exponential function

$$C = B + A \cdot \exp(-v/V)$$

where C is the raffinose concentration in the effluent,

v is the cumulated volume of effluent,

and B, A, and V are constants,

was fitted to the data by Atkins' (1971a) computer program. Values for the computed parameters are shown in Table 26 for nine raffinose wash-out experiments in different intestines. These results are surprising: the constant B, which is the asymptotic value to which C tends, is significantly greater than zero ($P < 0,001$), as though

TABLE 27

Tests of the goodness of fit of single exponential model to the kinetics of raffinose wash-out from vascular space

<u>Data point</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>	<u>5</u>	<u>6</u>	<u>7</u>	<u>8</u>
Mean residual	0.0336	-0.0509	-0.0280	0.0260	0.0694	0.0210	-0.0214	-0.0644
S.E.M.	0.0416	0.0683	0.0402	0.0294	0.0340	0.0316	0.0128	0.0414
D of F	8	8	8	8	8	7	7	6
t	0.807	0.745	0.695	0.884	2.044	0.666	1.670	1.558
P	NS	NS	NS	NS	>0.05	NS	>0.1	>0.1

$\log_e(\text{raffinose concentration in vascular effluent, mg/ml})$

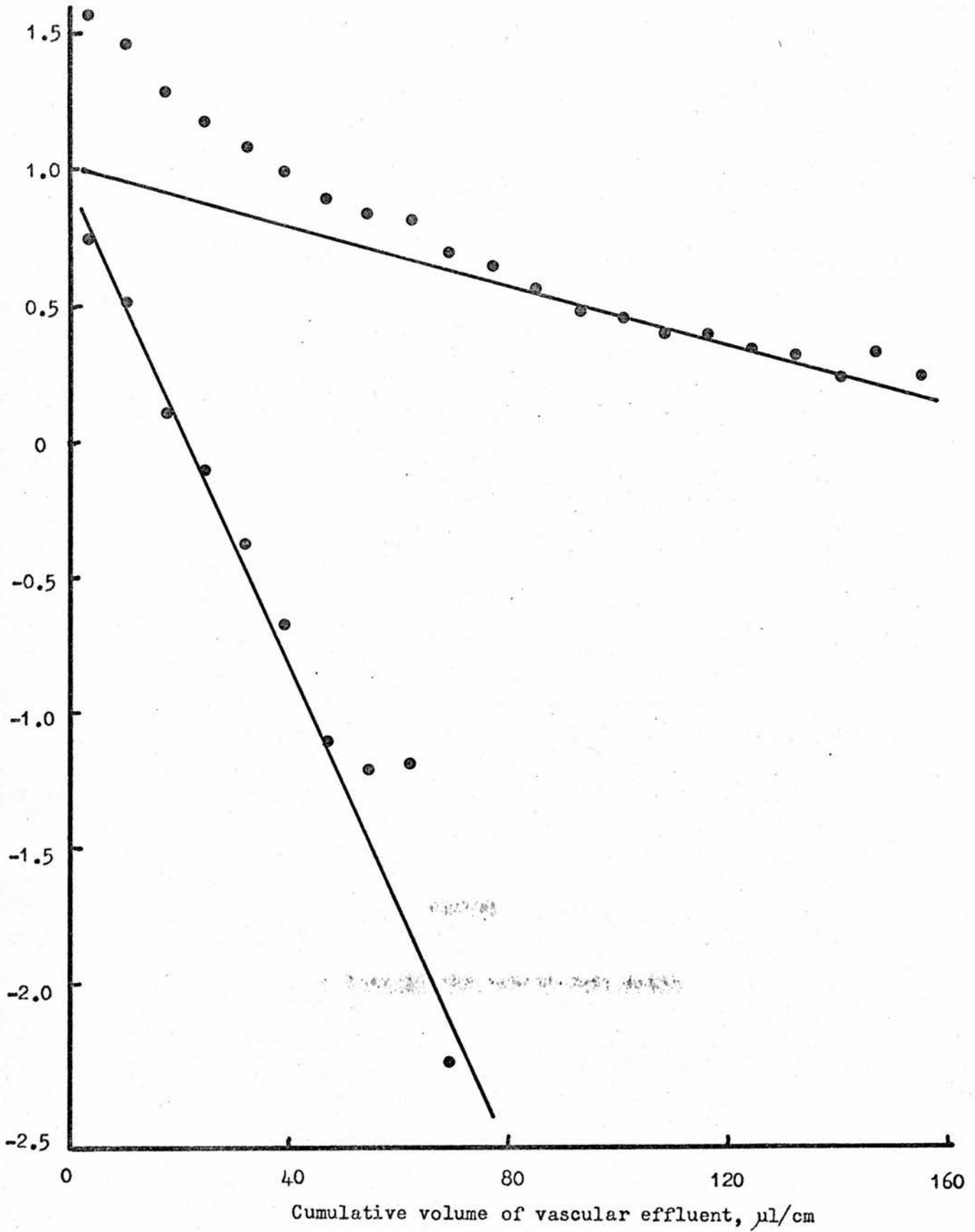


FIGURE 68

Raffinose wash-out data fitted by two semi-log plots

(one typical experiment)

there is a raffinose 'blank' in the vascular effluent when no raffinose is infused. However, this has already been disproved.

Therefore it seemed that the single compartment model was not a good fit to the experimental data. The test of goodness of fit which was described on page 84 was applied to these data, and Table 27 shows mean values of the residuals about each point. However there is no indication from this test that the data are poorly fitted since none of the residuals differ significantly from zero. This may suggest that the test of goodness of fit is a bad one, rather than that the model is a good one.

As a further check on the validity of the non-zero asymptote given by the curve-fitting method, the model

$$C = A \cdot \exp(-v/V)$$

was fitted to the data. That is, the asymptote was constrained to be zero. With this two-parameter model, the sum of squares of residuals and the standard deviation of the residuals were invariably greater than those for the three-parameter model. This confirmed that the better model was the one with the non-zero asymptote.

To examine in more detail the kinetics of raffinose wash-out, three further experiments were made with some 20 points on each wash-out curve. In each case the points on the semi-log plots did not lie on a single straight line. Therefore a graphical peeling-off technique (Atkins, 1969, page 102) was applied and two straight lines were obtained (e.g. Figure 68). This suggested that the observed kinetics were better described by a sum of two exponentials function viz.:-

$$C = B + A_1 \cdot e^{-k_1 \cdot v} + A_2 \cdot e^{-k_2 \cdot v}$$

Therefore the curve fitting was repeated using Atkins' computer program and this five parameter model. The results are given in

TABLE 28

Computed parameters for raffinose wash-out (two-exponential model)

$$C = B + A_1 \cdot e^{-k_1 \cdot v} + A_2 \cdot e^{-k_2 \cdot v}$$

B	A ₁	k ₁	A ₂	k ₂	SSD ₂	SSD ₁
0.01914	7.3787	0.04628	5.9324	0.008994	0.17297	0.25902
-0.00102	3.9783	0.04753	3.5489	0.009978	0.13815	0.07417
0.01011	4.0112	0.04681	4.7723	0.009547	0.10031	0.11779

SSD₂ & SSD₁ are the standard deviations of residuals for the 2- and 1-compartment models, respectively.

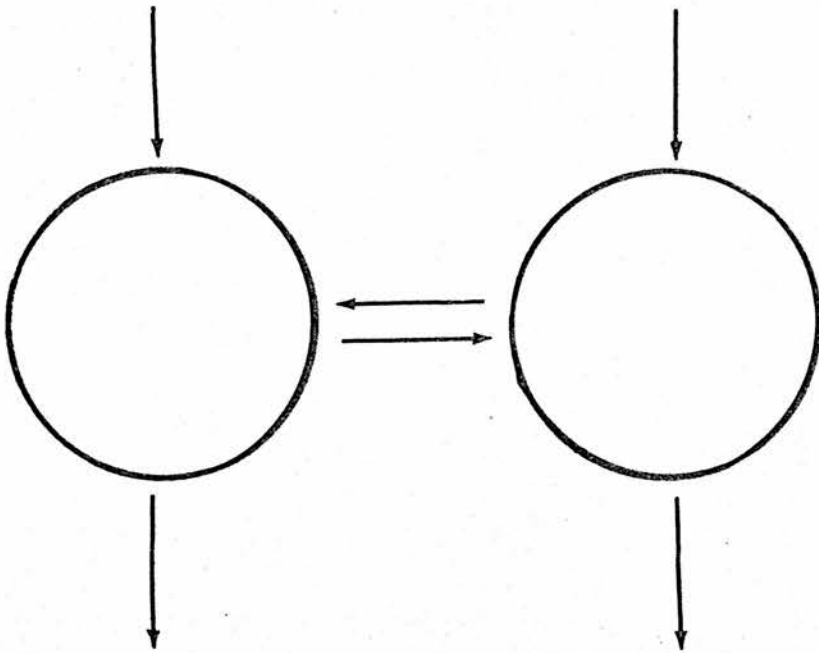


FIGURE 69

A general two-compartment model

Table 28 and in two cases the 2 exponential model gives lower standard deviation of residuals than the single exponential model does. Further, the asymptote term, B, is not significantly different from zero, and this is predicted for total wash-out from a tissue which does not have a measurable raffinose 'blank'. This model was also fitted to the earlier wash-out data, but in some cases the computation ceased before a minimum sum of squares of residuals was reached. This was hardly surprising since five parameters were being fitted to only eight data points. In six cases out of eleven the 2-compartment model gave a lower standard deviation of residuals than the 1-compartment model.

Such a sum of two exponentials is characteristic of a two compartment system. Figure 69 shows a general two compartment system. In specific cases of an open system up to three of the possible flux paths may be degenerate. However in the present case it is not possible to sample directly the two compartments and so one cannot distinguish between specific cases of the two compartment system. Further, it is not possible to estimate the compartment sizes from the exponential rate constants. Hence further interpretation of the kinetics is impossible and the mathematical compartments cannot be identified with anatomical or physiological compartments. However there is no reason to suppose that the intestinal extracellular space is homogeneous; for instance the capillaries to the epithelial cells and to the muscle layers may exchange infusate with two kinetically distinct compartments. Two-compartment kinetics for marker wash-out have been reported for the extracellular space in the heart (Morgan, Henderson, Regen & Park, 1961; Young, 1968; see also Cotlove, 1954). Young (1968) found that the faster component was affected by the degree of contractility of the heart while the slower component was not affected. It might therefore be

Glucose concentration in
vascular effluent, mg/ml

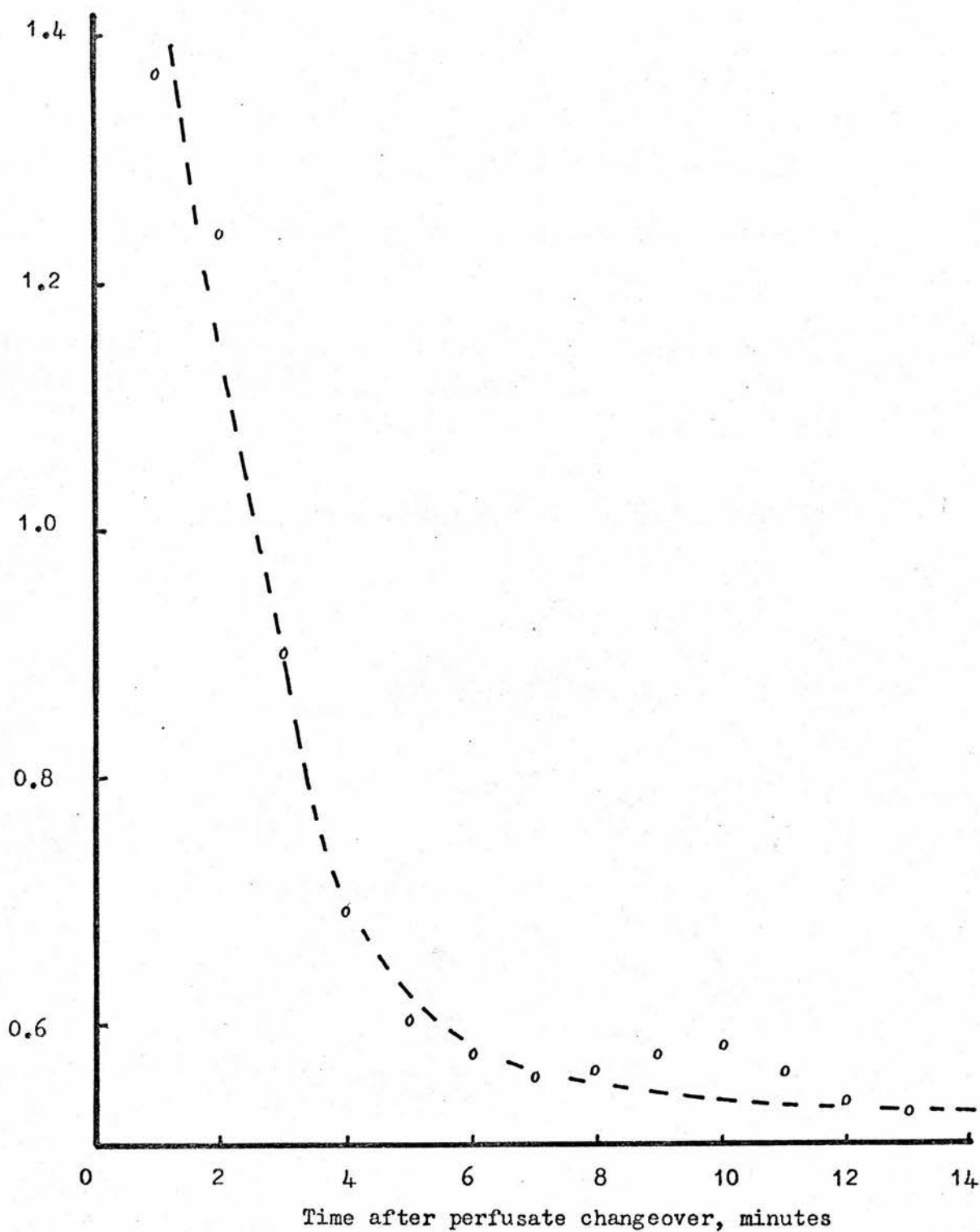


FIGURE 70

The rate of change of the glucose concentration in the vascular effluent following a sudden change in the glucose concentration in the luminal perfusate from 5 mg/ml to 1.5 mg/ml

interesting to see whether one component of the present kinetics of raffinose wash-out is affected by the intraluminal distension pressure. Morgan et al (1961) attempted to estimate the relative sizes of the two compartments, but the validity of their analysis is open to doubt.

However, the present kinetics cannot yet be regarded as satisfactorily explained.

The significance, if any, of the parameter V in Table 26 cannot be understood until the raffinose wash-out can be satisfactorily explained in terms of a model system. However, if the one compartment model had been upheld, as indeed was suggested by the tests in Table 27, V would have been a direct measure of the volume of the space with which the infused raffinose mixed. The value obtained here (33.456 $\mu\text{l}/\text{cm}$) is not significantly different from the value obtained in Chapter VI for the space with which intestinal secretion mixed in the unmodified preparation without arterial infusion. Regrettably it is fruitless to attempt to interpret this observation until the kinetics of raffinose wash-out have been resolved.

Whatever the form of the kinetics may be, it is clear from Figure 66 that a new steady-state can be attained in the vascular effluent within some 5 to 10 minutes at an infusion rate of 3 ml/min. Furthermore, Figure 70 shows the attainment of a new steady-state in the vascular effluent, subsequent to a change in the luminal glucose concentration from 5 mg/ml to 1.5 mg/ml. The glucose concentration in the vascular effluent became more or less constant within about 10-12 minutes; this contrasts with the results of Figure 39 where the steady-state was not reached in some 60 minutes in the unmodified preparation without arterial infusion.

Measurement of raffinose concentration in the effluent from the

TABLE 29

Rates of absorption etc. of glucose and water
in preparation with arterial infusion

	Rate of water absorption, $\mu\text{l/cm/hr}$	Rate of glucose absorption, mg/cm/hr	Rate of glucose secretion, mg/cm/hr	Rate of glucose utilisation, mg/cm/hr
Overall mean	124.0 ± 4.37 (78)	$3.99 \pm 0.255^*$ (23)	1.53 ± 0.10 (23)	2.43 ± 0.26 (23)
Mean over third collection period	134.0 ± 4.76 (78)			
Without oxygen therapy	120.1 ± 5.13 (49)	3.49 ± 0.133 (49)		
With oxygen therapy	131.8 ± 8.05 (29)	3.23 ± 0.097 (29)		
Unmodified preparation without arterial infusion (Table 10)	168.8 ± 4.4 (57)	3.17 ± 0.10 (57)	2.10 ± 0.07 (52)	1.19 ± 0.07 (52)

Notes: The values given are means \pm S.E.M. The number of experiments is in brackets.

The length of the segments was about 100 cm in the arterial infusion experiments, but only about 40 cm in these with the unmodified preparation.

The differences between absorption rates in animals with and without oxygen therapy are not significant.

* Further experiments gave: 3.40 ± 0.096 (78)

lumen showed that virtually no raffinose crossed the mucosa. The concentration in the lumen effluent was only about 0.1 mg/ml, and this corresponds to a rate of appearance into the lumen of about 180 μ g raffinose per cm length per hour. This is so small that it has been routinely ignored. This small loss of raffinose from the infusate would cause an overestimate of water absorption rates of as much as some 8%, and of glucose absorption rates of only about 1-2%.

Rates of water and glucose absorption

The mean rates of water and glucose absorption and of glucose utilisation are shown in Table 29 where the relevant data from Table 10 for the unmodified preparation without arterial infusion are also repeated. The rate for each intestine was taken as the mean of 3 consecutive measurements over the first fifteen minutes. As Figure 65 showed that the rate of water absorption did not become stable immediately, the mean rate of water absorption over the third five minute period is also given. According to Figure 65 this should be a reliable estimate of the steady-state rate.

Table 29 also compares the results from animals which had received oxygen therapy along with the anaesthetic (vide supra). This treatment had no significant effect.

Effect of phloridzin on glucose and water absorption

The effects of luminal phloridzin in the unmodified preparation have already been described (Figure 27). They have now been investigated in this new preparation, partly as a check on the use of raffinose as a 'marker' for water absorption. If raffinose were being metabolised by the intestine, then the estimates of the rate of water absorption would be spuriously high. Thus in the presence of a relatively toxic concentration of phloridzin in the lumen apparently significant rates of water absorption might be recorded.

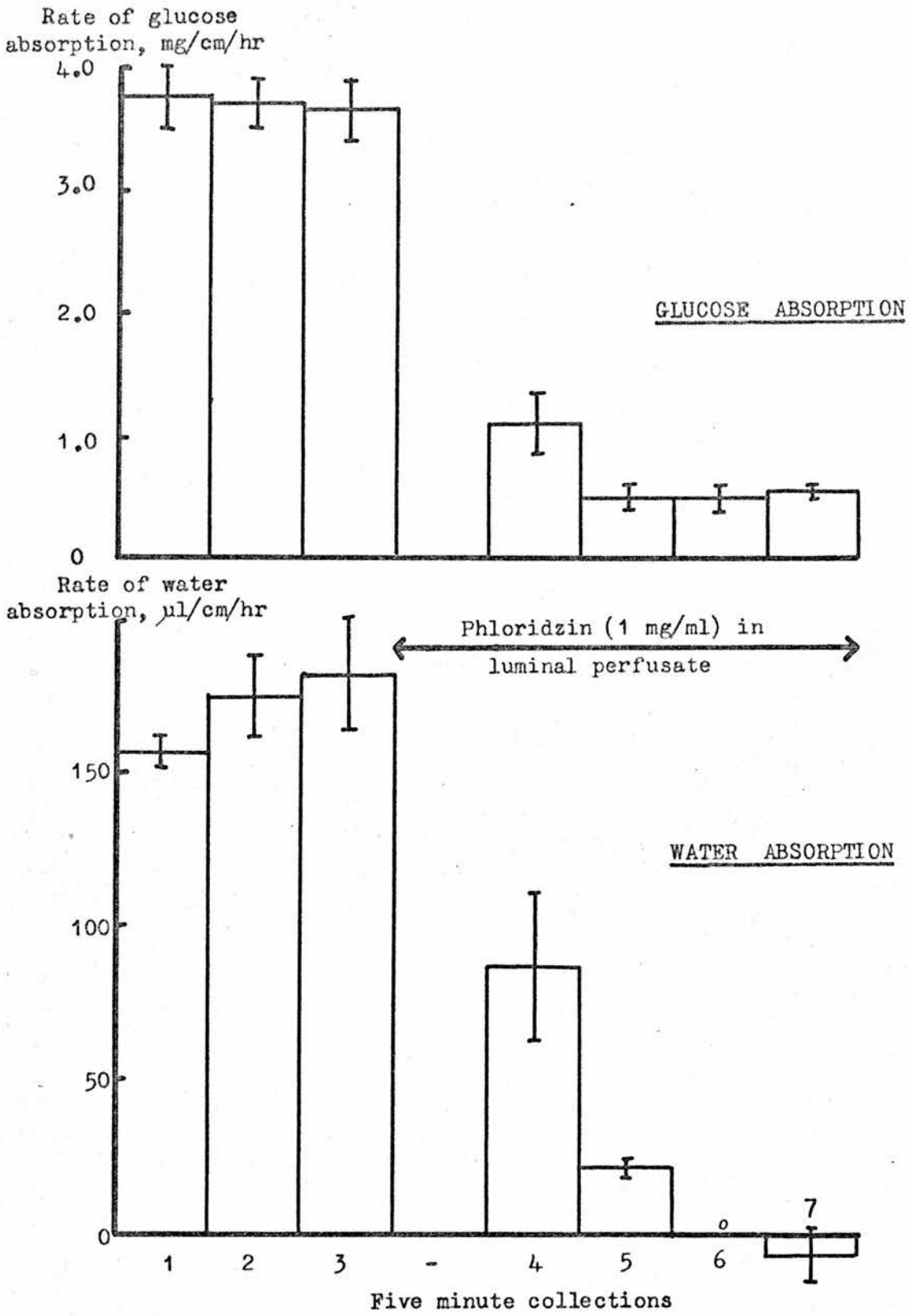
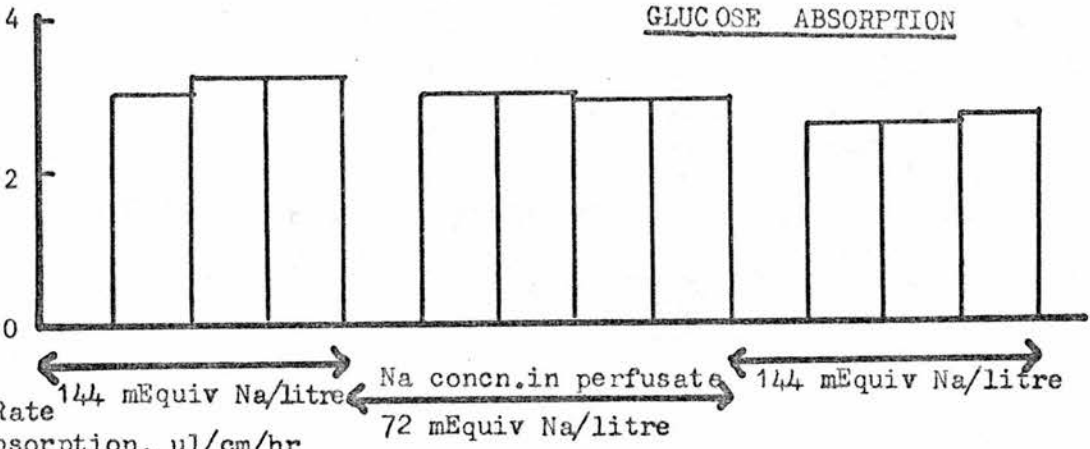


FIGURE 71

Effect of luminal phloridzin (1 mg/ml) in preparation with arterial infusion

Rate of glucose
absorption, mg/cm/hr



Rate
of water absorption, μ l/cm/hr

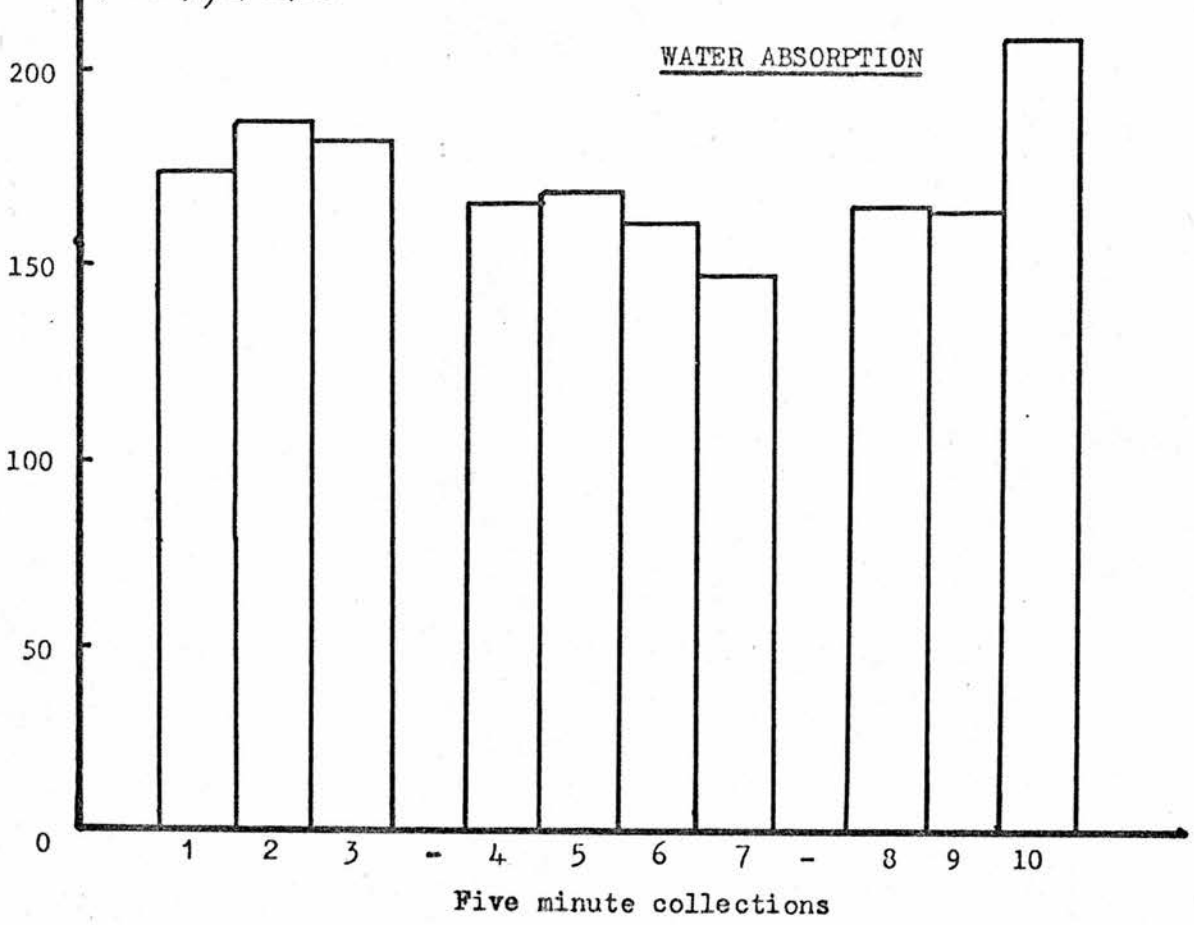


FIGURE 72

Effect of 50% replacement of luminal sodium by choline (one experiment)

Mean results from 3 intestines are shown in Figure 71. After three control periods each lasting 5 minutes, the perfusate was replaced by one similar but also containing phloridzin (1 mg/ml, 2.1 mM). Both glucose and water absorption were inhibited rapidly, and the water absorption rate became insignificantly different from zero. This suggests that raffinose was not metabolised, and in general supports its use as a marker for water absorption.

Effect of partial removal of luminal sodium

In preparation for some experiments on the effects of ouabain on the intestine which are reported in the next Chapter, it was desirable to know whether replacement of 50% of the luminal sodium by choline had any effect on glucose absorption.

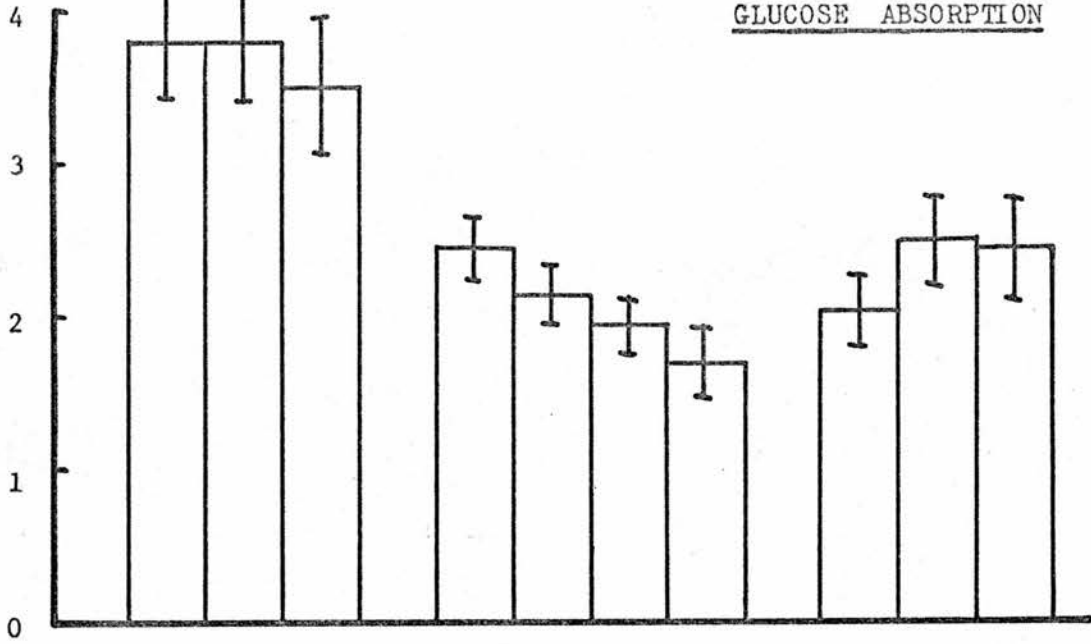
Figure 72 shows results from one experiment in which a normal lumen perfusate was replaced by one containing 72 mEq/litre sodium. Each collection lasted five minutes. Although water absorption was decreased by the reduction of luminal sodium ion concentration, no effect was noticeable on glucose absorption. Further experiments on separate intestines gave glucose absorption rates of 3.41 ± 0.096 (78) and 3.29 ± 0.316 (4) mg/cm/hr when the lumen sodium concentration was 144 mEq/litre and 72 mEq/litre respectively. These do not differ significantly.

Effect of total removal of luminal sodium

Experiments described in an earlier Chapter showed that the rate of glucose absorption fell only gradually after all the luminal sodium had been replaced by choline. The sodium concentration in the tissue fluid also fell progressively and it was remarked that the fall in glucose absorption might have been related to this.

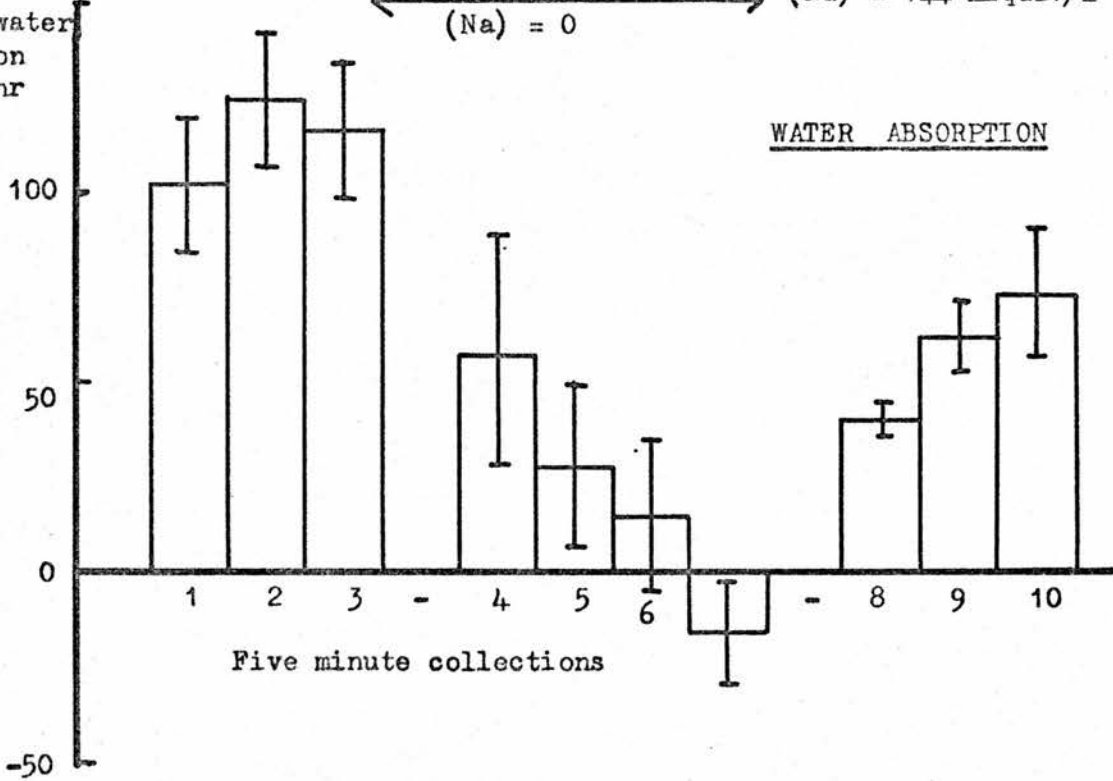
Therefore experiments were made in which all the sodium in the lumen perfusate was replaced by choline, but the tissue fluid

Rate of glucose
absorption, mg/cm/hr



GLUCOSE ABSORPTION

Rate of water
absorption
 μ l/cm/hr



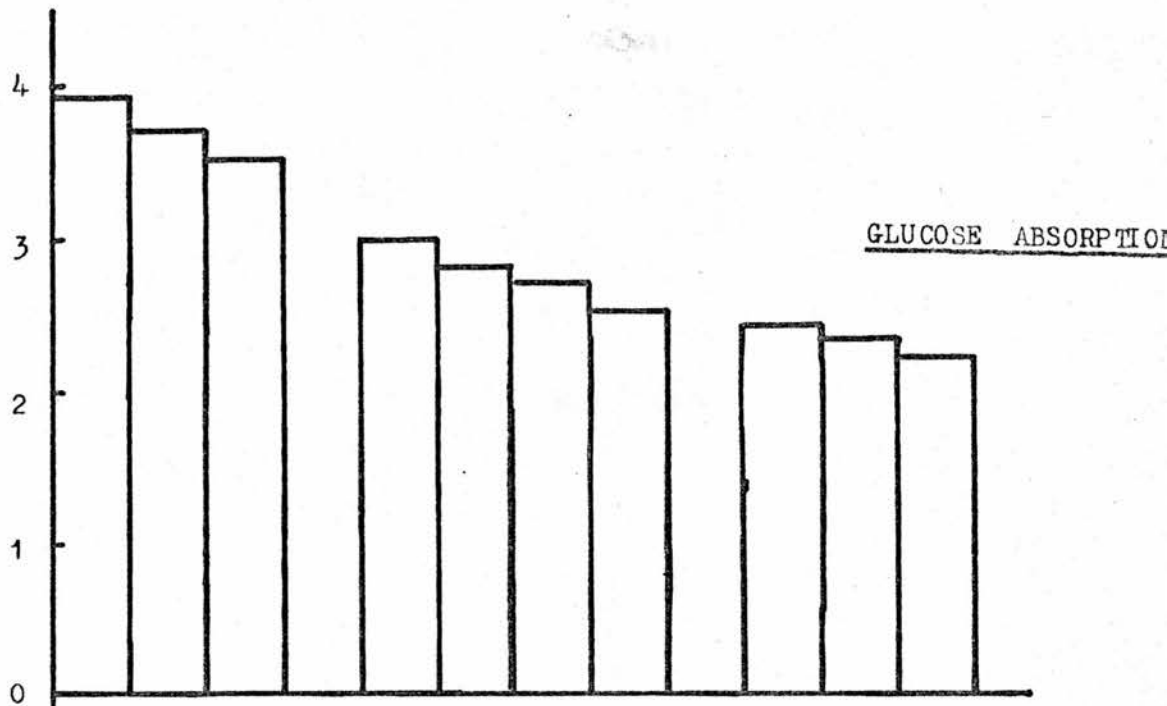
WATER ABSORPTION

FIGURE 73

Effect of total replacement of luminal sodium by choline. (Na) in arterial infusate = 144 mEq/litre.

(Mean of 3 experiments. The vertical bars show the S.E.M.)

Rate of glucose
absorption, mg/cm/hr



Rate of water
absorption, μ l/cm/hr

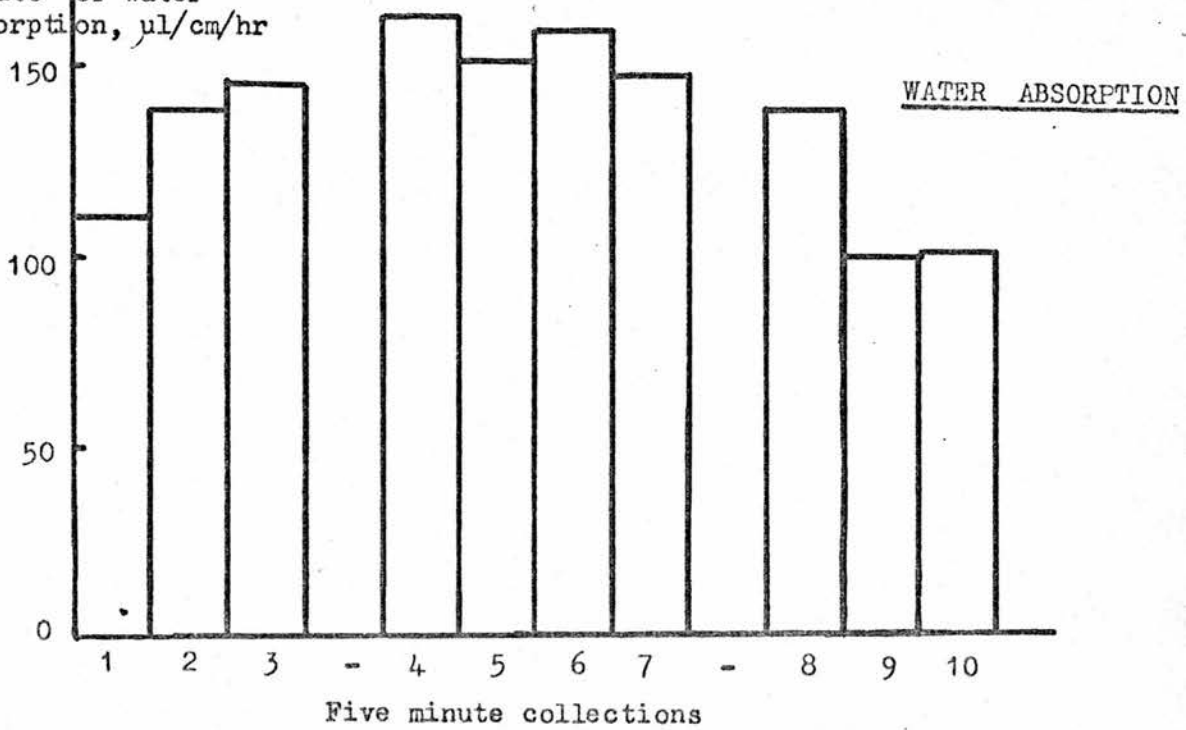
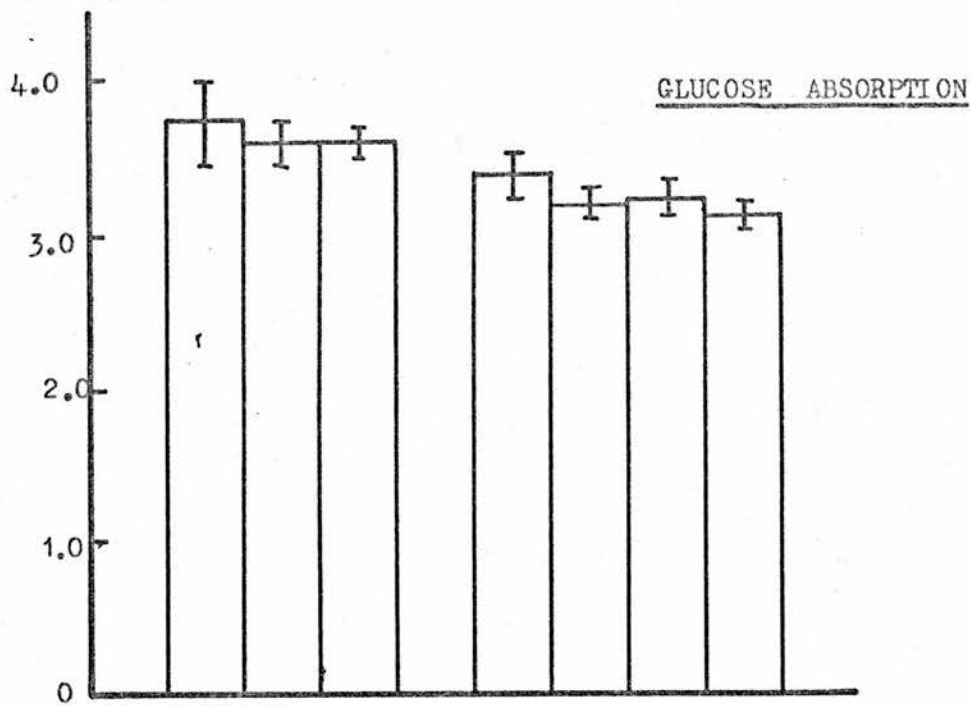


FIGURE 74

The effect of total replacement of sodium in arterial infusate by chol

(Mean of six experiments)

Rate of glucose
absorption, mg/cm/hr



Rate of water
absorption, μ l/cm/hr

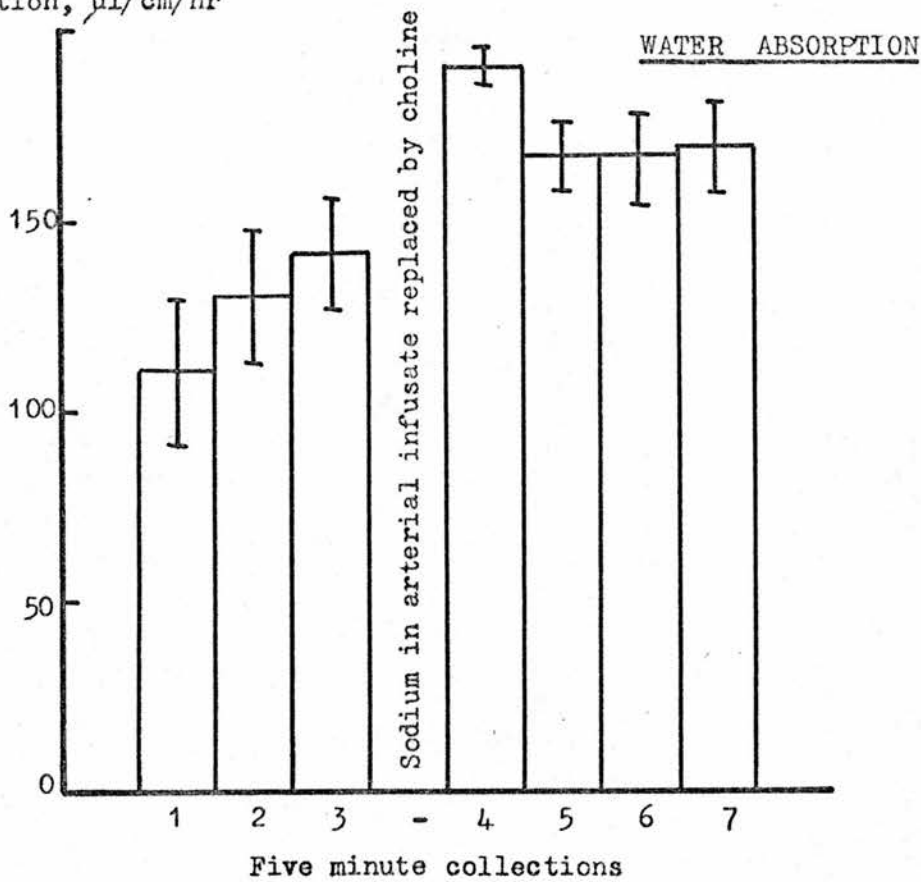


FIGURE 75

Effect of total replacement of sodium in arterial infusate by choline

(Mean of 3 experiments - the vertical bars show the S.E.M.)

sodium concentration was maintained constant at 144 mEq/litre by means of arterial infusion.

The results are shown in Figure 73, and clearly the rate of glucose absorption from the lumen again fell only gradually. However, it fell less rapidly than in the earlier experiments where the composition of the tissue fluid was not maintained.

Therefore it is unlikely that a reduction in the luminal sodium concentration produces its effect on glucose absorption by lowering the tissue fluid concentration of sodium per se.

Effect of total removal of sodium from the arterial infusate

Although the experiments just described have shown that depletion of luminal sodium can seriously inhibit glucose absorption even when the level of sodium in the tissue fluid was maintained, the possibility that depletion of the latter might affect glucose absorption has not yet been excluded.

Therefore, in six experiments, the normal arterial infusate was replaced by one containing choline salts in lieu of the sodium chloride and bicarbonate, while the lumen was perfused with a normal medium containing sodium (144 mEq/litre).

The results are shown in Figure 74, and show that there was no significant effect attributable to the removal of the sodium from the arterial infusate. Unfortunately there was a progressive falling off in activity throughout the whole experiment. More recently, and since more experience in the setting-up of the preparation had been gained, three further experiments were made. The results (Figure 75) confirm that total removal of the sodium from the arterial infusate, and hence from the tissue fluid, itself did not affect water absorption, and that any effect on glucose absorption was trivial.

Rate of glucose absorption, % of mean control

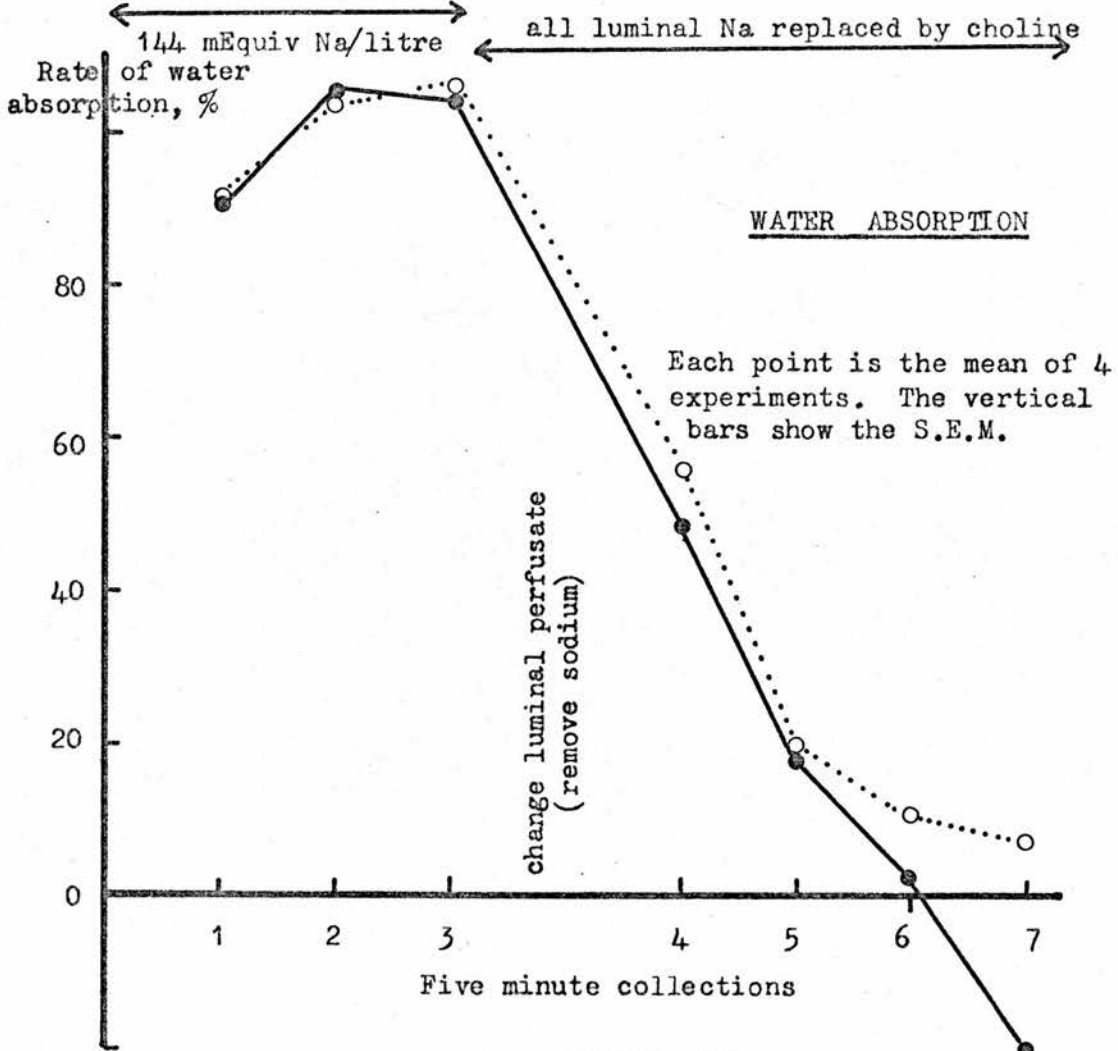
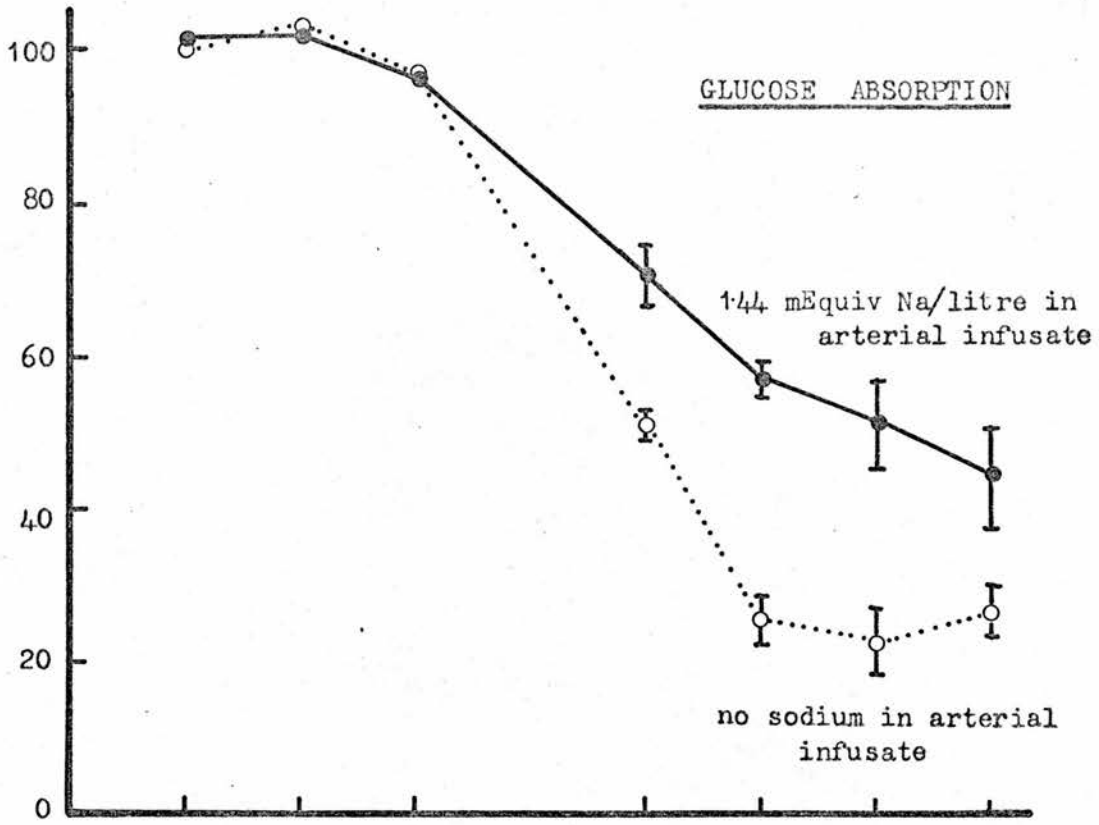


FIGURE 76

Effect of total replacement of luminal Na by choline, when arterial infusate contains either normal (●) or zero (○) Na.

Effect of total removal of both luminal and arterial infusate sodium

It has already been remarked that the rate of glucose absorption fell more rapidly in the earlier experiments with the unmodified preparation than in the recent ones with the preparation with arterial infusion. This might be a consequence of the maintenance of the sodium level in the tissue fluid by the arterial infusion.

Therefore the experiments on total removal of the luminal sodium were repeated, but with no sodium in the arterial infusate. All the sodium in the infusate was replaced by choline.

The results are shown in Figure 76 from which it is seen that the rate of glucose absorption fell significantly more rapidly when sodium was absent from the tissue fluid. All rates have been expressed relative to the mean 'control' rate during the first 3 collections for each intestine. Each collection lasted five minutes. In contrast, the rate of water absorption fell equally rapidly in each instance.

The possibility was mentioned in Chapter VIII that sodium might reflux from the tissue fluid into the lumen and so support 'co-transport' of sugar, even when a sodium-free perfusate was being pumped into the lumen. This might account for the delay in the attainment of a steady-state rate of glucose absorption when the sodium concentration in the perfusate was abruptly changed. However, in the absence of sodium from the tissue fluid the rate of glucose absorption still fell gradually in response to removal of the luminal sodium although more rapidly than when the arterial infusate contained sodium. Presumably the intracellular sodium concentration fell gradually in these experiments and some of the intracellular sodium ions would be discharged into the lumen. The lag in attainment of a steady-state of glucose absorption might be

(Na) in luminal
effluent, mEquiv/litre

- No Na in arterial infusate
- 144 mEquiv/litre Na in arterial infusate
- ▽—▽ unmodified preparation

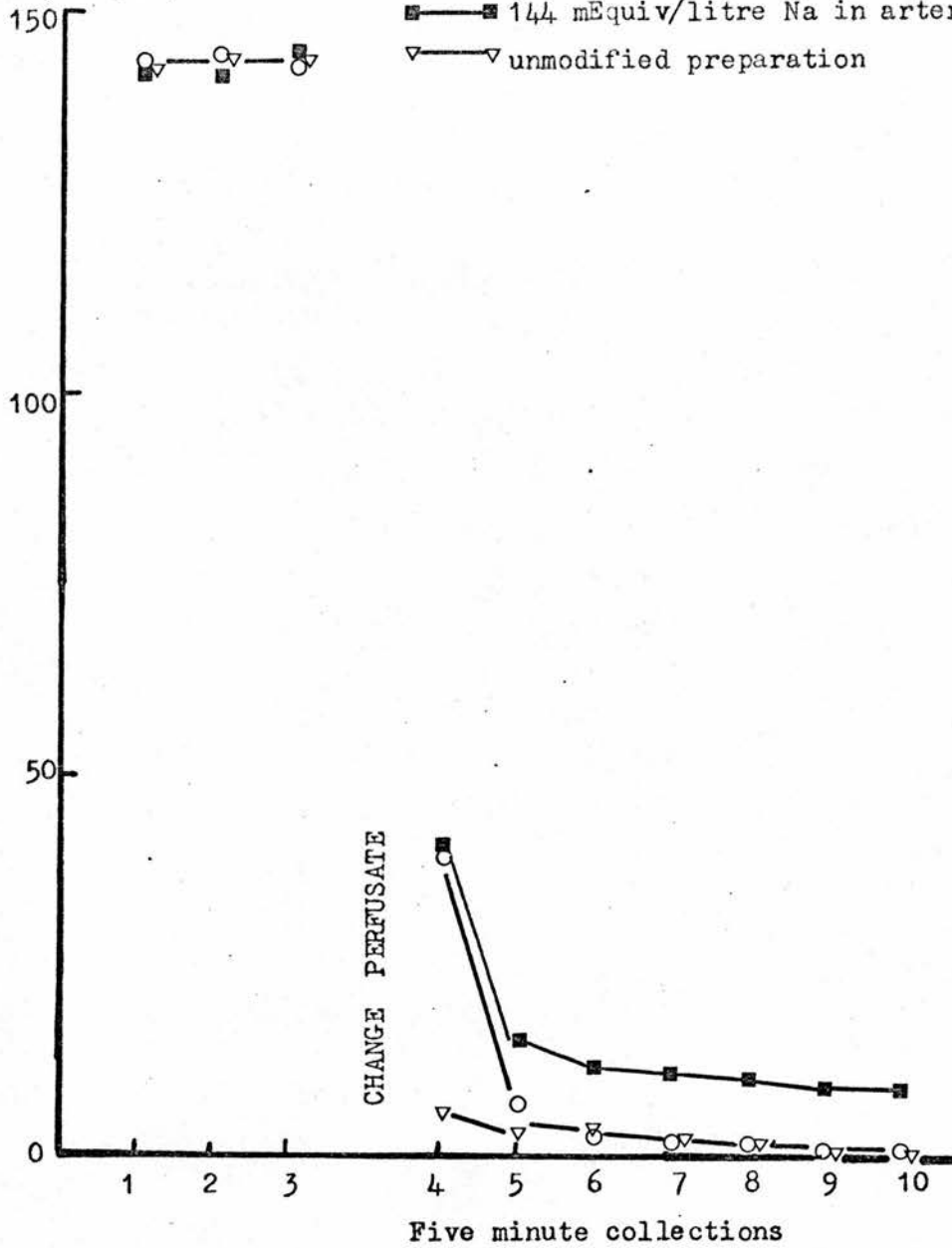


FIGURE 77

Sodium concentration in luminal effluent following replacement of a normal perfusate by a sodium-free perfusate

due to the gradual fall either in intracellular sodium level or in the luminal concentration of sodium ions which had originated in the intracellular water.

Therefore measurements were made of the sodium concentration in the luminal effluent in three types of experiment where the luminal sodium was removed. (A) unmodified preparation without arterial infusion, (B) with arterial infusion at normal (Na), (C) with arterial infusion at zero (Na). Figure 77 shows the results.

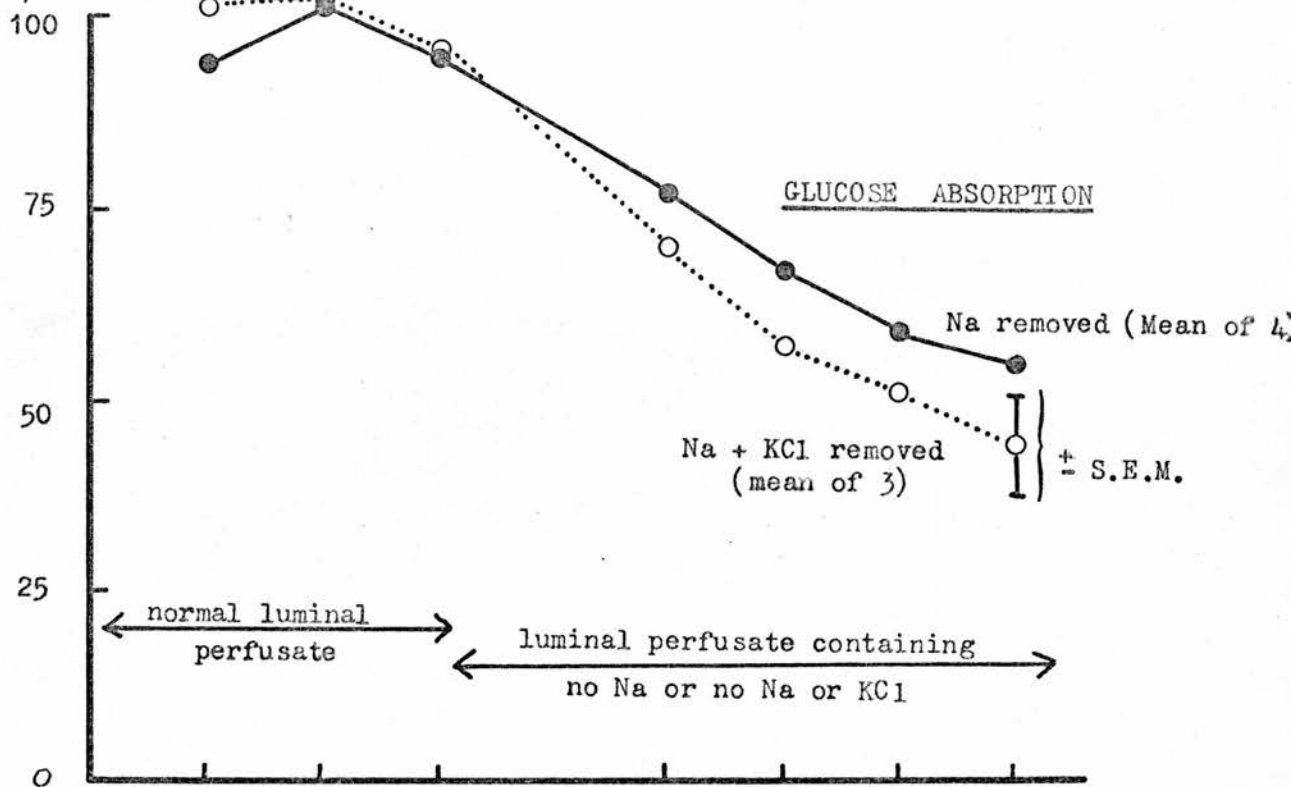
Clearly the luminal sodium concentration does not change as rapidly when the perfusate is abruptly changed as might be expected from corresponding results on the glucose concentration (Figure 24). Even when the arterial infusate contained no sodium there appeared to be back-flux of sodium into the lumen, presumably from the intracellular water (or from a part of the extracellular water to which the arterial infusate does not have access).

Effect of removal of luminal sodium AND potassium

Newey, Rampone & Smyth (1970) reported that the absorption of methionine from sodium-free media depended in some way on the potassium concentration in the media. There are several contradictory reports on the effects of potassium on sugar absorption. Riklis & Quastel (1958) found that an increase in the mucosal potassium concentration stimulated glucose absorption by guinea pig intestine, but Bihler & Crane (1962) noted the opposite effect in hamster intestine.

Sodium and potassium ions may act in antagonism on enzyme systems (e.g. see Dixon & Webb, 1964, page 424). Hence it may be held that a normal Na:K ratio is more important than the actual concentrations of these ions. The Na:K ratio in the normal perfusate is about

Rate of glucose absorption,
% of mean control.



Rate of water absorption, % of mean control

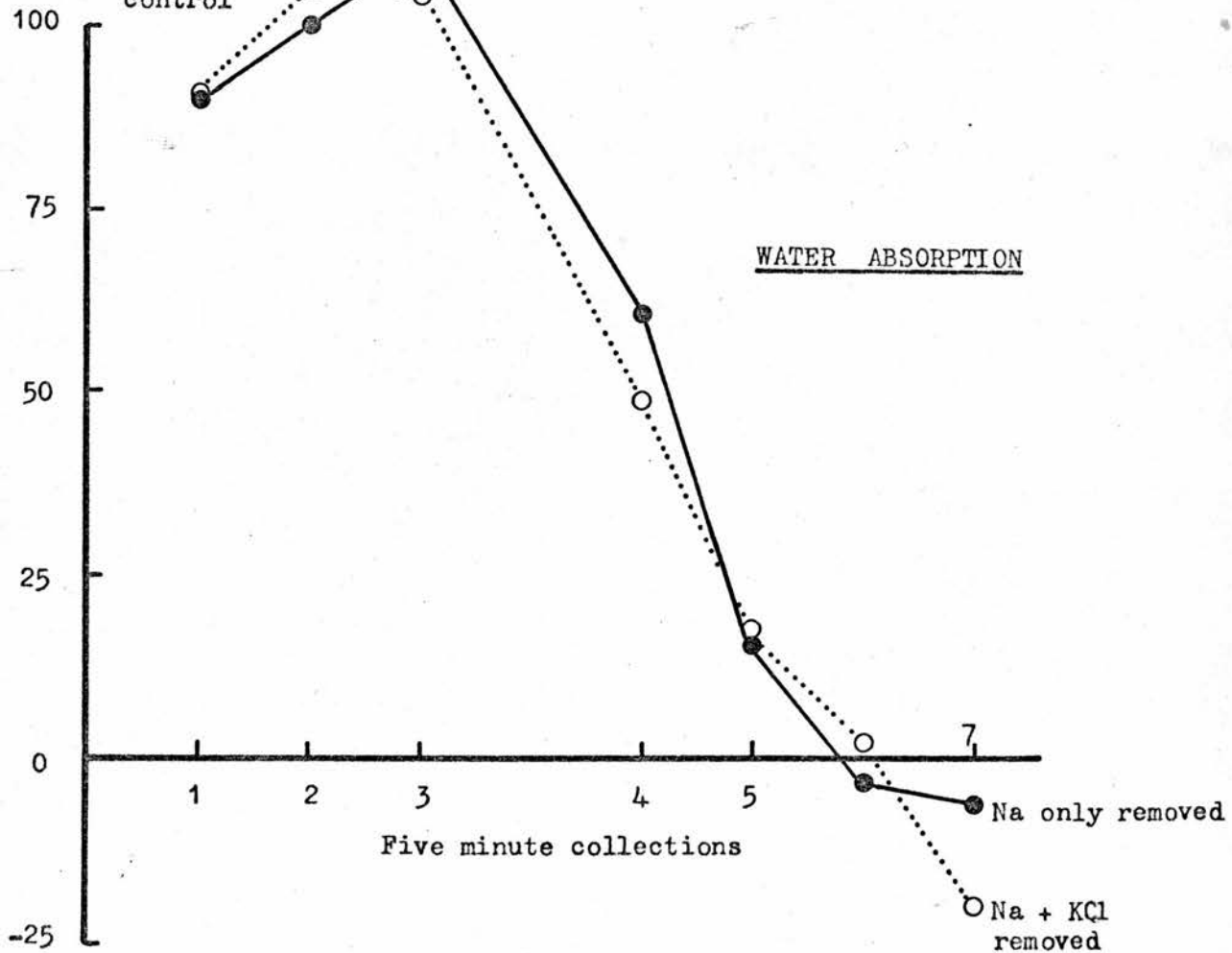


FIGURE 78

Effect of removal of all the sodium and the potassium chloride from luminal perfusate. Data for removal of sodium only are also shown.

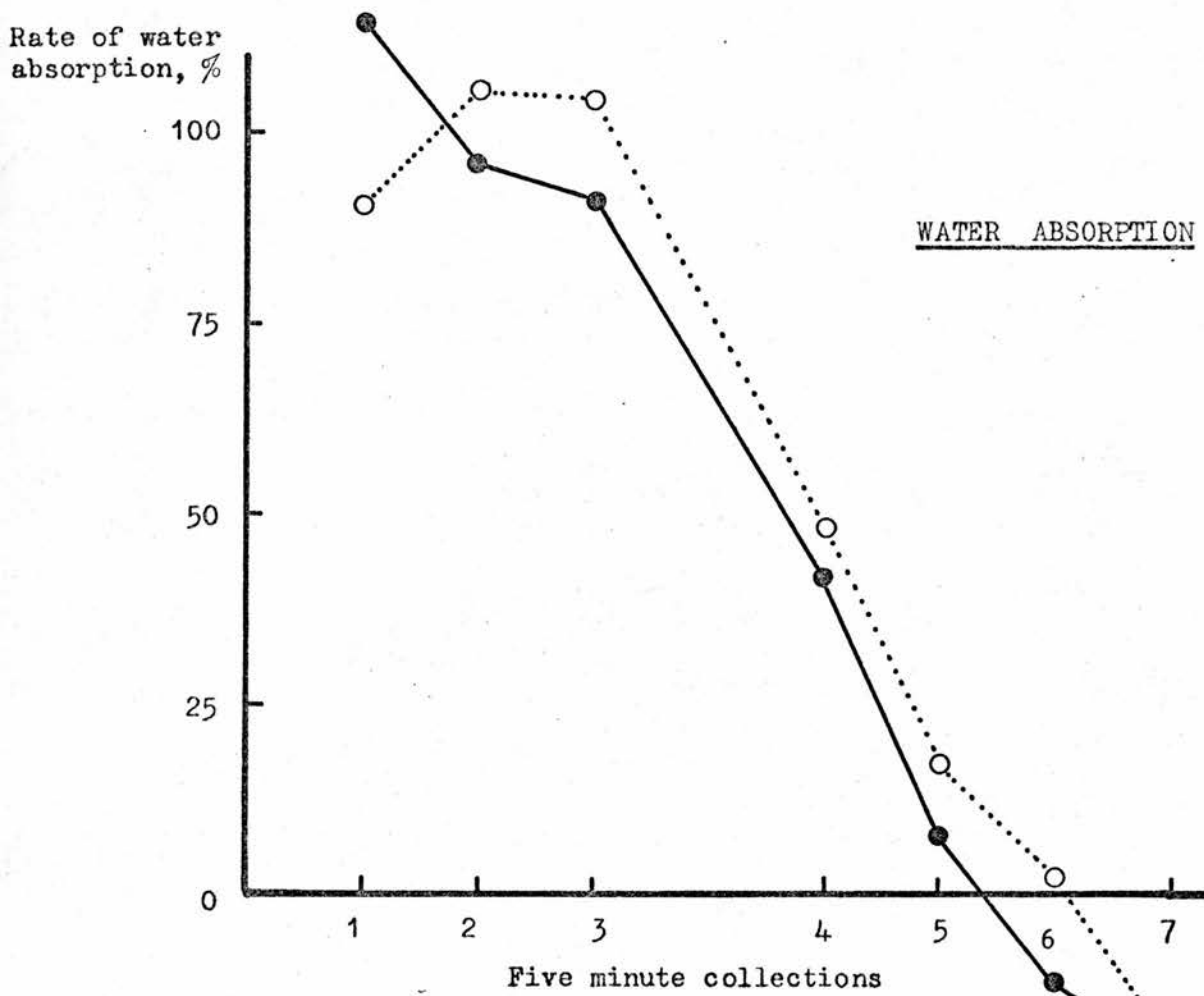
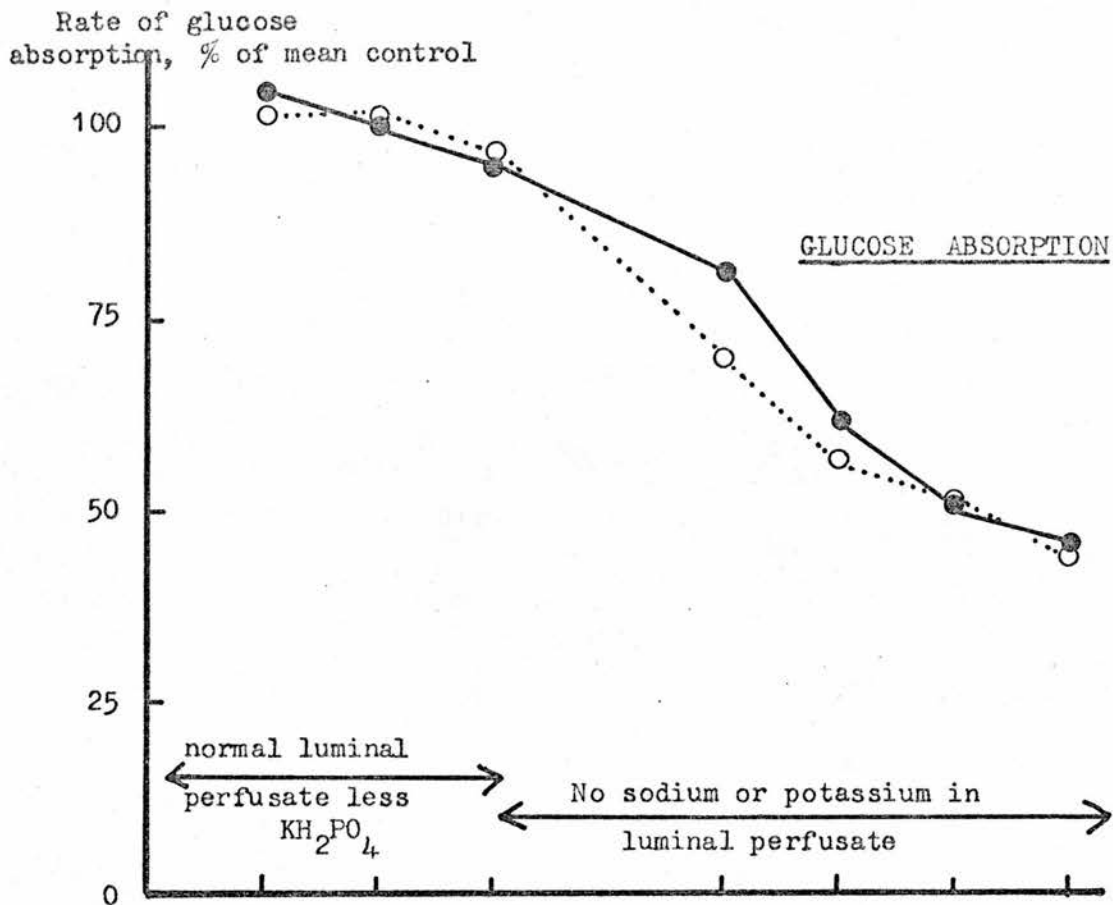


FIGURE 79

Effect of removal of all the sodium and all the potassium from the luminal perfusate (open circles). Data for removal of sodium only are also shown (closed circles - mean of 4).

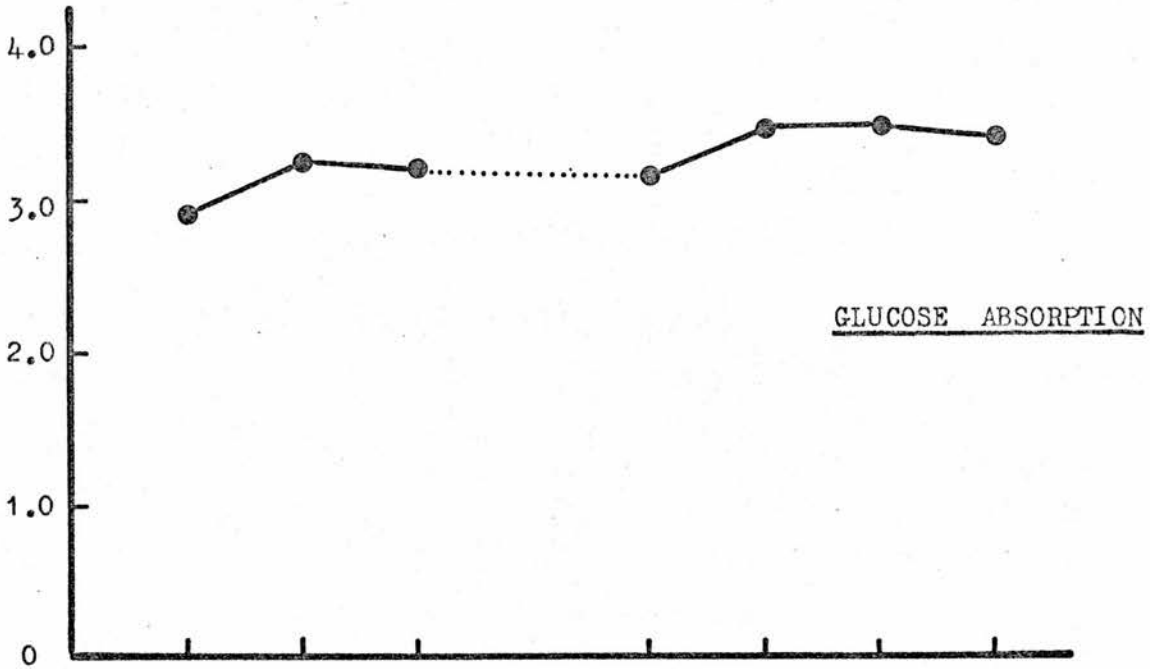
24:1, and when the sodium is totally replaced by choline, this ratio becomes about 1:155.

In order to test the possibility that it is this disturbance of Na:K balance which causes inhibition of glucose absorption, experiments were made in which the normal luminal perfusate was replaced by one which lacked both potassium chloride and all the sodium. The remaining potassium ion (present as KH_2PO_4) concentration was 1.18 mEq/litre. The results are summarised in Figure 78. The effects of removing all Na and most of the K are indistinguishable quantitatively from those of removal of all the Na alone.

However, the Na:K ratio, at about 1:31, is still highly abnormal. Therefore two experiments were made in which all the sodium and all the potassium were absent from the second luminal perfusate. As no choline phosphate was to hand, the KH_2PO_4 was omitted from both the control and the test media. In the control perfusate the Na:K ratio was approximately 30:1, while both ions were virtually absent from the test perfusate. The results are in Figure 79, and again the rates of glucose and water absorption fell at the same speed as when sodium only was removed from the perfusate. Hence it is unlikely that the effects of sodium removal on glucose and water absorption are related to a disturbance from the normal Na:K ratio in the lumen. Effects arising from a shift in the intracellular Na:K ratio cannot be excluded on this evidence.

It is noteworthy that glucose absorption was more or less normal during the control periods when the luminal perfusate contained no potassium phosphate. This merits further attention, since if the KH_2PO_4 is unnecessary for the stability or activity of the preparation this salt could be omitted routinely in future studies. It is desirable to simplify perfusion media and eliminate as many of the variable factors as possible.

Rate of glucose absorption, mg/cm/hr



Rate of water absorption, μ l/cm/hr

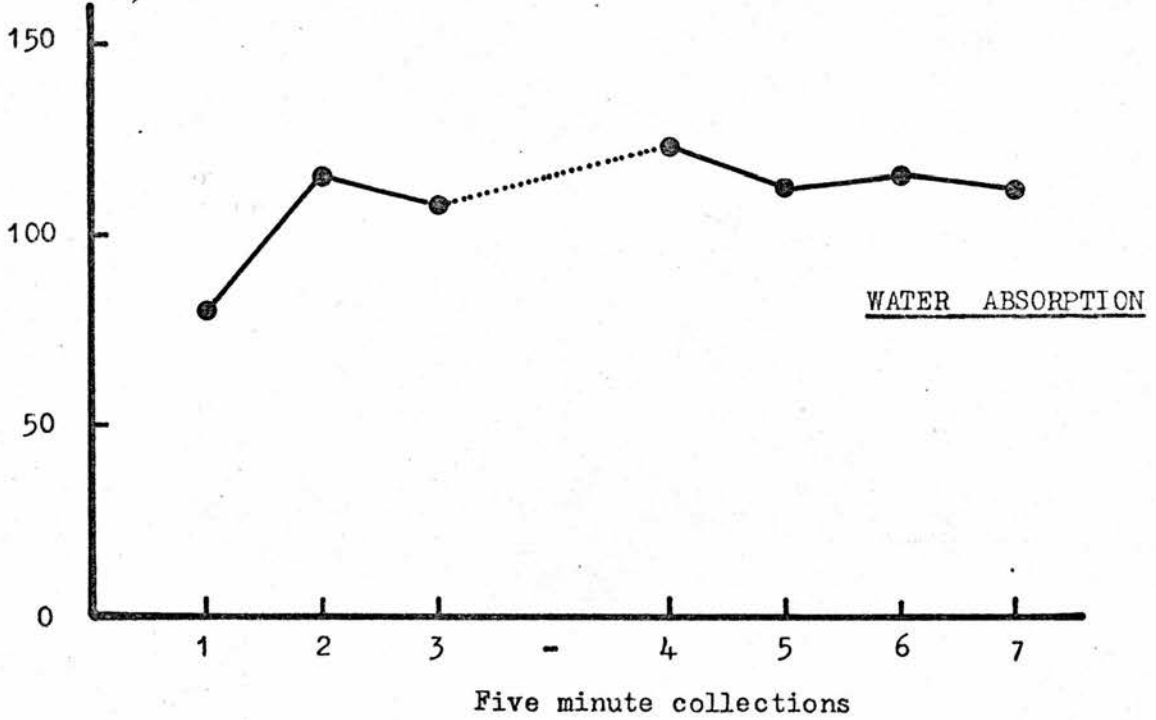


FIGURE 80

Effect of replacement of potassium chloride in arterial infusate by choline chloride

(Mean of two experiments)

Effect of partial removal of potassium from the arterial infusate

It was shown above that total removal of the sodium from the arterial infusate, and hence from the tissue fluid, has little or no effect on glucose absorption. However, the converse result might be expected if the potassium were removed from the tissue fluid, in view of the Na-K-activated-ATPase system, which is believed to be located at the serosal pole of mucosal cells and to require external potassium ions (Stein, 1967, page 228). Removal of the external (tissue fluid) potassium might inhibit the active efflux of sodium ions from the mucosal cells, as is the case for red blood cells (Garrahan & Glynn, 1966). Thus, removal of the external potassium can have similar effects to the application of external cardiac glycosides. An increase in the intracellular sodium concentration, and a subsequent decrease in the glucose absorption rate might be predicted. Such effects of ouabain have been documented quite well - see the next Chapter.

Figure 80 shows the results from two experiments in which all the potassium chloride in the arterial infusate was replaced by choline chloride. No inhibition at all was found. Indeed, in one experiment there appeared to be a slight stimulation of glucose absorption when the potassium concentration in the tissue fluid was reduced to some 1.18 mEq/litre (KH_2PO_4 only). Further experiments, preferably with total removal of the potassium from the infusate are desirable. In the absence of external potassium, Na-Na 'exchange diffusion' may occur, and it is possible that the stoichiometry is such that net efflux of sodium ions might still continue. This could be tested by the total removal of both sodium and potassium ions from the arterial infusate.

Resorption of glucose from the tissue fluid into the lumen

Because it seemed unlikely that the intestine *in vivo* receives its nutrient from the lumen rather than from the blood stream, experiments were made to test whether glucose passed into the intestinal lumen from an arterial infusate. It was hoped to extend the brief report of Duerdoth, Newey, Sanford & Smyth (1964) who had found that glucose, mannose, or fructose present in the serosal fluid inside everted sacs could be metabolised. These authors were using very high concentrations of these sugars (222 mM) to stimulate net water transfer from mucosa to serosa. They had noted further that this metabolism was dependent on the concentration of sodium in the mucosal fluid.

Experiments were made in which the intestinal lumen was perfused with a glucose-free medium, and the arterial infusate contained glucose (10 mg/ml, 55.5 mM). Glucose was subsequently recovered from the lumen effluent as well as from the vascular effluent. The mean rate of appearance of glucose in the lumen was 1.01 ± 0.25 (5) mg/cm/hr in the first five minutes after the preparation had been set up. It fell somewhat in the next 20 minutes. However, the apparent 'resorption' was probably caused by a leak in the mucosa since raffinose at a concentration of about 0.9 mg/ml appeared in the lumen effluent. This corresponds to a raffinose resorption rate of about 2 mg/cm/hr which has never been recorded when the luminal perfusate contained glucose. Consequently, the apparent rate of water absorption observed in these experiments (169 ± 27.9 (5) μ l/cm/hr) was a gross overestimate.

An experiment with phloridzin (1 mg/ml) in the luminal perfusate suggested that some glucose absorption from the lumen was occurring in these experiments: the rate of appearance of glucose in the lumen

Glucose concentration
in vascular effluent, mg/ml

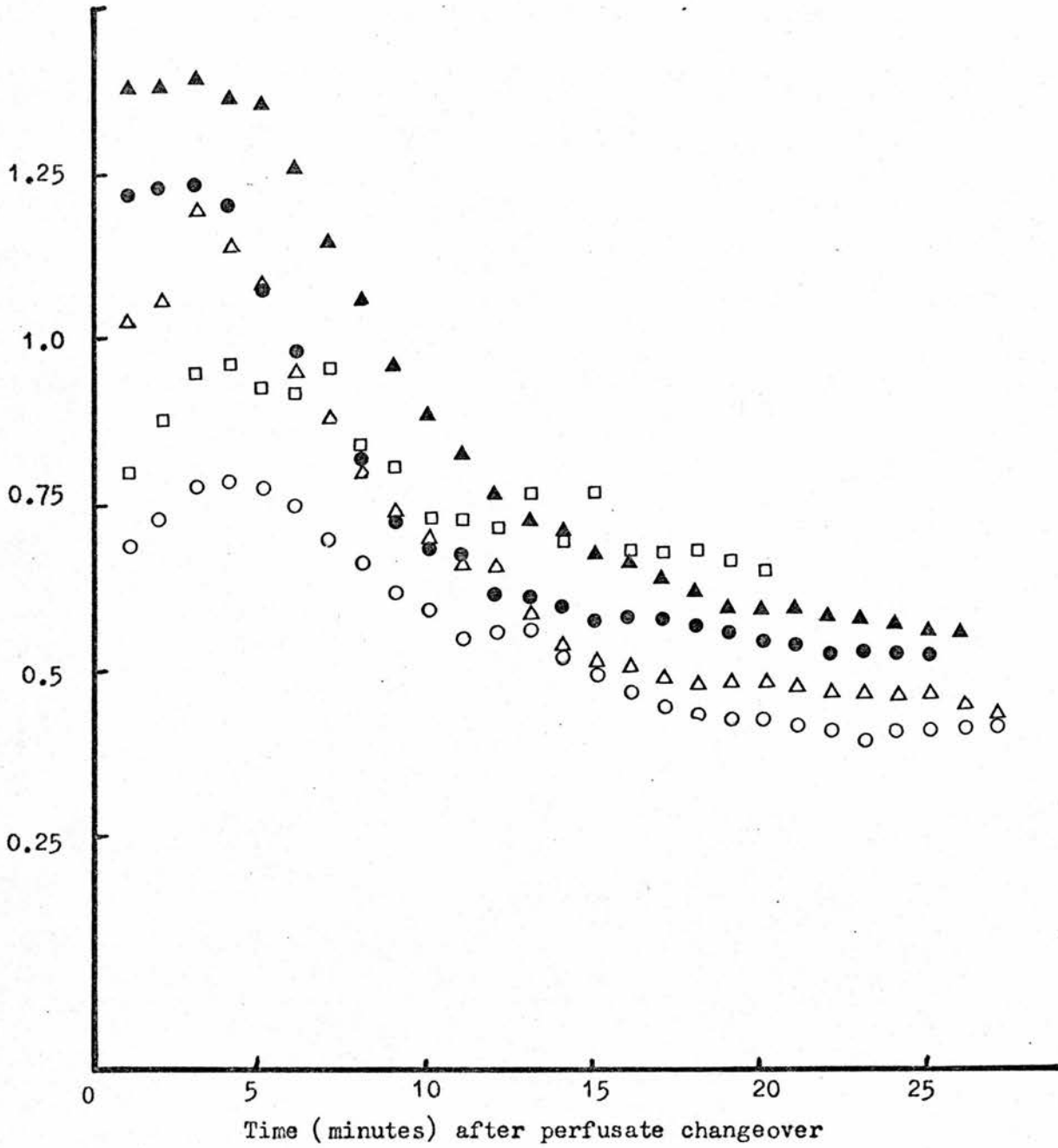


FIGURE 81

The rate of change in glucose concentration in the vascular effluent following a step change in luminal glucose concentration from 5 mg/ml to 1.5 mg/ml in preparations with vascular perfusion (open symbols) and with arterial infusion (closed symbols)

(1.01 mg/cm/hr) was in fact a net rate even although the lumen concentration of glucose was only about 0.6 mg/ml. When phloridzin was introduced into the lumen, the rate of appearance of glucose in the lumen effluent increased immediately by about 80%.

As a result of the leak of raffinose into the lumen, these experiments have been temporarily abandoned. In those already performed no evidence was obtained to show that lack of luminal sodium affected the loss of glucose infused into the tissue space.

A comparison between this preparation, and one with vascular perfusion

It has already been suggested that this novel technique of arterial infusion may possess advantages over conventional techniques of vascular perfusion. The distribution through the vasculature of a vascular perfusate is unknown and is subject to variation, both within and between experiments, owing to arterio-venous anastomoses: this might not be so for an arterial infusate which cannot escape via the venous system.

In an attempt to compare these two types of preparations, the kinetics of the change in glucose concentration in the vascular effluent were followed subsequent to a change in the luminal concentration of glucose.

Two infusion experiments were set up as usual - i.e. the superior mesenteric veins were ligated. Also, three vascular perfusion experiments were set up by a similar technique, except that the veins were cut, but not ligated. The concentration of glucose in the luminal perfusate was abruptly changed from 5 mg/ml to 1.5 mg/ml and collections of the vascular effluent were taken at one minute intervals.

The results are shown in Figure 81. No distinction between the two types of preparation can be seen. In both perfusion and

infusion experiments, the new steady-state in the vascular effluent was reached in some 10-12 minutes after the perfusate change-over (cf. 60-80 minutes for the unmodified preparation - see Chapter VI).

DISCUSSION

The tests on the new preparation have established that, in general, it fulfils the requirements. The stability of both water and glucose absorption is excellent over one hour, and this period is ample for most investigations. The rate of glucose absorption from a whole jejunum plus ileum (approx. 100 cm) was substantially greater than would be predicted for this length of intestine in the unmodified preparation without arterial infusion. The mean absorption rate of glucose for a 40 cm upper segment of small intestine in the unmodified preparation was 3.1 mg/cm/hr from a perfusate containing 5 mg glucose/ml (Chapter V). Using Fisher & Parsons'(1950) formula for the linear gradient of glucose absorptive activity down the length of the intestine, one can predict a mean rate of about 1.9 mg/cm/hr for a 100 cm segment. The relatively high absorption rate from the new preparation may be accounted for, at least in part, by the reduction in the glucose concentration in the intestinal tissue fluid. In this preparation the glucose level in the tissue fluid appears to be almost normal, whereas in the unmodified preparation exceptionally high values were recorded (approx. 13 mg/ml - see Chapter VI). Parsons & Pritchard (1968) showed that the rate of glucose absorption from amphibian intestine was proportional to the rate of vascular perfusion, and hence was inversely related to the glucose concentration in the vascular effluent.

The absorption rates in this preparation show much variability between animals. However, the stability is so good that each

intestine can again serve as its own control.

Contrary to expectation, no evidence was found to suggest that this preparation of arterial infusion is superior to ones with vascular perfusion. The data of Figure 81 show that, when the glucose concentration in the luminal perfusate was changed, the steady-state in the vascular effluent was attained as rapidly in a perfused preparation as in the one with arterial infusion. Thus, the occlusion of the superior mesenteric vein did not appear to increase the rate of exchange between the infused media and the tissue fluid.

Parsons & Pritchard (1968) found in their preparation of frog intestine with vascular perfusion that most of the perfusate escaped via the lymphatics rather than via the venous system when the intraluminal distension pressure exceeded some 15 cm of water. Perhaps, then, a perfused preparation with a high intraluminal distension pressure approximates to a preparation with arterial infusion.

While the measure of tying off the superior mesenteric vein may not have contributed materially to the success of this preparation, there is no doubt that it has been more successful than many previous attempts at vascular perfusion. For example, Windmueller, Spaeth & Ganote (1970) commented unfavourably on earlier work, and devised a means of sustaining absorption of glucose and water from perfused intestine (rat) over several hours. However, they claimed that the presence of a glucocorticoid and noradrenalin was obligatory. As judged from these authors' account, their intestine was subjected to severe hypoxia throughout the period of study, and this would account for the high degree of motility observed. Probably the present preparation with arterial infusion owes much of its success to the efficiency of oxygenation achieved via the segmented flow (see Chapter V).

Although the exact space to which the arterial infusate has access has not been defined, it seems that one now has direct access to both sides of a single layer of viable epithelial cells. The compositions of the fluids bathing both sides can be independently varied and determined. As was noted in the first paragraph of this Discussion, the composition of the sub-mucosal tissue fluid can be kept at approximately a physiological norm: this was not possible in the unmodified preparation.

Direct access to both sides of the epithelial layer makes possible precise studies of both transport and metabolism at a steady-state. Steady-states on both sides can be attained rapidly and confirmed. Conversely in the unmodified preparation the serosal steady state was attained only after more than one hour - see Chapter VI.

As with the unmodified preparation serial measurements over short periods are possible. Some of the studies already made have confirmed the value of following the kinetics of the intestine's response to an abrupt change in the conditions - for instance, the change in glucose absorption rate following a step change in luminal glucose or sodium concentration.

Again the segmented flow technique limits the new method to intestines of fairly small internal diameter. The time taken for the setting-up of the preparation and the difficulty in cannulation of the superior mesenteric artery could be construed as minor disadvantages. The main disadvantage of the new preparation is the inability to measure water absorption rates directly without recourse to a marker - raffinose in the present work. It is not always possible to verify the assumptions on which the use of the marker depends (see Chapter V) and small errors in the estimation of marker

can lead to relatively large errors in the measured rate of water absorption. A slow rate of infusion favours accurate measurement since the difference of marker concentrations between infusate and effluent is increased.

The experiments on sodium depletion showed that removal of the luminal sodium inhibited glucose absorption even when the tissue fluid level of sodium was maintained from a 'normal' arterial infusate. Also, removal of only tissue fluid sodium had little or no effect on glucose or water absorption. However, the level of sodium in the tissue fluid did affect significantly the rate at which removal of luminal sodium inhibited glucose absorption (Figure 76). A similar effect is seen in the data of Parsons & Pritchard (1971, their table 2). These authors did not give the kinetics of the effect of sodium depletion, and they did not attach any special significance to their finding which was in connection with studies on disaccharide hydrolysis and transport. When sodium was absent from the tissue fluid glucose absorption fell more rapidly in response to removal of the luminal sodium than when sodium was present in the tissue fluid. However the sodium level in the tissue fluid probably did not affect the steady-state rate of glucose absorption. This suggests that the intracellular sodium level is of importance for normal glucose absorption. When there is little or no sodium in the tissue fluid one can expect the intracellular sodium level to fall more rapidly than is normally the case: hence, glucose absorption falls more rapidly. Crane's (1962, 1965) hypothesis of 'gradient-coupling' only involves the intracellular concentration of sodium in as far as the transmembrane gradient of sodium concentration depends on both intracellular and luminal sodium concentrations. In this case, however,

a fall in intracellular sodium level would lead to an increase in the downhill gradient into the cell. Hence, a gradual increase in the rate of glucose absorption after an immediate sudden decrease would be predicted to result from sudden removal of the luminal sodium. Therefore these experimental results are more consistent with Csaky's hypothesis than with Crane's.

CHAPTER XI

THE EFFECT OF OUABAIN ON GLUCOSE ABSORPTION

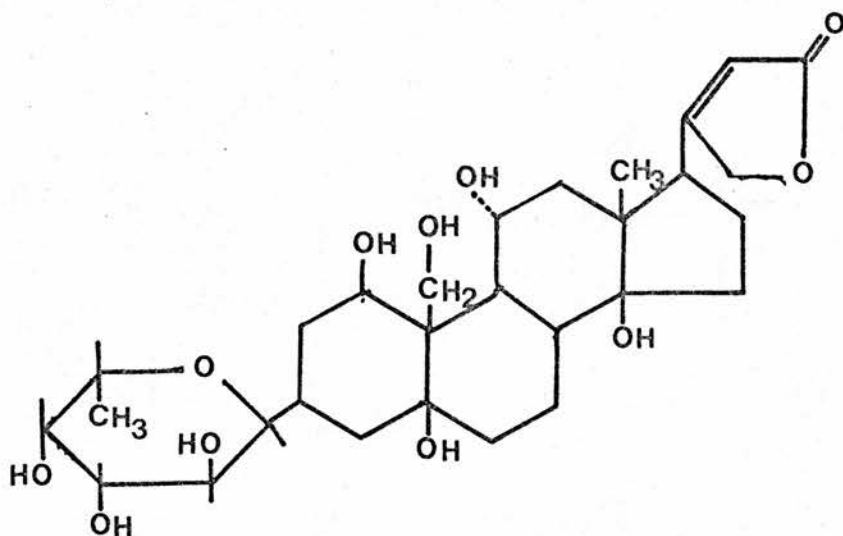


FIGURE 82

Ouabain (Strophanthin-G)

CHAPTER XI THE EFFECT OF OUABAIN ON GLUCOSE ABSORPTIONIntroduction

Inhibitory effects of the cardiac glycosides on active transport mechanisms have been widely documented. In particular, inhibition of sodium and potassium ion transport has been studied in many systems, e.g. red blood cells (Glynn, 1957), skeletal muscle (Conway, Kernan & Zadunaisky, 1961), cardiac muscle (Boyer & Poindexter, 1940), intestinal smooth muscle (Godfraind & Godfraind-de Becker, 1962), nerve axon (Caldwell & Keynes, 1959), eye lens (Kinoshita, Kern & Marola, 1961), frog skin (Koefoed-Johnsen, 1957), kidney (Schatzman, Windhager & Solomon, 1958), gall bladder (Diamond, 1962) and the alga *Nitella* (MacRobbie, 1962).

A large body of evidence now points to an ATP-ase enzyme which is activated by Na and K ions playing a central role in cation transport (see reviews by Glynn, 1964, 1968; Skou, 1965; Heinz, 1967). Comparison of the ATP-ase activity and of active transport in intact red cells and ghosts showed that the two processes shared many common features, with special regard to the kinetics of activation and inhibition. Bonting & Caravaggio (1963) and Glynn (1964) have pointed out that there is a good correlation between the ATP-ase activity in a particular tissue and the amount of pumping going on in that tissue. However, the molecular basis of the 'common or garden' sodium pump (as Glynn has called it) is still not understood fully.

The effects of ouabain (strophanthin-G), whose structure is shown in Figure 82, on intestinal transport have been investigated extensively. Many authors found that active transport of ions and sugars was inhibited by ouabain, but only if the drug was present in the serosal medium; little or no effect was demonstrable when

the drug was present in the luminal medium (Schultz & Zalusky, 1964b; Csaky & Hara, 1965; Parsons & Pritchard, 1968 - and others). Csaky & Hara (1965) gave evidence that this was because ouabain, unlike digitalis, can scarcely cross the mucosa, and they suggested that its site of action was either intracellular, or at the extracellular face of the serosal membrane. The latter is generally assumed to be the site of action, although not on strong evidence. In the giant axon of the squid, Caldwell & Keynes (1959) introduced ouabain directly into the cell and showed that it had no effect. In contrast, external ouabain severely inhibited efflux of sodium ions.

Schultz & Zalusky (1964a) showed that ouabain in the serosal compartment of a flux chamber gradually annihilated the net flux of sodium ions from mucosa to serosa in rabbit ileum. Their measurements of unidirectional flux rates showed that the flux from mucosa to serosa was substantially inhibited, but also that the flux from serosa to mucosa appeared to be slightly stimulated.

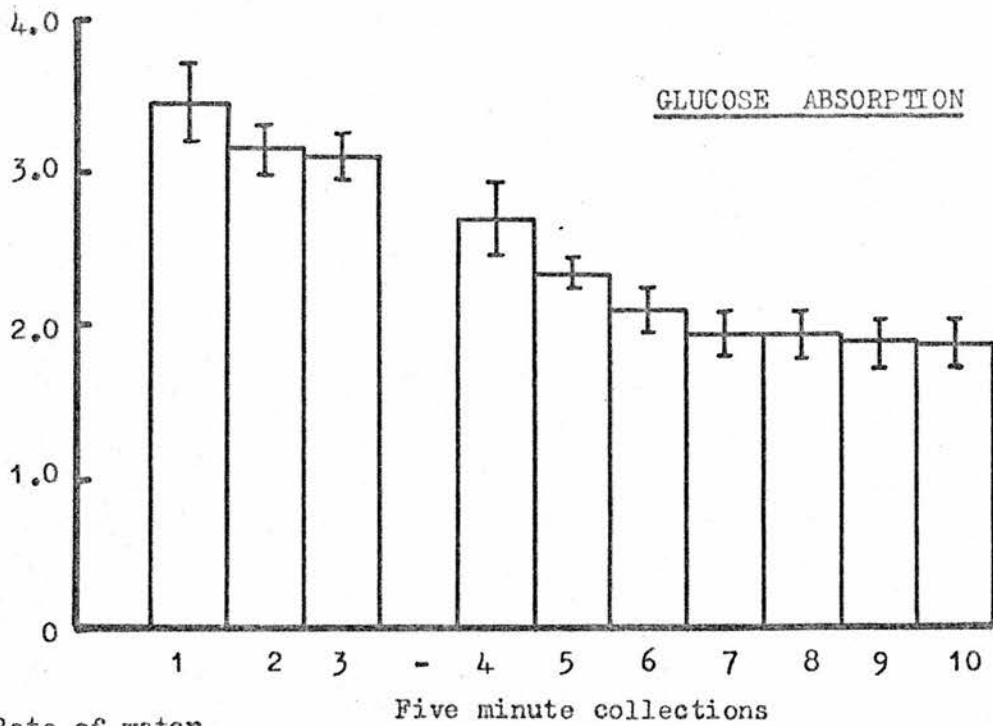
Most of these experiments on the intestine have been made using species other than the rat; there have been several claims that the tissue from the rat is affected little, or not at all, by ouabain (Faust, 1964; Robinson, 1967). Indeed, Robinson (1967, 1970) believes that there may be fundamental differences between the sodium pump mechanisms of different species. Nevertheless, Edmonds & Marriott (1968) found that serosal ouabain decreased the electrical potential difference across rat colon, in an *in vitro* preparation. In the absence of better evidence, it is hard to accept that fundamental differences exist between species. One reason which may have contributed to the failure of some authors to find an effect of ouabain in the rat may lie in the difficulty of ensuring that the drug is in fact in contact with the serosal

pole of the cells. This reasoning is supported by the observations of Csaky & Hara (1965) who estimated that the ouabain concentration in the submucosal fluid of the frog was about an order of magnitude less than that in the serosal medium in their flux chamber, even after some 2 hours.

The inhibitory effects on sugar transport might be related to either the inhibition of the sodium pump per se, or to the subsequent rise in the intracellular sodium level which is bound to occur. Schultz, Fuisz & Curran (1966) estimated that the intracellular concentration of sodium rose from 62 mEq/litre to 152 mEq/litre after exposure of mucosal scrapings of rabbit ileum to ouabain for 30 minutes. The intracellular potassium concentration fell from 143 mEq/litre to 60 mEq/litre. This increase in intracellular sodium level could affect sugar transport, perhaps because the cells' exergonic metabolism was inhibited at abnormal sodium concentrations. Alternatively, sugar transport might decline because the gradient of sodium concentration across the luminal membrane was reduced and ultimately abolished. The latter suggestion, embodied in the Crane (1965) hypothesis, has already been doubted. The evidence in the preceding Chapter suggested that the maintenance of intracellular sodium concentration itself might be important for glucose absorption.

This preparation with arterial infusion is probably ideally suited to studies on ouabain inhibition, especially since it seems that the arterial infusate has direct access to the sub-epithelial tissue fluid. Further, the stability is excellent, and control measurements can be made on each intestine before exposure to the inhibitor. In this Chapter the kinetics of ouabain inhibition of glucose absorption are studied. Experiments were made under a variety of conditions which might be predicted to affect any changes

Rate of glucose
absorption, mg/cm/hr



Rate of water
absorption, μ l/cm/hr

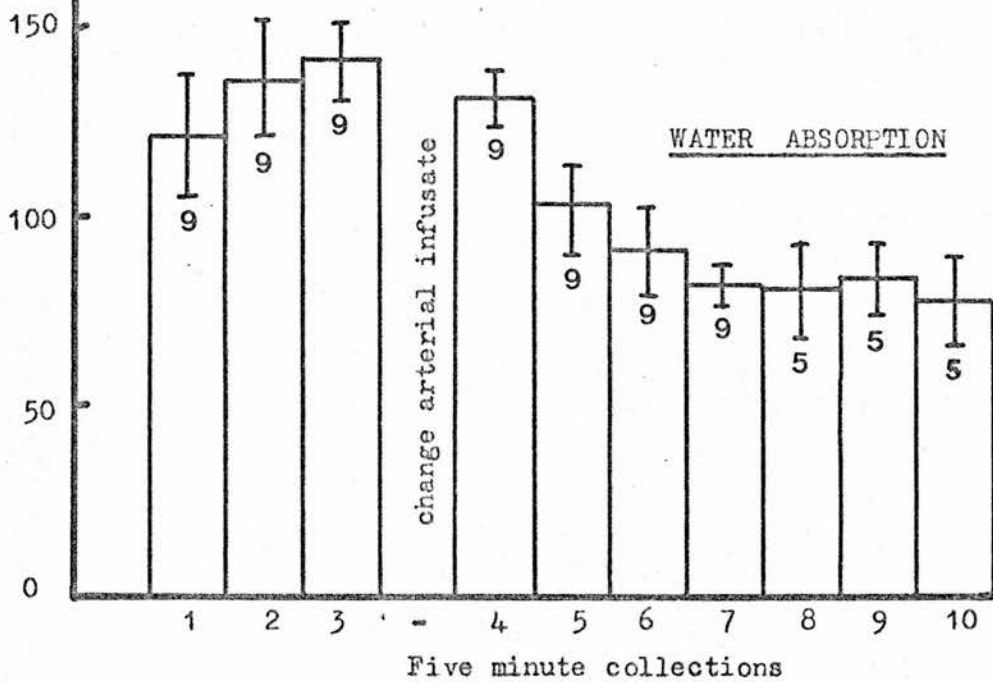


FIGURE 83

Effect of ouabain (10^{-4} M) in the arterial infusate

The ouabain was introduced after three 'control' measurements had been made. The sodium ion concentration was 144 mEq/litre in the luminal perfusate and in the arterial infusate. The vertical bars show the S.E.M. The number of experiments is shown within the blocks for water absorption.

in the intracellular concentration of sodium. Finally, an attempt was made to maintain an approximately normal concentration of sodium within the mucosal cells, while all sodium was removed from the luminal medium.

The kinetics of ouabain inhibition

After the first 15 minutes the normal arterial infusate was replaced by one which contained ouabain (72 mg/litre, $10^{-4}M$). The ouabain was supplied by the Sigma London Chemical Co. Ltd. Figure 83 shows estimates of water and glucose absorption rates measured over consecutive 5 minute periods. The onset of inhibition was gradual, but an apparently steady state was reached within about half an hour. This is substantially more rapid than in most other preparations; for example, the effect of $10^{-5}M$ ouabain was not noticeable for at least 40 minutes in Csaky & Hara's (1965) flux chamber experiment on frog intestine, and a steady-state had not been reached some 2 to 3 hours later. The gradual onset of inhibition in the present preparation is unlikely to be due to a delay in the ouabain reaching the extracellular face of the mucosal cells; it might be consistent with an intracellular site of drug action.

The steady-state rates of absorption are approximately 50% of the control rates in the absence of ouabain.

Effect of luminal sodium concentration on ouabain inhibition

It is well known that ouabain can cause a marked increase in the overall intracellular concentration of sodium (e.g. Schultz, Fuisz & Curran, 1966). One explanation is that the active extrusion of sodium is retarded by the ouabain inhibition of a sodium pump at the serosal pole of the mucosal cells, while Na ions continue to enter from the intestinal lumen into the cells. The steady-state

Rate of glucose absorption, % of mean control rate

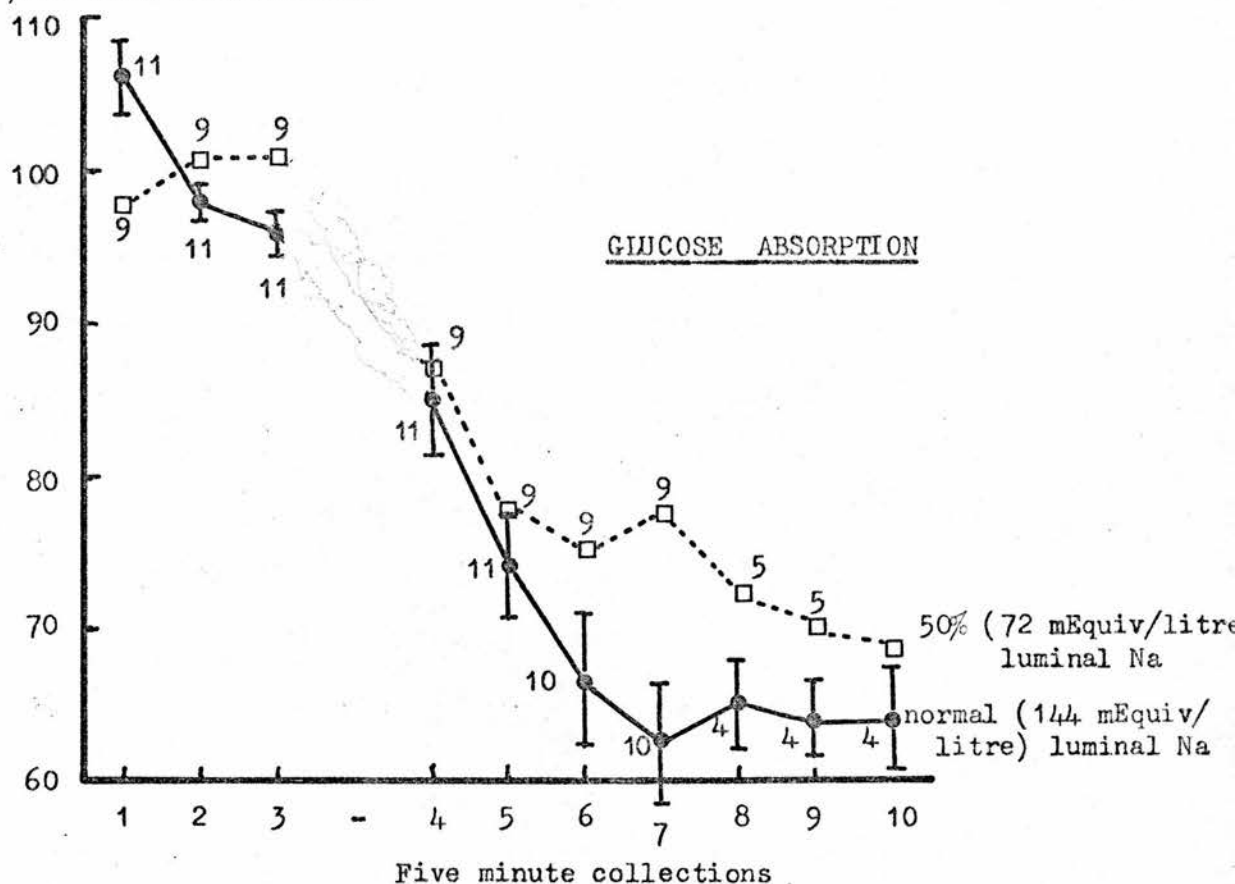


FIGURE 84

Effect of vascular ouabain on glucose absorption at normal and low luminal Na concentrations

The vertical bars show the S.E.M. They have been omitted from the 50% Na line for clarity. The number of experiments is shown beside each point.

would only be attained when intracellular and luminal activities of sodium became equal. If Crane's hypothesis of co-transport were valid, then the Na-dependent glucose absorption would be abolished ultimately when the sodium gradient became zero. If, however, the rate of glucose absorption depends on the intracellular sodium level itself, then the steady-state rate in the presence of ouabain would depend on the luminal sodium concentration. Experiments were made to test this.

It has already been found that the rates of glucose absorption were indistinguishable when the luminal concentration of sodium was 144 mEq/litre and 72 mEq/litre (page 142). The kinetics of ouabain inhibition were therefore studied while the lumen perfusate contained sodium at either 144 mEq/litre or 72 mEq/litre.

Figure 84 shows the results. Each rate of glucose absorption has been expressed as the percentage of the mean control rate for that intestine. (The mean control rate is the mean of 3 measurements over consecutive periods of five minutes made before ouabain ($10^{-4}M$) was present in the arterial infusate.) Although there was always slightly more inhibition with 144 mEq/litre luminal Na, the difference between individual points on the two curves was not significant, except in one case ($P < 0.01$ for collection No. 7). Both curves show the rate of glucose absorption becoming fairly steady by collection No. 7 - i.e. after 20 minutes exposure to ouabain.

When all the points for collections Nos. 7-10 are considered together, the difference between the two data sets is highly significant ($P < 0.001$) - i.e. the steady-state rate of glucose absorption is greater with the lower luminal concentration of sodium, under conditions of ouabain inhibition.

However, caution is necessary before this finding is accepted as confirmation of any hypothesis. Firstly, the difference between the steady-state rates of glucose absorption in the two sets of experiments is extremely small. A much larger difference might have been expected if all the assumptions were valid. Secondly, there was no evidence to show that the steady-state concentration of intracellular sodium did indeed depend on the luminal sodium concentration. Thirdly, the rates of absorption in the two sets of experiments differ significantly during the control periods. The reason for this is unknown.

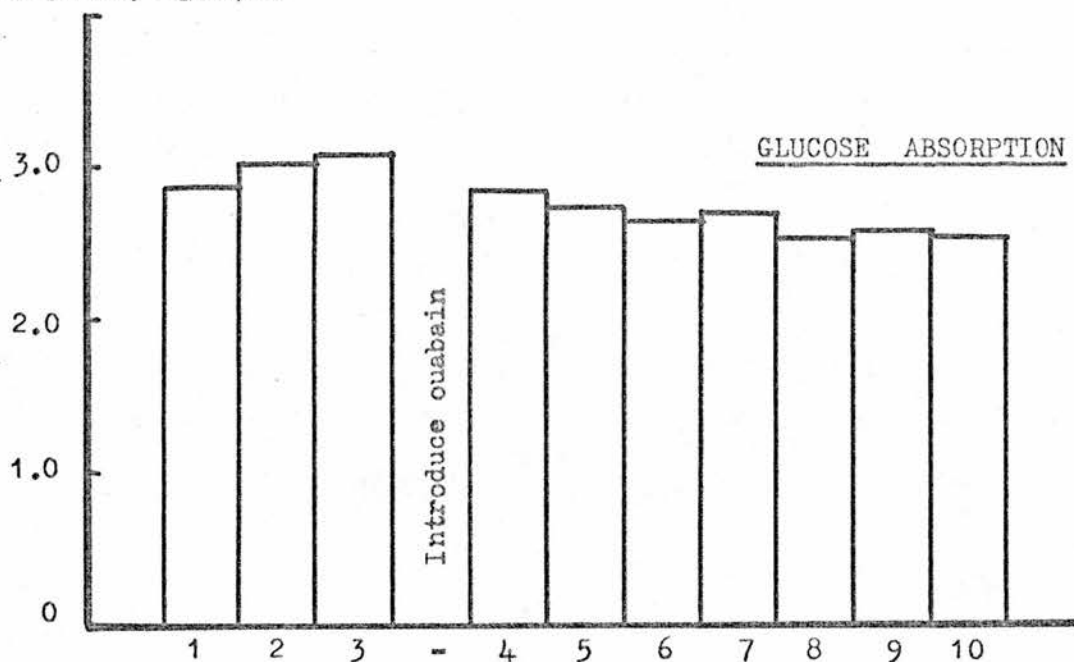
Especially for the latter reason, no conclusions can be drawn from these experiments.

Effect of sodium concentration in arterial infusate on ouabain inhibition

In the last series of experiments no measurements were made in support of the assumption that the intracellular sodium concentration would eventually equal that in the intestinal lumen. An alternative explanation for the increase in intracellular sodium concentration subsequent to ouabain inhibition of the sodium pump must also be considered.

It seems plausible to suggest that the source of the extra sodium in the cell might be the submucosal tissue fluid, or arterial infusate. Firstly, sodium ions from the tissue fluid might diffuse freely down their electrochemical gradient into the cells. While the sodium pump was inhibited by ouabain, they could not be extruded as rapidly as they enter. Hence, the steady-state would be reached when the activities of sodium in the cell water and in the tissue fluid become equal. If this were so, then no difference would be expected between the intracellular sodium concentrations at the

Rate of glucose
absorption, mg/cm/hr



Rate of water
absorption, μ l/cm/hr

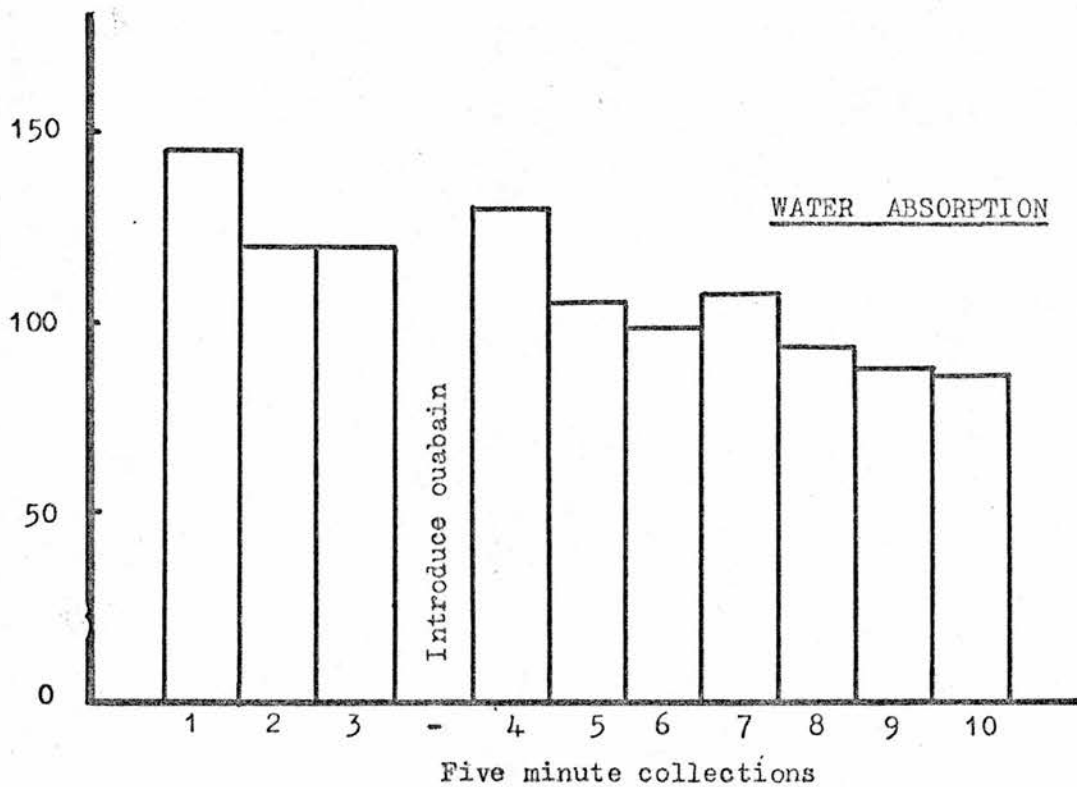


FIGURE 85

Effect of ouabain ($10^{-4}M$) in the arterial infusate when
the infusate sodium concentration was half-normal

(Mean of 3 experiments)

steady-state in the two sets of experiments shown in Figure 84. Consequently, no difference in glucose absorption rates would be predicted.

Secondly, in the absence of sodium in the arterial infusate, and hence in the tissue fluid, no sodium could enter the cell from the tissue fluid space. And any sodium entering the cell from the lumen might be able to diffuse freely out of the cell into the tissue fluid. Under these conditions with no uphill gradient of sodium activity, the active 'sodium pump' might be redundant for the maintenance of low cell Na and normal metabolism. If this were so, then ouabain would not affect glucose absorption if the normal effects of ouabain were secondary to an increased sodium level.

Therefore experiments were made to establish the kinetics of ouabain inhibition of glucose absorption when the arterial infusate contained less sodium than usual. Figure 85 summarises the time-course of ouabain inhibition when the sodium concentration in the arterial infusate was half-normal (72 mEq/litre). The sodium was iso-osmotically replaced by choline.

It is clear that at half the normal sodium concentration in the arterial infusate the inhibitory effect of ouabain on glucose absorption has almost been eliminated. This result is consistent with the hypothesis outlined above, namely that when ouabain is in the tissue fluid the inhibition of glucose absorption is only secondary to back-flux of sodium from the tissue fluid into the mucosal cells. When the tissue fluid sodium concentration is kept down to some 72 mEq/l only slight back-flux can occur before the sodium activities in the tissue fluid and the intracellular water become equal after inactivation, or partial inactivation, of the ouabain sensitive sodium pump.

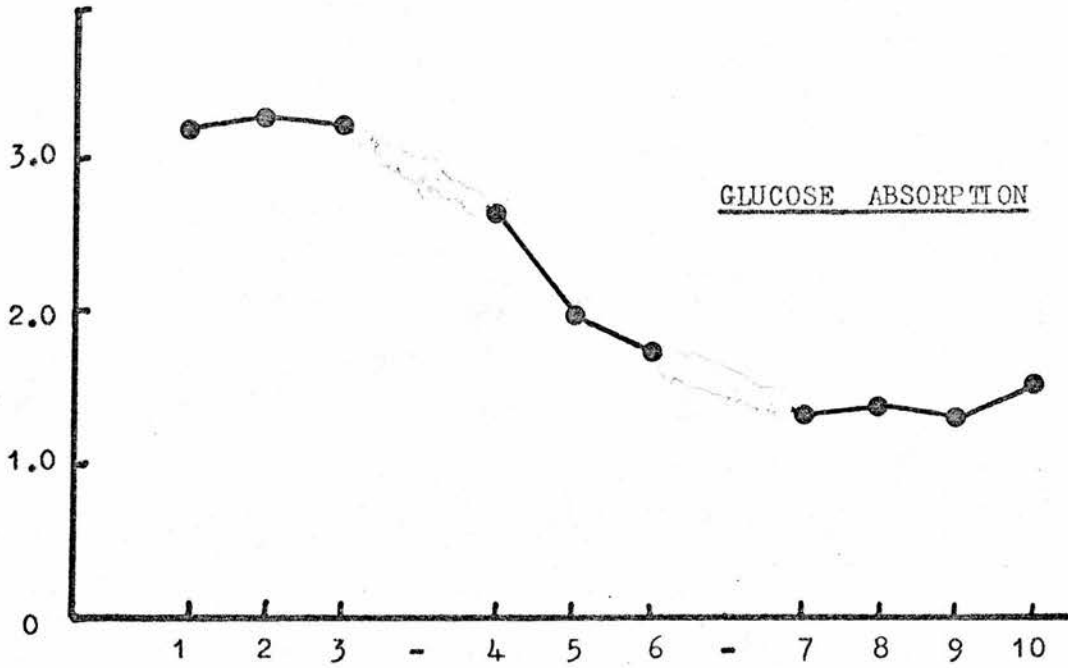
Although there is only slight inhibition of glucose absorption, the rate of water absorption fell as rapidly when the arterial infusate contained 72 mEq/l Na as when the sodium concentration in the infusate was normal (144 mEq/l). One single experiment was made in which all the sodium in the arterial infusate was replaced by choline. Again, any inhibition of glucose absorption was only trivial, but in contrast to the above experiments the rate of water absorption did not fall. The concentration of sodium in the vascular effluent was estimated in this experiment by atomic absorption spectrophotometry, and the rate of sodium appearance in this effluent (approx. 30 μ Eq/cm/hr) was apparently unaffected by the introduction of ouabain (10^{-4} M) into the arterial infusate.

Ouabain antagonism of the effects of luminal sodium depletion

Experiments described in Chapter VIII suggested that the decrease in the rate of glucose absorption following removal of the sodium ions from the luminal perfusate might be simply a consequence of depletion of the intracellular pool of sodium. The work discussed in the last section above supports the common notion that ouabain inhibits glucose absorption by increasing the intracellular concentration of sodium. Therefore it seems that both ouabain and low luminal sodium may act by changing the normal intracellular level of sodium, but in opposite directions. Hence, the effects of luminal sodium depletion might be antagonised by ouabain.

This possibility was tested in a single experiment. After 3 control collections of lumen and vascular effluents had been made, each over five minutes, the luminal perfusate was replaced by one which contained no sodium. After 3 further five minute collections had been made, the normal arterial infusate was replaced by one containing ouabain (10^{-4} M) and four further collections were made.

Rate of glucose
absorption, mg/cm/hr



Rate of water
absorption, μ l/cm/hr

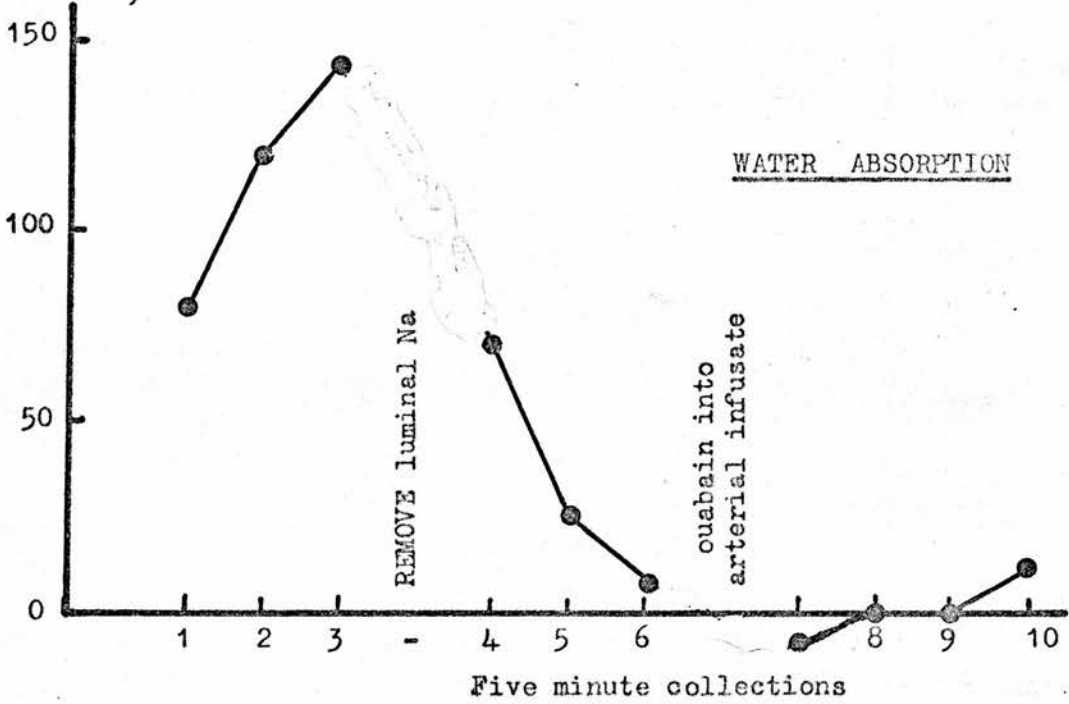
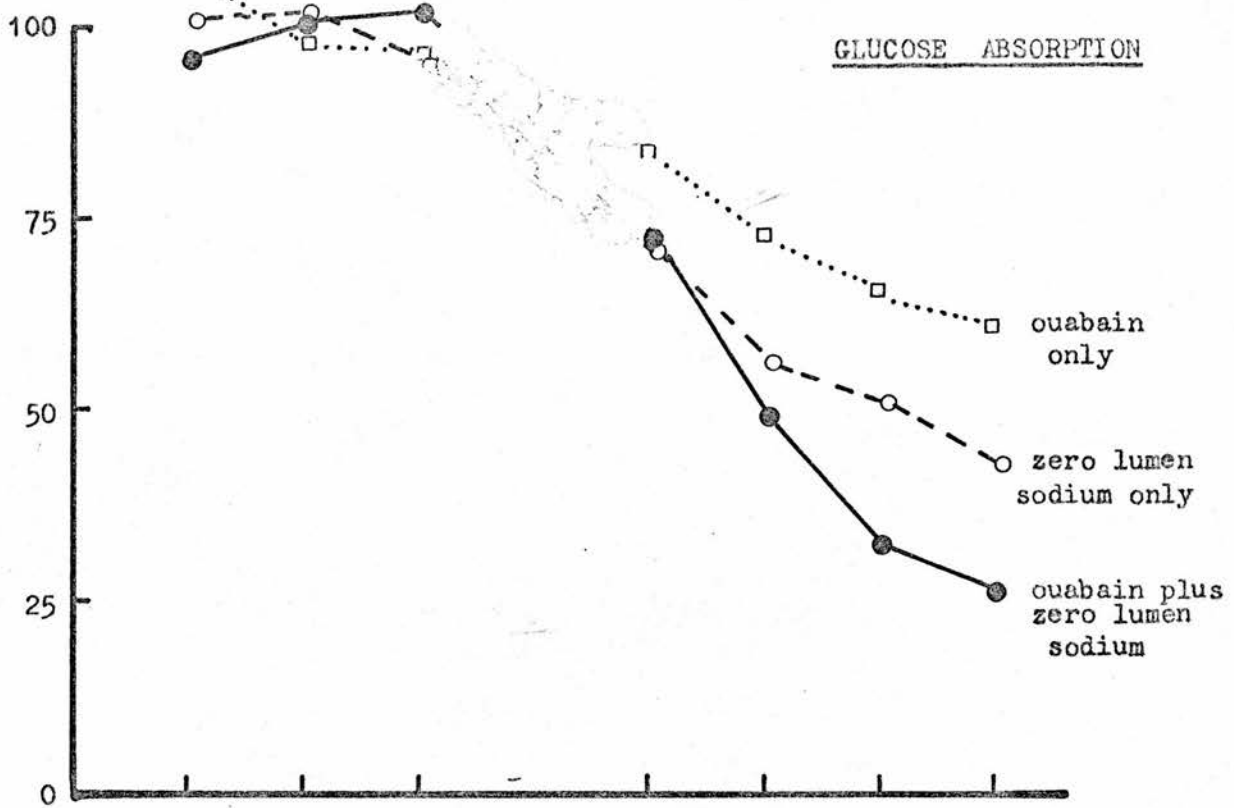


FIGURE 86

Test for antagonism of the effects of luminal sodium depletion by
ouabain ($10^{-4}M$) in the arterial infusate
(one experiment)

Rate of glucose absorption, % of mean control



Rate of water absorption, %

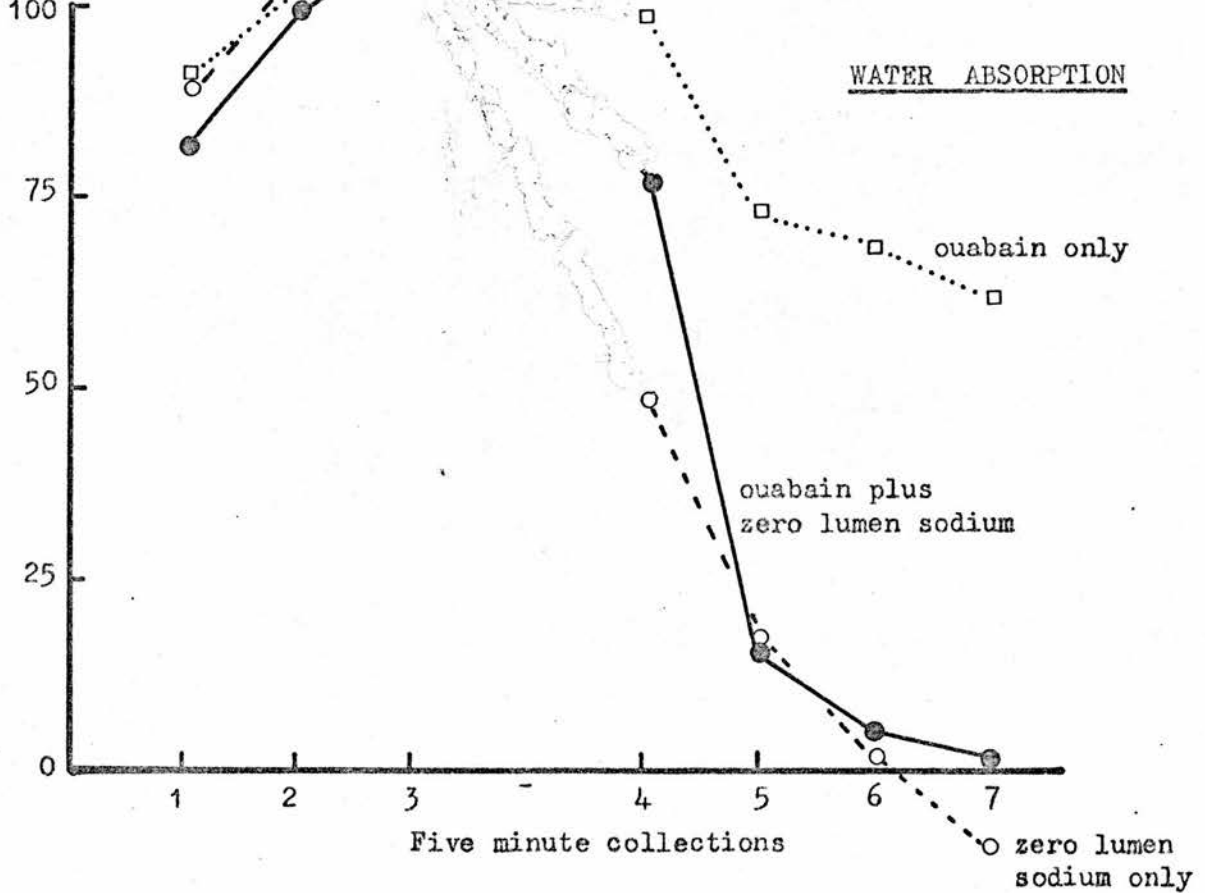


FIGURE 87

Simultaneous effect of ouabain in arterial infusate and zero luminal sodium

(mean of two experiments)

The results are shown in Figure 86, and within the time covered by this experiment there was no marked reversal of the inhibition of either glucose or water absorption. Figure 83 has already shown that the effects of ouabain on both glucose and water absorption normally become apparent within about 5 minutes.

As a second test for possible antagonism between luminal sodium depletion and ouabain, the following experiment was made on two intestines. After 3 control collections had been made, the lumen perfusate was replaced by a sodium-free medium and simultaneously the arterial infusate was replaced by one containing ouabain ($10^{-4}M$). If the predicted antagonism occurred then the rate of glucose absorption might be expected to fall more slowly than it does when only the luminal sodium is removed. However, the results (2 experiments) which are shown in Figure 87 show that this is not the case, but that glucose absorption is inhibited more rapidly than when either only the luminal sodium is removed or only ouabain introduced into the arterial infusate.

Therefore the suggestion that luminal sodium depletion and ouabain in the tissue fluid cause inhibition of glucose absorption by acting, in opposite directions, on the same pool (intracellular ?) of sodium cannot be accepted.

Effect of luminal 2, 4-dinitrophenol on glucose absorption

Schultz & Curran (1970) suggested that the effect of the uncoupling agent 2, 4-dinitrophenol on glucose absorption might be secondary to inhibition of the Na-K pump and the subsequent accumulation of sodium within the cells. This might be true if glucose absorption were driven by a downward gradient of sodium ion concentration into the cell and were not dependent directly on metabolic energy. Hence the effects of dinitrophenol would be similar to

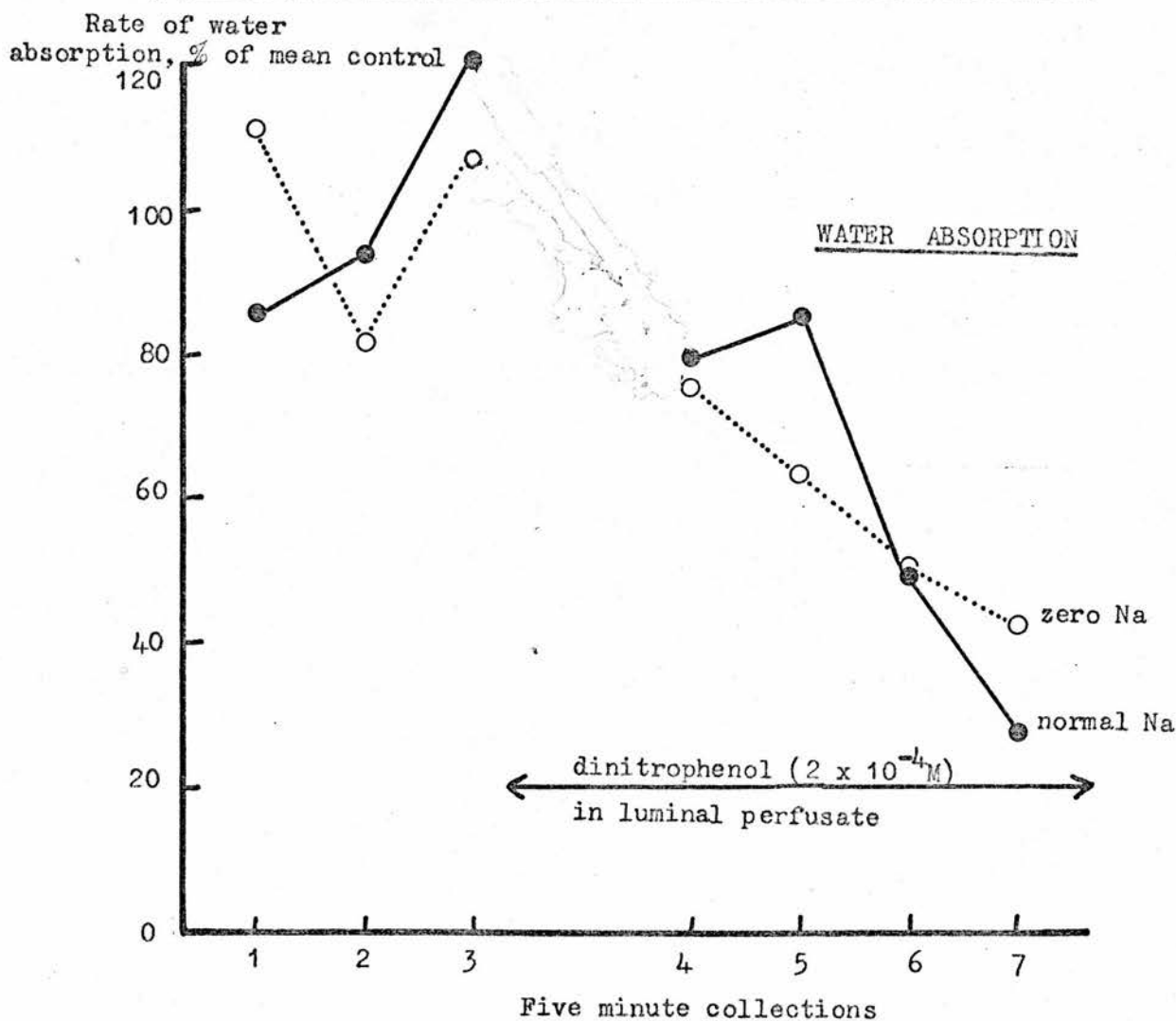
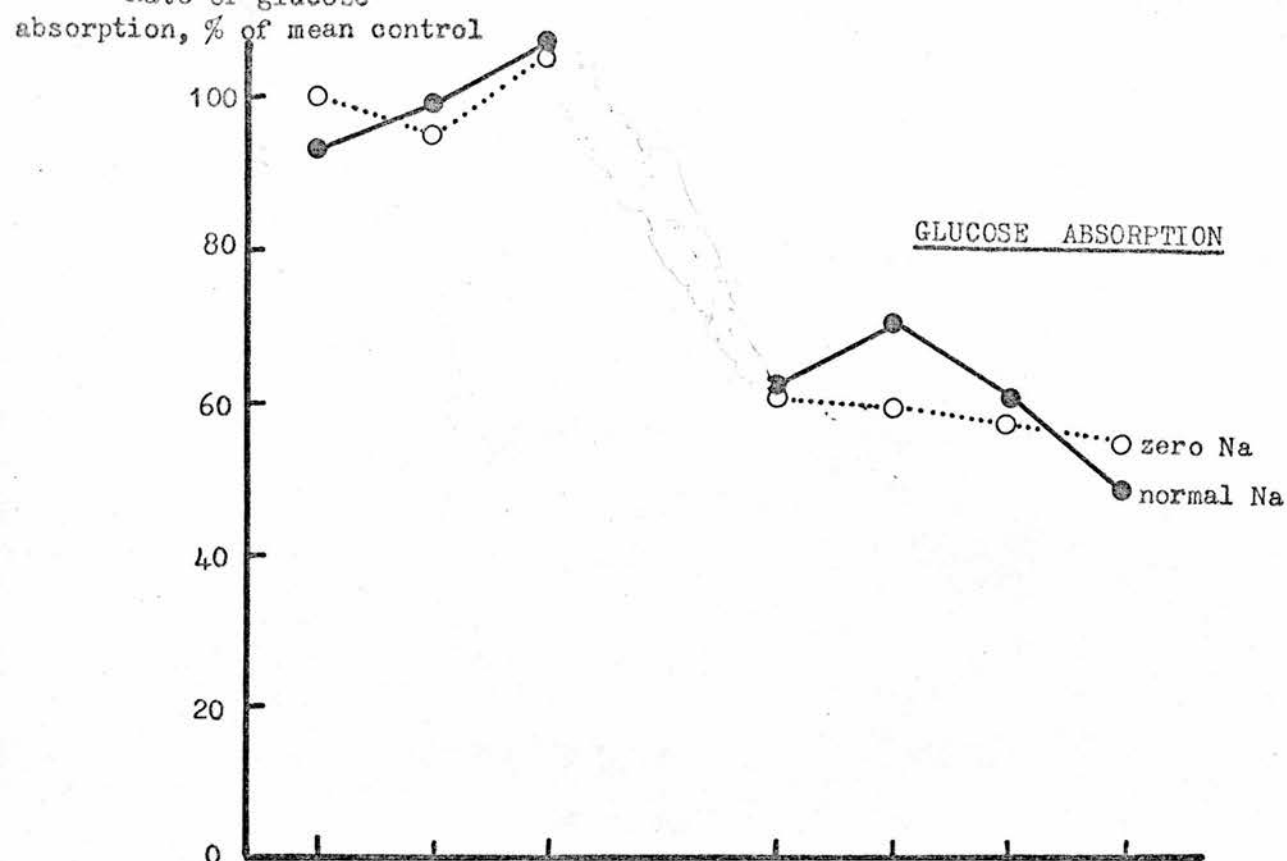


FIGURE 88

Effect of luminal 2, 4-dinitrophenol ($2 \times 10^{-4} M$) on glucose and water absorption

In one experiment the arterial infusate contained 144 mEq/litre Na (closed circles). In another experiment the arterial infusate contained

those of ouabain, and again no inhibition would be predicted when the tissue fluid sodium level is maintained low.

Therefore a pair of experiments was made to test the effect of 2, 4-dinitrophenol on glucose and water absorption. The inhibitor ($2 \times 10^{-4}M$) was present in the luminal perfusate, since Schultz & Zalusky (1964a) have shown rapid and marked inhibition of net sodium flux under these conditions.

Figure 88 shows the results from these experiments. The dinitrophenol inhibited both glucose and water absorption regardless of whether the arterial infusate contained sodium or not. This finding does not support Schultz & Curran's (1970) suggestion. However the results from this type of experiment must be interpreted with caution, since dinitrophenol may damage the tissue in other ways than simply by uncoupling oxidative phosphorylation (see e.g. Simon, 1953). Harris (1968, page 12) has made some pertinent remarks on the hazards attending interpretation of work with so-called specific inhibitors.

DISCUSSION

These experiments have shown clearly that rat small intestine is sensitive to ouabain. The findings of Robinson (1967), and others that ouabain did not affect rat intestine are probably a reflection on the types of preparation which these workers used. If the ouabain did not diffuse sufficiently rapidly into the tissue fluid from their serosal incubation media then no effect on absorption could be expected. No significance can be attached to the unlikely suggestion that the sodium pump mechanisms may be fundamentally different in, say, the rat and the mouse (Robinson, 1970).

Ouabain inhibition of glucose absorption was strongly dependent on the sodium concentration in the tissue fluid (Figures 83 & 85).

At 72 mEq/litre there was only slight inhibition of glucose absorption. This might reflect a need for sodium in the tissue fluid for binding to occur between ouabain and the Na-K-dependent ATP-ase (sodium pump) at the serosal membrane. Alternatively, inhibition of the sodium pump might have no effect on sodium ion movements when these are downhill and out of the cell. When the external sodium concentration is below that in the cell 'active' extrusion of sodium ions may be no longer necessary, since they can diffuse down their electrochemical gradient. Therefore an accumulation of sodium ions within the cell after application of ouabain can only occur when the external sodium concentration is greater than the intracellular concentration. This increase in the intracellular sodium level is often assumed to be the direct cause of the effects of ouabain on intestinal absorption (see e.g. Schultz, Fuisz & Curran, 1966), but the evidence has not been very firm. The present studies however support this view. They indicate that the inhibitor is probably not exerting a non-specific cytotoxic effect, since inhibition was trivial when the level of sodium in the tissue fluid was low. Furthermore, this observation suggests that it is the increase in intracellular sodium, rather than the concomitant decrease in intracellular potassium or the cessation of cation transport per se which causes the inhibition of glucose absorption.

The striking failure of ouabain to antagonise the effects of luminal sodium depletion suggests that the two agents do not act on the same pool of sodium ions, such as the intracellular pool. However there is no reason to suppose that the intracellular pool of sodium is in one homogeneous compartment, and in this respect the term 'intracellular sodium' can be rather misleading. The evidence

points to the removal of the luminal sodium causing depletion of one pool of sodium, while ouabain in the tissue fluid has the opposite effect on another pool of sodium.

Luminal dinitrophenol inhibited glucose and water absorption regardless of whether or not sodium was present in the arterial infusate. The experiments with ouabain have suggested already that inhibition of the active sodium pump does not affect the net extrusion of sodium ions from the cells when the tissue fluid contains sodium at a low concentration. Hence the action of dinitrophenol on glucose absorption cannot be explained readily in terms of inhibition of the sodium pump and subsequent accumulation of sodium within the cell, as Schultz & Curran (1970) proposed. The results here suggest that the absorption of glucose depends on the maintenance of metabolic energy rather than simply on the maintenance of a downhill gradient of sodium ion concentration across the luminal membrane. The latter is essentially what Crane's mechanism involves, while the former is partly in agreement with the views of Csaky (1963).

The effects of ouabain on glucose absorption may be related to interference with the supply of metabolic energy rather than to the abolition of a gradient of sodium ion concentration across the luminal membrane. For example, the elevated intracellular concentration of sodium which follows the application of ouabain (Schultz, Fuiss & Curran, 1966) may inhibit sodium-sensitive enzymes, and so restrict the formation of ATP. Ouabain is known to reduce the respiration of red blood cells (I. A. Nimmo, personal communication) and of intestinal strips (Stampa & Ponz, 1969). However, these effects might be subsequent to a reduction in substrate availability owing to initial inhibition of a transport mechanism.

CHAPTER XII

GENERAL DISCUSSION

The work presented in this thesis falls into two parts: technical developments for the precise study of absorption and metabolism, and the application of these methods to the investigation of the interaction between sodium ions and the mechanism for glucose absorption. The experimental results have already been discussed at the end of each Chapter. In this final Discussion, some especially pertinent findings and ideas have been briefly collected together.

The new preparations have been shown to enable precise measurements to be made under defined conditions. The major advantages of the new methods have already been discussed: they can be summarised as follows:-

(a) Simultaneous measurements of water absorption, glucose absorption from the lumen, glucose secretion into the tissue fluid, and glucose utilisation are possible.

(b) The preparations are highly stable over at least one hour as judged by the rates of absorption from the lumen. Hence control and experimental measurements can be made on one and the same segment of tissue.

(c) The rates of glucose and water absorption are relatively high compared with those from other preparations.

(d) Experimental conditions (e.g. composition of the perfusate) can be changed rapidly. Hence serial measurements can give a kinetic picture of the subsequent changes.

(e) In the unmodified preparation, the kinetics of appearance of solutes in the intestinal secretion could be accounted for on the basis of a simple one compartment model. This showed that the composition of the tissue fluid only slowly (exponentially) attained

a steady-state, and that the rate constant depended on the rate of water secretion.

(f) In the unmodified preparation the intestinal secretion appeared to be a fair sample of the tissue fluid. Therefore the compositions of the fluids bathing both sides of the single layer of epithelial cells could be determined.

(g) In the preparation with arterial infusion the composition of the tissue fluid could be directly controlled, and measured. i.e. one had access to both sides of the epithelial layer, and steady-states in each could be rapidly established.

A major feature to which these new methods must owe part of their success was the segmented flow technique. Even at rates of luminal perfusion which are low enough to permit accurate measurement of absorption, enough oxygen is supplied to the organ. There is probably additional benefit from the stirring effect of a segmented flow.

Although much information has been gained concerning the interrelation between sodium ions and glucose absorption, the problem has not been resolved. When the luminal glucose concentration was abruptly changed the rate of glucose absorption from the lumen changed abruptly. However this was not so when the luminal perfusate was replaced by one containing a lower concentration of sodium ions. Part of the reason for this was that the composition of the fluid inside the lumen did not abruptly change owing to the back-flux of sodium ions from the tissue fluid into the lumen. However, when the arterial infusate (and hence the tissue fluid) contained no sodium the rate of glucose absorption still fell only gradually. This slow decline in glucose absorption rate therefore appeared to be associated with the fall in the intracellular sodium

concentration which would be expected when the tissue is bathed in sodium-free media. However, although the concentration of sodium in the luminal effluent fell abruptly under these conditions (Figure 76) the possibility that intracellular sodium 'leaked' back into the lumen and was available for co-transport with glucose cannot be excluded.*

The marked dependence of ouabain inhibition on the sodium concentration in the arterial infusate supports, but does not prove, the view that ouabain inhibition of glucose absorption is only secondary to the accumulation of sodium within the cell. In the absence of sodium in the arterial infusate ouabain had no significant effect on the net transport of glucose or sodium. Therefore the effects of ouabain may be relatively specific rather than generally cytotoxic. Indeed it is possible that the drug never enters the cells, since Caldwell & Keynes (1959) showed that the site of action of ouabain on the squid giant axon was extracellular.

While the inhibitory effects of ouabain are consistent with Crane's theory, they are equally consistent with Csaky's view. Just as an increase in intracellular sodium level lowers the transmembrane electrochemical gradient of sodium ions, so it would be expected to inhibit metabolism. Sodium ions inhibit several enzymes, notably pyruvate kinase (E.C. 2.7.1.40), acetyl-CoA synthetase (E.C. 6.2.1.1) and pantothenate synthetase (E.C. 6.3.2.1) - (Dixon & Webb, 1964, page 422).

Although ouabain apparently causes an increase in the intracellular sodium level it did not antagonise the effects of luminal

* The relative importance of the intracellular and luminal concentrations of sodium might be assessed from experiments with no sodium in the infusate but with various rates of perfusion. Hence the rate of decrease of glucose absorption might be studied while the rate of change of the luminal sodium concentration was varied. Thus, one might be able to test whether the rate of glucose absorption were more dependent on the luminal or intracellular sodium level.

NOTE

AUTORADIOGRAPHY

I have proposed a modified technique of high resolution autoradiography with improved precision, resolution and speed over existing methods. The electrons from a beta-emitting isotope would be accelerated away from the specimen and focussed in a modified electron microscope onto a highly sensitive photographic (nuclear) emulsion. The microscope would have an additional anode between the specimen stage and the objective lenses, and a magnified image would be produced using electrons from the isotope rather than from the instrument's own electron gun. A picture taken subsequently by the instrument in the conventional mode could be superimposed onto the autoradiograph as a frame of reference.

Early attempts at electron emission radiography were only moderately successful, mainly because of relatively insensitive photographic emulsion, the absence of an accelerating anode between specimen and objectives, and the lack of variable electromagnetic focusing (see Barker, 1950; Barker, Richardson & Feather, 1950; Shapland, 1955). Modern resources make it probable that the technique could be developed. My ideas are in the hands of the Wolfson Microelectronics Liaison Unit, University of Edinburgh, for evaluation.

sodium depletion. Therefore, ouabain and sodium depletion probably act on different pools of sodium, although whether the pool affected by the latter is intracellular remains to be seen. The evidence of Chapter XI suggested that the source of the sodium ions which accumulate in the cell was the tissue fluid rather than the luminal perfusate. This should be confirmed and the site of the sodium pool identified by autoradiography (see Note opposite). This technique should make it possible to identify the cells whose sodium concentration is affected by ouabain, the sites of the sodium pool(s) concerned, and the source of the sodium ions.

If the accumulating sodium does originate from the tissue fluid then it might be possible to control the intracellular sodium level at a preset level by introducing ouabain into an arterial infusate whose sodium concentration is equal to the desired intracellular concentration.

No measurement has been made of the intracellular sodium concentration in the current work. The reasons are two-fold. Firstly, it is extremely difficult to measure accurately intracellular concentrations. Secondly and chiefly, any such measure only gives a mean value over the whole population of intestinal or mucosal cells. Especially since the mucosal cells form a mixed population such an estimate has little or no value. Note the vagueness of such terms as 'the cell' or even 'the mucosal cell' etc.

If energy for the active absorption of glucose came from a downhill electrochemical gradient of sodium ions into the cell as Crane suggested, then as Schultz & Curran (1970) predicted dinitrophenol would not necessarily inhibit glucose absorption. However, the experiments in Figure 88 showed that, even with no sodium in the tissue fluid, dinitrophenol inhibited absorption of glucose. Under

these conditions accumulation of intracellular sodium ions was unlikely since there was a downhill gradient out of the cell into the tissue fluid (see Chapter XI).

If on the other hand the energy for glucose absorption is supplied from metabolism (i.e. primary active transport - Stein, 1967) then these effects of dinitrophenol can be explained. Further, one would predict, as Csaky did, that the availability of energy from ATP would be restricted in conditions of intracellular sodium depletion since most ATPase enzymes are activated by sodium ions. (Post, Merritt, Kinsolving & Albright (1960) give the half-saturation concentration (K_m) of Na activation of erythrocyte ATPase as 20 mEq/litre in the presence of 5 mEq/litre K.) Further, Duerdoth, Sanford, Newey & Smyth (1964) found that removal of sodium from the mucosal fluid of everted sacs inhibited the 'resorption' and metabolism of mannose from the serosal fluid.

A further possible interrelation between sodium ions and glucose absorption must be considered. Crane, Forstner & Eicholz (1965) and others have shown that the Michaelis-Menten type of kinetics for glucose absorption were sodium dependent. Although great caution is necessary in extrapolating from the reaction kinetics of a simple system with purified enzymes to transport across a complex multi-cellular organ, the evidence mentioned on page 72 is striking. In the rat and hamster the K values both for the intestinal absorption of glucose and for the hydrolysis of sucrose by a partially purified preparation of sucrase are sodium dependent. But the V values are independent of the sodium concentration. However the complete reverse is true in the rabbit. Although this is only circumstantial evidence it does point strongly to the possibility that the glucose 'carrier' is modified by sodium, perhaps in an allosteric manner.

In conclusion, it seems probable that the energy for glucose absorption does not come from a downhill electrochemical gradient of sodium ions, but that the system for glucose absorption requires sodium ions for one or two reasons:-

(i) Normal exergonic metabolism requires intracellular sodium, and/or

(ii) Sodium ions may be necessary for binding or translocation at the glucose 'carrier' (by analogy with the sucrase sodium dependence). This may invoke an allosteric carrier (or multi-enzyme-carrier complex) which is what Crane (1965) originally envisaged.

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Corrections & Notes