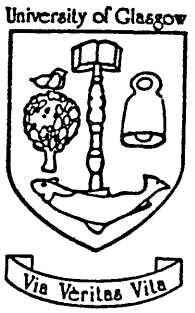

BIOCHEMICAL TESTS IN THE INVESTIGATION OF DIARRHOEA

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Thesis submitted to the Faculty of Medicine, University of Glasgow, Scotland, for the degree of Doctor of Philosophy. October 1990. The work was carried out in the Gastroenterology Laboratory, Glagow Royal Infirmary.

Dedicated to the women in my life.....

Mum, Liz, Ailsa and Mhairi.

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ABBREVIATIONS

Abbreviations not listed below are those recommended by the IUPAC-IUB commision on Biochemical Nomenclature (1)

BAID	bile acid induced diarrhoea
BAM	bile acid malabsorption
BDH	British Drug Houses
CA	cholic acid
cAMP	cyclic adenosine monophosphate
CCK-PZ	cholecystokinin-pancreozymin
CDCA	chenodeoxycholic acid
cGMP	cyclic guanosine monophosphate
CTMAB	cetyl trimethyl ammonium bromide
cv	coefficient of variation
DCA	deoxycholic acid
dpm	disintigrations per minute
FADBA	faecal aqueous dihydroxy bile acid
FPD	freezing point depression
GC	gas chromatography
GLDH	glutamate dehydrogenase
HMG	hydroxy methyl glutaryl
HPLC	high performance liquid chromatography
^{3}H-triether	tritiated glycerol triether
IBS	irritable bowel syndrome
IO	idiopathic oedema
KHP	potassium hydrogen phosphate
LCA	lithocholic acid
LID	laxative induced diarrhoea
NADH	reduced nicotinamide adenine dinucleotide

ns	not significant
ODS	octa-deca silane
OG	osmotic gap
PAT	percentage absorption of triolein
PAT-^{3}H	percentage absorption of triolein using ^{3}H-triether as nonabsorbable marker
PAT-^{51}Cr	percentage absorption of triolein using $^{51}CrCl_3$ as nonabsorbable marker
PCCD	post-cholecystectomy diarrhoea
PMSS	percentage of malabsorbed SeHCAT in stool supernatant
PVD	post vagotomy diarrhoea
PWC	percentage water content
QIP	quench index parameter
r	repiratory quotient
SCFA	short chain fatty acid
SeHCAT	tauro-23-[^{75}selena]-25-homocholic acid
sem	standard error of the mean
TBAH	tetra-butyl ammonium hydroxide
TLC	thin layer chromatography
triether	glycerol triether
UDCA	ursodeoxycholic acid
VPL	vapour pressure lowering
VIP	vasoactive intestinal polypeptide

SUMMARY

The idea behind this thesis developed during my employment at the Gastroenterology Laboratory at the Royal Infirmary Glasgow. The Laboratory is one of very few that has been established over the years as a centre for gastrointestinal research and also routine gastrointestinal investigations. In comparison to the 'high tech' biochemical investigations of other organs, the evaluation of gut function is decidedly 'low tech', frequently involving cumbersome tests, time-consuming analyses, and interpretative difficulties. This project concerns itself with the potential role of the clinical biochemistry laboratory in one aspect of gastrointestinal tests, namely the investigation of chronic diarrhoea.

For obvious reasons this is not a 'popular' area of academic interest and, perhaps as a result, much of the existing information is patchy and based on few and sometimes incomplete studies. The main objective was to fill some of the voids that exist in the current knowledge, with particular attention to methodological considerations. It consisted of four main themes.

The first aspect considered was the role of electrolyte and osmolality measurements of the stool water as a simple preliminary test. Electrolyte measurements on their own are of limited value but are thought to be useful when expressed in terms of the osmotic gap or potassium/sodium ratios.

The osmotic gap is considered to be of potential value in

the early diagnostic classification of the diarrhoea as either secretory or osmotic. However, there is doubt as to the most appropriate way of its calculation, namely whether the measured stool osmolality or the plasma osmolality should be used. This controversy was initially studied and convincing evidence was found to support the use of the latter approach. However, having established the best procedure and defined working reference ranges it was subsequently concluded that there was little value in measuring the osmotic gap: the concept of using only sodium and potassium concentrations to calculate the osmotic gap is too simplistic since it is also significantly contributed to by other cations; its use does little to narrow the diagnostic possibilities; and misleading results can be produced because of frequent large variations in results in consecutively collected stools. The only useful role that envisaged was in order to exclude a diagnosis of secretory or osmotic diarrhoea.

There has been a suggestion that the site of diarrhoea can be determined as being colonic or ileal depending on the potassium/sodium ratio. Our results have shown quite clearly that there is no clinical diagnostic benefit to be accrued from this investigation.

* * * * * *

The diagnostic possibility of laxative abuse is well documented but in this part of Scotland had not been widely considered in practice because of the lack of a screening

test. A thin layer chromatographic test was therefore established to provide both a routine service and information regarding the incidence of laxative induced diarrhoea. The test was found to be simple, cheap and effective in this respect and consequently a number of cases were detected which otherwise might have been missed. Methods for the diagnosis of saline laxative abuse were also established.

The incidence of laxative induced diarrhoea in new patients attending the Gastroenterology clinic for investigation of diarrhoea was found to be 4%, and in patients who had been diagnosed as having idiopathic diarrhoea, 20%. More importantly the diagnosis was clinically unsuspected in most instances being most frequently labeled as irritable bowel syndrome.

To avoid further missed diagnoses it is suggested that a laxative screening test be employed routinely as an early investigative test in all patients with diarrhoea of unknown aetiology.

* * * * * *

The Gastroenterology Laboratory in Glasgow Royal Infirmary has been responsible for evaluating a unique fat absorption test. This dual isotope method is based on the oral administration and subsequent measurement of stool ratios of isotopically labeled absorbable and non-absorbable markers. Further work was performed to assess the validity of using ^{3}H-triether and ^{51}chromium chloride as non-absorbable probes. We concluded that the dual isotope test

is the best available method for identifying patients with malabsorption and can justifiably claim to be the method of choice for evaluating alternative approaches.

The triolein breath test has been gaining popularity as a simpler and less unpleasant way of determining fat absorptive capacity. We expanded upon some of the limited studies already performed to evaluate this approach. Our conclusions place some doubt as to the role of this test. Although it was found to be capable of identifying patients with severe malabsorption it could not reliably detect patients with only mild degrees of malabsorption.

* * * * * *

Finally the diagnosis of bile acid induced diarrhoea was considered. Initially the measurement of the faecal concentration of the dihydroxy bile acids, chenodeoxycholic acid and deoxycholic acid, was established as the most appropriate means for its diagnosis. Because of the complexity of the analysis involved, an alternative test based on a modification of the SeHCAT test was evaluated but unfortunately found to be unreliable. The reason for the poor diagnostic capability of this test was subsequently found to be due to the non-physiological behaviour of the synthetic bile acid SeHCAT.

PREFACE

There will be few people who have not experienced diarrhoea from time to time, perhaps as a result of simple food poisoning or travellers' diarrhoea. Although these conditions are usually minor and short-lived they are unpleasant and may give some insight into the debilitating effect that chronic diarrhoea can have. Such patients usually suffer from more than the symptom itself. The urgency which frequently goes along with diarrhoea means that the victim must plan any activities carefully and so a simple trip to the shops requires a knowledge of the locations of public conveniences to avoid their being 'caught short'. Even the trip to the out-patients' department for investigations may be taken with some trepidation. Holidays, while not impossible, are often avoided because of the worry of unpredictable travel arrangements, lack of knowledge of toilet facilities and possibility of making matters worse by contracting travellers' diarrhoea. Social activities may be curtailed because of embarrassment over the frequency of having to 'excuse' oneself. It may be impossible to conceal the complaint in the workplace; one patient in this study was forced into giving up her work because the staff were concerned that they might 'catch something'.

The word 'diarrhoea' is derived from the Greek 'dia' (through) and 'rhein' (to flow) and is defined in the shorter English dictionary as being 'a disorder consisting

of the too frequent evacuation of too fluid faeces'. This definition is also close to the medical understanding of the term: an increase in the volume, frequency and/or liquidity of stool. However, it is probably more important to know what the patient presenting with diarrhoea understands by the term. Although many individuals complaining of diarrhoea have increased frequency, urgency and liquidity of bowel motions, many do not. Thus, well-formed motions passed frequently may be described as diarrhoea as may the relatively infrequent passage of poorly-formed stools. Similarly, the presence of blood and mucus with normal motions in patients with colonic carcinoma may also be described as diarrhoea (2) and people with faecal incontinence often euphamistically describe their symptoms as diarrhoea (3). Consequently it is not sufficient to accept the patients' description of their complaint without further enquiries or objective measurements.

The daily faecal output averages at just over 100g (4,5) in Westernised society but women tend to have substantially lower stool weights (4). The type of diet also affects stool volume: increasing dietary intake of bran for example results in increased stool weight and frequency (6). There is also evidence suggesting that faecal volume is affected by personality traits: people with positive outgoing personalities having a higher daily output (6)!

Stool frequency has been assessed mainly by means of questionnaires and has been found to vary quite widely: 99%

of two populations studied, a factory population and GP population, had more than 3 motions per week and less than 4 motions per day (7). Another study found that 52.7% of those asked passed one stool per day and 41% more than one daily (8).

With such a widespread variation in healthy subjects and differing concepts of normality, careful questioning is essential when investigating a patient presenting with diarrhoea. However, most patients will only become concerned when their bowel habit changes.

In the investigation of individuals with diarrhoea, the expertise of different specialities is often engaged. In acute infectious forms of diarrhoea the condition is usually short-lived and is allowed to 'run' its course. Few, if any, investigations are required except possibly the use of Bacteriology Laboratory to identify the infectious agent and occasionally the Biochemistry Department to monitor the acid/base and hydration status when the diarrhoea is severe.

The investigation of chronic diarrhoea offers more of a diagnostic challenge. This study specifically addresses the role of the clinical biochemisty laboratory in the diagnostic work-up of such patients.

CHAPTER 1

INTRODUCTION TO THE PHYSIOLOGY AND PATHOPHYSIOLOGY OF INTESTINAL WATER AND ELECTROLYTE TRANSPORT

PHYSIOLOGY OF INTESTINAL WATER AND ELECTROLYTE TRANSPORT

INTRODUCTION

To understand the mechanisms of diarrhoea more fully it is worthwhile to have a knowledge of the intestinal physiology for handling water. All movement of water within the gut, and indeed within other systems of the body, is almost certainly passive - that is does not require the input of energy. The transport of water follows osmotic gradients which are generated by the active movement of solutes, the most important of which is sodium. To understand the causes of diarrhoea the normal physiology of water absorption and ionic fluxes within the intestine will initially be illustrated.

Although the oral intake of fluid is in the order of two litres per day and faecal water losses are about 100ml the intestine handles in the order of nine litres of fluid daily and has the capacity to absorb up to double this volume (9). The missing seven litres in this equation are accounted for by gastrointestinal secretions of the salivary glands, stomach, gallbladder, pancreas, and duodenal Brünners' glands. The approximate daily volumes of these fluids and their electrolyte compositions are listed in Table 1.1 (10).

As well as these obligatory fluid secretions the mucosa of

the small intestine secretes and absorbs fluid and electrolytes in order to regulate the luminal contents in terms of its pH, osmolality and electrolyte composition.

	Volume (l)	Sodium (mmol)	Potassium (mmol)	Chloride (mmol)
Diet	2	150	50	200
Saliva	1	50	20	40
Gastric Juice	2	100	15	280
Bile	1	200	5	40
Pancreatic Juice	2	150	5	40
Succus entericus	1	150	5	100

Table 1.1 - Approximate daily composition of dietary and gastrointestinal secretions (10).

METHODS USED FOR ELUCIDATING ELECTROLYTE FLUXES IN THE INTESTINE

The current knowledge of electrolyte movement in the gut is based on several different experimental techniques and animal systems, and studies of gall bladder, trachea, kidney and bladder as well as intestine. The present understanding is based on an amalgam of knowledge from these heterogeneous sources and is by no means complete.

The *in vivo* method of intestinal perfusion has frequently been used (11). In this technique a segment of gut is perfused with a solution of known composition and the perfusate is collected and analysed. Altering the concentrations of the perfused electrolytes gives additional useful information. Studies of transmucosal electro-potential differences have been performed both *in vivo* and on isolated sheets of short-circuited mucosa using the classic Ussing Chamber. *In vitro* vesicles can be

prepared from basolateral or apical cell membranes allowing their properties to be assessed separately (12). The effect of drugs which modify specific protein functions has been used to obtain additional information. Drugs commonly used are acetazolamide (an inhibitor of carbonic anhydrase); the glycoside ouabain (inhibitor of Na^+/K^+-ATPase); propranolol (inhibitor of adenylate cyclase); and amiloride and frusemide (which block Na^+- and Cl^--specific ion channels respectively) (13, 14, 15)

MECHANISMS OF WATER AND ELECTROLYTE FLUXES WITHIN THE INTESTINE

There are five principal mechanisms which govern the intestinal absorption of electrolytes and water. To understand these systems it is useful to have a visual concept of a typical enterocyte as depicted in figure 1.1.

Enterocytes have microvilli on their mucosal surface in order to enhance intestinal surface area and so increase the efficiency of absorption. The luminal half of neighbouring cells are intimate having frequent interdigitations on their lateral surfaces. The intercellular spaces that separate adjacent cells are narrowest at the luminal junction where they are referred to as tight or occluding junctions (See figure 1.1). Tight junctions are approximately 300nms deep and 0.2 - 0.9nm wide.

The epithelial surface of enterocytes is covered with pores which allow the passive movement of water and solutes. The

size of these has been calculated by studying the transmucosal permeability of different sized molecules. By

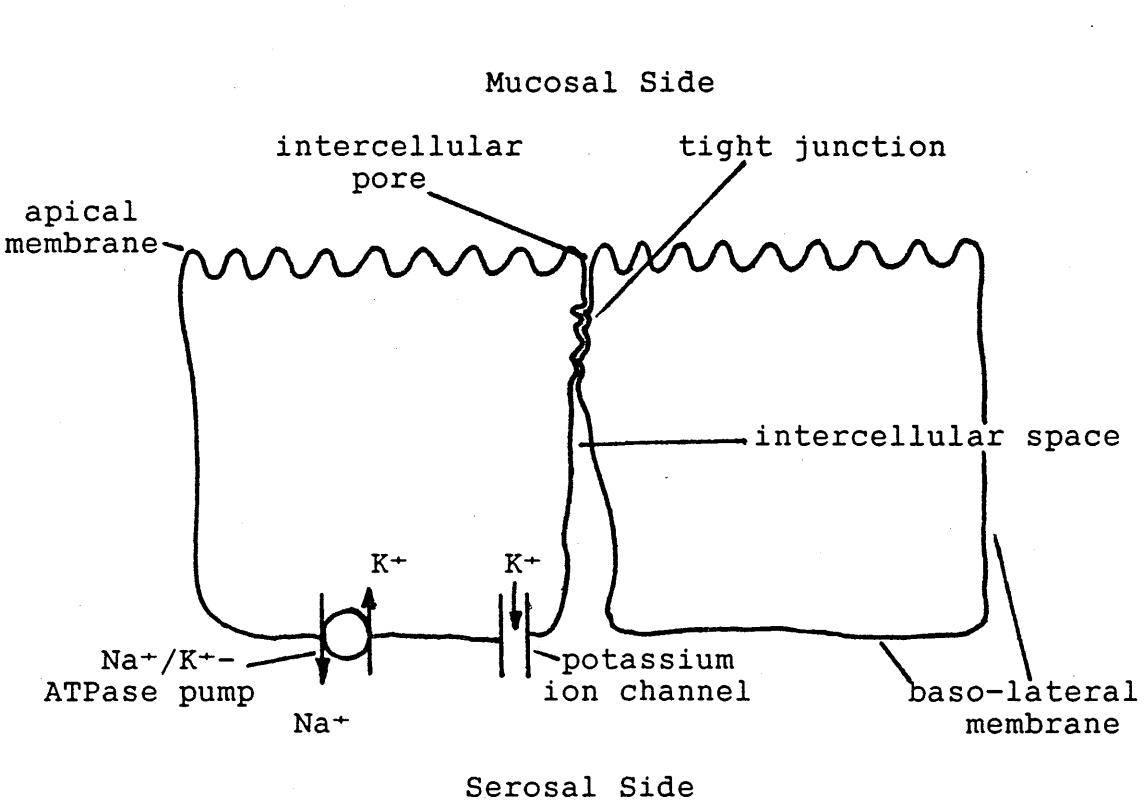


Figure 1.1 - Diagram of a Typical Enterocyte

this means the apparent pore size has been calculated at 0.7 - 0.85nm in the duodenum and jejunum, falling distally to 0.4nm in the ileum, and 0.2nm in the colon (16, 17) (1nm = 10Angstrom). There has been some uncertainty about the location of these aqueous pores: initially they were thought to be found on the enterocyte membrane but it is more likely that they are sited at the tight junctions.

1. Active Transport

Many intestinal transport processes require the input of

energy and exist as proteins located on both apical and basolateral membranes. The best studied and the most important one in the intestine is the Na^+/K^+-ATPase pump. This protein is located on the basolateral membrane of all enterocytes and directly influences absorption by the development of transmucosal electrochemical gradients.

2. Movement Across Electrochemical Gradients

The Na^+/K^+-ATPase system pumps three molecules of sodium out of the enterocyte for every two potassium molecules pumped in. Excess potassium which enters the cell by this process 'leaks' back into the interstitial space down K^+-specific ion channels in the basolateral membrane. By means of this pump the intracellular concentrations of sodium and potassium are maintained at approximately 15mmol/l and 75mmol/l respectively (18). As a consequence of the unbalanced ion movements of sodium and potassium, a potential difference of 30 - 40mV is generated across the apical membrane of the enterocytes with the interior of the cell being electronegative. There is also a transmucosal electropotential difference, the size of which varies inversely to the radius of the aqueous pores. Hence in the jejunum where the aqueous pores are relatively large the transmucosal electropotential difference is small, while in the colon the reverse holds (see figure 1.2). The electrochemical potential gradient allows the passive movement of certain ions - predominantly sodium - across the mucosa through specific protein systems called ion channels.

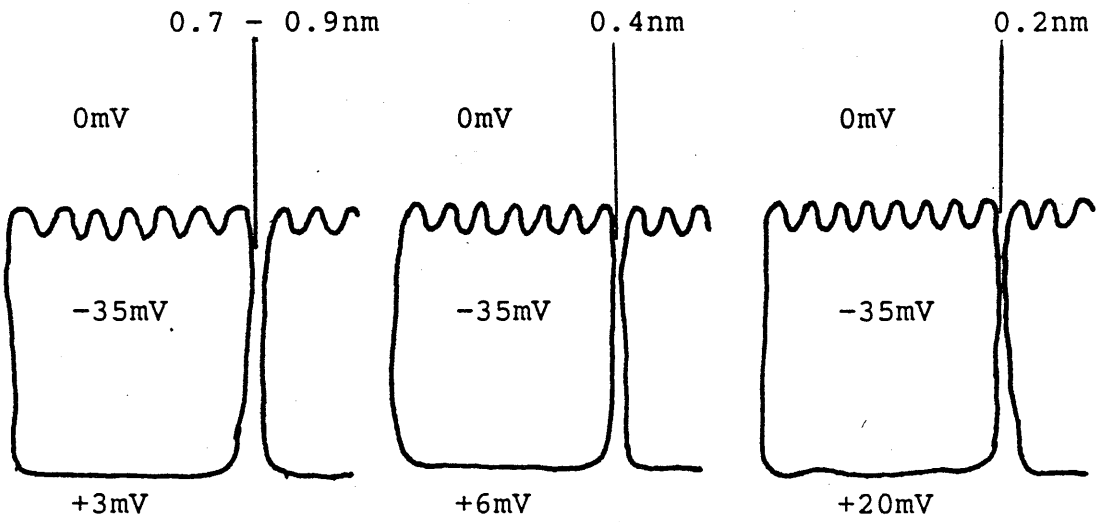


Figure 1.2 - Relationship of Transmucosal Electropotential Difference to Size of Tight Junctions in Jejunum, Ileum and Colon.

A current is invoked by this movement of electrically charged species and so this system of transport is referred to as electrogenic or rheogenic.

3. Osmotic Pressure

As previously mentioned the absorption of water is entirely passive being controlled by osmotic pressure differences across the mucosa. The osmotic gradient develops as solutes such as glucose and sodium are actively transferred across the semi-permeable mucosal membrane and subsequently into the lateral intercellular spaces. It is thought that relatively small osmotic differences - in the order of 3.5mosmol/Kg - are required to drive this movement of water (19).

The rate of this transmucosal flow of water is governed by

the size of aqueous pores. In the duodenum and jejunum the relatively large pores confer a low hydraulic resistance allowing the ready movement of water. This explains the rapid equilibration of hyperosmotic or hypo-osmotic fluids in the duodenum and jejunum. The sizes of the aqueous pores decreases distally and consequently in the colon where they are approximately 0.2nm water transport is much slower.

The direction of water flow - absorption or secretion - is dependent on the direction of the osmotic gradient. However, the intercellular spaces are smaller during secretion and so there is more resistance to the outflow of water (20, 21). Consequently even in circumstances in which the lumen is hyperosmotic there is a mechanism for the conservation of water.

4. Solvent Drag

Any movement of water also results in a passive non-specific 'drag' of solutes through intercellular spaces and across mucosal membranes. The efficiency of solvent drag (also called convective flow or entrainment) is dependent on the size of these pores in the same way as is the osmotically driven movement of water. Thus in the duodenum and jejunum, where the pores are largest, the majority of sodium absorption is accounted for by solvent drag (22). Further down the intestine, where the pore size decreases, solvent drag is less important as an absorptive mechanism. In general, cations and uncharged species are dragged

preferentially to anions because the intercellular spaces have a negatively-charged lining.

5. Hydrostatic Forces

Hydrostatic forces on either side of the mucosa also play a relatively minor role in governing water and electrolyte transport. Thus an increased serosal pressure, caused perhaps by hypertension, favours secretion, whereas volume depletion encourages absorption.

* * * * *

Each portion of the intestine has specific functions and this is reflected by differences in the handling of water and electrolytes. There is little net absorption of water above the duodenum and so ionic fluxes in the stomach and oesophagus will not be considered further. Ionic fluxes in the duodenum, jejunum, ileum, and colon which are mutually quite distinct, will be discussed separately. For practical purposes each part of the intestine is considered to be a uniform entity with similar functions throughout. However, in practice there are probably gradual changes through each system. The distal colon for example is known to have several quite different features from the proximal colon (23) and the same is probably true of the jejunum and ileum. In addition the gut has an impressive capacity to adapt to pathology. For example in cases of small bowel resection the colon can develop functions normally associated with the small intestine (24, 25). The converse holds with the small bowel of ileostomists whose colon is

effectively missing (26, 27).

IONIC FLUXES IN THE DUODENUM

The general function of the duodenum - rather than being one of absorption - is to adjust the pH and osmolality of luminal contents in preparation for digestion and absorption.

1. Control of pH

Gastric chyme entering the duodenum has a very acidic pH and so is potentially damaging. Neutralisation of pH is mainly achieved by dilution of the gastric outflow with pancreatic juices and succus entericus - the secretion of the duodenal Brünners Glands - both of which have a high bicarbonate content. The distribution of the Brünners glands - being numerous in the duodenal cap and proximal half of the descending duodenum - supports this concept of their function. The release of bicarbonate from the pancreas and Brünners glands is stimulated ultimately by the concentration of hydrogen ions in the duodenum. The individual cells of the duodenal mucosa also have the capacity to secrete bicarbonate, again in response to the presence of acid in the duodenal lumen (28) and this may well act as a fine control of pH.

There are two probable mechanisms for bicarbonate secretion from the enterocytes (29). One process is dependent on the presence of luminal chloride and exists as an electrically silent exchange of chloride for bicarbonate (figure 1.3a).

In the other there is secretion of bicarbonate, produced from carbonic acid, down its specific ion-channel into the lumen. Hydrogen ions which are produced at the same time

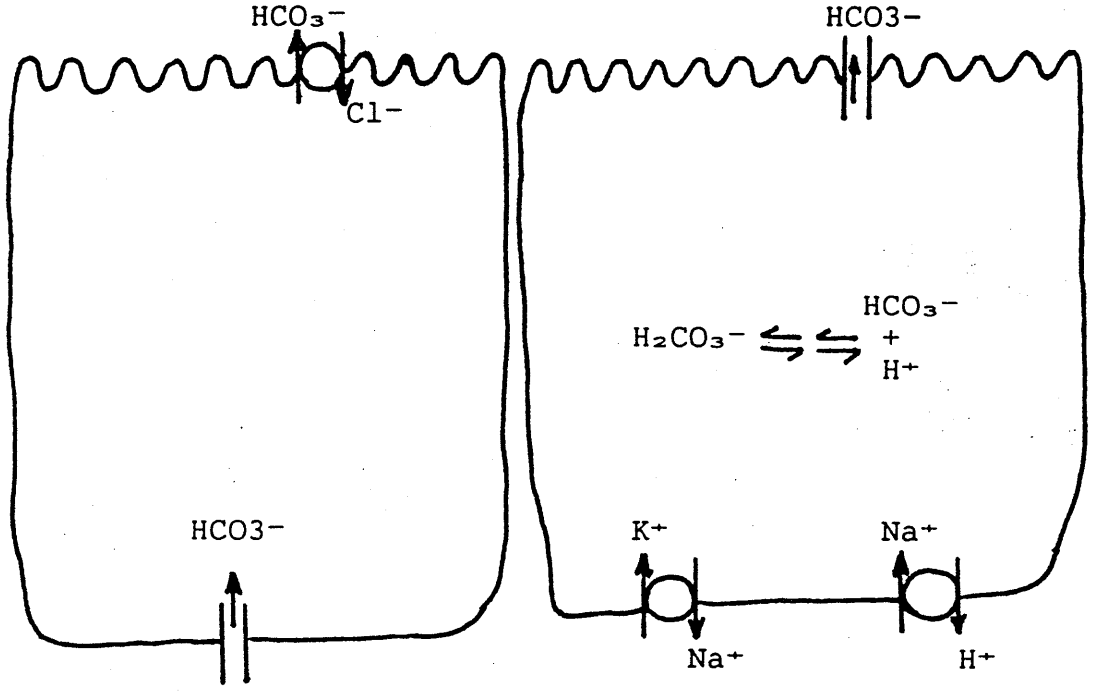


Figure 1.3 - Two Possible Mechanisms for Bicarbonate Secretion into the Duodenal Lumen.

as bicarbonate are exchanged for sodium on the basolateral membrane (See figure 1.3b). Although both of these mechanisms have been demonstrated it is likely that the former is the more important: acetazolamide which causes 99% inhibition of carbonic anhydrase does not inhibit secretion of bicarbonate implying that the source of secreted bicarbonate is extracellular (30) (see figure 1.3a).

To facilitate pH adjustment in the duodenum the emptying of

gastric contents is carefully controlled. The activation of duodenal pH receptors is one of a number of triggers for inhibition of gastric emptying. The overall result is the frequent release of small quantities of gastric contents into the duodenum.

2. Control of Osmolality

Although the osmolality of systemic fluids is regulated within very narrow specifications the intestine can readily cope with large ranges of osmotic intakes. For example water has zero osmotic pressure while pineapple juice has an osmolality of about 900mosmol/Kg. Furthermore, in the measurement of intestinal permeability a test solution with an osmolality of 1500 mosmol/Kg is taken orally with no untoward reaction.

The stomach seems to have little role in equilibrating osmolality of ingested foods. For example after a steak meal the gastric osmolality was measured at 243mosmol/Kg and this did not change over 90 minutes. Following a higher osmolality meal of doughnut and milk the gastric osmolality was 440mosmol/Kg with little change over a two hour period (31).

The duodenum on the other hand has a direct control on luminal osmolality. As described above the relatively wide intercellular spaces in the duodenum allow ready equilibration of any osmotic gradient over the mucosa. Consequently by the time luminal contents reach the ligament of Treitz the osmolality is nearly isotonic (32).

The duodenum contains osmoreceptors which, like duodenal pH receptors, have a feedback control on gastric emptying. Molecules which contribute the most to osmolality, such as amino acids, salt, sugars, and alcohol, stimulate these osmoreceptors.

The combined effect of duodenal pH and osmolality on gastric emptying is the controlled periodic release of gastric chyme into the duodenum. The importance of this gradual stomach emptying can be appreciated when the system is impaired. In dumping syndrome there is loss of duodenal sphincter control usually as a consequence of gastric surgery. The consequence of this is an uncontrolled release of gastric contents into the duodenum and subsequently an osmotically driven flow of fluid into the lumen. This may be severe enough to cause hypotension with up to 20% of the plasma volume being lost into the intestine. Symptoms can be controlled by adjusting the diet to small frequent meals of low osmotic load. This goes some way to restoring the normal physiological state of regular release of stomach contents.

JEJUNAL IONIC FLUXES

The jejunum is the predominant site for absorption of nutrients, water, and electrolytes. Sodium, the predominant cation, is absorbed both actively and passively. Most sodium is probably passively absorbed here by means of solvent drag (16). However, since this movement of sodium is passive an unfavourable

concentration gradient cannot be created and so when luminal sodium concentrations are less than about 135mmol/l sodium absorption will cease because of back diffusion through the relatively wide tight junctions.

Sodium absorption also occurs as a dual transport, or symport, system coupled to the absorption of a variety of nutrients such as glucose, galactose, diglycerides, triglycerides, some vitamins, bile salts, amino acids, and chloride (figure 1.4b). This process may be passive with the 'downhill' entry of sodium powering the uphill entry of the corresponding solute. The presence of these luminal nutrients markedly increases sodium and, in turn, water absorption. This seemingly minor fact has had major life-saving implications in the treatment of infectious watery diarrhoea in Third World countries. By the simple expedient of giving 'a little sugar and salt' the huge water and electrolyte losses associated with conditions such as cholera can be minimised. This modest therapy has been described as the worlds' most successful health programme (33). The continual Na^+/K^+-ATPase activity 'recharges the batteries' of the cell making this system possible. The concentration of solute in the cell is higher than the serosal solution and so the basolateral exit is probably passive. However, since the solutes are, in general, large molecules exceeding 0.4 - 0.5nm it is likely that diffusion occurs as a carrier-mediated facilitated process.

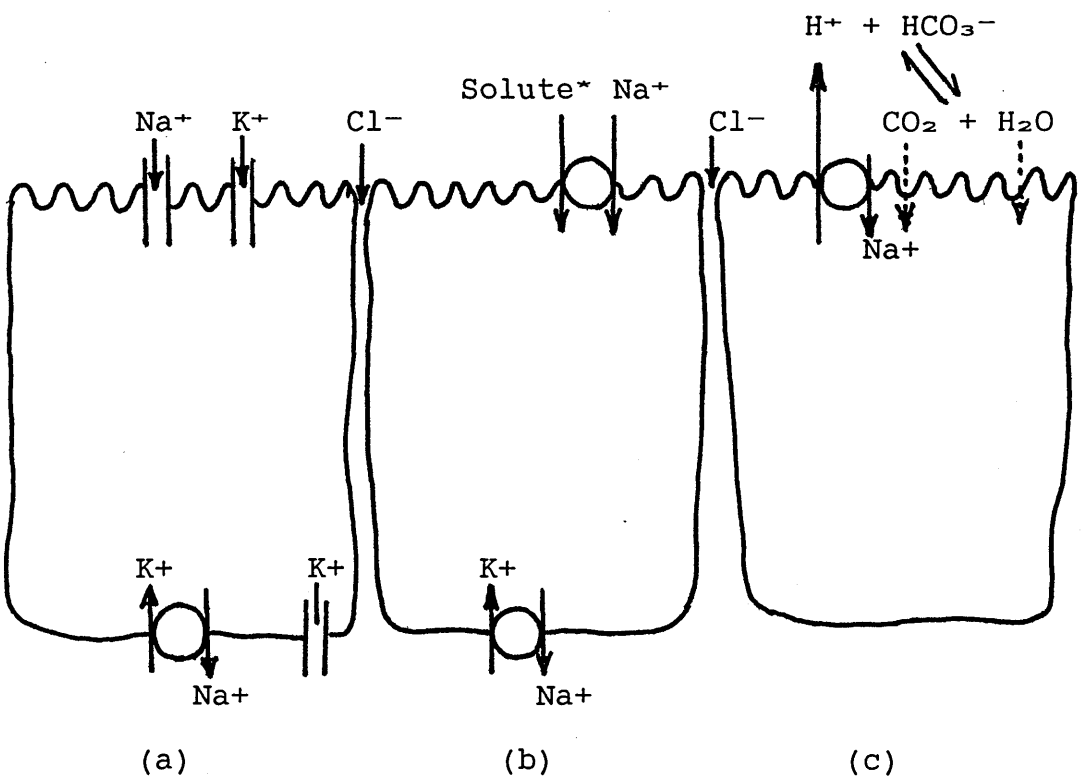


Figure 1.4 - Mechanism of Sodium Absorption in Jejunum: (a) electrogenic absorption; (b) dual transport system; (c) Na^+/H^+ pump. (* See text for appropriate solutes).

Sodium is also actively absorbed in exchange for protons, probably by an apical membrane Na^+/H^+-ATPase pump. This system requires intraluminal bicarbonate - which is in plentiful supply - and possibly results in the formation of carbonic acid which can then be reabsorbed as carbon dioxide and water as depicted in figure 1.4c (16, 34). This may account for the avid absorption of bicarbonate found in the jejunum.

Once sodium is absorbed into the enterocyte it is actively extruded by the Na^+/K^+-ATPase pump. This pump, by generating a transmucosal potential difference of 3mV - 5mV, powers the electrogenic absorption of chloride and other anions by way of the tight junctions and

intercellular spaces. Potassium is also absorbed passively into the cell by an electrogenic process.

IONIC FLUXES IN THE ILEUM.

The ileal lumen contains less in the way of digested foods such as glucose and amino acids and so the coupled transport of sodium with these nutrients is less important than in the jejunum. Some electrolyte absorption occurs by the solvent drag mechanism but this process is limited because the intercellular spaces are smaller in the ileum. Active mechanisms for electrolyte absorption are therefore more important. Sodium and chloride are absorbed in a mutually dependent manner which is achieved probably by a double ion exchange system involving Na^+/H^+ and Cl^-/HCO_3^- interchanges (figure 1.5). The result is absorption of

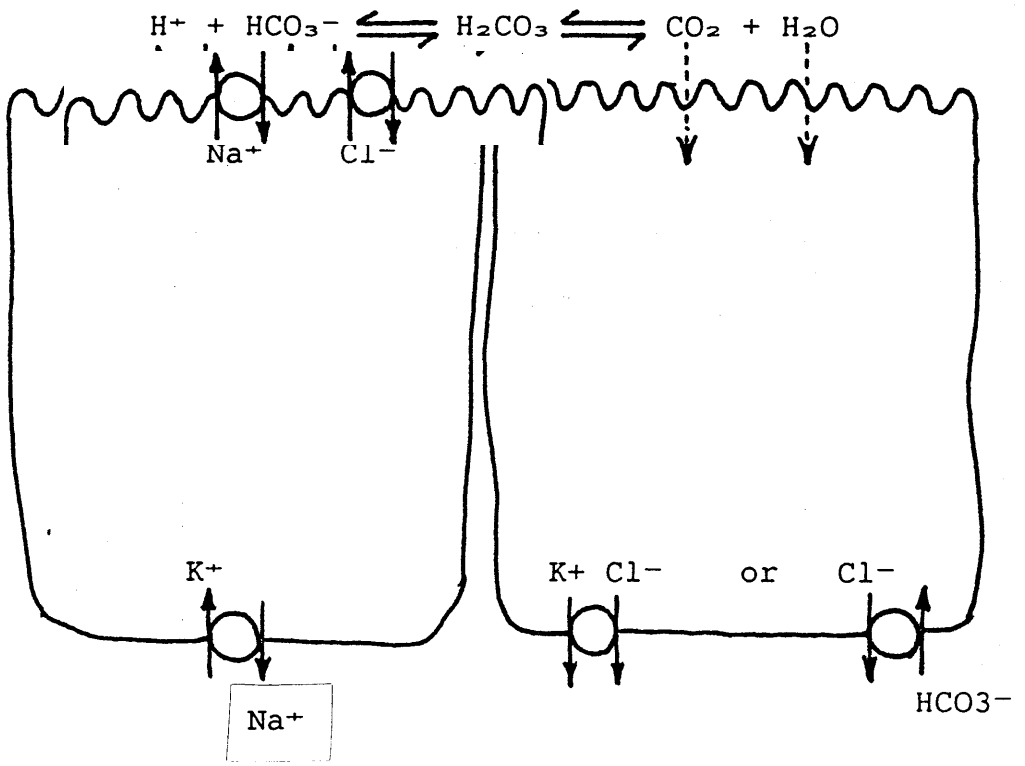


Figure 1.5 - Ionic Fluxes within the Ileum.

sodium chloride and secretion of carbonic acid (in the form of hydrogen ions and bicarbonate) which may be subsequently reabsorbed as carbon dioxide and water. Carbonic anhydrase which catalyses the interconversion of carbonic acid to water and carbon dioxide plays a role in this process which is blocked in the presence of acetazolamide - a carbonic anhydrase inhibitor (35). In the rare congenital condition of chloridorrhoea there is a large faecal loss of chloride along with a metabolic alkalosis (in contrast to the acidosis usually associated with diarrhoea). This is thought to be due to an absent or reversed Cl^-/HCO_3^- pump (36).

Chloride ions which are absorbed into the enterocyte are subsequently extruded over the basolateral membrane either by means of a potassium/chloride parallel system or a chloride/bicarbonate exchange mechanism (figure 1.5) (37).

Potassium absorption in the ileum, as in other parts of the small intestine, is passive via K^+-specific ion channels down a favourable electrochemical gradient.

The total absorptive capacity of the small intestine can be assessed with a knowledge of the composition of the luminal fluid reaching the terminal ileum. This was initially estimated by collecting fluid directly from patients with ileostomies (table 1.2) (38). However, these figures may

	Daily Ileal Fluid	Concentration
24 hour Volume	606 ± 287 ml/day	
Sodium	71 ± 36 mmol/day	117mmol/l
Potassium	5.1 ± 3.9 mmol/day	8.4mmol/l
pH	7.1 ± 0.9	

Table 1.2 Daily Composition of Ileostomy Fluid (38).

not be an accurate representation of the situation in normal subjects. The intestine has a remarkable capacity to adapt and in ileostomists the ileum, at least distally, probably functions more like the colon.

A more realistic idea of terminal ileal composition has been achieved by direct aspiration and measurement of caecal fluid (table 1.3). In total the small bowel absorbs some 80% of fluid introduced into the intestine. The one and a half or so litres which enters the colon has an electrolyte composition not dissimilar to that of plasma.

IONIC FLUXES IN THE COLON

In the small intestine as a whole, water and electrolyte absorption is rapid but incomplete. The converse is true in the colon which conserves water and electrolytes very efficiently but over a longer period of time. These functional variations between small and large bowel are reflected in their differing mechanisms of electrolyte and water absorption. Nutrient-associated sodium absorption does not occur in the colon (17) and solvent drag plays a minimal role.

	Terminal Ileal Fluid	Range	Mean Conc.
Volume	1524 ± 49 ml/day	(1255 - 1751)	
Sodium	196 ± 19 mmol/day	(139 - 243)	128mmol/l
Potassium	9.3 ± 0.9 mmol/day	(6.8 - 11.3)	6.1mmol/l
Chloride	103 ± 20 mmol/day	(63 - 123)	68mmol/l

Table 1.3 - Composition of terminal ileal fluid in normal subjects (39).

Sodium and water are absorbed in the colon by two principal mechanisms, an electrically-silent sodium chloride transport involving Na^+/H^+ and Cl^-/HCO_3^- exchanges as found in the ileum (figure 1.4), and an electrogenic process. The transmucosal electropotential difference is 20mV in the colon because the tight junctions, being very narrow here, limit the back diffusion of electrolytes into the lumen. Consequently the electrogenic mechanism is very effective in absorbing sodium against unfavourable concentration gradients and is of particular importance in the distal colon. However, if the tight junctions are widened by treatment with oxyphenisatin (a diphenylmethane laxative) or bile acid then sodium and water are pushed back into the lumen (40).

Sodium movement in the colon is controlled by aldosterone and glucocorticoids which, as in the kidney, promote its retention. Enhanced secretion of aldosterone results in an increase in the number of sodium-specific ion channels and a subsequent increase in activity of the Na^+/K^+-ATPase pump. There is a resultant increase in the transmucosal electropotential difference, the measurement of which has

been suggested as a rapid screening test for aldosteronism (41).

There is an active electrically silent absorption of potassium into enterocytes, probably by a K^+/H^+ exchange mechanism (42). This is opposed by an active potassium secretory mechanism in which potassium initially enters the cell by the Na^+/K^+-ATPase pump and is secreted through potassium-specific ion channels on the apical membrane. The balance between absorption or secretion of potassium in the normal colon is controlled by aldosterone and depends in part on the dietary intake of potassium. For example, with high intakes of potassium, secretion predominates (43). In addition to the active secretion of potassium there is a passive electrogenic leakage of potassium from serosal fluid via the tight junctions.

It is emerging that the colon has a role in nutrient absorption as well as water and electrolyte conservation. Dietary fibre has long been thought to pass through the gut unchanged, however both this, and other unabsorbed carbohydrates that reach the colon, are metabolised by bacteria with the production of short chain fatty acids (SCFA), primarily acetic, propionic and butyric acids. These are then efficiently absorbed by the colon, probably passively, (44) and may be a significant source of energy (45) particularly for colonocytes. The absorption of SCFA is associated with an increase in luminal bicarbonate and pH (46) suggesting that a HCO_3^-/SCFA exchange system may operate.

By comparing the composition of fluid entering the colon (table 1.3) with the faecal composition (table 1.4) the overall function of the colon with respect to handling of water and electrolytes can be gauged. This exercise has revealed that the colon absorbs 97% of the water and over 99% of the sodium and chloride presented to it (39). The colonic absorption of potassium is lower and is also highly variable (15% - 84%). It has since been established that faecal potassium loss is related to dietary intake and this probably explains the latter finding.

The capacity of the colon, however, is considerably greater than this and has been calculated by colonic perfusion to be 5700ml of water, 816mmol of sodium, and 44mmol of potassium per day (47). In the same study the daily flow of fluid into the caecum was estimated at 1940ml/day (1300 - 2660).

Analyte	Daily Faecal Loss (median ± sem)	Daily Colonic Absorption
Water	47 ± 5 (ml)	1190 - 1718 (ml)
Sodium	0.6 ± 0.3 (mmol)	137 - 242 (mmol)
Potassium	4.7 ± 1.0 (mmol)	1.3 - 7.2 (mmol)
Chloride	0.3 ± 0.1 (mmol)	62 - 123 (mmol)

Table 1.4 - Daily Stool Composition and Colonic Absorption of Water and Electrolytes (39).

PATHOPHYSIOLOGY OF WATER AND ELECTROLYTE ABSORPTION

From the above it was noted that the average small bowel absorbs 8l of fluid daily and the colon absorbs 0.9l with a total capacity of 3 - 4l. The colon therefore can compensate for abnormally high volumes of fluid from the small bowel. In this situation diarrhoea will occur only when the colonic capacity is exceeded. If colonic function is impaired however, there are no further distal compensating mechanisms and diarrhoea may result. (figure 1.6).

In practice, diarrhoea can be classified into three main types; secretory, osmotic, and altered motility. Despite the use of these convenient categories there are probably few examples of diarrhoea caused exclusively by a single mechanism. In exudative diarroea, which is often listed as a fourth category, there is loss of blood or interstitial fluid through inflamed or breached mucosa, but relatively

small volumes are lost by exudation and it is more likely that diarrhoea is the caused by one of the other three mechanisms.

SECRETORY DIARRHOEA.

This type of diarrhoea is caused by an abnormal electrolyte flux in which there is inhibition of electrolyte absorption, secretion of electrolytes, or both. The best understood and documented type of secretory diarrhoea is

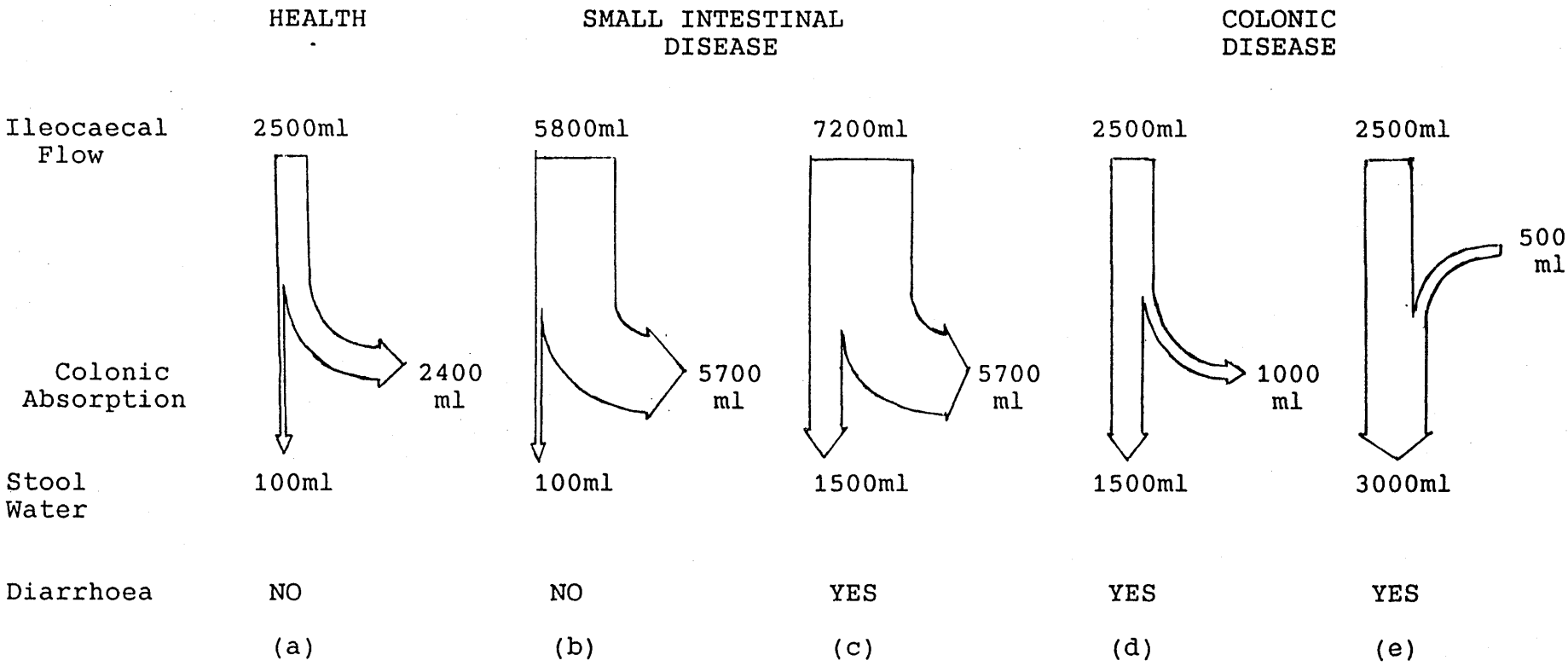


Figure 1.6 - Importance of the Colon in Diarrhoeal Disease. (a) Daily fluid volumes in healthy state. (b) Large ileo-caecal volume compensated for by increased colonic water conservation. (c) Large ileo-caecal volume which overwhelms colonic capacity for water absorption. (d) Decreased net colonic absorption (e) Net colonic secretion. (From Dobbins and Binder (97)).

the infectious diarrhoea caused by cholera. It is characterised by dehydration, metabolic acidosis, hypotension and severe diarrhoea frequently in excess of 1 litre per day. In cholera the pathogen *Vibrio cholerae* colonises the small bowel and produces an enterotoxin which binds to the mucosal cell surface. The enterotoxin splits into its two component subunits, the A unit of which enters the cell and stimulates

the adenylate cyclase complex on the basolateral membrane. The cyclic adenyl monophosphate (cAMP) whose production is catalysed by this enzyme, acts as the intracellular mediator for the abnormal electrolyte flux which follows. The abnormal secretion induced by *V. Cholera* in *in vitro* studies can be replicated by providing cAMP alone (48). Perfusion studies in patients with diarrhoea caused by cholera or *E. Coli* have demonstrated that sodium, potassium, chloride, and bicarbonate are secreted, predominantly into the jejunum but also into the ileum, with resultant fluid secretion (49). This fluid and electrolyte secretion continues after the diarrhoea is resolved indicating that the colon conserves sufficient water to avoid diarrhoea (cf. figure 1.6b).

It is likely that among these electrolytes only chloride is actively secreted in the small intestine. Normally the apical membrane is relatively impermeable to the movement of chloride, however, cAMP allows the ready loss of chloride down these channels by an electrogenic process. The chloride that is lost initially enters the enterocyte

by a co-transport system on the basolateral membrane involving simultaneous transfer of sodium and potassium (Figure 1.7). The driving force behind this passive flow of the three ions is the Na^+/K^+-ATPase pump. Cyclic AMP also causes an increase in the number or activity of potassium-specific ion channels on the basolateral membrane

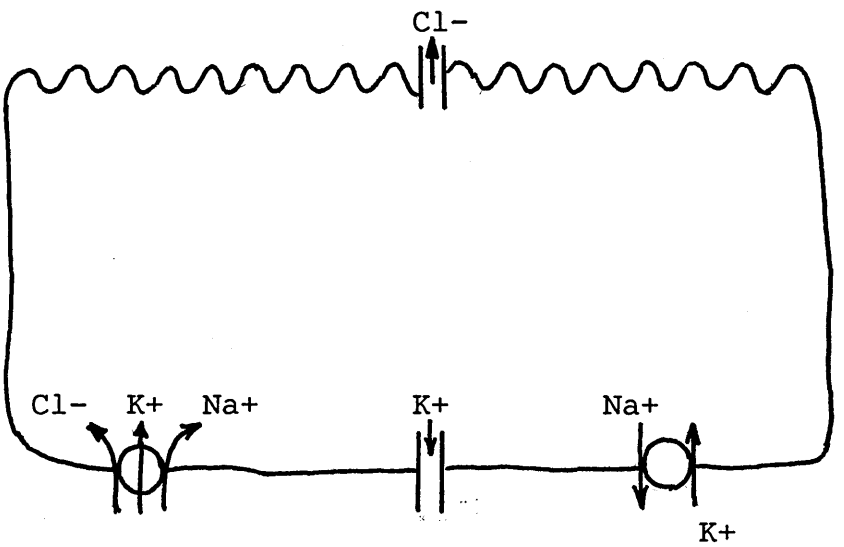


Figure 1.7 - Proposed Mechanism of Chloride Secretion.

and as potassium leaves the cell by this route the transmucosal electropotential difference increases, thus accelerating the secretion of chloride into the lumen (50).

Secretory diarrhoea which is induced by cAMP is also found in non-infectious diseases. A syndrome similar to that of cholera, in fact sometimes referred to as pancreatic cholera, consisting of watery diarrhoea, weight loss, and hypokalaemia was described and given the eponymous title Werner and Morrison syndrome (51). In the largest series of patients with this condition, elevated plasma concentrations of vasoactive intestinal peptide (VIP)

originating mainly from pancreatic tumours were found (52). Receptors for VIP are found on the basolateral membrane of enterocytes and its action is, like the cholera toxin, mediated by cAMP with secretion of chloride and inhibition of sodium absorption (53).

Cyclic AMP was the first intracellular mediator identified but it is now apparent that secretion can also be mediated by cyclic guanosine monophosphate (cGMP) and calcium. *Escherichia coli* and *Yersinia enterocolitica* colonise the small bowel producing toxins which invoke diarrhoea by a mechanism involving increased cellular cGMP (54, 55). The result, however, is similar to cholera toxin with increased chloride secretion and decreased sodium absorption (56).

The diarrhoea associated with the endotoxin from *Clostridium difficile* and with serotonin (carcinoid syndrome) is mediated by increased intracellular calcium concentrations. Studies using isolated rabbit ileum have shown that these two substances promote secretion of chloride and inhibition of sodium absorption. These effects were abolished by removal of serosal calcium or by verapamil - a calcium-channel blocker (57,58). Similarly an anthraquinone laxative has also been shown to cause colonic secretion which is mediated by calcium (59).

1. Secretion from Intestine with Blunted Villi

Mucosal cells have a relatively short life span and rapid turnover, being formed in crypts and then migrating to the villus. As they move their properties alter: Na^+/K^+-ATPase

activity declines, permeability to sodium increases, and the ability to absorb sodium in concert with actively transported nutrients develops (60). These changing properties reflect functional developments and, with regard to electrolyte fluxes, a convincing body of indirect evidence has been accumulating to suggest that absorption occurs via villus cells while secretion takes place through crypt cells (61). This evidence can be summarised as follows. (a) When jejunal mucosa is exposed to hypertonic solutions the absorptive capacity of villi is impaired while secretion can still be stimulated (62). (b) Exposure of mucosa to cycloheximide, which prevents mitosis, causes morphological damage to the crypt cells while villus cell structure is maintained. In mucosa subjected to this agent absorption can still be stimulated but secretion cannot (63). (c) Gall bladder epithelia and flounder intestine absorb electrolytes by similar mechanisms to mammalial mucosa, but these tissues do not contain crypts and cannot be made to secrete (64). (d) After pretreatment with theophylline, which induces secretion, electrolyte fluxes were detected in the intervillus but not villus cells (65). (e) When intestinal secretion is stimulated, the crypt cells can be seen to dilate (66).

The hypothesis that secretion occurs in the crypts has been more recently substantiated by two experimental studies (67): (a) When secretion was stimulated in the large intestine, droplets of fluid could be seen on the oil-covered mucosal surface directly over crypt duct openings;

(b) Using micro-electrodes to study the membrane potentials of villus and crypt cells, patterns associated with secretion were found only in the crypt cells.

The above theory has evolved following studies in which abnormal secretory states were experimentally produced. Less is known about the secretory status of crypt cells in healthy intestine, but there is probably a low-level secretion from crypt cells occurring at the same time as absorption from the villi (68).

If the ratio of villus cells to crypt cells fell then it would be expected that the balance of these two simultaneous processes would favour secretion. This situation has been demonstrated in conditions such as coeliac disease, tropical sprue (69) and viral enteritis (70). In these diseases where there is villus shortening and crypt hyperplasia, water and electrolytes are secreted into the intestinal lumen with resultant diarrhoea.

2. Bile acid induced diarrhoea

Diarrhoea commonly occurs after resection of the terminal ileum because of increased colonic concentrations of bile acids. The terminal ileum is the site of bile acid absorption and so in its absence more bile acids are delivered to the colon. The corresponding drop in the body pool of bile acids stimulates hepatic 7α-hydroxylase to increase bile acid production and this further increases the colonic concentration of bile acids. Hofmann originally postulated that malabsorbed bile acids caused

diarrhoea (71) which could be prevented by treatment with the binding agent, cholestyramine (72). This finding accorded with existing knowledge that deoxycholic acid impairs sodium and water absorption in both small and large bowel (73). It was subsequently demonstrated that the dihydroxy bile acids deoxycholic and chenodeoxycholic acids were most effective in promoting this fluid secretion (74). Increases in mucosal permeability (75) and enterocyte cAMP (76) have both been implicated in the mechanism although the former is probably of minor importance (77).

3. Fatty Acid Induced Diarrhoea

There is persuasive evidence to suggest that malabsorbed fatty acids may induce diarrhoea in the same way as bile acids. Hofmanns' group showed that the diarrhoea associated with ileal resection sometimes responded to replacement of dietary long chain fatty acids with medium chain fatty acids. They demonstrated that this subgroup of patients had high concentrations of faecal hydroxy fatty acids which fell after treatment was initiated (78). They later found that in patients with steatorrhoea secondary to gastric surgery, small bowel disease, or pancreatic insufficiency, the presence of diarrhoea was associated with high concentrations of faecal hydroxy fatty acids (79), although this finding was subsequently challenged (80). The possible implication of hydroxy fatty acids in causing diarrhoea is especially attractive because ricinoleic acid, the active ingredient in the powerful aperient castor oil, is an hydroxylated fatty acid.

Experimental evidence also supports this. During intestinal perfusion of the colon with ricinoleic and hydroxystearic acid respectively a decrease in water/electrolyte absorption by 75% and 57% occurred (81). Secretion of colonic electrolytes and water by hydroxy fatty acids is associated with stimulation of adenylate cyclase supporting the theory that cAMP is the intracellular mediator (82). However, as in bile acid-induced diarrhoea it is possible that increased colonic permeability may also have a role in the mechanism (82, 83).

Most studies on the mechanism of fatty acid induced diarrhoea have concentrated on the effects of the hydroxylated fatty acids. It seems, however, that non-hydroxylated fatty acids may play as important a role as the hydroxylated form (84). Although their potency is less they are present is much higher concentrations than the hydroxylated form.

4. Prostaglandins and diarrhoea

Prostaglandins have a cytoprotective role in the stomach and duodenum and have gained some credibility in the treatment of duodenal ulcers and prevention of intestinal lesions caused by non-steroidal anti-inflammatory drugs. However, one of the most common side-effects of this treatment is diarrhoea, which is almost certainly secretory in nature. Exposure of intestinal mucosa to prostaglandins results in fluid and electrolyte secretion (85) which is

associated with increased cAMP production (86). The diarrhoea associated with ulcerative colitis may also be caused by increased prostaglandin production (87) and can be effectively treated by inhibitors of cyclo-oxygenase. Similarly indomethacin has been useful in treating the secretory diarrhoea of villous adenoma which may also be caused by prostaglandins (88).

OSMOTIC DIARRHOEA.

As previously mentioned, the absorption of water is governed by the presence of an osmotic gradient from lumen to interstitium. Conversely, loss of water into the lumen would be expected if the osmotic gradient was in the opposite direction. This process occurs normally in the duodenum but, as nutrients are absorbed further down the gut, the luminal osmotic pressure decreases resulting in net absorption.

If a poorly absorbed substance is consumed in sufficient quantities then its presence in the intestine draws fluid into the intestinal lumen. This is the basis of the action of the saline laxatives such as magnesium sulphate whose two constituent ions are poorly absorbed causing a hyperosmotic luminal fluid. There is a subsequent rapid equilibration of osmolality by the movement of water from interstitial spaces. Electrolyte absorption in the small intestine continues and the larger volume of fluid reaching the colon is comprised predominantly of the unabsorbed ions; as the fluid passes through the colon little more

absorption of water or electrolytes can occur. If sufficient magnesium sulphate is taken the result is diarrhoea.

The diarrhoea often associated with disaccharidase deficiency is caused in a similar way. In subjects with lactase deficiency, intake of lactose results in a luminal osmotic load due to the unabsorbed sugar. Perfusion studies have demonstrated jejunal secretion of water and electrolytes (the latter presumably by solvent drag) and a rapid small intestinal transit time caused by the increased volume (89). The situation with disaccharidase deficiency is complicated by colonic fermentation of the sugar with the production of SCFA. It was originally thought that these moieties increased the osmotic load further but it has since been demonstrated (90) that the increase in faecal water is correlated to the unabsorbed sugar rather than the SCFAs which are in fact rapidly absorbed (44).

Non-pancreatic causes of malabsorption will also produce a diarrhoea which is due, at least in part, to the osmotic load caused by digested but malabsorbed nutrients. The diarrhoea found in dumping syndrome is also partially osmotic in nature (see page 33).

Congenital chloridorrhoea caused by a defective $Cl^-/HCO3^-$ pump is a rare cause of osmotic diarrhoea. The chloride ion which cannot be absorbed effectively acts as the osmotic agent and is found in very high concentrations in the stool water (36) (See page 37).

ALTERED MOTILITY

For the efficient absorption of water and electrolytes, as well as nutrients, intestinal fluid requires to be in contact with mucosa for an adequate period of time. Reduction of this time, as would be the case in hypermotility syndromes, might be expected to cause diarrhoea. There is a dearth of direct evidence to support this theory. However, it has been examined using an animal model in which saline solutions were infused into the small bowel of dogs. Predictably, as the rate of infusion was increased, diarrhoea (referred to as 'volumogenic' diarrhoea) resulted, the severity of which was proportional to the infusion rate (91). Conversely the passage of abnormally high fluid loads through the intestine results in a decreased intestinal transit time (81, 92). The ileo-caecal sphincter normally serves to control the entry of fluid into the colon in the same way as the pyloric sphincter allows small regular delivery of chyme from stomach to duodenum. However, ileal distension due to high fluid volumes causes its relaxation (93), preventing it from ameliorating diarrhoea in such situations.

Post-vagotomy diarrhoea may be a special case where there is rapid emptying of the terminal ileum caused by the ileo-caecal valve (whose action is controlled by the vagus nerve) not closing effectively. Consequently there is less time available for terminal ileal absorptive processes (including bile acid absorption). It is known that diarrhoea is associated with truncal vagotomy rather than

selective vagotomy in which the nerve fibres serving the ileo-caecal valve are maintained (94).

Intestinal smooth muscle activity is partly controlled by hormonal influences and it is probable that the diarrhoea associated with VIPoma, thyrotoxicosis, carcinoid syndrome and prostaglandins is partly due to hypermotility. Similarly bile acids, fatty acids, and some laxatives stimulate colonic motility, and indeed it seems likely that most causes of diarrhoea have a hypermotility component in their pathogenesis.

An obvious exception to this is the paradoxical diarrhoea associated with gut hypo-motility. An adequate flow of luminal contents is important in maintaining sterility in the small bowel (95). Consequently when the flow rate is compromised as in scleroderma or blind loop syndrome, small bowel overgrowth may occur resulting in diarrhoea due to fat malabsorption and/or toxin-stimulated secretion.

Lastly, premature emptying of the colon may partially account for the diarrhoea sometimes associated with irritable bowel syndrome in which there may be increased smooth muscle activity (96).

CHAPTER 2

PRELIMINARY INVESTIGATIONS OF STOOL WATER

INTRODUCTION

Most infectious causes of diarrhoea are relatively easily diagnosed by microbiological culture of organisms in a sample of faeces. In a similar way it is attractive to speculate that faeces from patients with other types of diarrhoea contains information which, by simple chemical analysis, might reveal the cause of diarrhoea.

Stool normally consists of between 65% and 80% water by weight. About a third of the dry weight of stool is comprised of bacteria and the remainder consists of undigested roughage, fat, mucus, desquamated epithelia and small amounts of protein. The relative proportions of these are predominantly dependent on the diet. In cases of diarrhoea it is unlikely that the composition of the solid portion will vary significantly. Perhaps some useful information can be gleaned by analysing the water phase of diarrhoeal stools. In Chapter 1 it was indicated that the excess water excreted in diarrhoea is ultimately controlled by electrolyte movements within the intestine. Consequently there has been some interest in the measurement of stool water electrolytes as a potential aid in the differential diagnosis of diarrhoea.

Water in faeces exists either in the intracellular compartment or as free intercellular water. The latter is in immediate contact with enterocytes and so is thought to be of particular relevance since its composition is directly affected by secretory or absorptive processes.

This component is variously described as stool water or faecal fluid.

PREPARATION OF STOOL WATER

Diarrhoeal stool water can readily be prepared by centrifugation of loose or watery faeces. Unfortunately comparative data from healthy individuals is more difficult to acquire since centrifugation of normal stools does not usually yield a supernatant. Alternative methods are required in such circumstances and four in particular have been employed.

1. Addition of water

In early studies of the electrolyte composition of stool water, a faecal sample was initially made fluid by adding water and homogenising. The electrolyte content of a protein-precipitated homogenate was measured and related to the water content which was estimated gravimetrically by evaporation of an aliquot to dryness (98). Unfortunately this method makes no distinction between intracellular water and free water.

In other studies sufficient water was added to normal stool so that an homogenate could be prepared and then centrifuged with the production of a supernatant (99, 100). However, without the use of a marker which does not enter the cell, this technique does not allow the original free water content to be calculated, so depreciating the value of subsequent analyses. Furthermore it is possible that

the addition of water may cause cell lysis resulting in contamination of the supernatant with intracellular fluid.

2. *In vivo* dialysis

In 1965 Wrongs' group devised an ingenious means of preparing stool water by an *in vivo* dialysis procedure (101). Small dialysis bags containing an inert colloidal solution were swallowed and after their passage through the intestine, were retrieved from stool. The composition of the faecal dialysate obtained was then analysed. Equilibration of dialysate with surrounding fluid was shown to be complete after an hours' incubation in an electrolyte solution. Since the bags were found to be within the colon for much longer periods of time, equilibration from colonic contents was assumed to be complete by the time of defaecation. Dialysates from bags inserted into the rectum of a healthy subject were also obtained. The electrolyte composition fell within the - very broad - normal range they had established using bags which had traversed the entire alimentary tract. Thus they concluded that the dialysate represented equilibration within the rectum rather than from proximal colon where the contents are more fluid. In addition the electrolyte composition in primary aldosteronism was shown to change in a predictable manner following surgery; the high potassium and low sodium concentrations found preoperatively were normalised post-operatively (102).

The results from this and further studies from the same

group have been widely quoted since and it is clear that they have played a significant role in the subsequent development of ideas and theories. The validation of this method of preparing stool water must, however, be considered as incomplete. It was difficult for the authors to achieve a more comprehensive evaluation because their studies were entirely original and so could not be meaningfully compared with other techniques. Some of their results seem surprising and could be interpreted as originating from an ineffective procedure. For example the concentration ranges found in normal subjects (n = 8) were very wide: 4.4 - 112mmol/l for sodium and 29 - 147mmol/l for potassium; there were wide intra-individual variations in dialysate composition; the composition of dialysates obtained from the same stool also varied widely.

Moreover, the validity of the *in vivo* technique has been questioned in a more recent study which compared it with a centrifugation method (103). Statistically significant differences were demonstrated for most of the analytes studied especially osmolality, SCFAs and bicarbonate. It also confirmed Wrongs' finding that there are significant differences in the composition of dialysates of bags taken from the same stool sample and concluded that the the dialysate is not in equilibrium with free faecal water. This conclusion concurs with results of an *in vitro* dialysis procedure in which it was shown that 18 - 24 hours was required for complete equilibration of dialysis fluid and faeces (104).

In another brief report comparing the composition of *in vivo* dialysates with ultracentrifuged supernatants significant difference were found for all analytes measured except sodium and potassium (105). However, the speed of ultracentrifugation (160,000g) may have caused some disruption to bacterial cells thereby altering the composition of the faecal fluid. Consequently the conclusions of this study are in question.

3. Ultrafiltration

The development of commercially available membrane filters has provided an alternative means of preparing stool fluid. This was initially evaluated by comparison with fluid obtained from dialysis bags after a 24 hour incubation with stool (104). The composition of faecal fluid by these two procedures was virtually identical except for diarrhoea samples when sodium and chloride were slightly (4%) lower in dialysate compared to ultrafiltrate. Several ultrafiltrates prepared from the same stool sample gave reproducible results (106). (This again contradicts *in vivo* dialysis results in which several dialysates from the same stool showed gross variations.) This system has been used to determine stool water characteristics in fifty healthy individuals (106).

4. *In vitro* dialysis

The preparation of stool water by this method is comparable to the *in vivo* technique except that dialysis bags are incubated for 24 hours in a refrigerated stool collection.

Results from this method were compared with those obtained using ultrafiltration (104) and high speed centrifugation (103). In both studies similar stool water compositions were found.

STOOL ELECTROLYTES AND OSMOLALITY

The main reason for developing a method of obtaining faecal fluid from normal stools is to have data on electrolyte composition for comparative purposes. At present five studies have been performed to provide this information but methodological problems probably invalidate all but one. In three, water or an aqueous solution was added to the stool to prepare the homogenate for centrifugation (98,99, 104) and another used the method of *in vivo* dialysis which is probably not a valid procedure and has certainly not been effectively evaluated. This leaves Costongs' data obtained from ultrafiltered stools (106) and these are shown in table 2.1 along with the figures obtained from the other studies mentioned. It is surprising that these results are broadly comparable to all of the other methods despite their inherent errors.

A faecal ionogram prepared using these data enables the composition of normal stool water to be visually assessed (figure 2.1). Potassium is the predominant cation in faecal water obtained from normal subjects, being present at about 80mmol/l concentrations as compared with sodium at about 20mmol/l. In patients with diarrhoea the concentrations of these cations varies. In general as the

Sample	Sodium (mmol/l)	Potassium (mmol/l)	Chloride (mmol/l)	Osmolality (mosm/Kg)
Ultrafiltrate (106)	17 (3 - 79)	80 (44 - 132)	11 (0 - 32)	308 (179 -472)
Dialysate (101)	32 (5 - 112)	75 (29 - 147)	16 (5 - 38)	376 (336 -423)
Supernatant (98)	36 (16 - 38)	99 (57 - 214)	nm	nm
Supernatant (99)	15 (15 - 79)	77 (44 - 131)	21 (5 - 31)	321 (254 - 464)
Supernatant (104)	19 sd = 2.4	79 sd = 7.1	12 sd = 1.4	324 sd = 16

Table 2.1 - Electrolytes in faecal fluids prepared by different methods. (Bracketed figures refer to ranges. nm - not measured.)

severity of diarrhoea increases the sodium and chloride concentrations rise while the potassium concentration falls slightly (figure 2.2) (107). This may be due to increased flow through the intestine resulting in incomplete sodium absorption and potassium secretion. Magnesium and calcium are also important faecal cations being dependent on dietary intake. Urea is the source of faecal ammonia, being digested by mucosal ureases or colonic bacterial enzymes. Ammonia exists either as NH_3 or NH_4^+ according to the equilibrium of the reaction:

$$NH_4^+ + HCO_3^- \rightleftharpoons H_2CO_3 + NH_3 \qquad (pK_a = 9.0)$$

Ammonium and bicarbonate concentrations and pH are interrelated: as pH falls, NH_3 is produced and then passively absorbed by mucosa; bicarbonate concentrations vary in proportion to those of ammonium (108).

It can be seen that the chloride and bicarbonate

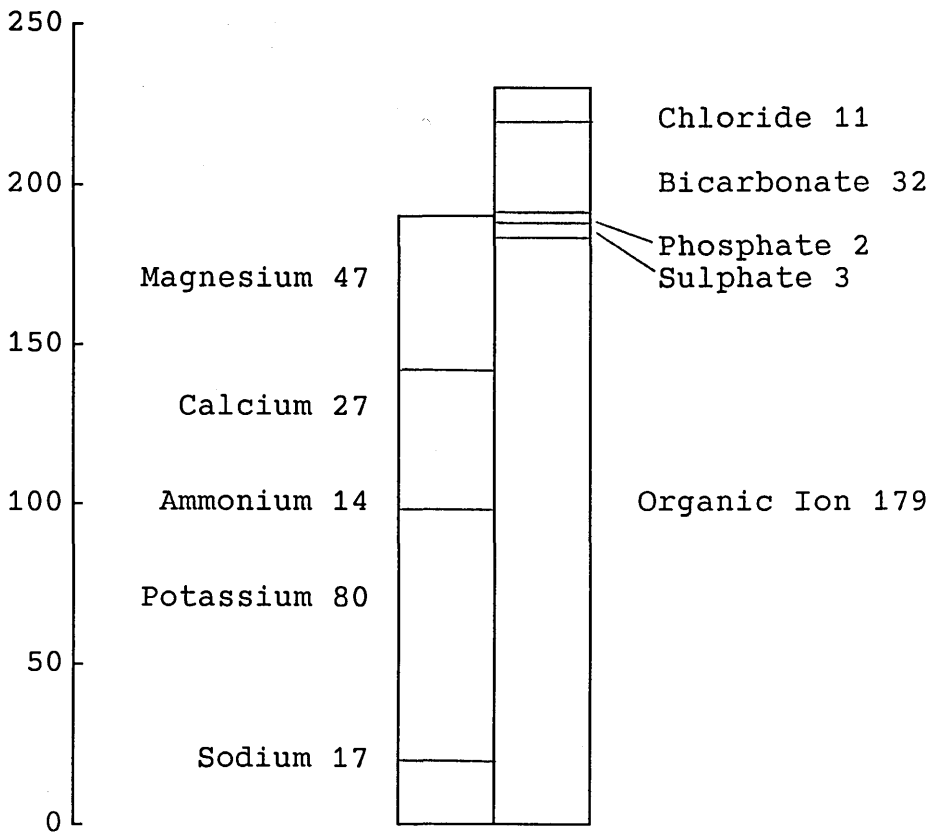


Figure 2.1 - Ionogram of Normal Faecal Fluid Derived from Mean Concentrations.

(Units in mmol/l)

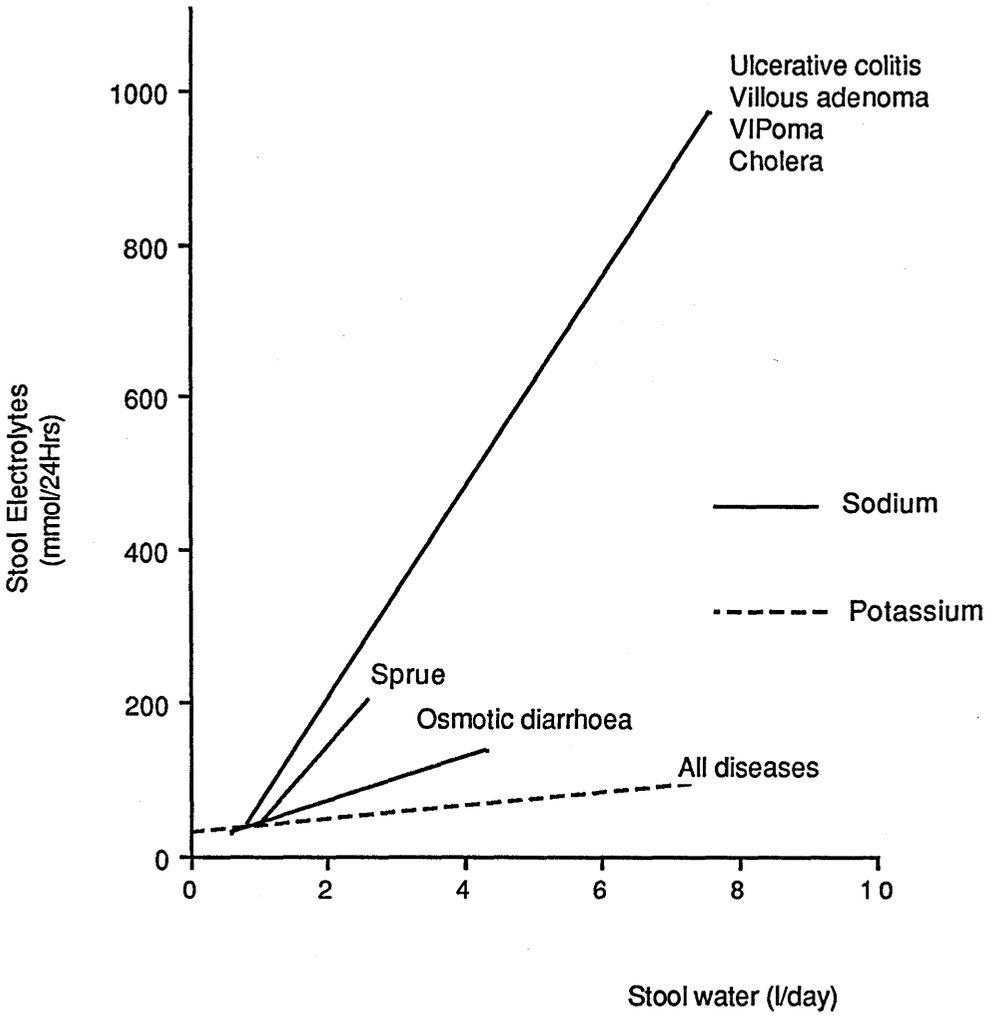


Figure 2.2 - Relationship between stool water and cations in different diarrhoea diseases (107).

concentrations only account for a fraction of the total anions. The 'missing' anions are the SCFAs, principally propionate, acetate, and butyrate. Sulphate and phosphate are minor electrolytes whose concentrations are dependent on diet.

The measured stool osmolality is usually hypertonic to plasma and tends to increase if a stool sample is left at room temperature, because of continuing bacterial degradation of carbohydrates. For this reason, in most studies faeces are refrigerated or frozen until analysis. One study using immediately processed samples has reported isotonic or minimally hypertonic results (285 - 330 mosmol/Kg) and suggest that results higher than this are due to improper storage (109). Interestingly this study measured osmolality by a decrease in vapour pressure rather than freezing point depression, which was the method used in other studies.

DIFFERENTIATION OF SECRETORY AND OSMOTIC DIARRHOEA - FAECAL OSMOTIC GAP

Chronic diarrhoea may have many different causes and the diagnosis can be difficult to make. In such circumstances an initial differentiation between secretory and osmotic diarrhoea would be useful. Such a classification is thought to be possible by monitoring the effect of fasting on the diarrhoea. Osmotic diarrhoea is caused ultimately by dietary factors such as milk in lactose intolerance or magnesium salts in saline laxative abuse. It is apparent therefore that fasting should resolve this type of

diarrhoea but should have no effect on secretory causes. Although the effect of fasting has been widely suggested as a useful preliminary test it is not foolproof: when patients are admitted to hospital, their diarrhoea tends to resolve irrespective of the cause; osmotic diarrhoea induced by saline laxative ingestion may continue during fasting (110, 109); and secretory diarrhoea caused by bile acids would not be expected to persist during fasting because of minimal bile acid release from the gall bladder. During the investigative work-up of 27 patients with chronic diarrhoea of unknown cause, the effect of fasting was found to be of no diagnostic value (111).

An alternative means of differentiating osmotic and secretory diarrhoea is to measure the osmotic gap (OG) in stool water prepared by an appropriate method. Sodium and potassium are the predominant cations present in faecal water in secretory diarrhoea and so by summing the sodium and potassium concentrations and doubling this figure (to account for anions) a calculated approximation can be made of its osmolality. When the diarrhoea is osmotic in nature the calculated figure is lower than the measured osmolality. Consequently, measuring the OG as shown in equation 1 (OG1) may be of value in the differential diagnosis of secretory and osmotic diarrhoeas.

Equation 1: Osmotic Gap (OG1) = Osmolality - $[2x(Na^+ + K^+)]$

One problem in measuring the OG is that stool osmolality tends to increase quite rapidly as any unabsorbed

carbohydrate is metabolised by faecal bacteria. It is thought that this may even occur in samples that are immediately refrigerated (112). As a result the OG1 may be overestimated. A possible solution to this problem is to subtract the calculated osmolality from the osmolality of plasma (equation 2). Since the colon has no capacity to concentrate or dilute, stool water should theoretically be isotonic in the absence of substrates such as carbohydrates for bacterial metabolism. The use of equation 2 to calculate OG (OG2) would seem to be a logical alternative especially since the use of antibiotics to prevent bacterial action *in vivo*, results in stool water that is isotonic (109, 113).

Equation 2: Osmotic Gap (OG2) =

$$\text{Plasma Osmolality} - [2x(Na^+ + K^+)]$$

The use of the osmotic gap has been pioneered by Fordtrans' group (114) who initially suggested using equation 1 for its calculation but subsequently favoured equation 2. Both methods of calculation have been used and several cut-off points have been suggested as shown in table 2.2. There is

Reference	Equation	Osmotic Gap	
		Osmotic Diarrhoea	Secretory Diarrhoea
111	1	> 100	-
115	1	> 50	< 40
109	1	> 160	< 0
112	2	> 50	-
116	1	> 40	0
117	1	> 15	-

Table 2.2. - Osmotic gaps suggested for distinguishing osmotic and secretory diarrhoea.

clearly a sizeable disparity in the various OGs suggested as cut-off points between secretory and osmotic diarrhoea.

There is currently some doubt as to the value of measuring the OG. It has been suggested that the osmotic gap may be of clinical value in the differential diagnosis of diarrhoea (109, 112) but only one evaluatory study has been carried out (118). In this study, diarrhoea was initially categorised as being secretory or osmotic on the basis of OG1 results. This classification was then compared with two alternative systems for distinguishing osmotic and secretory diarrhoea; the response of diarrhoea to fasting, and a classification based on current assumptions regarding pathogenesis of diarrhoea (128). However, neither of the two comparative methods can be considered as being definitive. Disadvantages of monitoring the response of diarrhoea to fasting have already been mentioned (page 66), while the theoretical classification according to current knowledge is over-simplistic, based on unjustifiable assumptions, and allows no differentiation into a mixed osmotic/secretory category.

* * * * * *

There is little information as to which electrolytes should be measured in order to reliably measure the OG. In plasma samples the OG can be calculated simply by doubling the concentrations of sodium and potassium. Because other cations and neutral solutes are present in such small quantities they do not require to be taken into account.

Whether the same is true with respect to stool water is open to question. All studies so far have considered only the sodium and potassium concentrations in computing the OG. However, as indicated previously (figure 2.1, page 63) magnesium, calcium and ammonium are also present in significant concentrations.

USEFULNESS OF FAECAL WATER POTASSIUM/SODIUM RATIO

In the luminal contents of the terminal ileum, sodium and potassium concentrations are close to those found in the plasma and the potassium/sodium ratio (K/Na) is relatively constant at about 0.06. The role of the colon in preserving sodium and secreting potassium, results in much higher K/Na ratios from 1.6 - 6 in stool water (99). It has been suggested that the measurement of this ratio may provide information as to the region of the gut predominantly responsible for diarrhoea - ileum or colon (117). In colonic disorders the ratio would theoretically be low and the converse would hold with ileal disease. For example in patients with a total colectomy the ratio was found to be low (0.05 - 0.1) but was 0.94 and 3.6 when 50% and 29% respectively of the colon was resected (119). Similarly, in patients with extensive ulcerative colitis the ratio was low (mean = 0.55, sd = 0.36) but when the disease was confined to the rectosigmoid area the ratio was nearer normal (mean = 3.64, sd = 2.2) (99). Apart from small incidental studies of this nature the usefulness of K/Na ratios has not been assessed.

OBJECTIVES

1. TO VALIDATE THE USE OF ULTRAFILTRATION MEMBRANES IN THE PREPARATION OF STOOL WATER.

The ultrafiltration method for the preparation of stool water has only been validated by comparison with an *in vitro* dialysis method (104). Unfortunately the latter method itself has not been evaluated, indeed the only approved method is by centrifugation. It was intended to compare the results of the ultracentrifugation and ultrafiltration methods using samples obtained from patients with loose or poorly formed bowel motions.

2. TO COMPARE FAECAL WATER OSMOLALITIES MEASURED BY FREEZING POINT DEPRESSION AND VAPOUR PRESSURE ELEVATION.

One study (109) has reported average stool osmolalities which are considerably lower than other studies. There were two notable differences in the way this group processed the results: osmolalities were measured by vapour pressure lowering rather than by freezing point depression; and samples were processed immediately after collection rather than being frozen.

In order to determine whether the different methodology for measuring osmolality caused this, several faecal fluids were assayed by both methods and the results compared.

3. TO DETERMINE THE EFFECT OF FREEZING STOOL COLLECTIONS PRIOR TO ANALYSIS.

As a follow-up to the previous objective, stool samples obtained from several patients with diarrhoea were aliquotted into two samples, one of which was analysed immediately and the other was initially frozen. Electrolytes, pH and osmolality were measured in each and the results compared.

4. TO DETERMINE WHICH EQUATION IS MOST APPROPRIATE IN CALCULATING THE FAECAL OSMOTIC GAP.

The main reasons for using equation 2 (page 67) when calculating the faecal osmotic gap are because of rapid changes in faecal osmolality even when samples are immediately frozen, and because equation 1 does not produce a result which correlates with the degree of diarrhoea. The appropriateness of each equation to calculate the osmotic gap was investigated by a) monitoring of the rate of change of osmolality in faecal homogenate and the effect of temperature on any change; b) calculating the osmotic gap by each method and comparing the results with the severity of osmotic diarrhoea; c) by comparing the intra-individual variabilities of OG1 and OG2.

5. TO DETERMINE THE CLINICAL VALUE OF THE FAECAL OSMOTIC GAP.

Before faecal osmotic gaps can be meaningfully used, an appropriate reference range must first be established, based on results from patients known to have a pure secretory or pure osmotic diarrhoea. Patients in these two

categories were recruited and the ranges calculated were used as reference points for further evaluation of the use of OG. Unfortunately there are few candidates who have purely osmotic or secretory diarrhoeas. Examples of a pure osmotic diarrhoea are found in patients with saline laxative induced diarrhoea or specific carbohydrate malabsorption such as lactose intolerance. As examples of pure secretory diarrhoea, patients with various infectious and hormone-induced diarrhoeas, and colonic stimulant laxatives abusers, were recruited.

To be of value the osmotic gap should be reproducible within patients with diarrhoea. For this reason the intra-individual variability of the osmotic gap in patients with diarrhoea was also measured.

The benefit of OG was also assessed retrospectively in patients presenting with diarrhoea.

6. TO DETERMINE WHETHER CATIONS OTHER THAN SODIUM AND POTASSIUM CONTRIBUTE SIGNIFICANTLY TO THE OSMOTIC GAP.

When calculating the faecal OG it has been assumed that only sodium and potassium concentrations require to be considered. That is, the contribution of other cations to the osmotic gap is believed to be relatively unimportant because they are present in small and constant concentrations. However, their concentrations have only been measured in the faecal fluid from healthy subjects. For this reason the relative importance of these previously neglected cations, such as calcium, magnesium and ammonium,

in calculating OGs was considered in patients with diarrhoea.

7. TO EVALUATE POTASSIUM/SODIUM RATIOS IN DETERMINING WHETHER DIARRHOEA IS PREDOMINANTLY ILEAL OR COLONIC.

Although the use of potassium/sodium ratios has been proposed as a useful preliminary investigation in order to distinguish ileal and colonic causes of diarrhoea (117), no evaluatory work has been reported. The ideal means of achieving this is by recruiting patients with diarrhoea known to be either ileal or colonic in nature and determining appropriate reference ranges. Examples of colonic causes of diarrhoea are ulcerative colitis, Crohn's disease limited to the colon, bile acid induced diarrheoa and infectious diarrhoeas such as those caused by *Shigella* or *Campylobacter* or acute *Eschericia coli* (120). Unfortunately diarrhoeal diseases associated with small bowel pathophysiology, such as *Vibro cholerae, Escherichia coli,* and *Giardia lamblia* are rare in this country. Therefore patients with small bowel causes of diarrhoea were limited to those with *Salmonella,* in whom the infection principally affects the ileum (120). Two other comparative groups were recruited; patients with a probable mixed ileal/colonic diarrhoea, and those with bile acid induced diarrhoea.

PATIENTS, MATERIALS AND METHODS

PATIENTS

Stool samples were collected from three categories of patients: inpatients and outpatients under investigation of diarrhoea; patients admitted to the Infectious Diseases wards of Monklands District General Hospital and Ruchill Hospital for investigation of suspected infectious diarrhoea; healthy volunteers in whom diarrhoea was induced by means of administration of saline laxatives (see pages 149 - 150).

METHODS

Patients were asked to defaecate, avoiding urinary contamination, directly into 2.5l plastic containers and to immediately transfer the container to a -20°C freezer. (A portable freezer was delivered to the homes of outpatients and volunteers for this purpose.) In the case of watery samples collected in Infectious Diseases wards, the patients defaecated into papier-mâché disposable trays and a nurse transferred an aliquot by means of a syringe into a 25ml universal container. The nurses were instructed to immediately place the sample into the portable freezer or into an insulated box or thermos flask containing dry ice.

Stool samples thus collected were semi-thawed and homogenised without the addition of water. Aliquots were transferred to appropriate tubes, weighed, and centrifuged

at 12,000rpm for one hour. The supernatant was decanted into a stainless steel filter unit and filtered through paper prefilter discs (Millipore Ltd, England). The centrifuge tube was reweighed and the weights of supernatant and sediment were calculated. The degree of liquidity of stool was expressed as the percentage by weight of supernatant compared to the total homogenate centrifuged (PWC).

PWC = (Weight of superantant) x 100/(Weight of Homogenate)

Ultrafiltrates were obtained by transferring a weighed aliquot into Centriflo membrane filter cones (Centriflo CF50A filters from Amicon Ltd, Stonehouse, Gloucestershire) and centrifuging at 1,000rpm for 30 minutes. A value equivalent to the PWC and coined the 'centrifugated water content' by the group who described this method of faecal fluid preparation (106) was also calculated by dividing the volume of the faecal fluid by the weight of the original faecal sample.

An HPLC method was established for the measurement of anions. By this method, anions in the injected sample displace light-absorbing counter anions, phthalate from potassium hydrogen phthalate (KHP, from BDH), from their attachment to an ion-pairing reagent, tetrabutylammonium hydroxide (TBAH, Sigma Chemical Co, Poole). Each anion-TBAH complex is separated on an ODS column and is measured as a negative absorption peak at 270nm. Using pure standards of bicarbonate and chloride linear responses were

produced up to concentrations of 1.5mmol/l. Stool supernatant was diluted 25 fold in distilled water and also injected onto the column, but many peaks were detected around the retention times for the anions of interest. Initial decolourisation of the samples by the addition of 500mg of activated charcoal effected some improvement, but there was still too much contamination to be able to measure the anions reliably. Standard techniques were subsequently used for the measurement of anions.

The various faecal analytes were measured as follows: sodium and potassium by flame emission spectrophotometry; chloride by electrometric titration with silver ions; calcium and magnesium by dilution and atomic absorption spectrophotometry (see page 144 - 146); osmolality by freezing point depression (Advanced Digimatic Osmometer Model 3DII from Advanced Instruments Inc.) and by vapour pressure elevation (5100C Osmometer form Wescor Inc.); ammonium by enzymatic assay (see below) (170-UV kit from Sigma Chemical Co, Poole, Dorset).

INACTIVATION OF PATHOGENIC BACTERIA IN STOOL

Since some of the stool samples analysed were obtained from patients with infectious diarrhoea, it was considered prudent to inactivate any pathogens prior to analysis. Two bacteriocidal methods were tested; pasteurisation (heating at 60°C for 30 minutes) and chemical treatment with 2% glutaraldehyde (ASEP from Galen Ltd, Craigallan, Northern Ireland). Each method was initially assessed using

supernatants from patients with non-infectious diarrhoea: bacteriology was performed on the treated specimen to determine how effectively bacteria were destroyed; electrolytes were measured in treated and untreated duplicate samples to determine whether results could be calculated accurately.

Both methods of bacteriocide were effective in destroying aerobic bacteria but in pasteurised samples the osmolality rose sharply, the pH fell, and the precision of electrolyte measurements deteriorated significantly.

The effect on the electrolyte content of ASEP was corrected for in ASEP-diluted supernatants (0.5ml of ASEP to 2ml of supernatant) by the following equation.

$$[analyte]_s = ([analyte]_{as} \times 5) - [analyte]_{asep})/4$$

where $[analyte]_s$, $[analyte]_{as}$ and $[analyte]_{asep}$ are the concentrations of analyte in the supernatant, ASEP-diluted supernatant, and ASEP respectively.

The correlations between the actual and calculated electrolyte concentrations were very good: r = 0.999, 0.999 and 0.998 for sodium, potassium and chloride respectively, but was poor for osmolality and pH.

Consequently electrolytes were measured using an ASEP-dilution of the supernatant but osmolality and pH were measured using the original supernatant. This was felt to be an acceptable procedure especially since sampling handling is minimal for the latter estimations and the

instrument could be easily decontaminated after analysis.

MEASUREMENT OF FAECAL AMMONIA

In general, the strong colour of faecal samples makes it difficult to measure analytes by means of spectrophotometric methods. For this reason an enzymatic method (121) using a commercial kit was used for the measurement of faecal ammonia. The method monitors the decrease in optical density caused by NADH (reduced nicotinamide-adenine dinucleotide) being consumed by the following reaction, catalysed by glutamate dehydrogenase (GLDH).

$$\text{2-ketoglutarate + Ammonia + NADH} \rightleftharpoons \text{Glutamate + NAD}$$

Briefly, to 3ml of NADH/buffer solution was added 0.2ml of sample (stool samples were diluted thirty-fold with 0.9% saline). After five minutes equilibration, the optical density was measured and then 0.02ml of GLDH was added to initiate the enzymic reaction. The absorbance fell, as NADH was consumed, until a final absorbance was reached after 5 - 10 minutes. The change in absorbance was proportional to the ammonia concentration.

When stool supernatants were used it was found that the absorbance fell gradually even before the GLDH was added, suggesting that NADH was being oxidised by other enzymes. In addition, with standards and controls the reaction reached completion within ten minutes, but when stool supernatants were used an end-point was not attained ie.

the optical density continued to fall. This could have been due to continued activity of urease and subsequent production of ammonia.

To avoid these problems a prior protein precipitation step was included to destroy any enzymes present. Three different precipitation methods were tested using sulphosalicylic acid (100g/l), methanol, and magnesium chloride (100mg/l). Stool supernatants were diluted 50:50 with each of the precipitants and then diluted fifteen-fold to reach a thirty-fold dilution in total. In the case of methanol and magnesium chloride, distilled water was used as diluent but with sulphosalicylic acid 0.2M phosphate buffer pH 7.4 was used in order to increase the pH to a suitable level for GLDH activity. A volume of 0.02ml was then added to the substrate/buffer solution and the ammonia method carried out as described above

With the use of methanol and magnesium chloride there was still a continual fall in absorbance before the GLDH was added and the reaction did not reach completion. The results using sulphosalicylic acid were successful in that an equilibrium was reached when NADH solution was added and the reaction reached completion after 5 - 10 minutes in the same way as for the standards and controls. This finding substantiated the supposition that the problems were caused by enzymes in the sample which consumed NADH and produced ammonia.

The method was modified to include this prior precipitation

step and recovery experiments were performed by spiking stool supernatants with 125µl of 100mmol/l ammonium chloride solution.

Because of the presence of urease in stool supernatants it was important to determine the stability of the samples. Accordingly, samples were divided into six aliquots of 1ml, and 0.05ml of 1mol/l hydrochloric acid was added to three in an attempt to stabilise the analyte as ammonium. Samples were stored at either room temperature, 4°C or -20°C.

The samples which were not treated with acid gave lower results suggesting that ammonia was lost. More surprisingly, the higher the temperature used for storage, the higher was the ammonia result. The probable explanation for this was that ammonia continued to be generated, presumably from urea or other nitrogenous substrates present in the stool sample.

A second stability experiment was then performed, with samples being diluted 50:50 with 100g/l sulphosalicylic acid to prevent enzymatic production of ammonia and then stored at either 4°C or 25°C. There was no significant change in ammonia concentration over a one week period when samples were stored at either 4°C or at 25°C. When further aliquots were measured after a month there was an increase in ammonia concentration at both temperatures.

A further experiment was performed in order to determine whether ammonia was sufficiently stable in stool samples

over the short period of time required for processing. A diarrhoea stool sample was aliquotted immediately after collection and one aliquot centrifuged immediately. The stool supernatant was diluted 50:50 with 100g/l sulphosalicylic acid. Other aliquots of stool were allowed to stand at room temperature for 30, 60, and 120 minutes before processing as above. One further aliquot was stored frozen and the following morning was thawed and processed as above. The ammonium concentrations were: 23.4, 23.4, 29.7 and 32.0mmol/l in samples processed at 0, 30, 60, and 120 minutes, and 15.8mmol/l in the frozen sample.

It was concluded that if stool samples are not processed within 30 minutes the ammonium concentration is overestimated. In addition, in stool samples that are frozen until analysis, the ammonia concentration is underestimated.

OSMOLALITY MEASUREMENTS

Osmolality results were compared on the same stool water samples by two methods, freezing point depression and vapour pressure lowering. The samples were analysed within ninety minutes of each other and were stored at 4°C to minimise any change in osmolality due to bacterial metabolism.

Recovery experiments were performed by measuring the percentage recovery in seventeen samples. A volume of 0.25ml of either 470mmol/l or 1264mmol/l sodium chloride solution was added to 1ml of faecal fluid. At these

concentrations the saline solutions have a fractional dissociation constant of 0.91, and so their calculated osmolalities were 850 and 2300mosmol/Kg, respectively.

RESULTS AND DISCUSSION

EVALUATION OF CENTRIFLO MEMBRANES IN THE PREPARATION OF STOOL WATER

The first step in any simple preliminary examination of stool for the purposes of investigation of diarrhoea, requires the preparation of faecal fluid. When the stool is watery there is little difficulty since a stool supernatant is readily prepared by centrifugation. Preparation of stool water from a semi-formed diarrhoeal sample is more difficult and there are pros and cons regarding existing techniques. As described earlier we concluded on the basis of existing literature that the *in vivo* dialysis procedure on which data from normal individuals is primarily based, is suspect. The most practicable method involves the use of commercially available membrane filters but unfortunately this method has not previously been evaluated. In the initial part of this study an assessment was made of this approach.

The composition of stool water prepared by filtration through Centriflo membranes was compared with that obtained by centrifugation/filtration. (To avoid confusion the resultant fluids are termed filtrate and supernatant respectively.) A filtration step was also included in the centrifugation step to ensure that no cell debris was present. We had initially compared the composition of filtered and unfiltered supernatants and found them to be similar.

After a thirty-minute spin through the Centriflo membrane the contents of the cone were usually still moist and so the filtrate was removed and the centrifugation step was repeated for a further thirty minutes and a second filtrate collected. Thus two filtrates were obtained from most of the samples. The data were compared to determine whether the composition of supernatant and filtrates were similar. The results of this experiment are tabulated (table 2.3).

	Supernatant Mean ± sem	Filtrate 1 Mean ± sem	Filtrate 2 Mean ± sem
pH	7.34 ± 0.24*	7.44 ± 0.26^	7.88 ± 0.33
Osmolality	378 ± 26*	366 ± 27	383 ± 32
Sodium	55.5 ± 6.7	55.6 ± 6.7	54.3 ± 9.0
Potassium	58.8 ± 5.2	58.1 ± 5.2	59 ± 8.3
Chloride	33.9 ± 3.5	33.8 ± 3.4	40.4 ± 4.5

Table 2.3 - Comparison of stool water composition when prepared by centrifugation and Centriflo filtration. (* - $p < 0.05$ vs filtrate 1, ^ - $p < 0.05$ vs filtrate 2). (n = 21 for the first two groups but only 14 for filtrate 2 group, therefore means are not directly comparable.)

There were no statistically discernible differences in the concentrations of sodium, potassium and chloride between the filtrates and supernatants. However, the osmolality of filtrate 1 was significantly lower ($p = 0.04$) and pH significantly higher ($p = 0.02$) than in the supernatant, and the pH of the first filtrate was significantly lower than that of the second ($p = 0.02$) (See Statistical Appendix, note 1).

The correlations between each group were high as expected (Statistical Appendix, note 2) and are illustrated in figures 2.3 to 2.7 (pages 85 - 89).

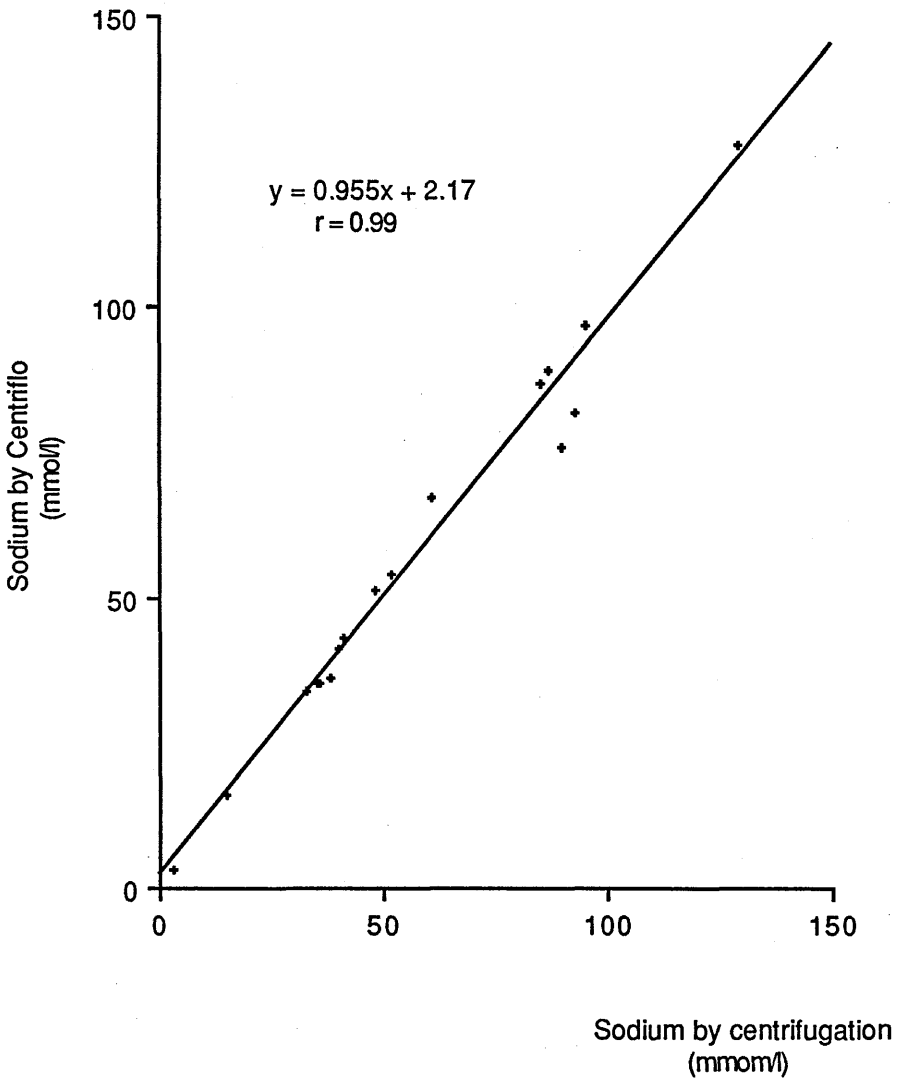


Figure 2.3 - Effect of method of stool water preparation on concentration of sodium.

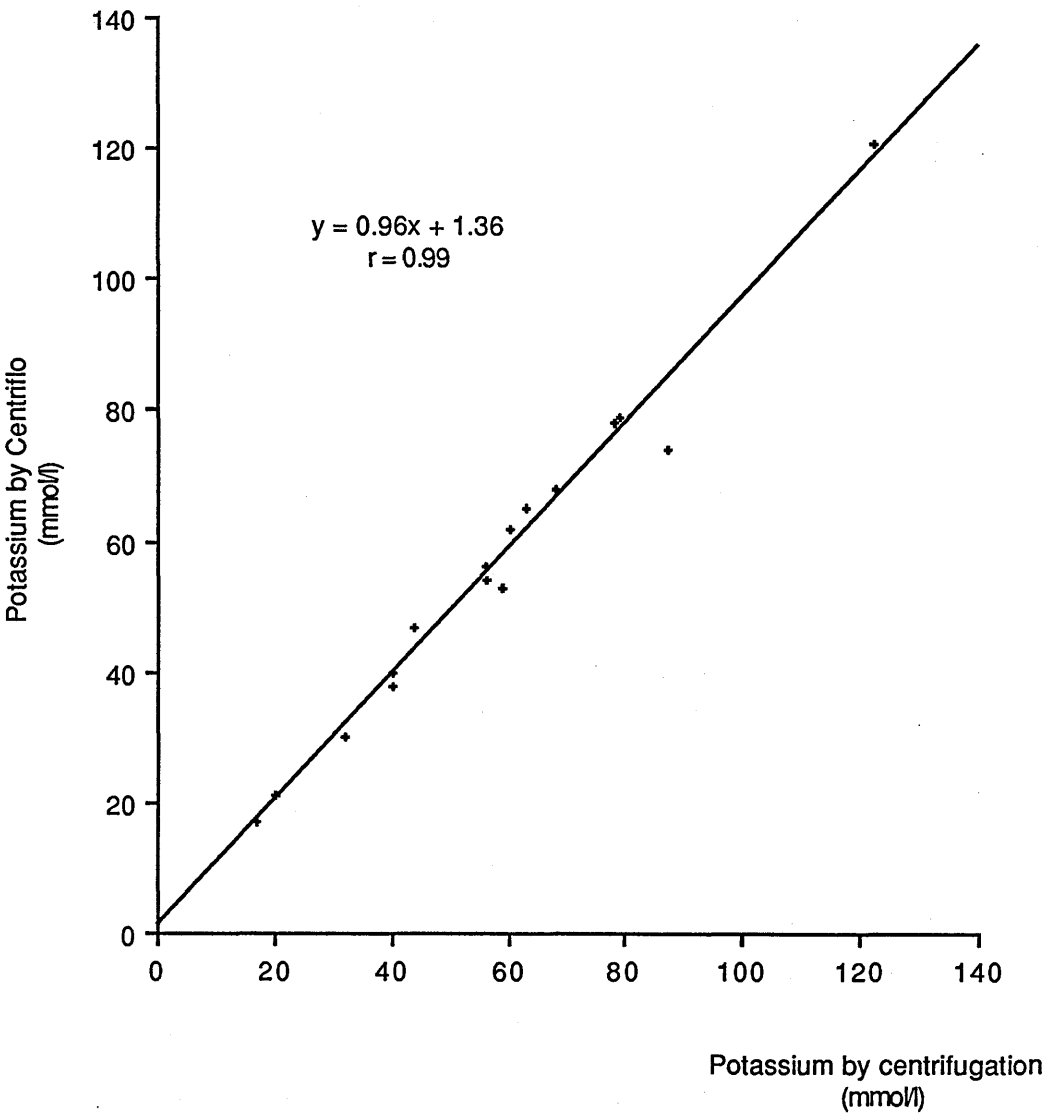


Figure 2.4 - Method of stool water preparation on concentration of potassium.

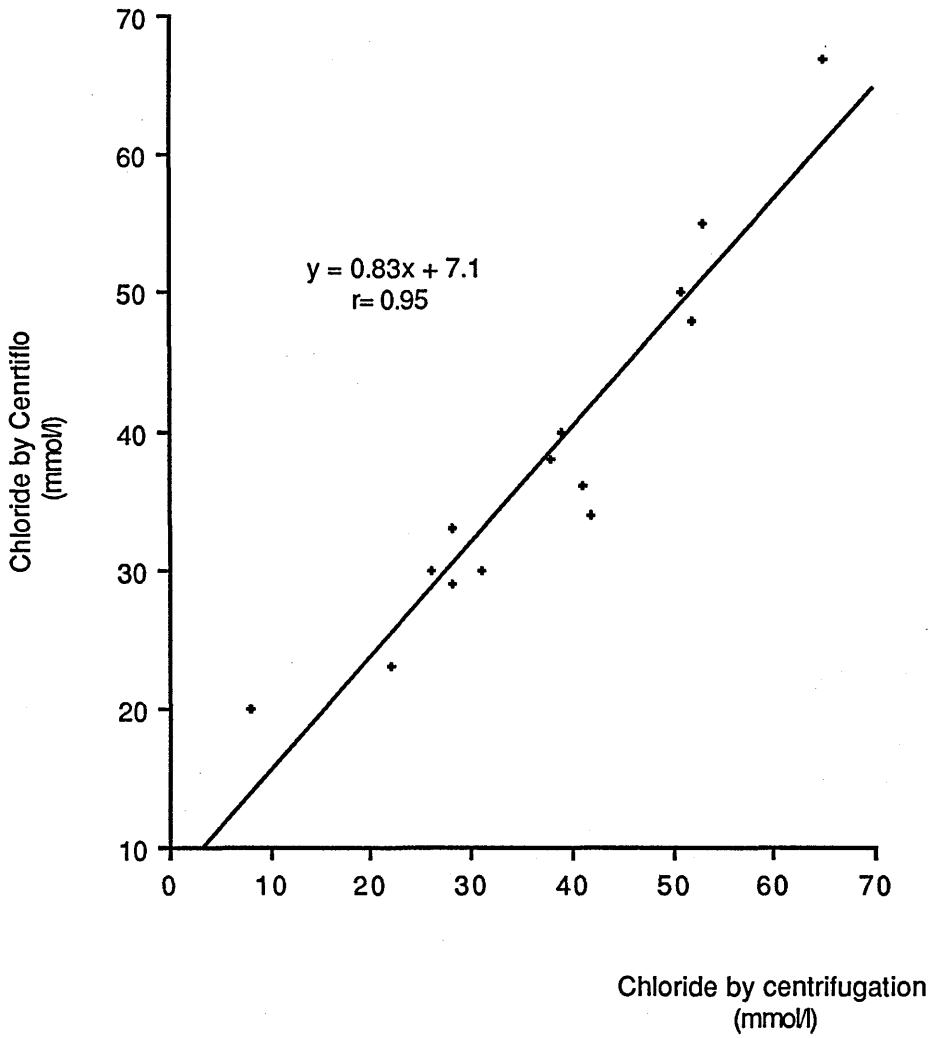


Figure 2.5 - Effect of stool water preparation on concentration of chloride.

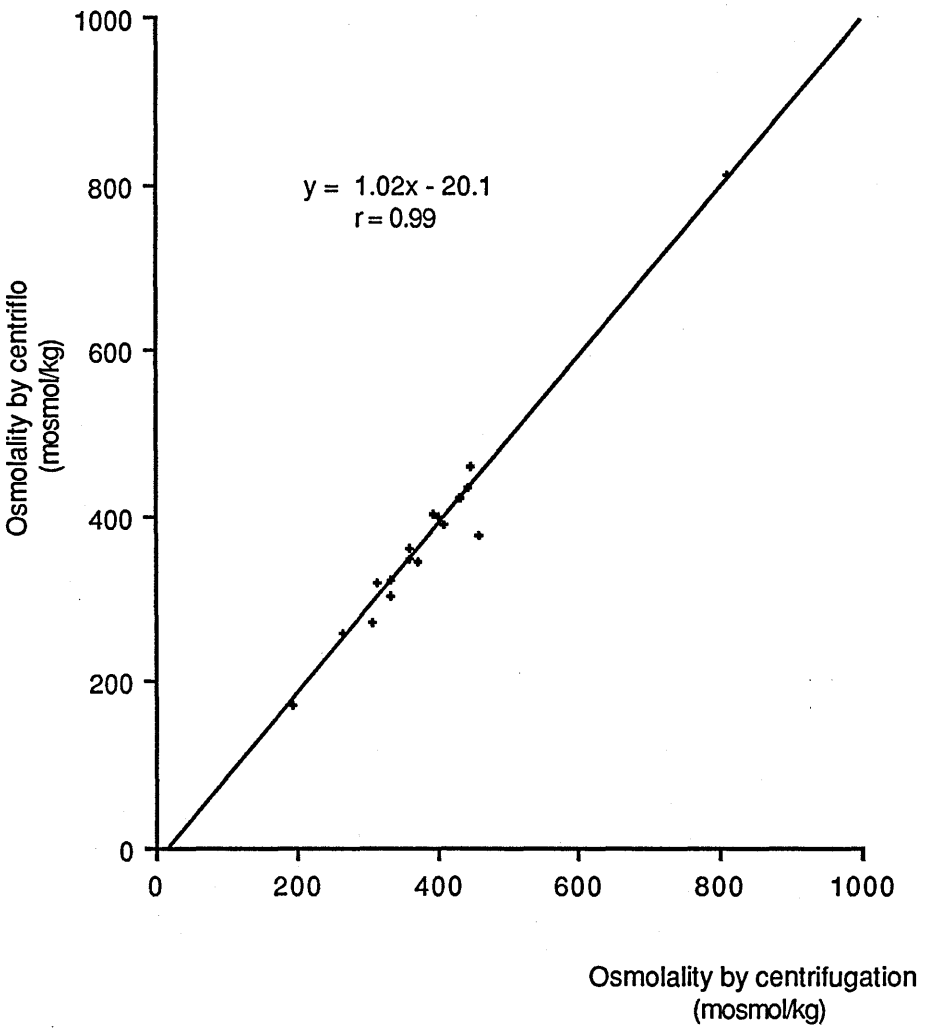


Figure 2.6 - Effect of stool water preparation on osmolality.

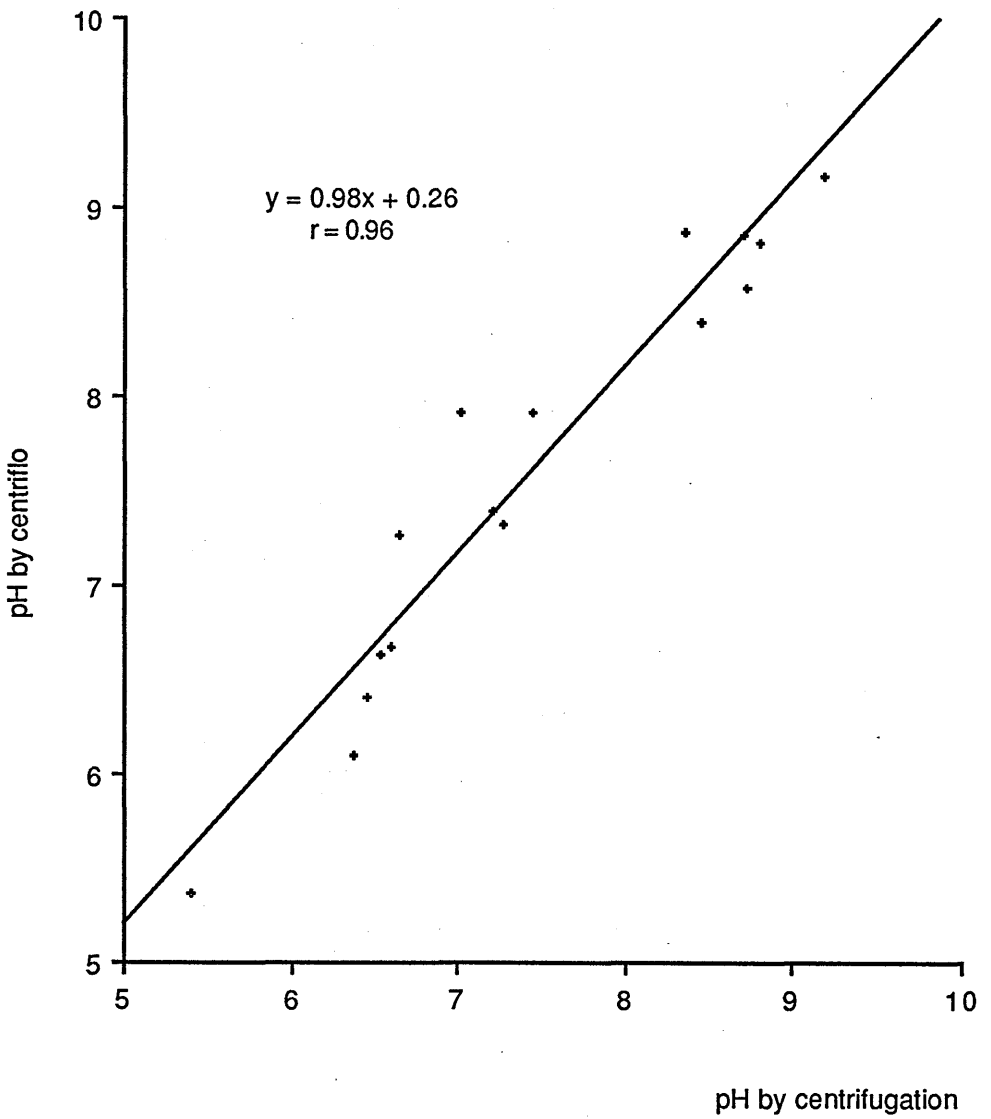


Figure 2.7 - Effect of method of stool water preparation on pH.

TECHNICAL FACTORS IN THE PREPARATION OF STOOL WATER

1. Rate of Change of Stool Composition

Some studies have demonstrated that the composition of faecal fluid varies if the stool is allowed to stand at room temperature for significant periods of time in excess of one day (103, 109). We investigated possible changes that may occur during the shorter time periods involved in sample preparation. Accordingly, several aliquots were made of three stools and these were then incubated at room temperature (approximately 24°C) for time periods of up to two hours.

Over the course of two hours there was no apparent change in the pH or concentrations of sodium, potassium and chloride but the osmolality rose by an average of 11.5mosmol/kg per hour (figure 2.8).

Although there was a definite rise in osmolality over short periods of time this was relatively small and was therefore considered unlikely to affect results in the time normally taken to process samples.

We also incubated stools at lower temperatures and confirmed previous findings (103, 109, 118) that stools could be stored refrigerated (4°C) for two days with no significant alterations in the composition of the resultant faecal fluid.

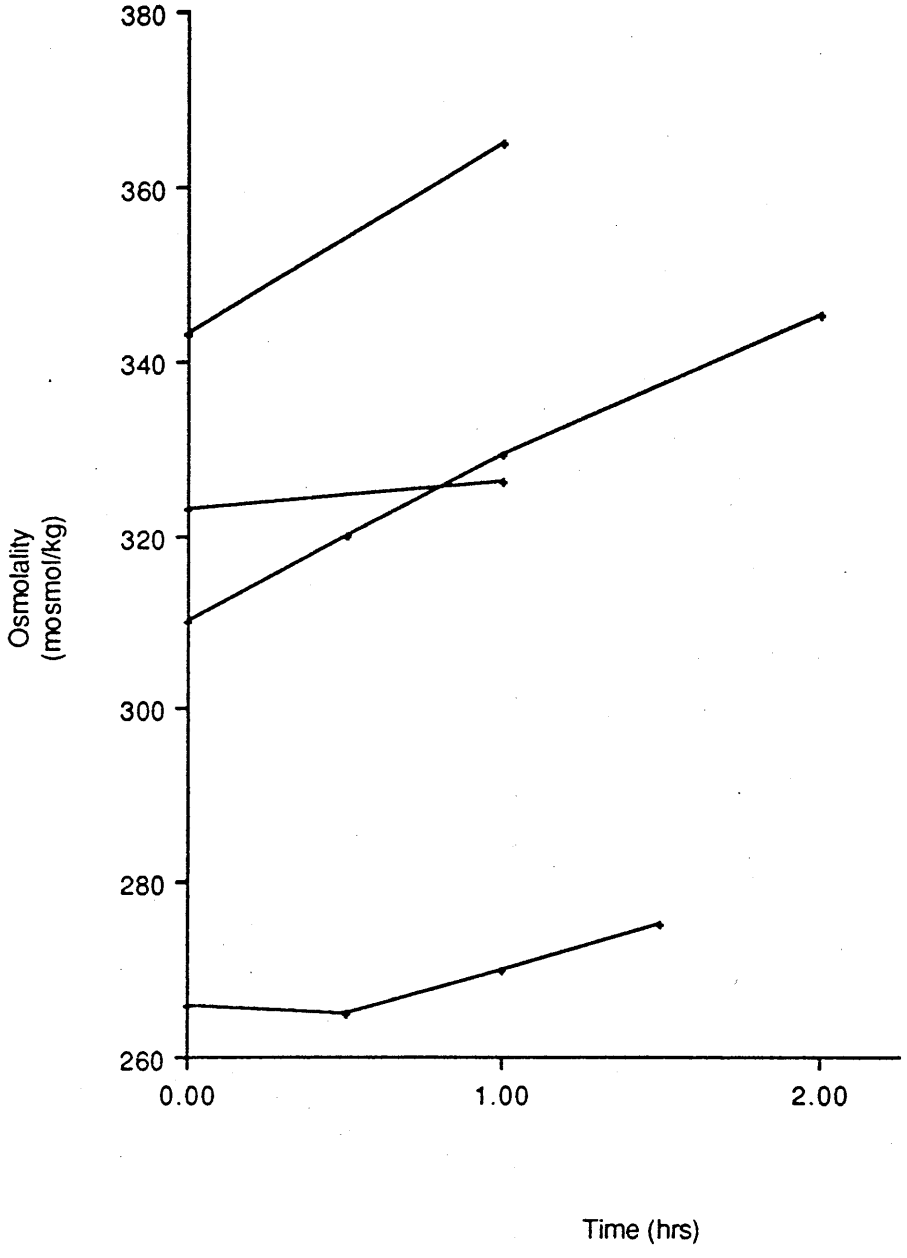


Figure 2.8 - Rate of change of stool osmolality incubated at room temperature.

When four stool samples were incubated at room temperature over a two day period of time, the change in osmolality was marked, increasing by an average of 128mosmol/kg/day. This concurs with a previous study which also demonstrated significant increases in osmolality over extended time periods (109). These changes in osmolality are caused by metabolic processes catalysed by faecal bacteria. For example Shiau *et al* showed that when stools were collected from patients receiving antibiotics, there was no change in osmolality when faeces were incubated for a two day period at 25°C (109). *In vivo* dialysates of faeces were also shown to be significantly lower (304 vs 357mosmol/kg) when normal subjects ingested antibiotics (122).

This latter study also recorded decreased ammonia and increased urea concentrations in dialysates obtained from subjects taking antibiotics, suggesting that bacteria normally metabolise urea with the production of ammonia.

We also found evidence to suggest that stool pH tends to decrease with time although this was not as marked as the drop in osmolality. When stools were left for two days at room temperature the pH of the resultant faecal fluid fell by approximately 0.15/day. This agrees with a previous study which documents a decrease in pH of 0.35/day in stools kept at room temperature (118). This probably reflects increased production of SCFAs by bacterial action.

2. Effect of Freezing on Stool Composition

For reasons of practicability it was more convenient to store stools so that they could be processed at a suitable time. Consequently, they were initially frozen to limit any changes in composition. However, subsequent thawing of stools could, depending on the size of the collection, take several hours. When Shiau *et al* processed samples immediately, they found considerably lower osmolalities than had previously been documented using samples that were frozen and thawed before preparation of stool water (109). For these reasons the effect of freezing and subsequent thawing on stool composition was studied.

Four stools were collected and aliquots taken for either immediate processing or freezing overnight before thawing and processing. Table 2.4 shows the results of this process, on the osmolality, pH, and electrolyte concentrations.

Analyte	Stool 1		Stool 2		Stool 3		Stool 4	
	I	F	I	F	I	F	I	F
Osmolality	310	280	418	431	343	360	265	266
pH	8.35	8.15	5.22	5.4	5.53	5.55	7.01	7.4
sodium	131	132	85	88	66	64	33	34
Potassium	34	39	60	50	30	27	56	52
Chloride	31	21	52	59	10	10	22	23

Table 2.4 - Comparison of analytes measured in stool water prepared immediately (I) and after freezing (F).

There was no apparent trend in any of the results although the reproducibility was poor for osmolality and pH. Therefore freezing and thawing of stools had little

apparent effect on the composition of faecal fluid.

In stools processed immediately the mean osmolality was 334mosmol/kg, but there was a large range in the osmolality results, from 265 to 418mosmol/kg. This would suggest that the cause of the lower osmolality of 295mosmol/kg (range of 285 - 330mosmol/kg) found by Shiau *et al* was not related to their practice of immediate processing of samples (109).

3. Measurement of Stool Osmolality

The study by Shiau *et al* (109), as well as processing samples immediately, also measured osmolality by a method monitoring vapour pressure lowering (VPL), rather than the more commonly employed technique of freezing point depression (FPD) used by ourselves and other workers.

As a preliminary experiment to determine if the two techniques of measuring osmolality, FPD and VPL, were producing different results fourteen stool water samples were analysed, in duplicate, by each method. Figure 2.9, which depicts the correlation achieved and the line of regression, shows that the results using the FPD method are, on average, 11.2% higher than the VPL method (see Statistical Appendix, note 2). The precision of each method was good: sd = 4.6mosmol/Kg, cv = 1.3% for FPD, and sd = 2.1mosmol/Kg, cv = 0.5% for VPL.

To determine whether this discrepancy was found only with stool samples, eighteen plasma or plasma/saline samples

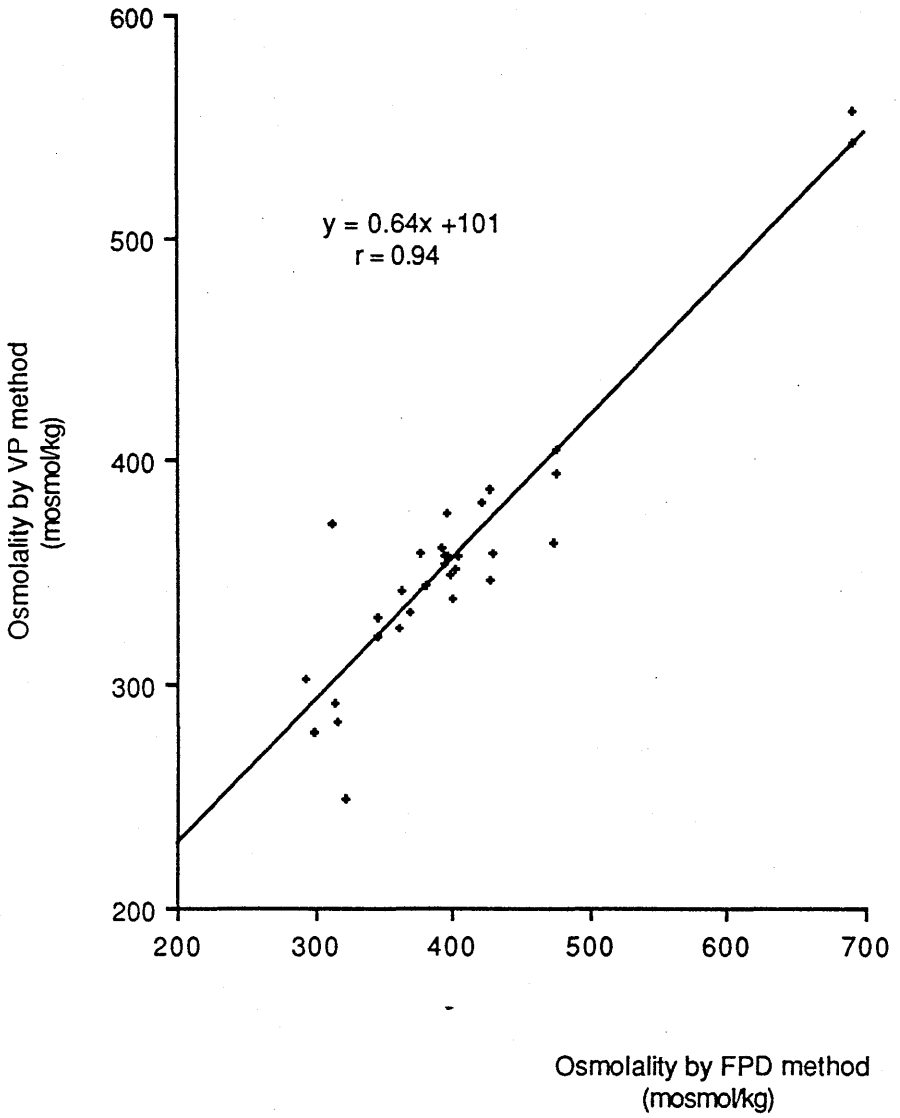


FIGURE 2.9 - Comparison of stool osmolality results measured by FPD and VPL methods.

were measured by each method. Since the osmolality of plasma is within a tightly defined reference range, some plasmas were spiked with saline in order to assess other ranges of osmolality.

There was a very close agreement between the two methods using these samples, with results from the FPD method higher by just 1%. This initially suggested that the error found with stool samples was due to a matrix effect by one of the methods.

In order to determine which of the two methods was inaccurate, recovery experiments were performed. However, this demonstrated that the accuracy of both methods was very high: 101.4% (sd = 2.03%) for the VPL method, compared with 99.6% (sd = 2.87%) for the FPD method.

Despite the relative bias between the two methods both were very reliable, giving excellent precision and accuracy when stool samples were used. One of the few ways of interpreting this seeming paradox is that the methods measure different osmotically active species. The reason for this became apparent when the variations in methodology were considered. The VPL method measures the depression of dew point temperature caused by particles in solution. However, the presence of volatile species in solution have little effect on this property. The reason for the difference between the two methods can therefore be explained by the presence of volatile substances in stool water, such as SCFAs, ammonium, and bicarbonate.

stool water, such as SCFAs, ammonium, and bicarbonate. Since the VPL method only measures non-volatile substances these components are not detected.

MEASUREMENT OF OSMOTIC GAP

Before considering the potential value of osmotic gap measurements a decision had to be made on the most appropriate way for its calculation, ie. the use of a measured stool osmolality or a figure of 290mosmol/kg (see page 67). We decided to opt for the latter measurement for the reasons quoted below.

a) As mentioned above, VPL methods underestimate the true osmolality because they do not account for the presence of volatile species. However, Shiau argues (123) that these components are not the result of altered intestinal electrolyte transport and so are irrelevant when considering the cause of diarrhoea. However, without knowledge of the extent and variability that VPL methods 'ignore' the various volatile components, this conclusion is very speculative.

A simple experiment was performed to investigate this assumption of Shiau, that the VPL method does not measure osmotically active species that are the result of bacterial metabolism. Six stool supernatants were aliquotted into pairs and then incubated for two days at either 24°C or 0°C. The osmolalities of samples were then measured by both methods. The osmolalities of the samples incubated at 24° should increase because of bacterial

metabolism. If Shiau's theory is correct then there should be no increase in osmolality measured by the VPL method. However, the results in table 2.5 demonstrate that there were significant increases in stool osmolality

	Increase in Osmolality (mosmol/kg/2days)	
Sample	VPL method	FPD method
1	16	26
2	106	159
3	38	59
4	14	16
5	21	36
6	13	22

Table 2.5 - Increase in stool osmolalities, measured by VPL and FPD methods, after a two day incubation at 24°C.

by both methods. The increase in osmolality was less for the VPL method (by an an average of 34%) suggesting that some of the products of metabolism were volatile in nature.

This experiment did not demonstrate whether the VPL method measures only a proportion of volatile substances, or whether bacterial action also produces non-volatile substances. However, since the experiment demonstrated that the VPL method is inappropriate for the determination of stool osmolality, this point is largely academic.

b) Krejs and Fordtran concluded on the basis of unpublished observations (112) that osmolality increased rapidly when a stool collection was made, even if a sample was processed immediately. However, the present study found that osmolality in fact only increases slowly with

time and insufficiently to account for the large stool osmolalities sometimes encountered. This would imply that the bacterial action that causes the production of more osmotically active species is occurring in the colonic lumen and that insufficient water is drawn into the lumen to normalise osmolality. This would make teleological sense since it would be inappropriate for the body to loose water solely for the purpose of maintaining iso-osmolality of the distal colonic contents and faeces which are of no physiological importance. It is also known that the rectosigmoid colon is capable of extracting small amounts of water from the faecal material. Therefore the longer that the faeces are stored here the higher the osmolality is likely to rise.

Shiau *et al* suggest that the distal colon in patients with diarrhoea has little opportunity for bacterial metabolism and that this explains the lower osmolality that they found in diarrhoeal stool water (109). However, since their VPL method of measuring osmolality does not 'see' all of the predominantly volatile products of bacterial metabolism, this conclusion is unfounded. In conflict with this assumption we found a high faecal fluid osmolality (418mosmol/kg) in one of our patients whose sample was processed immediately.

c) Two of the patients who were found to have a secretory cause of diarrhoea (see page 107) produced stools which gave negative OGs (-46 and -4) when a figure of 290 had been used for its estimation, but gave high figures of 64

and 153 when the stool osmolality had been used in the calculation of the osmotic gap.

d) If it is assumed that the mechanism of diarrhoea remains the same in individual patients, then it would be expected that the OG would not vary significantly from one stool to the next. We therefore compared the variability of the OG measured by each method in intra-individual stool collections from 34 patients. We found that the OG1 measurements varied significantly more than OG2 values ($p = 0.0004$) (see Statistical Appendix, note 3).

The reason for the greater variability in OG1 measurments is presumably due to varying degrees of changes in osmolality caused by differing activities of bacterial metabolic processes.

e) In cases of osmotic diarrhoea, it would be expected that a relationship between the severity of diarroea and OG would exist (112). We therefore measured the correlation of the severity of diarrhoea (expressed as PWC) with the OG1 and OG2 values in our patients with pure osmotic diarrhoea (see Statistical Appendix, note 2). The regression graphs (figures 2.10 and 2.11) illustrate clearly that the correlation of PWC with OG2 ($r = 0.81$) was considerably better than PWC with OG1 ($r = 0.25$) again suggesting the OG2 measurement to be superior.

f) For any test to have clinical applicability it must be practicable and potential sources of error must be controllable. During the course of this study we

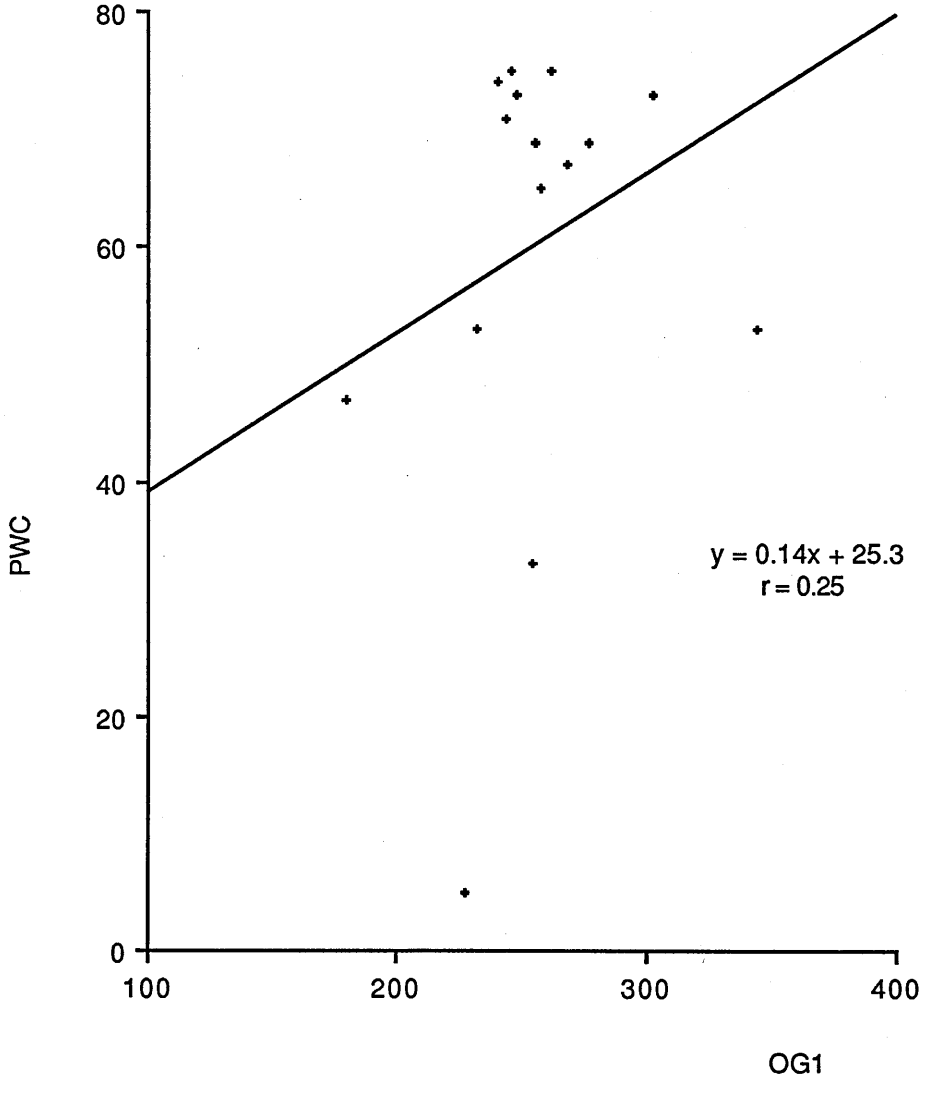


Figure 2.10 - Correlation of PWC with OG1.

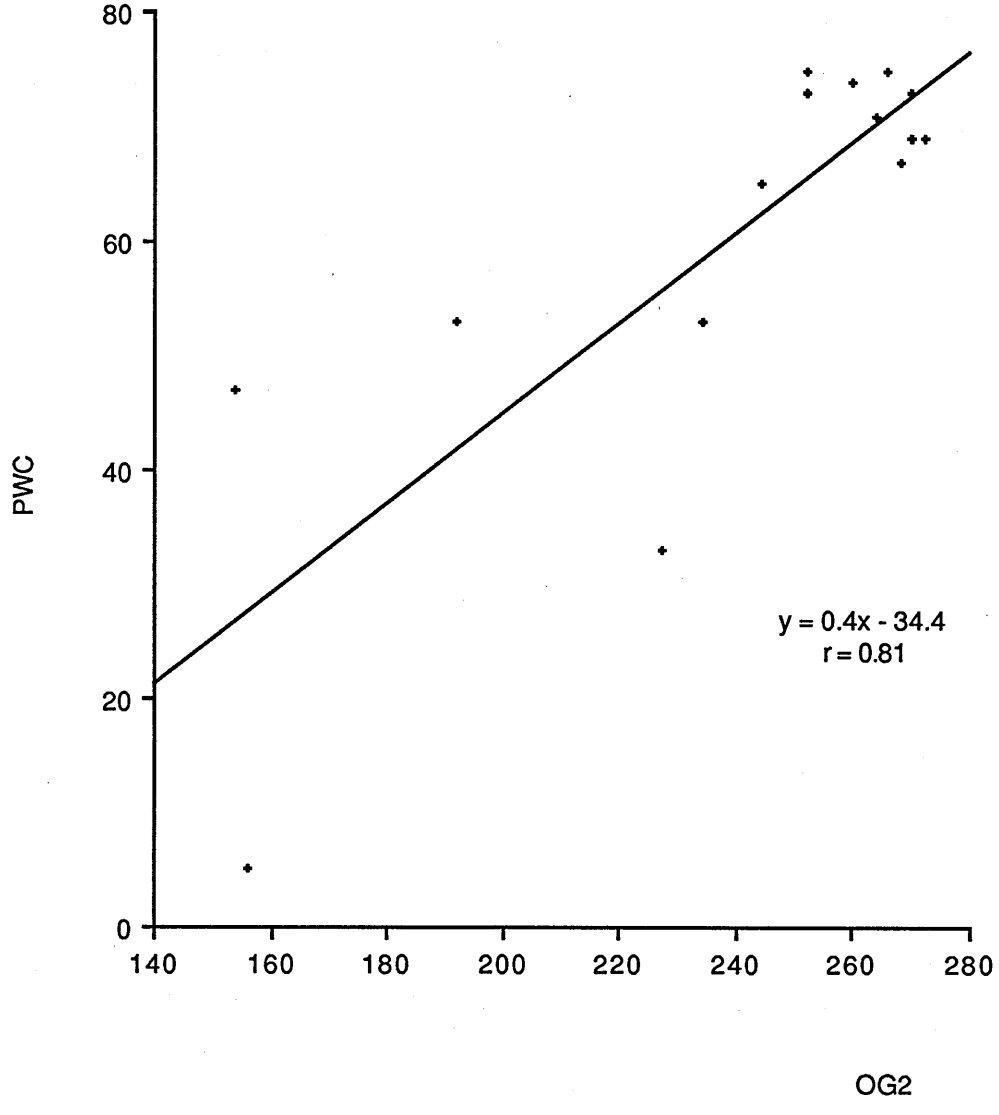


Figure 2.11 - Correlation of PWC with OG2.

experienced practical difficulties in freezing samples immediately after their collection. Since patients cannot defaecate 'on demand', co-operation in collecting and freezing stool samples was required. Nursing staff and/or patients were informed of the importance of freezing samples immediately and a portable freezer was conveniently located for this purpose. With out-patients, the freezer was delivered to the patients home and responsibility for immediate freezing of the sample, rested with the patient. With in-patients, nursing staff were often responsible for freezing the sample. In spite of the instructions given, some stool samples had very high osmolalities (sometimes in excess of 500mosmol/kg) because of uninhibited bacterial activity. Thus it was evident that stools were not always frozen immediately.

In this regard the use of OG1 to express the osmotic gap was concluded to be impracticable since samples were not always properly collected. Furthermore a procedure for assessing the quality of collection was difficult to implement.

* * * * * *

For these six reasons we based the following conclusions regarding the clinical value of osmotic gap measurements on the use of OG2 calculations.

Since only sodium and potassium, which are stable in stool homogenates (102, 109), require to be measured to calculate OG2, the opportunity arose of including OG2

results on stool samples that had previously been collected. It is routine practice in the Gastroenterology Laboratory, to store frozen aliquots of stool homogenates. It was possible therefore to calculate OG2 on those homogenates which had been prepared without the addition of water. To ensure that sodium and potassium results were accurate, seven stools which had known electrolyte concentrations were also thawed and re-analysed. The repeat results of sodium and potassium concentrations were in very close agreement to the original values which had been measured up to two years previously.

VALUE OF MEASURING THE OSMOTIC GAP

Before assessing the clinical usefulness of measuring OG2, reference ranges in secretory and osmotic diarrhoea were determined. These reference figures could only be determined using samples from patients with diarrhoeas known to be either secretory or osmotic in nature. Although the mechanisms of many diarrhoeal conditions are not fully established, certain pathologies are well recognised as causing secretory or osmotic diarrhoea (112) and so only patients with these conditions, which are listed in table 2.6, were recruited.

Over the course of the study five cases of pure secretory diarrhoea and nine cases of pure osmotic diarrhoea were recruited. Of the patients with secretory diarrhoea the diagnoses were tubular villous adenoma in two, abuse of phenolphthalein in two and carcinoid syndrome. All

Secretory Diarrhoea	Osmotic Diarrhoea
Hormonal VIPoma Carcinoid Medullary Carcinoma of thyroid Villous adenoma **Drug-Induced** Phenololphthalein Bisacodyl Anthraquinones Ricinoleic acid Cytotec **Infectious** *Cholera* *E. coli* **Idiopathic**	**Saline Laxative Induced** Magnesium sulphate Magnesium hydroxide **Carbohydrate malabsorption** Lactose intolerance Lactulose ingestion

Table 2.6 - Recognised causes of secretory and osmotic diarrhoea.

osmotic diarrhoeas were artificially induced, seven with magnesium hydroxide, and one each with magnesium sulphate and lactulose.

Fifteen stools were collected from the five patients with secretory diarrhoea and nineteen from the ten patients with osmotic diarrhoea. The summary statistics are documented in table 2.7.

Analyte	Osmotic Diarrhoea	Secretory Diarrhoea	p
Osmolality	311 ± 38.7	342 ± 59.5	ns
Sodium	8.3 ± 15.1	104.1 ± 18.8	$p < 0.0001$
Potassium	20.4 ± 16.3	48.8 ± 25.6	$p = 0.0005$
Chloride	18.3 ± 4.9	36.7 ± 10.2	$p = 0.0002$
OG 1	253.8 ± 40.6	6.8 ± 74.4	$p = 0.0001$
OG 2	233.9 ± 48.8	-15.7 ± 35.1	$p < 0.0001$

Table 2.7 - Comparison of analytes and osmotic gaps in osmotic and secretory diarrhoeas (mean ± sd).

There was no apparent statistically significant difference between the osmolalitites of the two groups, but the

sodium, potassium and chloride concentrations were all significantly higher in patients with secretory diarrhoea. As expected there was a highly significant difference in the OGs of the two groups whether these were calculated using equation 1 or 2 (ie. OG1 or OG2)

When OG1 results were plotted there was an overlap between patients with secretory and osmotic diarrhoea because of high osmotic gaps in two patients with secretory diarrhoea (figure 2.12). On both occasions the stool water osmolality was high (447 and 460mosmol/kg). In contrast, when OG2 results were plotted there was a clear discrimination between patients with osmotic diarrhoea and those with secretory diarrhoea (figure 2.12).

Using these data, provisional cut-off points were selected, an OG2 value of over 100mosmol/Kg being consistent with osmotic diarhoea and an OG2 result of less than 30mosmol/Kg suggestive of secretory diarrhoea.

The only other study in which OG2 results have been measured suggests that osmotic diarrhoea is associated with OG2 values of greater than 50mosmol/Kg, while OG2 results of less than 20 are disregarded (112). It is possible that with further experience our cut-off values of less than 30mosmol/Kg for secretory diarrhoea and greater than 100mosmol/Kg for osmotic diarrhoea, may be raised or lowered respectively. The range for secretory diarrhoea is also consistent with OG2 figures of -10 ± 5 (sem) that were calculated using data from a study in

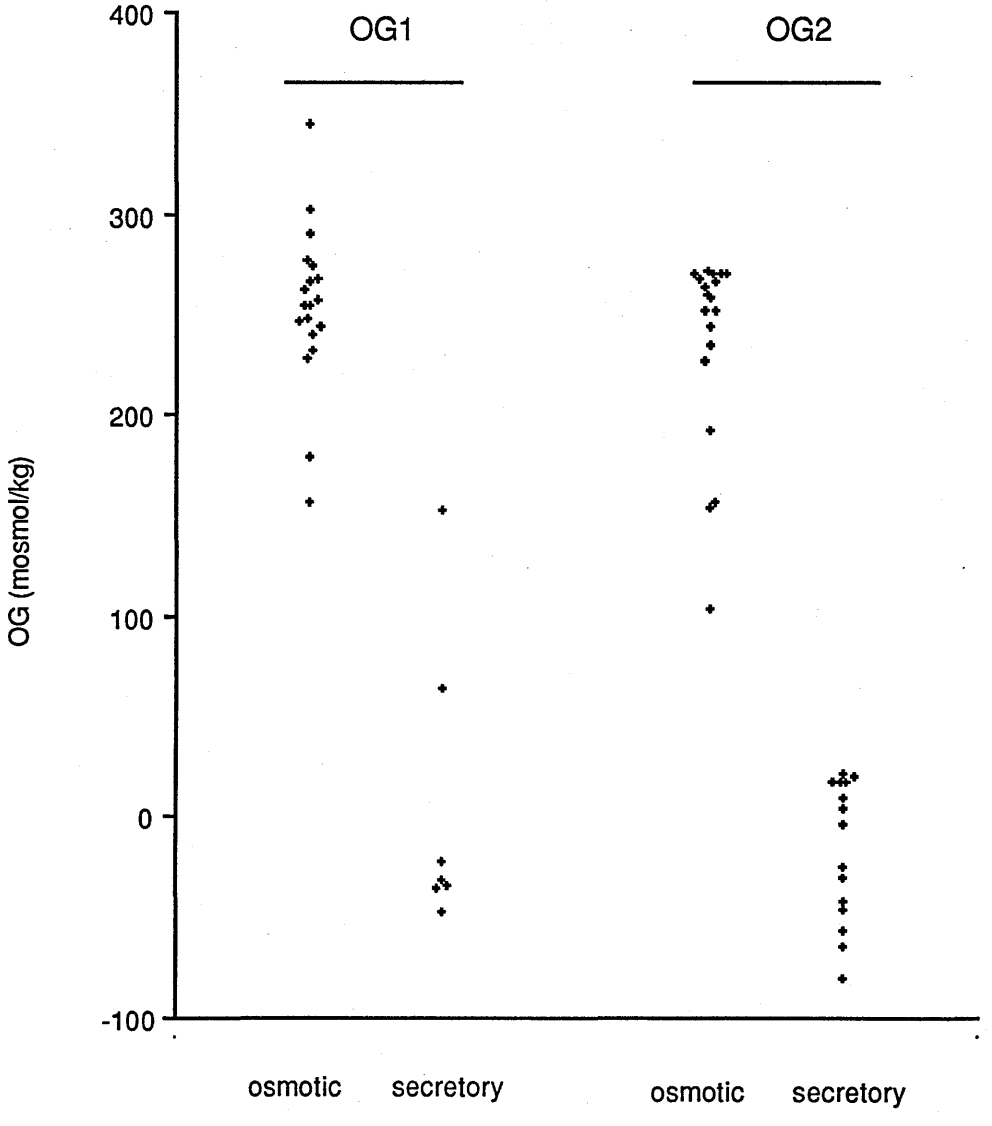


Figure 2.12 - Comparison of OG results in osmotic and secretory diarrhoea.

which secretory diarrhoea was induced in healthy subjects by VIP infusion (124).

Several of the OG2 results gave negative values. This seemingly anomalous finding is readily explained: simple doubling of the sum of potassium and sodium concentrations results in an overestimate because of incomplete ionisation of electrolytes, and the presence of divalent anions.

Before OG2 measurements can be employed usefully in a clinical setting, it is important that it is known which conditions give rise to osmotic diarrhoea and which cause secretory diarrhoea. At the present time the cause of diarrhoea in many different illnesses is not definitely known and is limited to the list compiled in table 2.6. Perusal of this list will show that most of these conditions are relatively uncommon in this country. It is clearly advantageous to know in what category other more commonly encountered diarrhoea states can be classified. For this reason we measured the OG2 in several groups of pathology; chronic pancreatitis, coeliac disease, small bowel Crohn's disease, infectious disease, post gastric surgery, bile acid induced diarrhoea, ulcerative colitis, Crohn's colitis, and alcohol induced diarrhoea. Figure 2.13 shows the OG2 results found in these groups.

The large range of OG2 results in most of the disease categories is probably indicative of the heterogeneity of each group and the multifactorial nature of its cause.

For example, the diarrhoea of Crohn's disease may be attributable to a variety of mechanisms, for example exudation, active disease, carbohydrate malabsorption, malabsorption of bile acids or fatty acids etc.

Figure 2.13 also demonstrates that within each pathology group, diarrhoea was associated with OG2 values in the secretory (< 30mosmol/kg) and osmotic (> 100mosmol/kg) range. Table 2.8 lists the various pathologies associated with secretory and osmotic OG2 results encountered during this research.

Secretory Diarrhoea	Osmotic Diarrhoea
Phenolphthalein ingestion Irritable bowel syndrome Seminoma Systemic lupus Alcohol abuse *Campylobacter enteritis* Small bowel overgrowth Villous adenoma Carcinoid syndrome Chronic pancreatitis Coeliac disease Small bowel Crohn's disease *Salmonella* Post vagotomy diarrhoea Ulcerative colitis Radiation enteritis Chronic pancreatitis Bile acid induced diarrhoea Crohn's colitis	Coeliac disease Irritable bowel syndrome Small bowel Crohn's disease Dumping syndrome *Salmonella* Insulin dependent diabetes Crohn's colitis Bile acid induced diarrhoea Ulcerative colitis

Table 2.8 - Pathologies associated with either secretory or osmotic diarrhoea on the basis of OG2 results.

With such a large list of possibilites, to which can also be added the diseases listed in table 2.6, the measurement of OG2 is unlikely to provide any further diagnostic information in cases of chronic idiopathic diarrhoea.

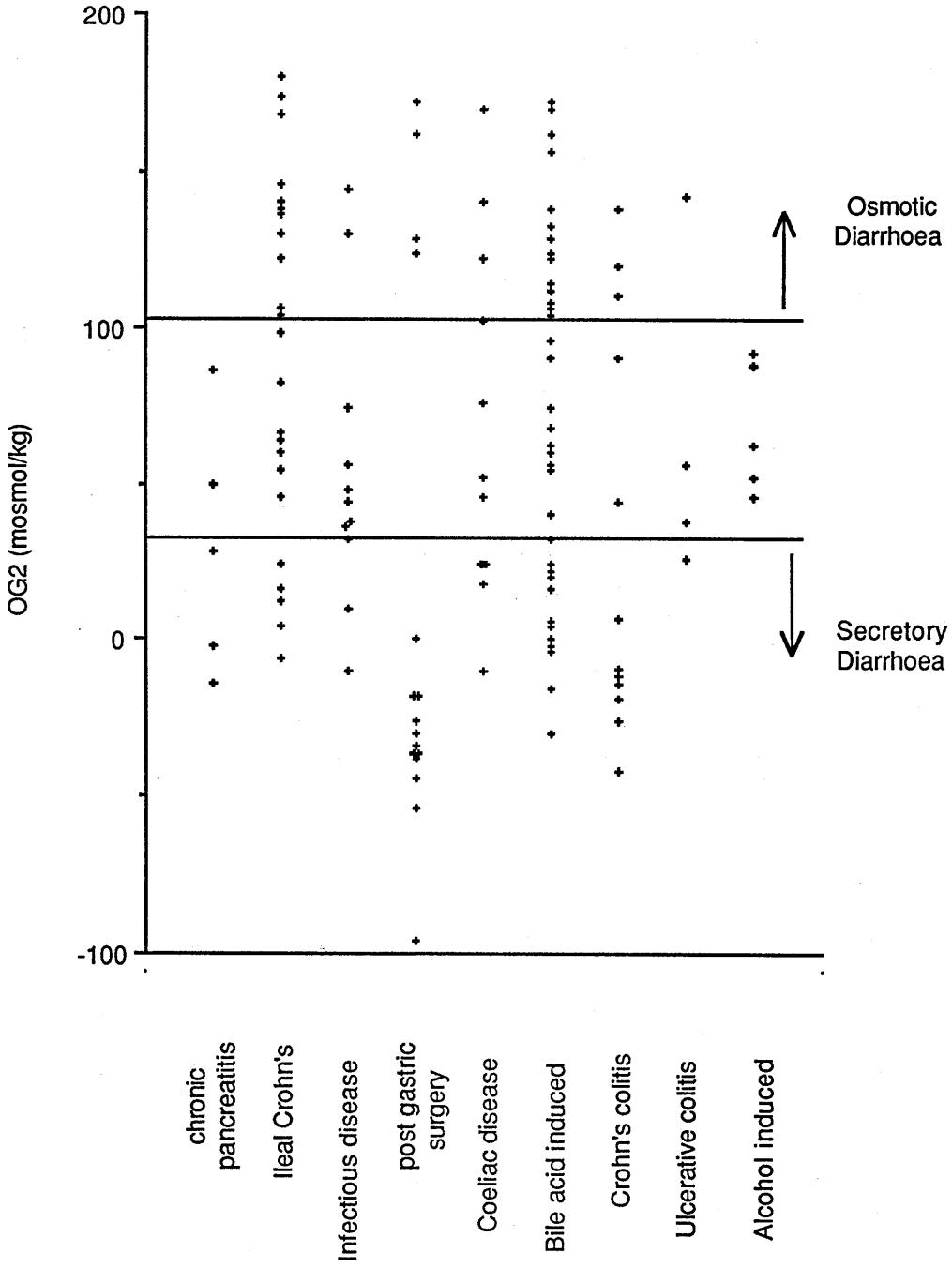


Figure 2.13 - OG2 results in various pathologies associated with diarrhoea

* * * * * *

The clinical value of measuring OG2 was also assessed retrospectively in twenty-six patients who presented with chronic diarrhoea of unknown cause. The results and the subsequent diagnoses are listed in table 2.9.

Patient	OG2	Diagnosis
1	24,16,22,10	Idiopathic, ?IBS
2	112	Malabsorption, unknown cause
3	201,144,121,52	Alcohol-induced
4	86,-2	Malabsorption, chronic pancreatitis
5	16,122,108,156	Crohn's disease
6	14	Malabsorption, unknown cause
7	50,28,-14,20,	malabsorption, unknown cause
8	14,92,14	IBS
9	-10,-56,-64,-46,	Carcinoid syndrome
10	10,4,18,20	Phenolphthalein abuser
11	134	Idiopathic
12	122,102,140,170	Malabsorption coeliac disease
13	46	Alcohol induced
14	40	
15	30	
16	0	Malabsorption, unknown cause
17	52,76	Malabsorption, coeliac
18	18,52	? Post infectitious IBS, ? Alcohol-induced
19	2	Small bowel overgrowth
20	108	? Alcohol-induced
21	-30	Tubular villous adenoma
22	192	Malabsorption, unknown cause
23	26	IBS
24	22	Phenolphthalein abuser
25	-4	Tubular villous adenoma

Table 2.9 - OG2 results in patients with diarrhoea.

In six patients (numbers 3, 4, 5, 7, 8, and 18) in whom more than one stool had been analysed, the intra-individual results varied such that no classification into secretory or osmotic diarrhoea could be made. In a further three (numbers 13, 14, 17) OG2 results fell between 30 and 100 and so again no classification could be

made. Eleven patients (numbers 1, 6, 9, 10, 15, 16, 19, 21, 23, 24 and 25) had results of 30 or less suggestive of secretory diarrhoea. Two of these patients had malabsorption, two had possible irritable bowel syndrome and one had small bowel overgrowth secondary to systemic sclerosis. The OG2 results would have been of no value in the first two instances since both malabsorption and IBS can be associated with secretory or osmotic diarrhoea. Similarly the mechanism of diarrhoea in patients with small bowel overgrowth is dependent on the cause and is multifactorial in nature (125). Hence a knowledge of the OG2 would not have been informative in this patient.

The other five patients with secretory diarrhoea were the patients mentioned earlier with known secretory diarrhoea caused by laxative abuse, carcinoid syndrome, or villous adenoma. In these cases the OG2 could have played a minor role in the differential diagnosis of diarrhoea. However, in the case of the two laxative abusers, more specific and simpler alternative tests are available. The patient with carcinoid syndrome was also readily diagnosed following urinary estimation of 5-hydroxyindole acetic acid concentrations and so did not pose a particularly difficult diagnostic problem. Similarly the diagnosis of villous adenoma in two patients was not difficult, the condition being recognised after sigmoidoscopy and histology.

Of the five patients with osmotic diarrhoea on the basis of high OG2 results (numbers 2, 11, 12, 20, and 22), three

had malabsorption (two of unknown cause and one secondary to coeliac disease), and one had probably alcohol-induced diarrhoea. In one case a diagnosis was not made.

* * * * * *

Several conclusions can be reached regarding the clinical value of OG2 measurements. a) There is a large intra-individual variability in the results in many patients with diarrhoea. Consequently, diarrhoea cannot be classified as being secretory from a single result of less than 30. Similarly osmotic diarrhoea cannot be diagnosed on the basis of a single OG2 result greater than 100. Instead, a series of OG2 results from the same patient is necessary. A possible exception to this is when a very low value (e.g. less than -50mosmol/kg) is found.

b) Even when diarrhoea is classified as being osmotic or secretory using OG2 results, the search for the eventual diagnosis is not advanced significantly because of the long list of possible differential diagnoses (see table 2.8).

c) The diagnosis of the cause of diarrhoea can often be made following simpler and more specific tests than OG2.

d) The only apparent clinical place for measuring a single OG2 result is in order to exclude a diagnosis. For example a result of greater than 30 would exclude secretory diarrhoea, while a result of less than 100 would exclude osmotic diarrhoea.

CONTRIBUTION OF OTHER ELECTROLYTES TO THE OSMOTIC GAP

In order to determine what significance calcium, magnesium and ammonium had in contributing to the composition of cationic electrolytes, these components were also measured. Unfortunately it was not practicable to measure ammonium because its concentration increases rapidly unless samples are processed immediately and the analysis done within a few days (see page 81).

The results of calcium and magnesium measurements are tabulated in 32 patients along with the contribution to the osmotic gap that these electrolytes would have made (table 2.10). Their contribution to the osmotic gap was calculated by doubling the sum of the magnesium and calcium concentrations. (This may in fact underestimate the OG if magesium is associated, as is likely, with two monovalent anions rather than one divalent anion.)

Although the average magnesium and calcium concentrations are relatively small there is a very large range. Consequently the contribution to the osmotic gap in some cases may exceed 100. It is probable that elevated

	Magnesium Concentration	Calcium Concentration	Osmotic gap (2 x [Ca + Mg])
Mean	6.5	4	25
sd	15.6	6.1	35.3
Range	0.2 - 55	0.1 - 21	0.6 - 128

Table 2.10 - Concentrations of magnesium and calcium in stool water in diarrhoea, and their contribution to the osmotic gap.

concentrations of calcium and magnesium in the stool water

are factors of dietary intake and/or absorptive capacity. When the group was subdivided into patients with and without malabsorption the average contribution to the OG was 44 (range of 2.4 - 112) in the malabsorption group, and 32 (range of 2 - 128) in patients with normal absorptive capacity. Hence the presence of high concentrations of calcium and magnesium in stool water cannot be attributed solely to those patients with malabsorption. There was no significant difference between these results (see Statistical Appendix, note 1) implying that variations in diet are more important in deciding the amount of calcium and magnesium excreted. This conclusion is supported by results showing that the concentrations of these substances decrease when patients are fasted (118).

None of the 32 patients abused magnesium laxatives and so there were no cases of diarrhoea induced solely by these. However, it is likely that high magnesium and/or calcium concentrations may be a contributary cause of diarrhoea in some patients. The measurement of OG2 in these cases would correctly reveal a high osmotic gap. On the other hand, lower concentrations of magnesium and calcium (e.g. in the order of 30 - 60) would be unlikely to play a significant role in the cause of diarrhoea, yet large enough to produce an erroneously high OG2 result.

These conclusions were reached without measuring ammonium and unfortunately there is no published data on ammonium

concentrations in stool water obtained from diarrhoea specimens. Two ammonium results were available from this study; 23mmol/l in a patient with carcinoid syndrome and 23mmol/l in a patient with moderate malabsorption of unknown aetiology. Therefore it is likely that ammonium concentrations, as with calcium and magesium concentrations, should be considered when calculating the osmotic gap.

MEASUREMENT OF K/NA RATIOS

A total of ten patients with diarrhoea due to a colonic pathology were identified; two with *Campylobacter* enteritis, four with ulcerative colitis, three with Crohn's disease confined to the colon, and one each with *Shigella sonnei* infection and tubular villous adenoma. Recruiting patients with a purely ileal cause of diarrhoea was more difficult. Although there were several patients with small bowel lesions and a healthy colon (patients with Crohn's disease affecting only the ileum, or patients with coeliac disease), their diarrhoea was probably related, at least in part, to the effects of malabsorbed fatty acids and bile acids on the colon. As a group of patients with ileal diarrhoea we used five patients with salmonella. The diarrhoea in these patients is due to the effect of the toxin on the small bowel (112). As an additional comparative group, sixty-seven stool supernatants from thirty patients with diarrhoea due to a mixed ileal/colonic dysfunction were used. The patients in this group consisted of six with malabsorption,

fourteen with Crohn's disease affecting colon and ileum, and one each with carcinoid syndrome, dumping syndrome and alcohol induced diarrhoea. A fourth group consisted of forty stool supernatants from ten patients with bile acid induced diarrhoea. This last group should also have colonic diarrhoea.

The summary statistics of each group are shown in table 2.11.

Cause of diarrhoea	median	sem	inter quartile range
Mixed ileal/colonic	0.75	0.15	0.57 - 1.33
Colonic	0.66	0.15	0.4 - 1.05
Ileal	0.31	0.34	0.24 - 1.11
BAID	0.8	0.25	0.52 - 1.3

Table 2.11 - Summary statistics of K/Na ratios in various diarrhoea groups.

Figure 2.14 shows the distribution of the K/Na ratios in each group. No statistical significance was found between the groups.

The intra-individual variability of K/Na ratios in stool water collected consecutively from the same patient, was also studied. For the K/Na to be of potential value, the results should not vary significantly. Seven stool samples were collected from a patient with Crohn's colitis and the K/Na ratios were found to vary from 0.36 to 2.43. A similar wide spread of K/Na ratios was also found in stool supernatants prepared from consecutively collected stools from patients with a mixed ileal/colonic cause of diarrhoea or with BAID (eg. 0.32 - 5.76, 0.44 - 8.1).

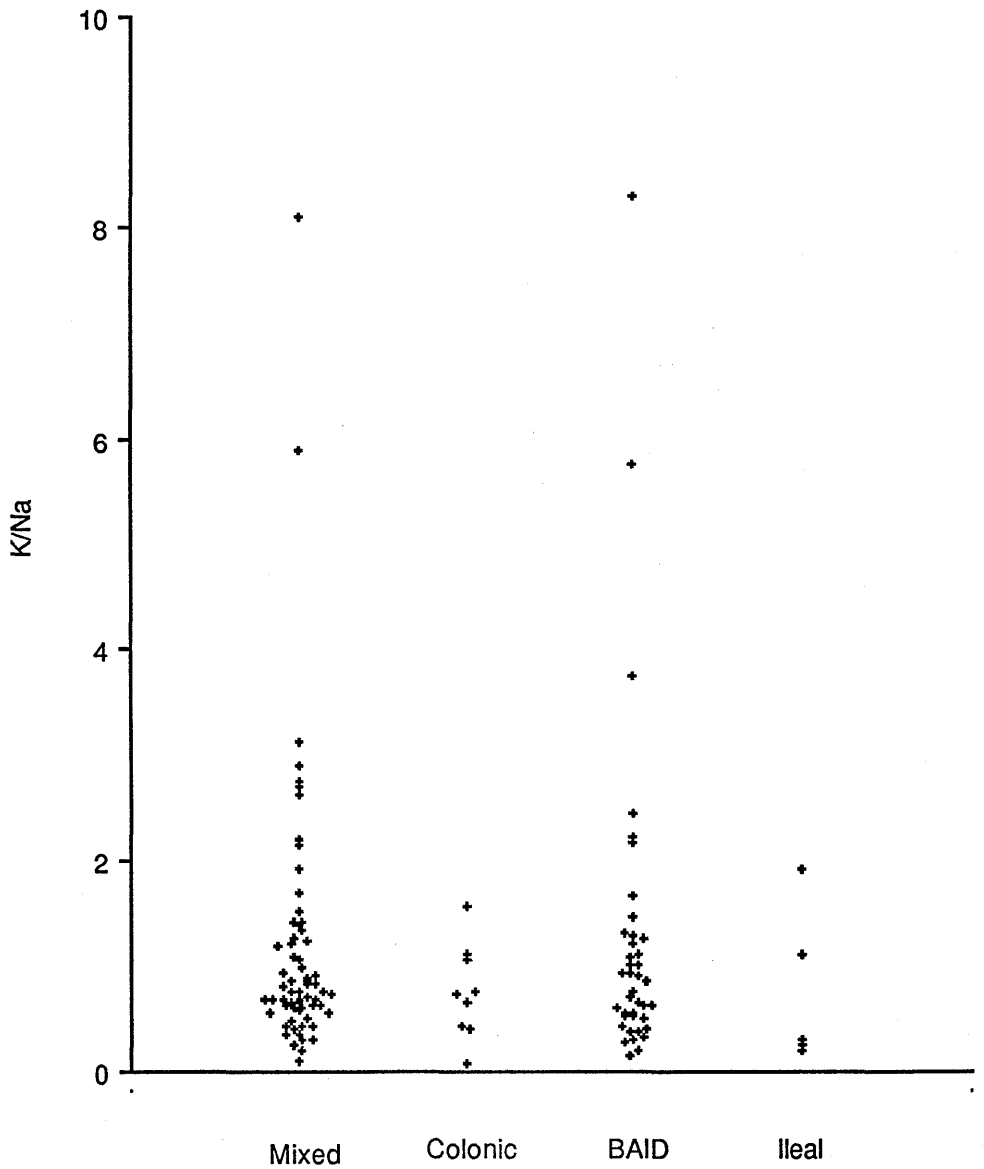


Figure 2.14 - Potassium/sodium ratios in different diarrhoeal states

This offers clear evidence that the calculation of K/Na ratios provides no information regarding the site of diarrhoea between small and large bowel. Other workers have suggested, however, that it may be of value in classifying patients with inflammatory bowel disease affecting the colon. In patients with ulcerative proctitis the ratio is significantly higher (3.6) compared with patients with chronic ulcerative colitis (0.55) or Crohns' colitis (0.9) (99).

The main concept of using K/Na ratios an a clinical aid, was based on the notion that the main colonic mechanism governing the luminal concentrations of these electrolytes is a simple exchange of the two. Evidence has accumulated in the 1980s that another major factor controlling potassium fluxes in the colon is the body's potassium stores. In the rat, at least, colonic perfusion studies have shown that a high dietary potassium intake stimulates the secretion of this ion in the colon (126). Conversely; when animals are fed a potassium-deficient diet, active potassium absorption in the colon helps to maintain homeostasis (127). Consequently the concentration of potassium in stool water is, in part, decided by dietary intake. Therefore, a patient with a colonic cause of diarrhoea, would be expected to have a high K/Na ratio if his body stores of potassium were replete.

CHAPTER 3

DIAGNOSIS OF LAXATIVE INDUCED DIARRHOEA

INTRODUCTION

'Bleed him and purge him; if he dies bury him'

(Spanish proverb)

HISTORICAL ASPECTS OF LAXATIVE USE

The use of purgatives in medicine has been documented since ancient times. Several herbal laxatives are listed in Eber's Papyrus - a pharmacopoeia dating from ancient Egyptian times (ca 1570 BC). The use of purgation as a therapeutic mode for a wide variety of medical conditions has been attributed to Melampus who was a Greek shepherd-cum-healer. He successfully treated the daughters of Proteus, the King of Argos, for hysterical monomania using hellebore, a herb which he observed caused diarrhoea in his goats. Along with bloodletting, purgation has been a mainstay in medical therapy since these times and until surprisingly recently. The philosophy behind the use of purging has been encapsulated by Moses ben Maimen a twelfth century physician....

> *'man should always strive to have his intestines relaxed all the days of his life and that bowel function should approximate diarrhoea. This is a fundamental in medicine namely whenever the stool is withheld or extruded with difficulty grave illnesses result'.*

The belief that regular bowel movements were essential to remove dangerous 'humors' from the intestine has persisted

until this century. As recently as 1918 Dr. Arbuthnot Lane advocated laxation for a wide range of conditions: high and low blood pressure, asthma, neuritis, depression, rheumatism, goitre, tuberculosis and cancer; even flat feet and loss of feminine contours (129). The following extracts from a prestigious medical textbook written in the 1920's also suggest that the use of laxatives was widespread at that time...

> *'Diarrhoea very frequently results from the habit of taking aperients, either in excess of what is required for the correction of chronic constipation, or even when the bowels left to themselves would act quite normally. Thus many of the symptoms often ascribed to constipation are really due to the diarrhoea caused by purgatives, as it results in the absorption of excess of poisons from the too fluid faeces.'*
>
> *'The most common cause of chronic catarrhal colitis* is the habitual use of purgatives which are frequently taken even in the absence of constipation.' (130)*

(* Obsolete terminology for irritable bowel syndrome.)

Medical thinking regarding the use of laxatives has changed through this century and now their prescribed use is reserved for the cautious treatment of severe constipation or as a bowel washout prior to intestinal surgery, barium radiology or colonoscopy (131).

Nevertheless laxatives are easily purchased over the counter and consequently are freely available for ingestion by those so inclined. Certainly they constitute a considerable source of income for the pharmaceutical industry. In 1975 $130 million were spent on laxatives bought over the counter in USA (132). Similarly the proprietary sales of laxatives in Great Britain in 1988 were £15 million (133). More insight into the usage of laxatives by the general public has been drawn from questionnaire studies. For example the use of laxatives in a GP population was found to be 6.4% of patients in their twenties and increased to 39% of patients in their fifties (134). In a Swiss study 18% (15% women and 3% men) of ambulatory patients admitted to regular laxative use (135). In this country 22% of healthy people interviewed took regular laxatives other than bran and 6% took laxatives at least twice per week (136). The motives for this habit are probably related to the individuals' concept of bowel normality, but aperients are also used as slimming aids. A questionnaire given to 1728 fifteen year old Americans, revealed that a considerable number took laxatives to control their weight: 1.1% of females and 0.2% of males took laxatives several times per week and 6.1% of females and 4.1% of males used laxatives at least monthly (137).

There is further evidence suggesting that laxatives are widely used. In 1937 of 1000 patients who presented to a medical clinic with gastrointestinal symptoms phenolphthalein was found in the faeces of 177 (138); in a

study published in 1958 the authors managed to amass a series of 887 patients with melanosis coli, a discolouration of the rectum caused by regular intake of anthraquinone laxatives (139).

ABUSE OF LAXATIVES

Although chronic laxative use is undoubtedly common, this practice does not usually cause significant pathology. However, since the 1930s an increasing number of reports have appeared which represent an extreme in the use of laxatives and can be considered as laxative abuse. Laxative abuse is now a well recognised clinical term which can be simply defined as a disease process which has been induced by the ingestion of laxatives.

There is no justifiable clinical reason for prescribing large, frequent doses of laxatives and so it is likely that the abused laxatives are purchased by the patient over the counter. As table 3.1 shows there is a sizeable choice.

Laxative abuse is subdivisable into four distinct categories which are based partly on the motives for ingestion. 1) Control of bowel habit; 2) In emotionally or psychiatrically disturbed patients who may be seeking attention or sympathy by inducing illness; 3) Weight control by anorexia nervosa and bulimia nervosa patients; 4) Child abuse.

LAXATIVE	PROPRIETARY DRUG
PHENOLPHTHALEIN	Boldomint, Carters Little Pills, Ex-lax, Fam-Lax, Feen-a-Mint, Juno Juniper Tablets, Kest, Nylax Reguletts, Sure-Lax.
BISACODYL	Nylax.
SENNOSIDE	California Syrup of Figs, Eucarbon, Nylax, Sennokot, Boots Senna Tablets.
ALOIN	Beecham's Pills, Carter's Little Pills, Nylax.
CASCARA	California Syrup of Figs, Nylax,
FRANGULA	Normacol,
MAGNESIUM SALTS	Andrews Liver Salts, Kest, Kruschen Salts, Milk of Magnesia, Mil-par, Epsom Salts
SULPHATE SALTS	Juno Juniper Tablets, Kruschen Salts, Epsom Salts, Glaubers Salts

Table 3.1 - Laxatives Available Over the Counter

1. Laxative Abuse as a Means of Controlling Bowel Habit

The overzealous use of laxatives for the purpose of treating constipation, or perhaps more correctly inducing regular loose motions, can result in pathology. The patient typically presents with symptoms of muscular weakness and malaise and investigations demonstrate hypokalaemia and sometimes hypocalcaemia (140, 141, 142, 143, 144, 145). Paradoxically they sometimes present with severe constipation caused by a colon which has become spastic from long-term laxative ingestion. These patients seem to have an unusual concept of what constitutes normal bowel frequency. Consequently they do not necessarily

complain of diarrhoea despite the fact that typically they pass several loose motions per day. Clearly the treatment is to stop taking laxatives and the outcome then is usually successful.

It is probable that in Westernised societies the incidence of this form of laxative abuse is decreasing as traditional cultures and medical knowledge change. It is difficult to imagine that the advertisement in figure 3.1 could have an impact today. The situation in other cultures may be different, however. In India, for example, there is still a degree of paranoia about bowel frequency and laxatives and enemas remain in vogue (146).

2. Laxative Abuse as a Means of Inducing Illness

This group of laxative abusers usually present with diarrhoea and/or abdominal pain and less often muscular weakness and weight loss. Most investigations are normal although hypokalaemia or low-normal potassium may be present. There may be some degree of steatorrhoea or protein-losing enteropathy, and osteomalacia has also been reported (147, 148). The condition is sometimes erroneously diagnosed as irritable bowel syndrome and there are some similarities in that the patient has diarrhoea, is typically not unwell, and is often of a nervous or anxious disposition. However, some patients have been extensively investigated, having multiple invasive tests and even laparotomy (149, 150, 151, 152). If the patient is asked about the use of laxatives there is usually a prompt

Figure 2.1 - Advertisement from May edition of Women's Weekly, 1952.

denial. As a result the diagnosis is difficult to make and requires either a search of the patients' belongings or chemical identification of the drug in urine or stool.

This type of laxative abuse is more common in women and is a form of Munchausen syndrome where the patient is hoping for a gain - perhaps of attention or sympathy - by inducing illness. The patients are frequently anxious or nervous individuals and the case notes often include a passing reference to an unusual psyche or bizarre personality. Confronting a patient with a positive finding invariably elicits a strong denial. On persisting, some patients do reluctantly admit to the fact, enabling some form of psychiatric treatment to be initiated. Nevertheless, many continue to deny purgative abuse. In either case the response to treatment is poor and even if the purgative habit is stopped the patients' psychiatric condition may present itself in another form.

The extent of this form of laxative abuse is not known although Fordtran found it to be the main diagnosis in patients referred to his unit with extensively investigated chronic idiopathic diarrhoea (111). Another study concluded that the incidence of this type of laxative abuse may be more common than is generally believed (148).

3. Laxative Abuse for Weight Control

Purgatives are sometimes used, along with diuretics, as slimming aids, and advertisements for laxatives sometimes appear in slimming magazines. Their use has been reported

in patients with anorexia nervosa and bulimia nervosa (153). Again the laxative abuse may be surreptitious (154) although it is common for these patients to be quite open about the habit (155).

4. Child Abuse by the Administration of Laxatives

Perhaps the saddest form of laxative abuse is Munchausen syndrome by proxy when parents, seemingly quite devoted, administer laxatives to their children (156).

* * * * * *

In the last three categories laxatives are taken, or given, surreptitiously. If it was not for the fact that phenolphthalein, one of the more popular laxatives, turns a bright violet colour in an alkaline medium, these syndromes might well be unrecognised. The discovery of laxative abuse in patients presenting with diarrhoea and in children was initially quite fortuitous. French et al 'accidentally' diagnosed the syndrome in two patients when their stool samples were alkalinised during the van de Kamer procedure for faecal fat estimation (157). Similarly the reported cases of laxative abuse in children were recognised only because of the pink-staining of nappies or bed-clothes (156, 158).

DIAGNOSIS OF LAXATIVE ABUSE

This study is concerned mainly with the surreptitious laxative abuse associated with induction of illness and control of weight. Since the patients' ingestion of the

laxatives is usually secret, simple questioning of the patient may not be informative. Instead a more devious approach is required such as searching the patients' belongings or by a chemical test. The former method has ethical and indeed legal implications (159,160) and the search has to be done while the patient is in hospital. In addition, a negative search does not necessarily exclude laxative abuse. A Dutch group has established an effective, albeit rather complex, thin layer chromatography method for the detection of most over-the-counter laxatives (161) and this method has been recently simplified (162). By thin layer chromatographic methods, the so-called colonic stimulant laxatives such as phenolphthalein, bisacodyl, sennosides, cascara and aloin are detectable, but saline laxatives like magnesium sulphate and sodium sulphate are not. Of the two hundred or so case reports published on laxative abuse only five describe saline laxative abuse (110, 115, 118, 163, 164) and in three of these, colonic stimulant laxatives were also taken. The reason for the relative paucity of such reports may be that saline laxatives are not as frequently abused. (They are certainly less palatable than irritant laxatives.) An alternative possibility is that the diagnosis of saline laxative abuse is easy to miss. Saline laxatives are indistinguishable from endogenous constituents and so interpretation of a measurement can only be made by comparison with reference ranges from normal subjects and laxative abusers. At present such reference ranges have not been established although cut-off points for magnesium

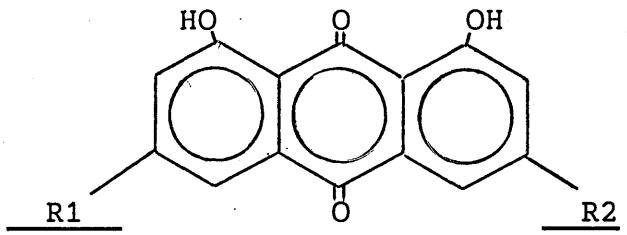
in urine and stool water have been suggested (149, 165).

PHARMACOLOGY OF LAXATIVES

It is useful to have some knowledge of how laxatives work in order to diagnose their surreptitious use.

1. Anthraquinones

The anthraquinone group of laxatives include the main vegetable purgatives such as cascara, senna, aloes, and frangula. The anthraquinones occur naturally either as the free molecules, as dianthrones, or more commonly as glycosides. Glucose is the predominant sugar residue forming either O-glycosides at positions 8 and/or 10 or β C-glycosides at position 10 (166). The anthraquinone glycosides are not digested by the alpha-glycosidases in the small intestine and so they pass through the small bowel virtually unchanged. Any free anthraquinones are absorbed and then excreted into the urine or bile as glucuronides. Once in the colon, the glycosides cannot induce laxation directly but must initially be hydrolysed to the free form (see figure 3.2) by bacterial enzymes. Thus in neomycin-treated animals little laxative effect is seen. The purgative action is thought to be caused by a local effect on the intramural nerves resulting in peristalsis (167). It has also been demonstrated that free anthraquinones cause sodium and water secretion (168, 169). Only the free anthraquinone can be absorbed and this is metabolised and excreted by the kidney as a glucuronide conjugate. (see figure 3.3, page 134)

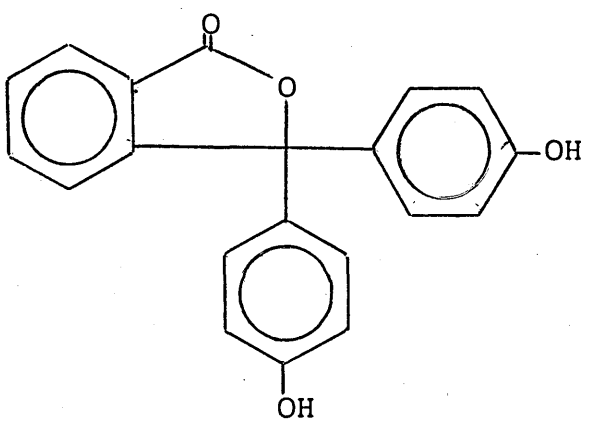


R1		R2
-COOH	RHEIN	-H
$-CH_2OH$	ALOE-EMODIN	-H
$-CH_3$	FRANGULA-EMODIN	-OH
$-CH_3$	CHRYSOPHANOL	-H
$-CH_3$	PHYSCION	$-OCH_3$
-H	DANTHRON	-H

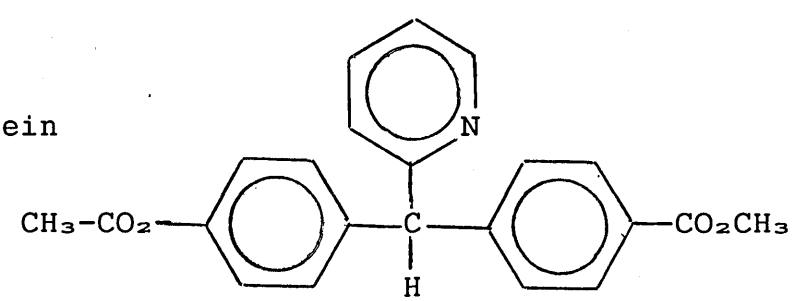
Figure 3.2. - The Chemical Structure of Anthraqinone Laxatives.

2. Diphenylmethanes

Phenolphthalein, bisacodyl and oxyphenisatin are the main laxatives in this group although the last has been withdrawn from the market because of liver toxicity. They are structurally similar (see figure 3.4) and probably effect laxation by the same mechanism. They are absorbed



Phenolphthalein



Bisacodyl

Figure 3.4 - The Chemical Structure of Diphenylmethane Laxatives.

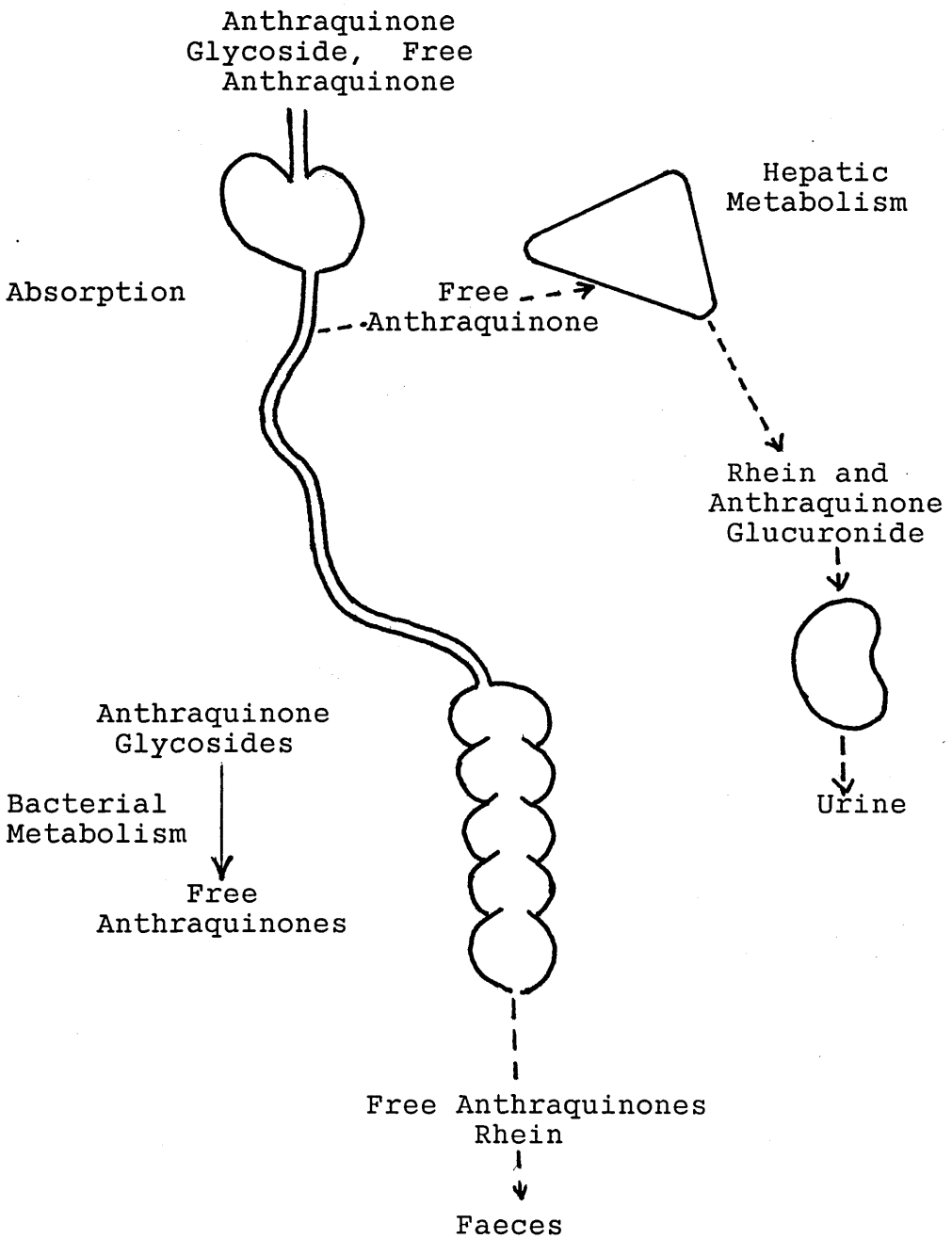


Figure 3.3 - Metabolism of Anthraquinones

from the intestine and excreted in the bile as glucuronide conjugates. They seem to have little effect systemically since obstruction of the bile duct prevents their action. There is a direct action on the colonic smooth muscle causing peristalsis and in addition there is secretion of sodium and water, mediated by inhibition of Na/K-ATPase, and the stimulation of prostaglandin E2 and cyclic AMP production (168).

3. Saline Laxatives

This group of laxatives which are also called osmotic laxatives, are thought to draw fluid into the lumen of the intestine by an osmotic effect. Certainly magnesium, sulphate and phosphate, of which the saline laxatives are comprised, are poorly absorbed and so increase the osmolality in the gut lumen. An alternative theory, however, has been proposed by Harvey and Read, who suggested that magnesium sulphate stimulates the release of cholecystokinin which in turn causes increased peristalsis and intestinal hurry (171).

OBJECTIVES

1. DEVELOPMENT OF A SUITABLE SCREENING METHOD FOR LAXATIVES.

At present the screening methods for laxative abuse are qualitative only. It was intended to develop a high performance liquid chromatography method so that laxatives could be quantitated. This would enable future study of the pharmacokinetics of the colonic irritant drugs as well as providing a laxative screening technique.

2. DETERMINATION OF THE SPECIFICITY OF THE LAXATIVE SCREEN.

Since anthraquinone laxatives are derived from plants, a false positive laxative screen might occur if a patient consumes a vegetable containing such compounds. The patients' denial of laxative ingestion would be quite appropriate in such a circumstance. Nevertheless a denial would be in keeping with laxative abuse and would probably be taken as such. Clearly it is essential that the specificity or false positive rate of the test is known. In order to determine this, possible dietary sources of anthraquinones in foods associated with diarrhoea (such as rhubarb, liquorice, chillies, prunes, and figs) were investigated. Urine samples taken after consumption of these foodstuffs were screened for the presence of laxatives.

The possibility of false positive results caused by drug interference has already been considered and of 73

commonly prescribed drugs none were found to interfere with the TLC method (161). It is possible that other drugs not tested in this study may interfere. Rather than attempt to exclude all drugs exhaustively by performing TLC on each, drugs known to be structurally similar to laxatives were tested. Thus, drugs such as chrysarobin, dithranol, podophyllum, daunorubicin and doxorubicin which have anthraquinone moieties, were analysed.

3. TO ESTABLISH METHODS FOR MEASURING FAECAL MAGNESIUM AND SULPHATE CONCENTRATIONS.

Magnesium and sulphate are present in most over-the-counter saline laxatives. These substances can be measured readily in plasma and urine but their measurement in faeces, especially sulphate, poses particular difficulties. Methods were developed for their measurement in stool using atomic absorption spectrophotometry for magnesium, and high performance liquid chromatography for sulphate.

4. TO DETERMINE REFERENCE RANGES IN THE APPROPRIATE SPECIMEN FOR THE DIAGNOSIS OF SALINE LAXATIVE ABUSE.

Magnesium and sulphate are normal body components and so reference ranges must initially be established before saline laxative abuse can be diagnosed. In addition it is not known what sample, stool or urine, is most appropriate to use. These aspects were investigated in a group of volunteers in whom diarrhoea was induced by ingestion of saline laxatives.

5. TO DETERMINE THE EXTENT OF LAXATIVE ABUSE.

The diagnosis of laxative induced diarrhoea can only be made reliably by a chemical test which is not routinely available at most hospitals. As a result it is possible that the diagnosis of laxative abuse may be missed. Some idea of this eventuality can be made by calculating the prevalence of laxative abuse. However, it would be logistically difficult to measure the incidence in a random population unless it were fairly common. For this reason the incidence in a selected population, patients presenting to a Gastroenterology Clinic with diarrhoea, was studied.

Another way of gaining information on the possible underdiagnosis of laxative abuse is to investigate the requesting pattern for laxative screening tests in the areas where this test is available. For this purpose requesting patterns in Scotland were studied.

6. ACCUMULATION OF A SERIES OF LAXATIVE ABUSERS FOR CLINICAL AND THERAPEUTIC INVESTIGATION.

After establishing a laxative screening method a service was offered to Biochemistry and Gastroenterology departments throughout the West of Scotland. It was hoped that sufficient cases would be diagnosed to build up a series of patients on whom more information about the natural history of the condition could be gleaned. In addition a unified approach to the psychiatric management could be adopted in order to determine the effectiveness of this form of therapy.

7. TO STUDY THE MECHANISM OF DIARRHOEA CAUSED BY SALINE LAXATIVES.

Two postulates on the mechanism of saline laxative action have been proposed: secretion of water into the gut by an osmotic effect or release of cholecystokinin with consequent peristalsis. This was studied by measuring the osmotic gap in stool, and plasma cholecystokinin concentration in samples obtained from volunteers in whom diarrhoea was induced by ingestion of saline laxatives.

MATERIALS AND METHODS

PREPARATION OF ANTHRAQUINONES

Anthraquinones were initially prepared as standards by oxidative hydrolysis of their glycosidic form using ferric chloride in concentrated hydrochloric acid (172). It was later found that the recovery of anthraquinones could be considerably improved by oxidising with 4% periodic acid. The final method was as follows. 100mg of anthraquinone glycoside and 25ml of water was added to a stoppered test tube and boiled for 15 minutes. The supernatant was transferred to a second tube and 5ml of 4% periodic acid was added and the mixture boiled for 5 minutes. A volume of 1ml of concentrated hydrochloric acid was added and the anthraquinones extracted into 3 x 20ml of ether. Glycosides used were sennoside A, sennoside B, rhein glycoside (gifted by Reckitt and Colman, Dansom Lane, Hull), aloin (gifted by Parke Davis and Co, Pontypool, Gwent) and frangula (gifted by Norgine Ltd, Headington, Oxford).

The following commercially available substances were also used: emodin, chrysophanic acid, rhein (Aldrich Chemical Co, Gillingham, Dorset), bisacodyl (gifted by Dr Karl Thomae GMBH), sodium picosulphate (gifted by WB Pharmaceuticals, Bracknell, Berkshire), oxyphenisatin (gifted by Sterling Winthrop Group Ltd, Aldwych, London), phenolphthalein (British Drug Houses, Poole) and danthron (gifted by Riker Laboratories, Loughborough,

Leicestershire).

To determine the specificity of the laxative method, dietary constituents which are sometimes associated with diarrhoea (rhubarb, prunes, figs, liquorice, and chilli) were analysed. Lyophilised aliquots of 100mg were oxidatively hydrolysed by the above method prior to TLC analysis.

Two groups of drugs were also studied: those with a structural similarity to anthraquinone laxatives (chrysarobin, dithranol, podophyllum, adriamycin, plicamycin, dactinomycin, and methotrexate) and the drugs taken by patients who gave a positive laxative screen. Minimum patient doses were extracted into chloroform/isopropanol (9:1) and separated by thin layer chromatography (TLC).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) of laxatives was performed using an isocratic system consisting of an Altex 110A pump, Altex Sample Injector, Hitachi 100-10 ultra-violet/visible spectrophotometer, Altex 155-00 analytical flow cell (path length 1cm, cell volume 20µl), 24 x 0.5cm metal column packed with 5µm microparticulate octadecasilane (ODS, Shandon Southern Products, Astmoor, Runcorn), and a 5 x 0.5cm guard cell with similar packing. Chemicals used were HPLC grade methanol and HPLC grade acetonitrile (Rathburn Chemicals Ltd, Walkerburn, Peeblesshire) and cetyl trimethyl ammonium bromide (CTMAB

Aldrich Chemical Co, Gillingham, Dorset). Mobile phases were degassed prior to use by vacuum and filtered through membrane filters (GVWP filters from Millipore).

The conditions of the HPLC method developed are shown in table 3.2.

Mobile Phase	25% methanol, 25% Acetonitrile 0.0075% CTMAB and 50% 0.2M acetate buffer pH 5.8
Flow Rate	0.5ml/min
Detection	UV absorbance at 254nm
Column	5µm ODS
Volume	20µl

Table 3.2 - HPLC Conditions for Separation of Colonic Stimulant Laxatives.

EXTRACTION OF LAXATIVES

Purification of laxatives is an essential preliminary step prior to their analysis in urine and faeces by HPLC. Commercially available columns were used in the development of a solid phase extraction system. Type W columns (Du Pont,) and XOD columns (Analytichem,) were tested. The basic method was as follows. Columns were activated by flushing with 1ml of methanol followed by 1ml of water. To 10ml of urine was added 1ml of acetate buffer (0.2mol/l, pH 4.5) and 50µl of glucuronidase (Sigma Chemical Co) and this was heated for 2 hours at 60°C. A volume of 4ml of solution was placed on the top of the column which was spun at 1800rpm for ten minutes and then

washed with 2ml of water. Laxatives were eluted from the column with either chloroform:isopropanol (9:1) or methanol.

Urine and stools from subjects who had ingested laxatives, or from positive patient material were used. On some occasions normal samples were spiked with laxative to produce a final concentration of 250mg/l to 500mg/l. In these cases similar concentrations in mobile phase were also prepared so that recoveries could be calculated. When chloroform/isopropanol (9/1) was used to elute columns the recoveries from type W columns were 90% for phenolphthalein, 15% for rhein and 30% for picosulphate. Using XOD columns the recoveries were 80%, 0% and 30% respectively. The recovery of bisacodyl could not be accurately measured since a negative urine gave a peak corresponding to the R_f of bisacodyl. When methanol was used as the eluting solvent the recoveries were 80%, 50%, and 0% from type W columns for phenolphthalein, rhein and picosulphate respectively and 70%, 80%, and 0% from XOD columns.

Using an aqueous standard it was found that most picosulphate passed through the column without being retained by the solid phase. By making the standard solution 0.5g/l with CTMAB, almost all was retained on the columns and could be eluted with methanol. Unfortunately when the sample was injected on to the HPLC column the Rf values were prolonged because of the additional injected CTMAB. When a standard picosulphate solution was prepared

using lower CTMAB concentrations (100 and 250mg/l) only a small amount was retained on the column.

THIN LAYER CHROMATOGRAPHY

Laxatives were detected in urine by an adapted TLC method (162). Briefly, to 20ml of urine was added 2ml of acetate buffer (pH 5.0) and 50µl of β-glucuronidase. After overnight incubation at room temperature or two hour incubation at 60°C, the laxatives were extracted into 20ml of chloroform:isopropanol (9:1). The lower solvent layer was dried and then redissolved in 100µl chloroform before applying a suitable volume (5 - 50µl) to the TLC plate (LHP-KF plates, Whatman Laboratory Sales). The laxatives were initially separated using the published solvent system of chloroform:acetone (4:1). However, it was found that pure rhein remained at the origin rather than having a mobility of 0.5 as reported. In addition a patient sample which was positive for rhein (patient CL who took Sennokot) gave a mobility of 0.04. To avoid potential confusion, an alternative solvent system was used: 4-methyl-2-pentanone:chloroform:acetic acid (5:2:1). To visualise the laxatives the plate was sprayed with 5M sodium hydroxide and then heated at 100°C for 10 minutes. TLC was also employed as a confirmatory method by using a solvent system of hexane:toluene:glacial acetic acid (3:1:1).

MEASUREMENT OF FAECAL MAGNESIUM

Magnesium in faecal homogenate or faecal lyophylate was

easily measured by atomic absorption spectrophotometry following digestion and dilution of the samples. The magnesium concentrations were measured using the conventional 265nm magnesium peak as well as the less sensitive 202nm peak. The reason for using the latter wavelength was to obviate the necessity of the second dilution step.

The precision of duplicate assays was measured using each of the two wavelengths and were acceptable for a faecal assay, as shown in table 3.3. In addition the correlation of the two methods was good (r_s = 0.979, $Y_{202} = 1.02X_{265} + 0.23$). (See Statistical Appendix, note 4.)

Although there was little to choose between the two methods the 265nm wavelength method was marginally preferred because the baseline was steadier and the precision slightly better. The magnesium results which follow are based on the method using a wavelength of 265nm.

	Magnesium at 265nm	Magnesium at 202nm
Number of Pairs	31	29
Mean (mmol/Kg)	22.9	31.1
Standard Deviation	1.8	2.9
Coefficient of Variation	7.9%	9.4%

Table 3.3 - Precision of two Magnesium Methods

* * * * * *

In brief, the following methods were developed for measurement of magnesium in the various stool samples.

Magnesium in Homogenate: About 500mg of faecal homogenate or 50mg of lyophilised stool (weighed accurately) was added to a 50ml graduated stoppered test tube. To this was added 5ml of concentrated nitric acid and 1ml of concentrated sulphuric acid. The tube was capped lightly, slowly heated to 150°C, and refluxed for one hour. The cap was removed and nitric acid evaporated off. After cooling, 0.5% lanthanum chloride was added to the 50ml mark. A seventy-fold dilution was then made with 0.5% lanthanum chloride and the magnesium was measured at 265nm by atomic absorption spectrophotometry. Alternatively to avoid the need for the dilution step the sensitivity of the method could be diminished by using the minor magnesium peak at 202nm. Each sample was measured in duplicate.

Magnesium in Faecal Supernatant: Concentrated hydrochloric acid was added to freshly prepared stool supernatant (50µl to 1000µl) to ensure that magnesium did not precipitate out of solution. The supernatant was initially diluted 1:20 (or 1:100 if a high result was expected) and then 1:70 with 0.5% lanthanum chloride, before aspirating into the atomic absorption spectrophotometer.

EFFECT OF STANDING ON MAGNESIUM CONCENTRATION

It is well recognised that pH falls in stool samples left at room temperature. Since magnesium is more soluble in acid conditions it seemed likely that the magnesium

concentration in stool supernatant might increase in samples left at room temperature. To test this supposition five stool samples were aliquotted and left at room temperature for 24 hours before being centrifuged. The results illustrated in table 3.4 show that the magnesium concentration in supernatant increased significantly in samples that were not processed timeously.

	Magnesium Concentration		Percentage Increase
	0 Hours	24 Hours	
Stool 1	101	147	46%
Stool 2	134	147	10%
Stool 3	140	173	24%
Stool 4	20	22	10%
Stool 5	20	27	35%

Table 3.4 - Effect of Incubation at 25°C on Concentration of Magnesium in Supernatant.

MEASUREMENT OF FAECAL SULPHATE.

Some time was spent in developing an assay for measuring sulphate in faecal water in order to cater for the diagnostic possibility of sodium sulphate laxative abuse. Two spectrophotometric methods and an HPLC method (173) were tested for the measurement of faecal sulphate. Sulphate was measured using the reversed-phase ion-pair HPLC system described previously (see page 75). The conditions employed in this HPLC system are outlined in table 3.5.

A published spectrophotometric method for sulphate (174) subsequently modified by Wrong *et al* (101) for measurement of faecal sulphate was established. When the recovery of

Mobile Phase	0.065% TBAH adjusted to pH 8.0 with KHP
Flow Rate	1.5ml/min
Detection	UV absorbance at 280nm
Column	5µm ODS
Volume	20µl

Table 3.5 - Conditions for HPLC of Sulphate.

sulphate in faecal water was measured, highly variable results were found. Wrong *et al* used as standards, solutions of sulphate which had not been pretreated in the same way as stool water (by decolourisation with activated charcoal, and passing through a cation exchange column to remove interfering cations). We found that when standards were treated in the same way as samples, a obvious linear response was no longer found: instead there was little apparent trend.

In view of these findings which could be attributable to very poor precision this method was abandoned.

A second spectrophotometric method using sodium rhodizonate as chromophore was also tested (175). Again the precision of this method was very poor and the addition of phosphate, at concentrations found in stool water, produced significant interference. This method was also abandoned.

* * * * * *

The main problem encountered in these methodologies was

the strong stool colouration which interfered with the spectrophotometric end-point. Attempts to decolourise the stool with charcoal helped but were not curative. In view of the success of the enzymatic method which was developed for measuring faecal ammonium (page 78), this approach, perhaps using the enzyme aryl sulphatase, might prove successful in the measurement of sulphate. The advantage of an enzymatic method in this situation is that a change in optical density is monitored, making the intensity of background colour immaterial.

The prevalence of sodium sulphate induced diarrhoea is probably very low: sodium sulphate is unpalatable and OTC sales are minimal (information provided by Intercontinental Medical Statistics Ltd, Hanover House, Lyon Rd, Harrow, Middlesex); sales are less than magnesium based laxatives which are themselves probably abused very rarely. For these reasons it was considered inexpedient to invest further effort in the development of a sulphate assay.

INDUCTION OF DIARRHOEA BY SALINE LAXATIVES

After an overnight fast six healthy volunteers were given an oral dose of 5.86g magnesium hydroxide in 100ml of water.

Stool was collected for the next 36 hours into separate plastic containers and stored immediately in a freezer. Blood samples were collected into 10ml heparin tubes containing 200µl of Trasylol (20,000 units/ml) at 0, 30 and

60 minutes. Aliquots of 1ml were lyophilised and cholecystokinin measured by radioimmunoassay (Professor S Bloom, Hammersmith Hospital, London). Magnesium was measured in plasma and in a 24 hour urine collection by atomic absorption spectrophotometry.

Stool samples were semi-thawed and homogenised without addition of water. An aliquot was centrifuged at 14000rpm and the supernatant produced was filtered through paper filter discs (AP25 pre-filters from Millipore). The supernatant was frozen until analysed or refrigerated if the assays were performed on the same day. The following analyses were made on the faecal supernatant: magnesium by the method described above; osmolality using a freezing point depression osmometer; sodium and potassium by flame photometry; chloride using a silver electrode chloride meter.

Sulphate induced diarrhoea was induced in two healthy subjects by the oral administration of 13.2g of anhydrous soldium sulphate or 35g of magnesium sulphate ($MgSO4.7H2O$) as Epsom Salts.

It was intended to measure the daily output of magnesium using an non-absorbable stool marker such as cuprous thiocyanate (176) in order to make appropriate corrections. Unfortunately new legislation prohibiting human consumption of non-BP chemicals, such as stool markers, was introduced at the time that this study was carried out and so this could not be done.

As control samples stool and urine collections were obtained from patients being investigated for diarrhoea.

Ethical permission for this part of the study was granted by the Ethical Committee of the Greater Glasgow Health Board, East 1.

PATIENTS

The casenotes of out-patients due to attend the Gastroenterology clinic were screened in order to select those patients who were a) GP referrals for the investigation of diarrhoea, or b) existing patients who were being investigated for chronic diarrhoea of unknown origin. The patients were asked to return a random urine specimen (collected at a time when they had diarrhoea) at their next appointment or to provide a urine sample during the clinic. These urines were screened for colonic stimulant laxatives by the TLC method.

LAXATIVE SCREENING SERVICE

The laxative screening service was offered in writing to all Gastroenterologists and Biochemists in the following hospitals:

Royal Infirmary, Glasgow;
Victoria Infirmary, Glasgow;
Western Infirmary, Glasgow;
Southern General Hospital, Glasgow;
Stobhill Hospital, Glasgow;
Royal Alexandra Hospital, Paisley;
Royal Hospital for Sick Children, Glasgow;
Falkirk and District Royal Infirmary;
Dumfries and Galloway Royal Infirmary;
Vale of Leven Hospital, Dumbarton;
Stirling Infirmary;
Inverclyde Hospital, Greenock;

Crosshouse Hospital, Kilmarnock;
Monklands DGH, Airdrie;
Law Hospital.

These are the principal hospitals in six of the health boards in Scotland (Greater Glasgow, Argyll and Clyde, Forth Valley, Lanarkshire, Ayrshire and Arran, and Dumfries and Galloway), covering approximately 52% (2,751,600 at June 1987) of the countrys' population (177). In addition, all physicians within the Royal Infirmary, Glasgow were invited to use the laxative screening service.

TELEPHONE SURVEY

The main biochemistry laboratories throughout Scotland were contacted by telephone and the following information gleaned from an appropriate source: whether the laboratory provided a routine laxative screening service; if so the annual request rate.

RESULTS AND DISCUSSION

METHODOLOGICAL ASPECTS

HPLC of Stimulant Laxatives

HPLC was initially chosen as a way of detecting laxatives to enable their quantitation and so facilitate possible future pharmacokinetic studies. An HPLC method was readily established such that colonic stimulant laxatives could be reliably measured from standard solutions.

Using the HPLC system as described (page 141) it was possible to separate the colonic stimulant laxatives tested from a standard solution. They were eluted in the order of bisacodyl, oxyphensitin, phenolphthalein, picosulphate, rhein, and danthron. The retention times and precisions of standard solutions of each laxatives are shown in table 3.6.

Laxative	Retention Time (Minutes)	Precision (CV)
Bisacodyl	4.6	2.8%
Oxyphenisatin	5.2	1.1%
Phenolphthalein	6.4	2.2%
Picosulphate	12.1	11.8%
Rhein	14.0	3.8%
Danthron	38.0	4.7%

Table 3.6. - Retention times and Precisions for Laxative Assay

The retention times could be readily adjusted by simple modification of the mobile phase: increasing the proportion of non-polar solvent resulted in shorter retention times, and *vice versa*. Picosulphate was most

sensitive to alterations in the acetonitrile concentration followed by rhein, danthron, aloin, phenolphthalein, oxyphenisatin and bisacodyl. Similarly, picosulphate was highly sensitive to changes in the ratio of methanol followed by oxyphenisatin, phenolphthalein and bisacodyl. The retention times of rhein and picosulphate, and to a lesser extent aloin and danthron, varied in proportion to the CTMAB concentration. Since ion-pairing played no part in the separation of phenolphthalein, bisacodyl and oxyphenisatin, their retention times were unaffected by alterations in CTMAB conceentration. The retention times of all laxatives were prolonged by increasing the methanol:acetonitrile ratio.

The detector response was linear up to a concentration of (at least) 1mmol/l for phenolphthalein, bisacodyl, and oxyphenisatin and up to 0.7mmol/l for picosulphate, rhein and danthron.

However, it was clear that in order to measure laxative concentrations in urine and faecal homogenates a 'clean-up' procedure was required. By using liquid/liquid and solid phase extraction procedures a purified extract was obtained and, although the laxative recovery was good, there remained a number of extra chromatographic peaks which were caused by impurities. Two possible solutions to this problem were considered; the use of other solid phase extraction columns in an attempt to purify the sample further, or replacement of the non-specific UV detector with a more specific detection system.

There have been recent commercial improvements in the area of solid phase extraction with newer and more specific matrices being developed. There are about twenty different materials now available and, although the choice can be narrowed down to a handful by chemical considerations, final selection of the optimum column often requires individual evaluation of each.

UV detection is versatile but unfortunately very non-specific. Consequently several UV absorbing species in the urine and stool samples interfered with the laxative method. A possible alternative spectrophotometric method is alkalinisation of the eluent and measurement of the more specific reddish chromophores spectrophotometrically. Alkalinisation can be achieved either by post-column addition of base or by use of an alkaline mobile phase and a resin column which can withstand the conditions of elevated pH. Unfortunately financial and temporal constraints did not allow either of these possibilites to be explored.

In retrospect, TLC is a superior method to HPLC for the detection of drugs. With the latter, the presence of some spurious extra peaks made it difficult to be entirely confident that a peak of an appropriate Rf value was indeed a laxative. If HPLC had been used to detect laxatives a confirmatory method such as TLC would have been essential. In addition, HPLC is a more 'temperamental' technique requiring fastidious attention to detail. In brief, HPLC is useful when quantitation

rather than identification is required while TLC is the preferable method in the converse situation.

TLC of Stimulant Laxatives

TLC was chosen as the most appropriate screening method for detection of laxatives because of the problems in their measurement by HPLC. Figures 3.5 and 3.6 show chromatographs of typical separations using the main solvent system of 4-methyl-2-pentanone:-chloroform:acetic acid (5:2:1) and the confirmatory method using hexane:toluene:acetic acid (3:1:1). The colours produced and their Rf values are shown in tables 3.7 and 3.8.

In the first screening procedure published, by a Dutch group (161), two separate TLC separations using different solvent systems were required in order to achieve adequate resolution. However, Morton demonstrated that all laxatives can be satifactorily separated by employing a single, and simpler, solvent system (chloroform:acetone - 4:1) (162). This method was initially chosen for the present study and proved to be effective for separating all laxatives with the exception of rhein. The reported Rf for rhein was 0.5, but in our hands the pure rhein standard did not migrate from the origin, while a positive rhein control had a slow mobility (Rf = 0.04). The reason for this difference could not be accounted for, especially since both sources of rhein gave similar mobilities using the Dutch system and the one finally chosen for this study (4-methyl-2-pentanone:chloroform: acetic acid - 5:2:1).

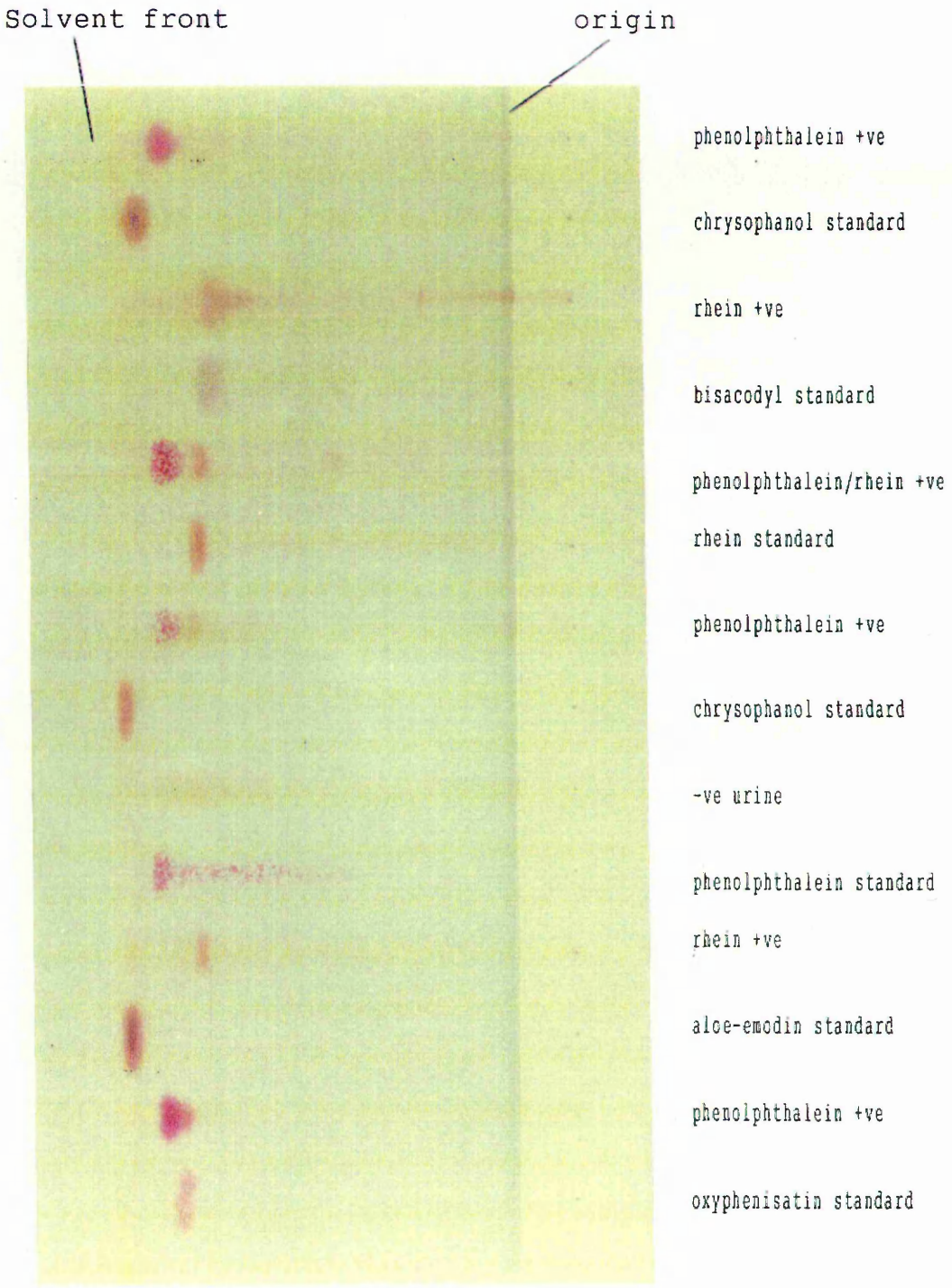


Figure 3.5 - Typical thin layer chromatograph of laxatives using main solvent system (methyl pentanone-:chlororform:acetic acid).

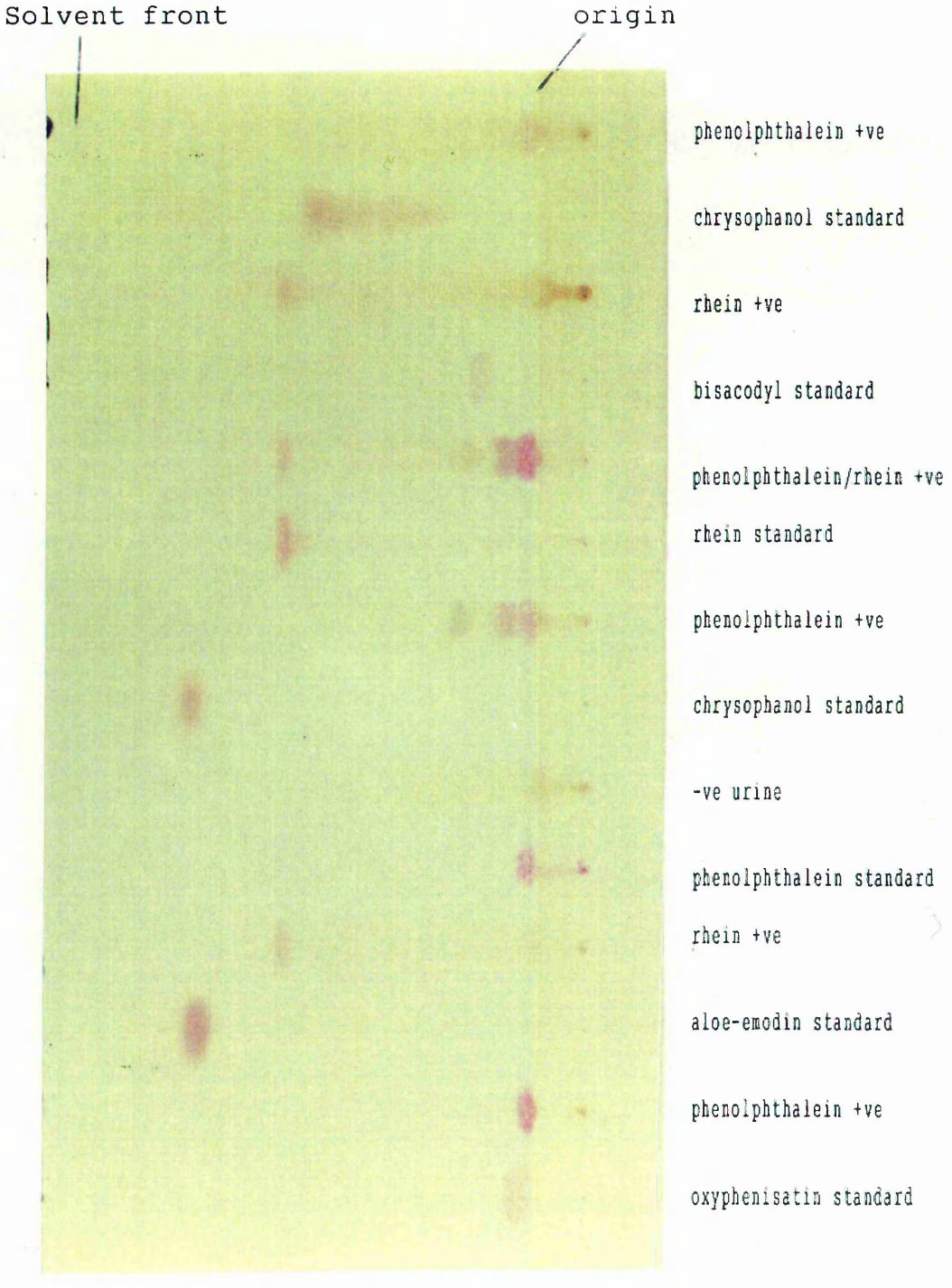


Figure 3.6 - Typical thin layer chromatograph of laxatives using confirmatory solvent system (hexane:toluene:acetic acid).

Laxative	Rf	Light	Alkali	Heating
Bisacodyl	0.68	-	-	Blue/purple
Biscodyl Metabolite	0.39	-	-	Blue/purple
Phenolphthalein	0.89	-	Violet	Violet
Phenolphthalein metabolite	0.86	-	Blue/purple	Blue/purple
Rhein	0.81	Yellow	Orange	Orange
Emodin	0.93	Yellow	Purple	Purple
	0.86	Yellow	Red/brown	Red/Brown
Chrysophanol	0.93	Yellow	Pink/purple	Pink/purple
Danthron	1.0	Yellow	Orange	Orange
Oxyphenisatin	0.46-0.98	-	-	Pink/purple
Frangula	0.92	Yellow	Pink/purple	Pink/purple

Table 3.7 - Mobilities and colour reactions of colonic stimulant laxatives separated by TLC (methyl pentanone:chloroform:acetic acid).

Laxative	Rf	Light	Alkali	Heating
Bisacodyl	0.14	-	-	Blue/purple
Biscodyl Metabolite	0.0	-	-	Blue/purple
Phenolphthalein	0.04	-	Violet	Violet*
Phenolphthalein metabolite	0.04	-	Blue/purple	Blue/purple
Rhein	0.53	Yellow	Orange	Orange
Emodin	0.44	Yellow	Purple	Purple
	0.	Yellow	Red/brown	Red/Brown
Chrysophanol	0.73	Yellow	Pink/purple	Pink/purple
Danthron	0.7	Yellow	Orange	Orange
Oxyphenisatin	0.0-0.15	-	-	Pink/purple
Frangula	0.83	Yellow	Pink/purple	Pink/purple

Table 3.8 - Mobilities and colour reactions of colonic stimulant laxatives separated by confirmatory TLC method (hexane:toluene:acetic acid).

It was felt that the unexplained difference in movement of standard and sample rhein could cause confusion and this was later shown to be the case. A colleague in Edinburgh using the chloroform:acetone solvent system contacted me for assurance that a result he had obtained was not due to the presence of a laxative. The substance in question gave a colour reaction typical of rhein but had a different mobility from the standard (0.04 compared to 0). When the run was repeated in the Gastroenterology laboratory in the Royal Infirmary similar results were found. Although the sample spot was distinct from the standard it had the same Rf as the positive control. When either of the solvent systems quoted in the present study were used, the band in question was readily identified as rhein.

This episode revealed one of the arguments for providing a regional service for detection of laxatives, namely the availability of experience. Another potential hazard in using TLC is that by applying too much urine extract to the plate, ironically perhaps in the hope of not missing a positive response, the presence of a laxative can be concealed. In addition, the phenolphthalein colour, while being very vivid and characteristic, fades quite rapidly and in inexperienced hands may well be missed.

With the low numbers of laxative screens requested at present, it is not a sensible use of hospital resources to duplicate an infrequently used test in each laboratory. Even with this laboratory acting as a regional centre for

the detection of laxatives the low request rate has resulted in most requests being analysed individually.

Predictive Value of TLC Laxative Screening

The predictive value of the TLC laxative screening test in terms of sensitivity and specificity cannot be accurately evaluated in the absence of a definitive method for identification such as mass spectrometry. With respect to the sensitivity of laxative screening, it was impossible to determine how many, if any, laxative abusers were missed. However, de Wolff *et al* have provided some insight into the sensitivity of the test by performing TLC on urine extracts obtained at different times after ingestion of the lowest commercially available dose of each laxative (161). They found that all the laxatives were detectable in urine up to 18 hours, and in most cases up to 32 hours, after ingestion. In a second similar study (164) positive results were found for all urine samples from sixteen patients who were taking laxatives in doses ranging from one to six tablets per day. This second study also concluded that the method was sufficiently sensitive to detect bisacodyl, phenolphthalein or anthraquinone at least 32 hours after ingestion of a single dose (178).

It is also possible to miss a laxative abuser if the urine sample is collected at a time when the patient has not been taking laxatives. In Bytzers' study (164) four of seven patients with laxative induced diarrhoea were taking

laxatives intermittently and so if a single urine sample had been collected false negatives might have resulted. In contrast, if the patient has diarrhoea at the time that the sample is collected then one sample will suffice. Although in the present study one urine was usually collected, this was done at a time when the patient was complaining of diarrhoea.

It is particularly important that a laxative screening test is specific, since a false positive could result in a patient being misdiagnosed as a laxative abuser. While the patient would, quite appropriately, deny taking laxatives, the clinician would take such a denial as being in keeping with the diagnosis. The specificity cannot be determined in the conventional way by comparison with a gold standard method since none exists. Although complete assurance cannot be given regarding the specificity of the laxative screening test, de Wolff and colleagues have tried to exclude the possibility of false positive results by demonstrating the absence of substances which might give a positive response. Hence they performed the screen on 73 commonly prescribed non-laxative drugs, none of which gave positive results. (161).

Two possible sources of anthraquinone substances which could result in false positive results have so far escaped investigation. All of the purgative substances that have been used through the history of medicine were derived from plants, indeed the anthraquinone laxatives still sold today are obtained on a commercial scale from plants

rather than being synthesised. It is quite plausible then, that dietary sources of anthraquinones might be detected in the urine. In addition anthraquinones and some chemically related substances have therapeutic qualities other than laxation. Examples are drugs such as dithranol, podophyllum, and chrysarobin for the treatment of skin conditions, and mithramycin, daunomycin, and adriamycin as antibiotics. The possibility of these drugs interfering is strengthened by the observation that some colour the urine red similarly to some laxatives.

These two potential causes of false positive results were investigated in this study.

Of the foods analysed only rhubarb contained a substance which produced a characteristic although faint colouration corresponding to chrysophanol. Using the confirmatory TLC method the Rf of the rhubarb band was 0.51 in comparison to chrysophanol whose Rf was 0.73. Urine samples from four volunteers who had taken 85g (a generous helping) of rhubarb eight hours before, were all negative.

In contrast, several of the drugs gave positive responses by TLC (table 3.9) although only one, dithranol, gave an

Drug	Rf by TLC screen	Rf by confirm-atory method	Colour
Plicamycin	0.02	0.0	Yellow
Podophyllum	0.85	0.0	Yellow
Doxorubicin	0.0	0.0	Blue
Dithranol	0.92	0.2-0.75	Brown/purple
Dactinomycin	0.46	0.07	Yellow

Table 3.9 - Laxative screen of potentially interferent drugs.

Rf and colour reaction that was likely to cause confusion.

Whenever a positive laxative result was produced, the other medications that the patient was taking were also obtained and tested by TLC. All of the following drugs which were tested to exclude a possible interference gave a negative result: metochlopramide, imodium, paracetamol, codeine, frusemide, hydrochlorthiazide, bendrofluazide, bumetanide, amiloride, triamterine, cyclopenthiazide amitryptiline, ibuprofen, rifampicin, isoniazid, nitrazepam, ponstan, and hydroflumethazide.

In contrast to the anthraquinones it is unlikely that the presence of phenolphthalein in the urine could be caused by anything other than laxative ingestion. Phenolphthalein is not a naturally occurring substance, neither is it a food additive (although at the turn of the century it was tested as a red colouring for wine) and so would not be expected to be consumed as a normal constituent of the diet.

Diagnosis of Saline Laxative Abuse

Saline laxative abuse is more difficult to identify because the constituent ions of these substances are normally found in body fluids. Consequently appropriate reference ranges in a suitable sample must be determined initially. The first diagnostic consideration then, is to decide the sample in which saline laxative ions should be measured. In order to facilitate this objective, diarrhoea was induced in seven healthy volunteers by oral

administration of magnesium hydroxide, and in two further volunteers by magnesium sulphate and anhydrous sodium sulphate. These doses provoked borborygmus in all subjects followed by 1 - 4 loose bowel motions after four hours.

Plasma magnesium concentrations did not vary significantly before or 30 and 60 minutes after oral administration (figure 3.7). Urinary excretion of magnesium is shown in figure 3.8 compared with 24 hour concentrations in seven patients with diarrhoea and the normal 24 hour value quoted by the Clinical Biochemistry Laboratory.

Magnesium was measured in seventeen stools collected by the seven volunteers and in 60 stool collections from 39 diarrhoea controls. The concentrations of magnesium in the stool homogenate, lyophylate, and supernatant, are shown (figures 3.9, 3.10, 3.11).

There is an obvious difference in the magnesium concentrations between the two groups in all stool samples and the 24 hour urine collections. However, in only the stool supernatant is there a complete separation between the two groups: in the magnesium group the minimum magnesium concentration is 65 mmol/l (mean = 123, sem = 6.1) and in the control group the maximum magnesium concentration is 26mmol/l (mean = 9.1, sem = 0.77).

Previous studies have suggested the measurement of magnesium in urine, stool homogenate or stool water (149,

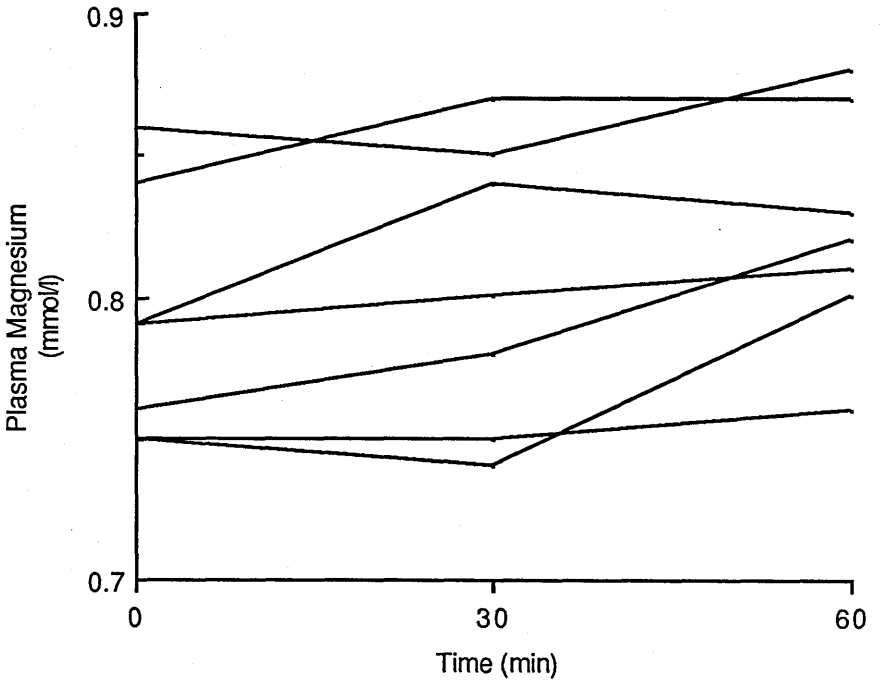


Figure 3.7 - Plasma magnesium concentrations following oral magnesium hydroxide.

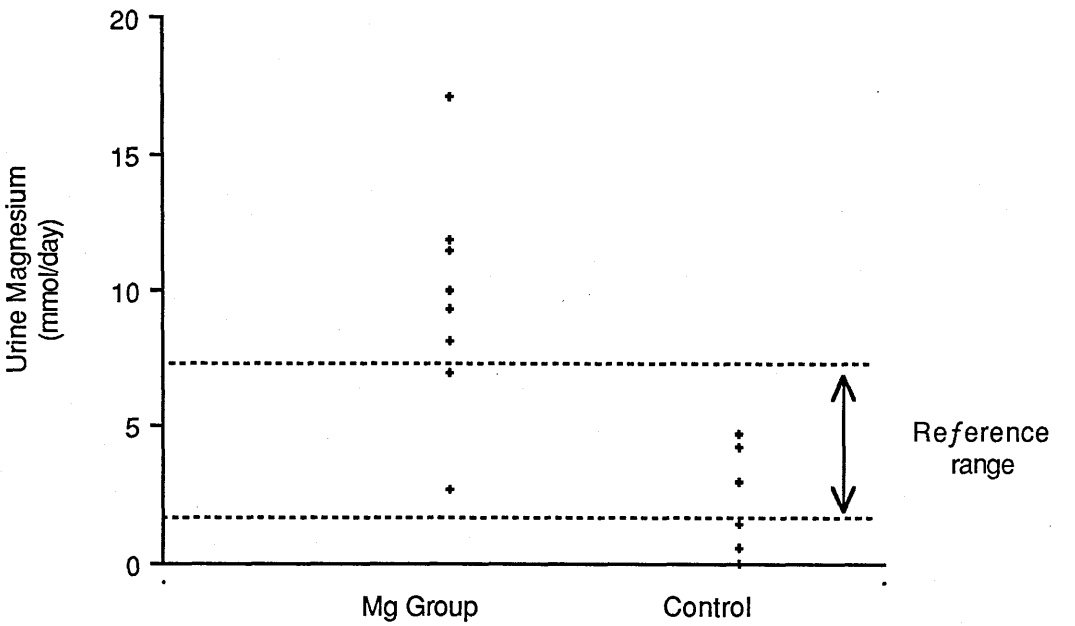


Figure 3.8 - Urine magnesium output in magnesium group compared to control group.

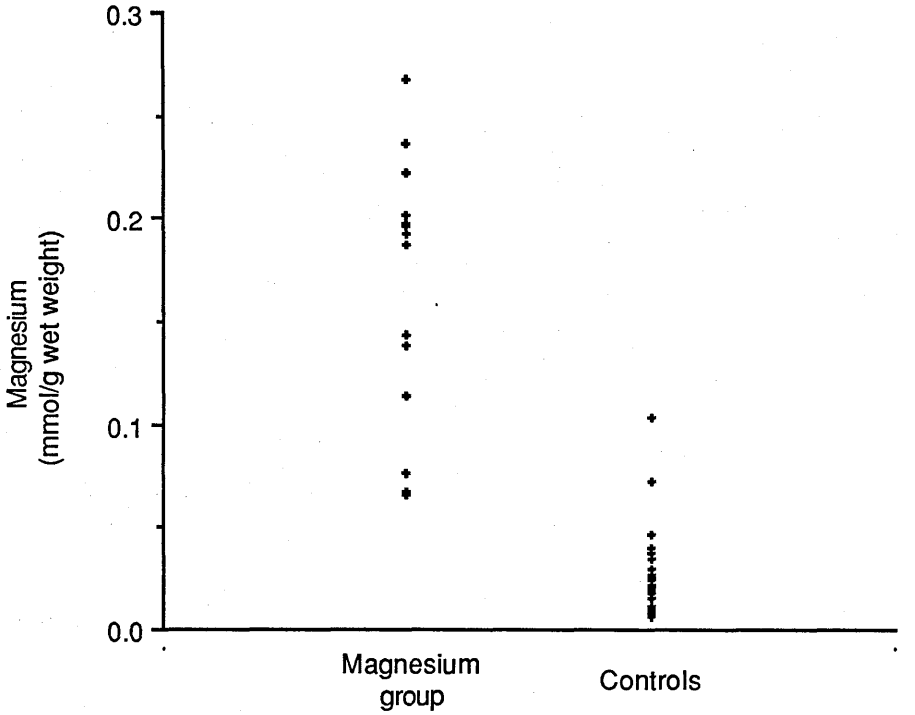


Figure 3.9 - Magnesium concentration in homogenate of magnesium group compared with controls

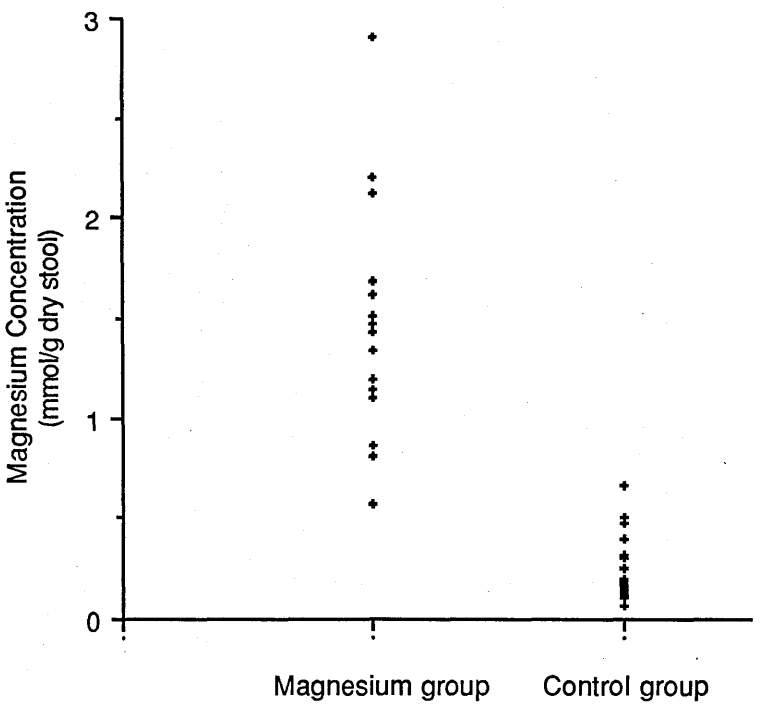


Figure 3.10 - Magnesium concentration in stool lyophylate of magnesium group compared with controls.

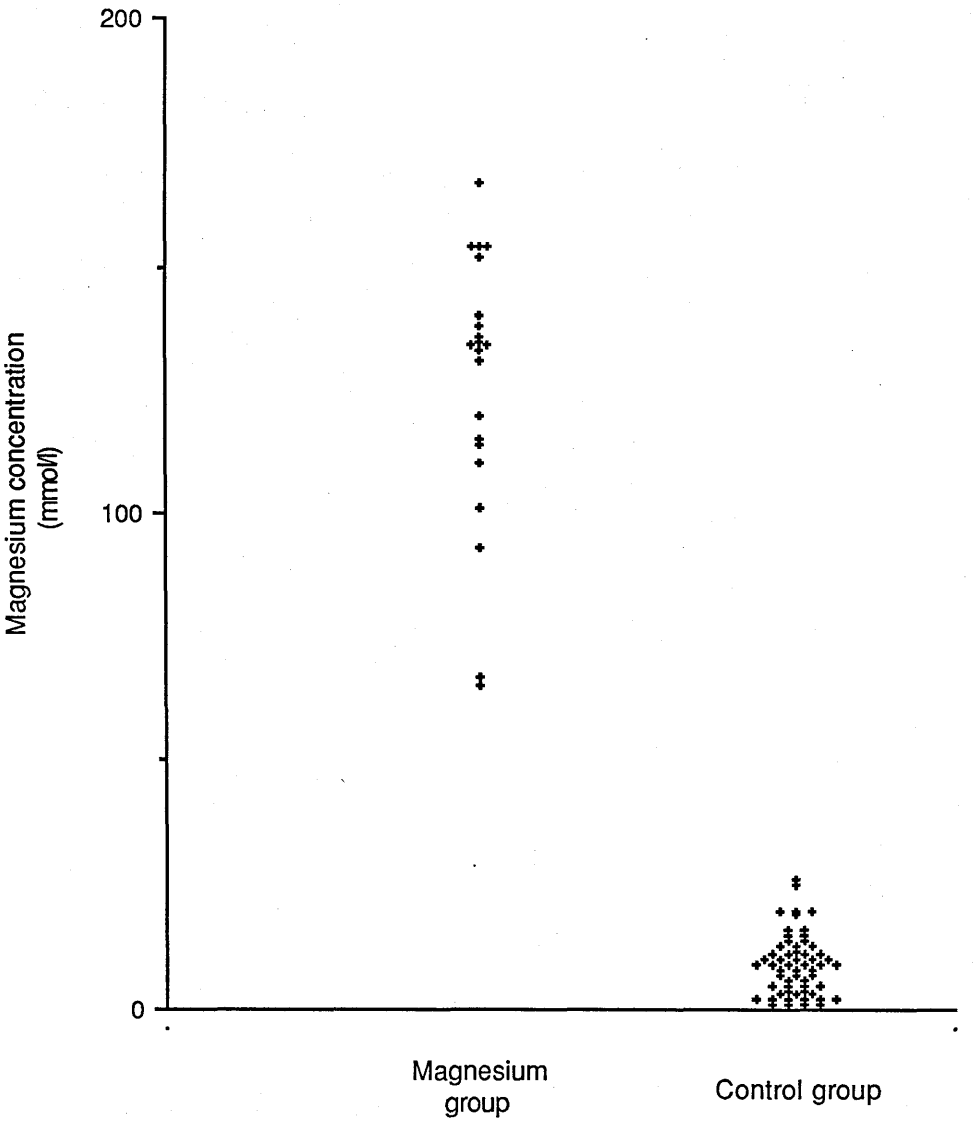


Figure 3.11 - Magnesium concentration in supernatant compared in magnesium and control groups

163, 164, 165). Although our findings show that a urinary output of more than 7mmol/d may be consistent with saline laxative induced diarrhoea, it cannot be considered as diagnostic (see figure 3.8, page 166). Similarly the measurement of magnesium in the faecal homogenate, expressed either with respect to the wet weight or the dry weight of the sample, cannot be used to diagnose magnesium salt laxative abuse (see figures 3.9 and 3.10, page 167). This is not surprising since the faecal output of magnesium is a factor of dietary magnesium intake and percentage absorption of magnesium. Since about 60 - 70% of magnesium consumed normally escapes absorption (179), a high dietary magnesium intake will give elevated outputs of faecal magnesium. Malabsorption syndromes, of which hypomagnesaemia is a common consequence, would also be expected to be associated with an increased faecal output of magnesium. Unfortunately the daily output of magnesium could not be assessed in this study because of the legal problems of using a non-absorbable stool marker (see page 150).

The present work demonstrates that the measurement of magnesium in the stool water gives the only clear separation of subjects with magnesium induced diarrhoea from controls (see figure 3.11).

The next diagnostic consideration was to determine the concentration of magnesium in stool water that separates cases of magnesium induced diarrhoea from other diarrhoeal illnesses. Results from this study show that the maximum

stool water magnesium concentration in the control group was 26mmol/l (mean = 9.1mmol/l, sem = 0.77) compared with a minimum concentration of 65mmol/l (mean = 123mmol/l, sem = 6.1) in the magnesium-induced diarrhoea group. On the basis of these results 30mmol/l would be a suitable cut-off concentration.

Morris and Turnberg (163) suggested a cut-off figure of 20mmol/l but gave no scientific reason to support this choice. Some previous studies have used 49mmol/l as the upper limit of normal, this being the maximum faecal dialysate concentration found in healthy individuals (101). However, the validity of using the *in vivo* dialysis procedure to prepare stool water has been questioned (see page 58). In addition, rather than use healthy subjects as a control group, it is more appropriate to recruit patients with non-laxative causes of diarrhoea.

Our control results were considerably higher than those from a study by Fortran *et al* in which the range of magnesium in a diarrhoea control group was 0.1 - 11.3mmol/l (mean = 2.1, sem = 0.6). They suggested a cut-off concentration of only 12mmol/l (112). However, in their study only a relatively small number of patients with undefined causes of diarrhoea were used to obtain this figure. In our control series some patients were specifically selected because they had fat malabsorption. In these patients a combination of decreased absorption of magnesium and increased solubility, because of lower stool

pH, would be expected to give higher faecal water concentrations. This may explain why our results were higher.

It is worthwhile remembering that the volunteers used in the present study were given a single dose of purgative and so the resultant diarrhoea might not be entirely typical of magnesium laxative abuse. It is also possible that the diarrhoea was more severe than patients with purgative abuse. One volunteer, however, had only minimal diarrhoea with a faecal water magnesium concentration of 65mmol/l which was still quite distinct from the control group (less than 26mmol/l).

Five cases of magnesium induced diarrhoea have been reported and the stool water magnesium concentrations were measured in all. Results of 166, 58 and 21mmol/l were found by Morris and Turnberg in three successive daily stools from a patient with magnesium induced diarrhoea (163). The last result of 21mmol/l can be excluded from consideration because the patient added water to this stool in an attempt to mislead clinicians. In another case report, elevated magnesium concentrations of 148 and 41mmol/l were noted before and after fasting (110). In the other three studies stool water magnesium of 140mmol/l, 120mmol/l and 31mmol/l were noted in patients with magnesium induced diarrhoea (115, 118, 164). Although by our criteria the last result is borderline, the diagnosis was probably correct since the patient was also abusing bisacodyl (164).

Our work also demonstrates the importance of freezing stools until analysis or processing them immediately since the magnesium concentration can rise significantly in stools which are left at room temperature.

Predictive Value of Saline Laxative Measurements

Less can be said about the predictive value of measuring saline laxatives. The severity of diarrhoea correlated with the magnesium concentration suggesting that these purgatives act by an osmotic effect. Consequently it is highly improbable that a patient with saline laxative induced diarrhoea could have a normal concentration of the salt in the stool water. Hence there is little likelihood, assuming the result is technically correct, of producing false negative results. On the other hand, the constituents of saline laxatives are normal body ions and so a false positive result is perhaps feasible, albeit unlikely. Consequently in patients with only a slightly elevated result it would be sensible to exclude a) malabsorption as a possible cause of a high result, and b) a dietary idiosyncrasy which might result in a high intake of magnesium, (such as an addiction to dried fruit, brazil nuts or winkles!).

Mechanism of Diarrhoea

The faecal supernatant electrolyte concentrations of the subjects taking magnesium were measured and are shown in table 3.10.

Faecal Analytes	Median	Interquartile range
OG2	260	234 - 270
Magnesium (mmol/l)	134	114 - 140
PWC value	67%	46 - 73%
Sodium (mmol/l)	5	2 - 8
Potassium (mmol/l)	14	9 - 40
Chloride (mmol/l)	18	16 - 24
Bicarbonate (mmol/l)	152	141 - 170
pH	8.4	8.2 - 8.5

Table 3.10 - Concentrations of faecal electrolytes in supernatants in magnesium hydroxide induced diarrhoea.

The electrolyte composition of the supernatant was quite different from normal faecal fluid: magnesium and bicarbonate concentrations were both considerably higher; the sodium, potassium, chloride and hydrogen ion concentrations were relatively low.

When the severity of diarrhoea, as assessed by the PWC value (see page 75) was studied, no correlation with the magnesium content of faecal fluid was found as had been expected (r_s = -0.04, see Statistical Appendix, note 4). Of the volunteers studied, six produced more than one diarrhoeal stool sample and so it was possible to assess the individual correlation of severity of diarrhoea with magnesium concentration (see Statistical Appendix, note 5). When the data was analysed in this way there was a correlation between PWC and magnesium concentrations (r_s = 0.59), implying a direct porportional relationship. The disagreement in results produced by these two statistical approaches, suggests that the subjects had differing sensitivities to high concentrations of magnesium in the intestinal lumen.

As was previously mentioned (page 102) there was a good correlation between the OG2 and severity of diarrhoea (r = 0.81) suggesting that the diarrhoea is osmotic in nature. The faecal water in these patients has a very large osmotic gap which largely comprises magnesium and its associated anions.

Cholecystokinin was measured and gave the results shown in figure 3.12. However, there was difficulty in solubilising some of the lyophylates and so doubt was cast on the reliability of these results.

CLINICAL ASPECTS

Requesting Pattern

The laxative screening service was offered to Gastroenterologists and Biochemists in the main hospitals of six Scottish Health Boards, covering a population of 2.7 million. None of the regions previously had ready access to such a facility.

Over a two year period, sixteen requests for the screen were made, three of which originated from hospitals other than those originally notified. The breakdown of the figures is given in table 3.11.

Of the sixteen requests made, ten (63%) were for investigation of unexplained diarrhoea, three (19%) in patients with eating disorders, and the remaining three for investigation of infantile diarrhoea, red urine, and of long-standing unexplained hypokalaemia.

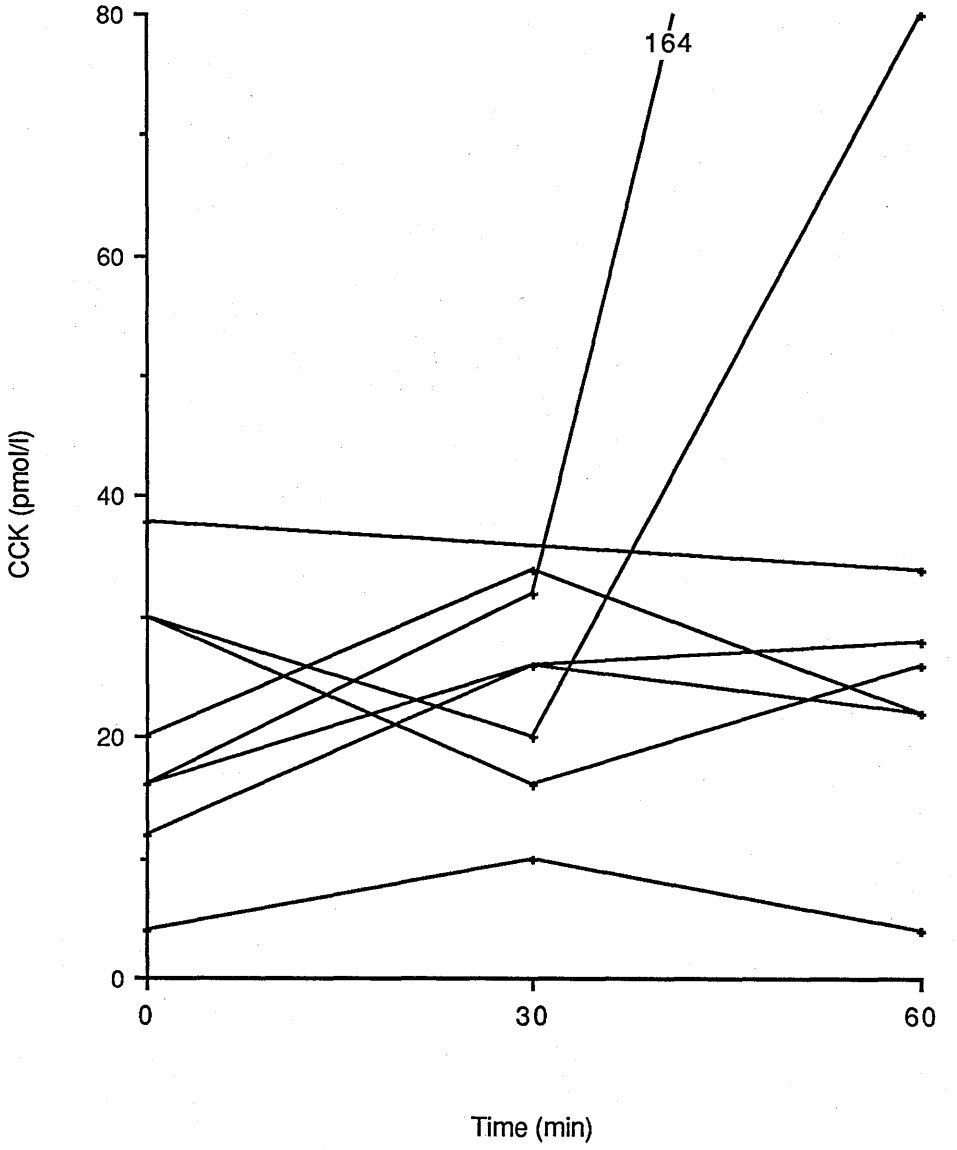


Figure 3.12 - Plasma cholecystokinin following magnesium hydroxide ingestion

Hospital	Source	No.
Glasgow Royal Infirmary	Gastroenterologist	2
Royal Hospital for Sick Children	Physician	1
Raigmore Hospital, Inverness	Psychiatrist	2
"	GP	1
Lewis Hospital, Stornoway	Psychiatrist	1
Monklands DGH, Airdrie	Physician	1
Falkirk Royal Infirmary	Gastroenterologist	3
Stobhill Hospital, Glasgow	Gastroenterologist	1
Royal Alexandra Hosptial, Paisley	Physician	1
Western Infirmary, Glasgow	Gastroenterology	3

Table 3.11 - Requesting Pattern for Laxative Screen over a Two Year Period

Over the same two year period none of the physicians within the Glasgow Royal Infirmary who were informed made use of the facility.

Despite the apparent clinical usefulness of this screening test it was surprisingly infrequently used, as reflected by the request rate of eight per annum. In addition many of the requests came from the same sources (frequently Falkirk and Inverness) where there was presumably more awareness of laxative induced diarrhoea (LID) as a diagnostic possibility.

This low rate of requests prompted the telephone survey in order to determine the pattern of laxative screen requesting throughout Scotland. This exercise revealed a similar request rate with a total of only about twenty laxative screens being performed annually in Scotland.

Two laboratories were found to have carried out tests for phenolphthalein in the past but did not provide a comprehensive service. A full screening service, using

TLC, was only available in two other centres.

Prior to 1987 only one centre in Scotland (Wolffson Gastrointestinal Laboratory in Edinburgh's Western General Hospital) offered a laxative screening service. It is of interest that of the three screening services that have been initiated since then, only one of these was in response to a request from clinicians (at Raigmore Hospital in Inverness, whose samples are now analysed in the Gastroenterology Laboratory at Glasgow Royal Infirmary). The other two were instigated by Biochemists as research projects.

Prevalence of Laxative Induced Darrhoea

One of our principal objectives of the study was to the determine the prevalence of laxative abuse. However, it was clearly impracticable to screen the general population to this end. Consequently the prevalence of laxative abuse was estimated in a sub-population who were unselected apart from having diarrhoea and being referred to the Gastroenterology Department of Glasgow Royal Infirmary for its investigation. We discovered in 4% (2 of 49) of patients presenting with diarrhoea, that the complaint was self-induced.

It is interesting to gauge the incidence of laxative induced diarrhoea in Scotland using this statistic. Almost 7,000 new patients were seen at Scottish Gastroenterology outpatients clinics in 1989 (information provided by Information and Statistics Division, Scottish

Health Services Common Services Agency, Trinity Park House, South Trinity Rd, Edinburgh). Since about a third of new patients seen present with diarrhoea (181) the prevalence of LID in Scotland can be extrapolated to be approximately 82 cases per year.

The prevalence of LID was also measured in patients who were already being investigated for chronic diarrhoea of unknown cause or on whom the diagnosis was not definite. Many of these patients had complained of diarrhoea for many years. In 20% of these patients (2 of 10) laxatives were detected in the urine. This figure is broadly comparable with the frequency of laxative abuse in unselected patients who were referred to specialist Gastroenterology Centres with long-standing diarrhoea: 26% by Fordtran *et al* (111) and 13% by Lodefoged *et al* (118).

It was entirely coincidental that a diagnosis of LID was happened upon in two further patients who were also being investigated for chronic diarrhoea. Both had been taking phenolphthalein and during alkalinisation of stool samples the characteristic colour of this laxative was noticed.

It has been widely assumed that LID is relatively uncommon. Although most publications of laxative abuse are in the form of single case reports a few series of patients have been accumulated. However, in each case several years have been taken to amass these. For example Cummings' series of seven patients were diagnosed over a period of five years with some of these having been

investigated for much longer (149). Fordtrans' group found that laxative abuse was the commonest diagnosis, at 7 of 27 (15%), in patients referred to them for investigation of chronic idiopathic diarrhoea (111). These patients who were highly selected having previously been investigated elsewhere, were gathered over a period of six years. Fordtran *et al* extended this study by determining the diagnosis in 87 subsequent patients who were referred from other physicians for investigation of chronic diarrhoea (180). Of these patients, 20 (23%) were surreptiously ingesting drugs, predominantly laxatives. In a similar study performed over a five year period, 3 of 23 (13%) with chronic diarrhoea were found to be laxative abusers (118). The largest documented series was obtained retrospectively with 17 LID patients being uncovered during a five year period from a large referral centre with 1000 beds (150).

It is difficult to extrapolate the real prevalence of LID from these reports. In some studies highly selected groups of patients with severe chronic diarrhoea were recruited, while in others only patients suspected of laxative ingestion were studied. None examined the prevalence in unselected patients with diarrhoea. A more recent study has given more insight into the prevalence of LID (164): of 200 unselected patients attending a Gastroenterology department 54 had diarrhoea of which 47 were screened for ingestion of laxatives (including magnesium laxatives). Of these, seven gave positive

results putting the prevalence at 15% in this population. It is possible that this figure is an underestimate since four patients with diarrhoea refused to be included in the study when they were informed (surprisingly) that their excretions would be analysed for laxatives.

While the relatively high prevalence rate of 4% in our study was unexpected, we were further surprised at the 15% figure quoted by the Danish group. The discrepancy between the two studies is large considering that the design of the investigations was broadly similar. However, one important difference was the way that the two studies tried to avoid missing the diagnosis in patients who only took laxatives intermittently. In the study by Bytzer *et al* several urines from each patient were usually collected (on average two per patient). Although we only requested one urine sample per patient we asked that it be taken at a time when diarrhoea was occurring. Since we put the onus on patient co-operation in this respect, it is possible that our result may be underestimated. Nevertheless, it was felt that this was probably not the main factor: using their figures it was calculated that two out of the seven laxative abusers might have been missed had they collected only one urine. This would still put the prevalence in their study at 11%. Other factors, possibly cultural differences, between the two populations could be involved.

It was interesting that in the Danish study the type of laxatives ingested were anthraquinones in 71% (5 of 7

patients) and bisacodyl in 29% (2 of 7 patients), while in our experience phenolphthalein is the laxative most usually taken at 86% (6 of 7 patients) followed by anthraquinone at 14% (1 of 7 patients) with no patients taking bisacodyl. This preference for phenolphthalein was also found in America (180). This may be due to a combination of different preferences between the two populations and differing availabilities of 'over the counter' laxative preparations. On the other hand the high prevalence found by Bytzers' group could also be explained by false positive results, which are more likely to be encountered with anthraquinone laxatives.

Case Histories

During the two year course of this study nine patients were discovered to be taking laxatives, seven of whom were being investigated or followed up in a Gastroenterolgy clinic for diarrhoea.

A brief case history of each of the nine diagnosed cases of laxative abuse follows.

1. MD, a 64 year old lady, presented to the Gastroenterology clinic with a two month history of diarrhoea, abdominal pain, insomnia, fluid retention, and general pruritis amongst other complaints. She was anxious, highly-strung, and hyperactive with a poor appetite. After several investigations a diagnosis of irritable bowel syndrome was made and she was treated with amitryptiline and ibuprofen and was regularly seen at the

outpatient clinic.

About a year and a half after her initial presentation she was admitted for investigation of weight loss in the presence of continuing diarrhoea and abdominal pain. A three day stool was collected for analysis of faecal fat. During the alkalinisation step of this assay the sample turned a purple colour which was subsequently shown by TLC to be phenolphthalein. She emphatically denied taking this despite several further positive urine analyses.

Her symptoms remained and she was continued to be seen and investigated at the Gastroenterology clinic for several months but was eventually discharged.

2. IH, a 50 year old woman, had made numerous prior visits to Gynaecology, Orthopaedic, Surgical, Psychiatric and General Medical clinics over an eighteen year period. In 1972 she was initially investigated in Gynaecology clinics for lower abdominal pain which was thought to be related to her menstrual cycle. After sterilisation she complained of pain in the lower back, right iliac fossa, and right thigh. Investigations revealed little and she was treated symptomatically.

Since then she frequently complained of pain which was variously located in the back, thigh, head, right iliac fossa, and upper abdomen. A large functional component was suspected especially since she had many emotional difficulties and at different times suffered from depression, anxiety, insomnia, lethargy, and poor

appetite. She was taking many medications for these ailments and at one time had been prescribed danthron for the treatment of constipation.

Over the years more tangible diagnoses were found and treated accordingly: thyroidectomy for simple cystic adenomata; bilateral oophorectomy for polycystic ovaries; hysterectomy because of dysmenorrhoea; and cholecystectomy for gall stones.

In 1978 following one of many check-ups she was found to have hypokalaemia ($K^+ = 1.6$) and had predictable symptoms of muscular weakness, tiredness and lack of energy. She also complained of vomiting and this was thought to be a contributory factor to her low serum potassium, along with inadequate nutrition and a suspicion of laxative abuse. Although she had admitted to taking laxatives in the past this was contemporarily denied. Other members of her family, however, confirmed that she took sennoside laxatives and on occasions she also admitted to the intermittent use of laxatives to control her constipation.

Latterly she complained of urinary incontinence which was though to be voluntary, and polyuria which was caused by massive intake of fluids especially Cola which she consumed in quantities of several litres each day.

The subsequent laboratory confirmation of laxative intake (on these occasions phenolphthalein was detected) served to corroborate clinical suspicions but this information did not elicit any therapeutic benefit. This lady's

medical and psychiatric problems continue unabated and she remains a frustrating clinical challenge.

3. CL, a 41 year old casualty sister, presented with diarrhoea of two years duration dating from the birth of her last child. She had suffered from post-partum depression and also developed a phobia about going out. The presence of melanosis coli was noted at sigmoidoscopy but she denied taking laxatives. When informed about the association of melanosis coli and laxative use she explained that her mother had given her laxatives as a child because of a 'lazy bowel'.

Despite this story laxative abuse was still suspected because of her unusual personality, and a laxative screen was positive for rhein. Initially she again denied taking purgatives but eventually admitted to taking Senokot. She was referred to a psychiatrist for management.

4. MK had already been undergoing long-term treatment of fluid retention syndrome or idiopathic oedema, a condition which other family members also suffered. She was a difficult patient with an unstable and emotional personality and could be very manipulative and hysterical. During a period of poor psychiatric status she had been admitted with nausea, vomiting, and diarrhoea and was found to have hypokalaemia and hypochloraemic alkalosis. A butterfat test demonstrated that she had malabsorption and a three day stool collection was made for measurement of α_1-antitrypsin clearance. While the glassware was

being cleaned of faecal contamination using an alkaline detergent, a strong purple colour typical of phenolphthalein, was noticed. The presence of phenolphthalein was confirmed by TLC.

When confronted with this finding she vigorously denied taking laxatives to the extent that her physician was persuaded to accept her story and subsequently made a diagnosis of irritable bowel syndrome. A few months later her diarrhoea spontaneously resolved. Shortly after she presented with breast pain and urge frequency of micturition.

5. GH had been diagnosed with coeliac disease in 1966 but had not taken a gluten-free diet because of financial restraints. He presented in 1983 with epigastric discomfort and a variety of non-specific gastrointestinal problems including loose motions but had no symptoms strongly suggestive of continuing coeliac disease. He was described as having a bizarre personality, claimed to have stigmata, and had a previous psychiatric referral.

A urine sample was taken as part of the study to determine the prevalence of laxative-induced diarrhoea and this, and further samples, were positive for phenolphthalein. He repeatedly denied taking laxatives and has been treated symptomatically since.

6. LF, an 18 year old girl who was thought to be suffering from anorexia nervosa, presented to her family doctor concerned that she might be passing blood because of red

colouration of her urine. No blood was detected by dip-stick analysis and her astute GP thought that she might be taking phenolphthalein. The patient denied this during her initial GP visit but when the laxative screen detected phenolphthalein and she was asked again, she readily admitted taking Sure Lax laxative tablets in an attempt to control her weight.

7. GS, a strange 55 year old man, had a laxative screen when he presented to the clinic with a six month history of diarrhoea and abdominal pain. This was positive for phenolphthalein and he admitted that he had taken laxatives. This man was considered to have a low intellectual capacity and a short time later he again presented to the clinic complaining of diarrhoea. A laxative screen was not requested.

8. KO, a Ugandan girl of Asian descent, presented in 1981 with a six week history of constant diarrhoea, vague upper abdominal pain, nausea and vomiting. She was a very nervous individual and after several investigations and unsuccessful treatments with lactose-free and high-fibre diets she was diagnosed as having irritable bowel syndrome.

She continued to be seen at the Gastroenterology clinic for the next two years but because of continuing diarrhoea and abdominal pain, as well as weight loss and slight anaemia, she was admitted for investigation. She was thin on admission and several investigations, including Mantoux

test, were negative. A presumptive diagnosis of Crohn's disease was made but response to sulphosalazine and steroids was poor. Subsequent colonoscopy was normal and she was readmitted for laparotomy which excluded Crohn's disease.

Despite negative tests for tuberculosis a trial of isoniazid and rifampicin was started with some improvement in symptoms. For the next six years she had many intermittent courses of treatment for tuberculosis but continued to have diarrhoea and abdominal pain. In 1989 she was seen at the Rheumatology clinic because of arthropathy and neurological problems and was diagnosed with osteomalacia. In the same year a routine urine sample was collected as part of this study and this gave a strongly positive result for phenolphthalein. She denied laxative intake and despite many subsequent positive urine tests and prompts, continues to disclaim this.

9. HP presented as a very pleasant 34 year old lady for investigation of abdominal pain. Past medical history revealed that she had been troubled with constipation since the birth of her second child. She was found to have iron-deficiency anaemia caused by a combination of gastric erosions and heavy periods. She developed watery diarrhoea defaecating about five times every three or four days. Initially she was thought to be suffering from irritable bowel syndrome but a previous finding of amyloidosis made intestinal amyloidosis a diagnostic possibility. A urine sample collected as a routine part

of this project was strongly positive for phenolphthalein. On her next two clinic appointments she denied self-induced diarrhoea or that laxatives could have been ingested accidentally. However, she subsequently made a tentative admission conceding that she had taken laxatives prior to her present appointment and also in the past in order to contol constipation. The cause of the latter was thought to be pelvic floor damage during childbirth or descending perineum syndrome. On referral for surgical assessment she was found to have a normal proctogram but a very slow transit constipation only defaecating every 10 - 14 days when laxatives were avoided. At present she is considering colectomy.

* * * * * *

Table 3.12 summarises some of the more relevant findings in the seven patients who had laxative induced diarrhoea.

Patient	Time to Diagnosis	Denial of Laxative	Diagnosis Suspected	Steato-rrhoea	Diagnosis
MD	18 months	YES	NO	MILD	IBS
CL	1 month	YES/NO	YES	?	?LID/?IBS
MK	6 months	YES	NO	MILD	IBS
GH	6 months	YES	NO	?	IBS
GS	9 months	NO	YES	NO	?IBS/?LID
KO	8 years	YES	NO	MODERATE	IBS/Crohns /TB
HP	9 months	YES/NO	NO	NO	?IBS/ ?Amyloid

Table 3.12 - Summary of findings in patients with laxative induced diarrhoea.

Rate of Missed Diagnosis of Laxative Induced Diarrhoea

An important incidental finding of this study was that in five of the seven LID patients (71%) the diagnosis was clinically unsuspected. This figure accords with Bytzer *et al*'s report (164) in which the diagnosis was unsuspected in four of seven (57%). In particular we found that the diagnosis was often mistaken for irritable bowel syndrome (IBS): of our seven patients a diagnosis of IBS had been made in three and was, or had been, considered in four others (table 3.12).

IBS is one of the most common conditions seen by Gastroenterologists: of 2000 consecutive outpatients attending a Gastroenterology clinic, an eventual diagnosis of idiopathic painless diarrhoea was made in 214 (10.7%) and a further 998 (45%) were diagnosed with IBS (abdominal pain and altered bowel habit) (181). In view of the incidence of IBS and possibility of LID being mistaken for IBS, it would be prudent to perform a laxative screen on these patients. Indeed, since IBS is considered to be a diagnosis of exclusion, it is mandatory to perform a laxative screen before the diagnosis can be confidently made.

LID has been well documented over the years as a clinical entity and textbooks invariably warn of the possibility of surreptitious ingestion of laxatives in patients with chronic diarrhoea. Nevertheless, as the low request rate for laxative screens testifies, there seems to be little clinical awareness of the condition. In contrast,

requests in this country for VIP measurement are relatively common in the diagnostic workup of patients with chronic idiopathic diarrhoea, yet the incidence of VIPoma is rare. For example, of the patients referred to Fordtran's unit for investigation of diarrhoea (111) the possibility of VIPoma had been suggested in most patients by the referring physicians (None of these patients was eventually diagnosed as such.)

It is interesting to speculate the reason that LID is infrequently considered as a diagnosis. Typically, patients with LID, and indeed IBS, are not unwell but tend to be nervous or anxious and are often quite demanding. They are often referred to as 'heart-sink' patients because of the feeling they invoke as the doctor sees them entering his consulting room. In the eyes of the clinician, the demands of such patients with an apparently functional illness may outweigh the severity of their symptoms. Consequently they may not command as much respect, interest, or sympathy as would a more severely ill patient with organic disease. Such a reaction is understandable in view of frequent understaffing and the consequent time constraints on doctors in typically busy outpatient clinics.

* * * * * *

Another unforeseen situation in this study was a reluctance by the doctor to accept the laboratory finding of laxative ingestion. This happened on one occasion when

the physician, caught between laboratory evidence of laxative abuse and his patients' denial, accepted the latter, presumably assuming that the laboratory test was erroneous.

Screening for Laxative Induced Diarrhoea

It has been suggested that it may be a worthwhile exercise to screen for laxatives in all patients presenting to Gastroenterology clinics with diarrhoea. For example de Wolff advised that 'the cost of the laboratory screen is negligible in comparison with the cost of hospital admission or other advanced diagnostic tests' (182). The criteria for instigating screening procedures can be itemised as follows. i) For treatment to be effective the disease should be diagnosed early so that initiation of therapy is prompt. While this is true of most diseases screened, such as cervical and breast cancer, congenital hypothyroidism, phenylketonuria etc, early diagnosis is not crucial for the well-being of patients with LID, especially since response to treatment is relatively poor. ii) The disease should not be readily diagnosed clinically. In the case of LID the only effective way to make the diagnosis is by a chemical test. iii) The benefits of a screening procedure should outweigh the cost associated with it. In the case of laxative induced diarrhoea this is the most telling argument in favour of screening and indeed the main benefit is financial. Bytzer *et al* demonstrated that the cost of a screen (186DKK) was about one hundred times less than the money

actually spent on potentially preventable costs (18,931DKK per patient). In total, the cost of their screening exercise was 20,790DKK compared to 132,519DKK, the costs of outpatient and inpatient visits and investigative tests that were considered in retrospect to be irrelevant (164). Had the cost of inappropriate treatment also been considered these financial arguments would be even more persuading.

It is important to exclude LID so that further unnecessary tests, with corresponding financial and health-care implications, can be avoided. One patient (KO, page 186) in this series highlights this: over an eight year period she had, in retrospect, many irrelevant investigations, including laparotomy and had been variously treated as having IBS for two years, Crohn's disease for several months, and intestinal tuberculaosis for six years. Whereas this patients' initial condition was mainly psychiatric, she now has medical problems in the form of osteomalacia.

* * * * * *

There is less justification in screening for saline laxative abuse: the prevalence is relatively low and such patients, who also usually take colonic stimulant laxatives, would probably be detected by the TLC screen.

Potential Aids in the Diagnosis of Laxative Induced Diarrhoea

Apart from diarrhoea there are a number of signs and

symptoms which may also be part of the clinical picture of LID: abdominal discomfort, weight loss, nausea and vomiting, hypokalaemia with resultant muscular weakness and lassitude, melanosis coli, and steatorrhoea.

Although the diagnosis of LID can only reliably be made by a chemical test, it was thought that 'at risk' patients might be identifiable by a study of their clinical details. For this reason the following symptoms, signs and other clinical factors which were thought to be potentially helpful were chosen: abdominal pain, hypokalaemia, nausea and vomiting, previous psychiatric referral, weight loss, loss of appetite and non-specific psychological symptoms. These factors were selected because of their apparent frequency in our seven cases of LID. The inclusion of the relatively unscientific term of non-specific psychological symptoms was considered useful and included symptoms such as depression, anxiety, insomnia, phobia, and hysteria, and clinical observations such as unusual personality. Table 3.13 compares the incidence of these factors in LID and other patients whose

Symptoms	LID	Diarrhoea Controls	Probability
Abdominal Pain	75%	73%	ns
Hypokalaemia	38%	0%	p = 0.014
Nausea & Vomiting	50%	18%	ns
Psychiatric Referral	38%	18%	ns
Weight Loss	88%	35%	p = 0.015
Poor Appetite	50%	24%	ns
Non-Specific Psychological symptoms	88%	35%	p = 0.015

Table 3.13 - Frequency of potentially useful diagnostic factors in patients with laxative induced diarrhoea and other diarrhoea patients.

diarrhoea was not caused by laxatives. When analysed statistically (see Statistical Appendix, note 6) three factors were significantly more common in the group of patients with LID, the presence of hypokalaemia, weight loss and non-specific pyschological symptoms.

Bytzer *et al* also found that psychiatric disorders were more common in LID (164). Therefore it is particularly important that LID is excluded in patients with psychiatric disorders and those with psychologically related symptoms such as anxiety, nervousness, insomnia, and/or depression.

Other Relevant Clinical Findings in Laxative Induced Diarrhoea

The reported association with steatorrhoea (159) was found in three patients of the four in which faecal fat was measured, with the fat malabsorption being severe enough in one case to cause osteomalacia (KO, page 186). Hypocalcaemia has been documented previously in patients with LID (183, 184, 185, 186) but only once has osteomalacia been reported (183), being induced following twenty years of phenolphthalein intake. Simply by avoiding laxative intake this documented patient improved radiologically, biochemically and symptomatically.

Melanosis coli, a brown discolouration of the colonic mucosa caused by anthracenes, was found in the one patient who took sennokot, an anthraquinone laxative. When she was informed of the association of melanosis coli and

laxative intake she claimed that she had been given laxatives as a child by her mother. However, this explanation was misleading since melanosis coli is known to disappear within a year after discontinuation of anthraquinones (139).

It is widely held that the diarrhoea induced by laxatives is severe (159, 165) and individual reports often quote patients with gross diarrhoea; for example, Read *et al* found the average daily stool weight to be over one litre (1.1l, range = 325 - 2561g/day) (111). However, in most of our patients in whom stool weights were measured the diarrhoea was not copious (mean = 331g/day ± 53, n = 4).

* * * * * *

The motive that drives patients to self-induce diarrhoea is difficult to understand although a desire to induce sympathy from family or friends or to gain attention has been demonstrated in some individuals (159). Consequently the condition can be considered to be a form of Munchausens syndrome. Since many patients (86% in this study) deny taking laxatives they will clearly not volunteer their reasons, even if rational motives exist. In most of these patients, however, there seem to be complex underlying psychological factors of relevance. Table 3.14 documents some personal circumstances of our patients with co-existing psychological conditions which may be pertinent. Anxiety, depression, insomnia and emotional lability are frequently present. It is possible

PATIENT	RELEVANT SOCIAL AND DOMESTIC HISTORY	ASSOCIATED PSYCHOLOGICAL COMPAINTS
MD	Very lonely, unsatisfactory living accommodation	Insomnia, Anxious, Hyperactive, Depression, Fluid Retention
IH	Bossy overbearing mother	Functional Back Pain, Insomnia, Manipulative, ? Self Induced Vomiting , ? Volunary Urinary Incontinence
CL	Physically and verbally abused by 1st Husband, Concerned about weight	Puerperal Depression, Agarophobia, Insomnia, Anxious, Enuretic to age of 12
MK	Marital problems	Idiopathic Oedema, Hysterical, Depression Anxious, ? Pyschogenic Polydypsia
GH		Insomnia, Nervous, Depressed, Impotent, Stress Incontinence, Stigmata
GS		Bizarre Personality
KO	Looks after aging mother	Very nervous
HP		None

Table 3.14 - Relevant Social and Psychological Factors in Patients with laxative Induced Diarrhoea

that with an underlying psychiatric condition, the existence of unmanageable personal or social circumstances may have been the prompt for inducing disease. However, this possibility could not be reliably demonstrated in any of our patients. Two of the LID patients in this study were underweight and anorexia nervosa was a diagnostic possibility although this had not been established.

It is particularly relevant that some of our patients had other medical manifestations of a psychiatric illness. One of the LID patients in this study (MK, page 184) had a primary diagnosis of fluid retention syndrome or idiopathic oedema (IO), and another (MD, page 181) had symptoms consistent with this condition.

The psychiatric make-up of patients with IO has several parallels to those with LID, with psychological symptoms such as lability of mood, hysteria, phobia, anxiety and depression. Indeed, it has been postulated that the pathogenesis of this disorder may be related to diuretic and/or laxative abuse (187). There is a strong local interest in IO (Dr M Dunnigan, Stobhill Hospital) and this enabled a co-operative study to be performed, of the possible role of diuretic and laxatives in the pathogenesis of IO. Preliminary results of this investigation have revealed that laxative abuse is unlikely to be a factor. Of 100 urines from patients with IO, laxatives were found in only four, of whom only one was abusing laxatives.

Treatment of Laxative Induced Diarrhoea

In the treatment of LID one of the first considerations is whether the patient should be confronted with the laboratory findings of laxative ingestion. In most published studies this has usually been done although some psychiatrists doubt its value. All of the patients in our series were confronted with the results of the laxative screen but only one readily admitted to taking laxatives, with two others eventually admitting after further questioning at subsequent consultations. If patients initially deny laxative intake then it is less likely that they will admit to this at a later date. Indeed the more that a patient is questioned about laxative ingestion the more vehement their denial may become. Slugg and Carey believe that heated confrontations can be avoided by explaining in a non-judgmental manner, the result of the test and what it means (150). An alternative approach is to play down the central issue of laxative ingestion and to control the atmosphere and direction of the consultation such that the patient is not embarrassed to admit to taking laxatives. If this fails then an alternative tactic is to avoid confronting the patient with laboratory evidence of laxative ingestion but rather make it tacitly evident in conversation that it is known that laxatives have been taken and so give the impression that the diagnosis has been accepted.

Unless a patient admits to taking laxatives it is difficult to persuade him/her to accept a psychiatric

referral. This is considered to be a central aspect of management. Medical management may, nevertheless, still be required if the patient has electrolyte abnormalities, or other organic illnesses, such as cathartic colon induced by long-term laxative intake. An additional clinical problem is the development of oedema which may occur when the patient stops laxative intake. This is thought to be caused by secondary hyperaldosteronism induced by sodium depletion (188). The latter has been well documented in these patients and is controlled by limiting fluid intake (189).

If psychiatric referral is not accepted by the patient the outcome is generally poor: the patient may continue to attend the clinic for symptomatic treatment or discharge themselves from the clinic and present with the same or other symptoms elsewhere. Alternatively the patient may stop taking laxatives and perhaps present with another medical problem which is secondary to their underlying mental condition.

Ultimately in most cases of LID the clinical outcome is unsatisfactory. This was the case in most of the patients detected in this study. However, despite this unfavourable conclusion LID should still be considered, if for no other reason than to avoid subsequent needless investigations.

CHAPTER 4

DIAGNOSIS OF STEATORRHOEA

INTRODUCTION

Malabsorption of dietary nutrients is a non-specific finding in a wide range of gastrointestinal diseases. Whereas malabsorbed carbohydrates and proteins are metabolised by colonic bacteria, malabsorbed fat passes through the gut relatively unchanged resulting in steatorrhoea, the excretion of excess fat in the stool. Tests for the diagnosis of malabsorption usually focus on the ability of the intestine to absorb fat, rather than carbohydrate, protein or any other nutrient. The physiology of fat absorption is complex, requiring the normal functioning of liver and pancreas as well as intestine and so a test of malabsorption can be used as a broad screening test which reflects the function of these organs. Consequently it is useful to have a full understanding of the normal physiology of fat absorption.

PHYSIOLOGY OF FAT ABSORPTION

Westernised societies have a fat intake of approximately 50-100g, most of which is in the form of triglycerides. The digestion of fat begins in the duodenum, where its presence stimulates the release of secretin and cholecystokinin-pancreozymin (CCK-PZ) from epithelial cells and also inhibits the further emptying of gastric contents into the duodenum. Secretin causes the pancreas to secrete bicarbonate which neutralises the gastric chyme to pH 6.0 - 6.5, the optimum for most digestive enzymes. CCK-PZ acts upon the pancreas to release digestive enzymes and also

stimulates gallbladder contraction and relaxation of the sphincter of Oddi.

Triglyceride digestion requires the presence of pancreatic lipase which hydrolyses the ester bond at positions 1 and 3 of triglycerides releasing β-monoglycerides and free fatty acids. The efficiency of lipase action is greatly improved by the presence of bile salts and lecithin, which are normal components of bile. These substances act as surfactants reducing the surface tension at fat/water interfaces. Pancreatic colipase is a cofactor of MW 10,000 which binds to the interfaces of bile salts and lipids and is necessary for the efficient action of lipase. Other pancreatic enzymes which play a role in fat digestion are phospholipase, which hydrolyses phospholipids within micelles, and cholesterol esterase, which digests cholesterol esters.

The second phase of absorption involves the delivery of the products of lipase digestion - monoglycerides and fatty acids - to the mucosal cells. These digested fats being insoluble in water are not readily absorbed but their solubility is increased by their association with bile salts to form micelles. Digested fats form the centre of micelles, and bile salts which are amphipathic (ie have hydrophobic and hydrophilic areas) surround this core.

In micellar form, fats can more readily penetrate the two layers which cover mucosal cells (the unstirred water layer, and mucous coat layer) so gaining access to the

absorptive villi. In free form, the non-polar fatty acids and monoglycerides would find these layers relatively impermeable. For micelle formation the concentration of aqueous bile salts must exceed a specific value (the critical micellar concentration) of around 4mmol/l (190). In the normal duodenum and jejunum, bile salt concentrations of 3 - 12mmol/l ensure that micelle formation is rapid.

Once delivered to the microvillus membrane the fats are absorbed into epithelial cells where triglycerides are reformed and then incorporated with specific proteins into chylomicra. These proteins (apoB, apoA-1 and apoA-IV) are produced in mucosal cells and are required for the production of normal chlyomicra.

The intraluminal breakdown of micelles results in the release of bile salts into solution again. Although there is some passive absorption in the upper intestine of bile salts, mainly monohydroxy and dihydroxy bile salts, most bile salts are absorbed in the terminal ileum by an active process. A small proportion of bile salts will escape absorption in the small bowel and are rapidly deconjugated and 7α-dehydroxylated by anaerobic bacteria of the colon. Despite the secretion of large amounts of bile salts into the intestine, the efficient enterohepatic circulation, together with some jejunal and colonic absorption, ensures that the faecal loss of bile salts is limited to about 500mg daily.

The final stage of fat assimilation is the release of chylomicra into the lamina propria and their transport through the lymphatic system and thoracic duct to the circulatory system.

PATHOPHYSIOLOGY OF FAT ABSORPTION

The physiology of fat assimilation is summarised diagramatically in figure 4.1. Disease can disturb this physiology in the following ways giving rise to malabsorption.

1. Decreased mucosal release of CCK-PZ and secretin eg. in conditions where the duodenum is by-passed, such as Billroth II anastamosis and gastroenterostomy.

2. Decreased amounts of lipolytic enzymes eg. chronic pancreatitis or obstruction of ducts (pancreatic carcinoma).

3. Inactivation of lipase due to low duodenal pH eg. Zollinger- Ellison syndrome (low pH also causes precipitation of bile salts and damage to mucosal cells).

4. Decreased hepatic release of bile acids eg. chronic hepatic disease causing poor uptake of bile acids from portal circulation, and decreased *de novo* synthesis.

5. Decreased delivery of bile acids to the duodenum eg.

biliary obstruction.

6. Depleted bile salt pool eg. impaired reabsorption of

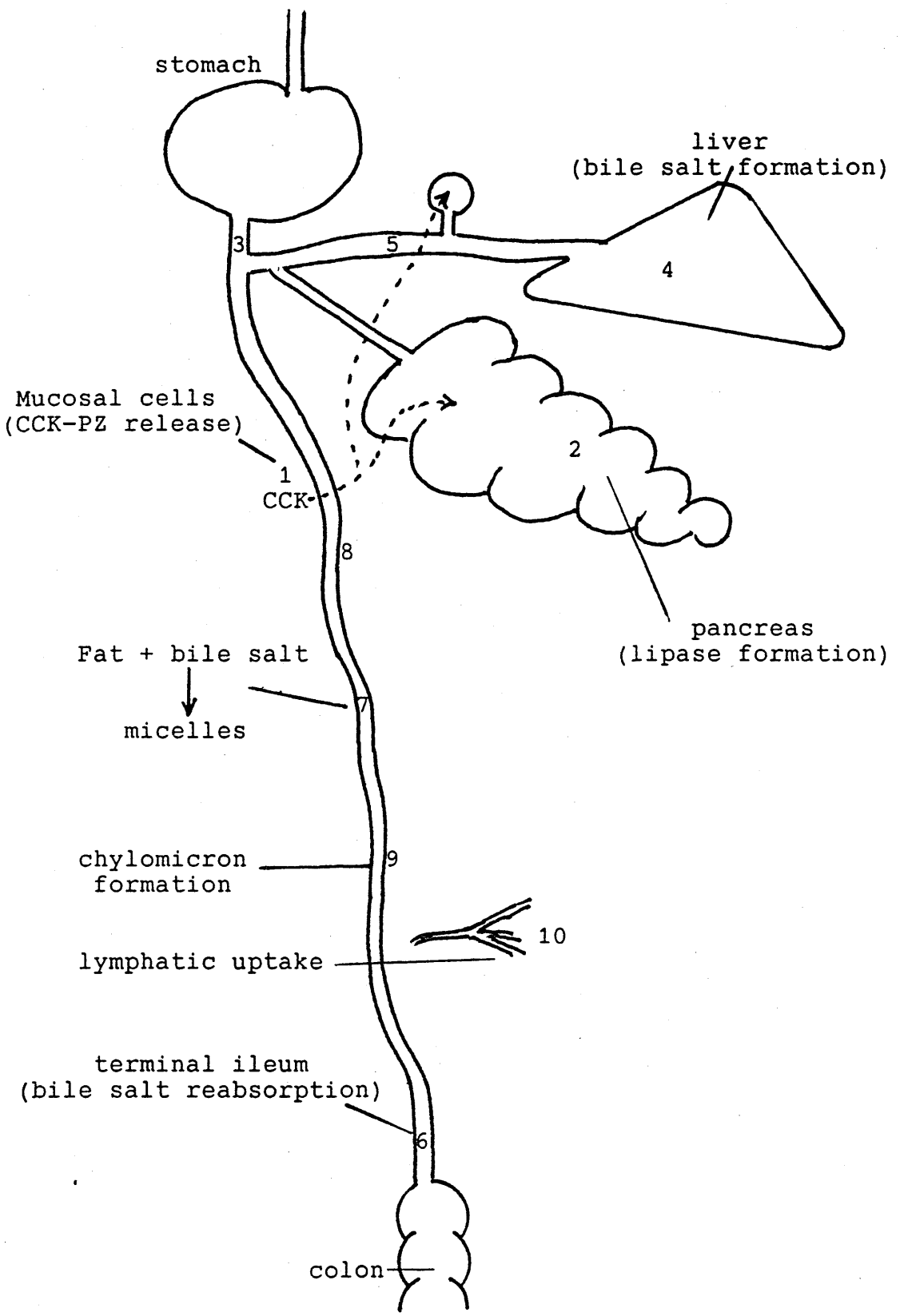


Figure 4.1 - Physiology of fat absorption. Numbers refer to sites of pathophysiological mechanisms in fat malabsorption (see text).

bile acids due to terminal ileal disease such as Crohn's disease, radiation enteritis or terminal ileal resection.

7. Intraluminal deconjugation of bile salts eg. small bowel overgrowth, which can be caused by blind loop; scleroderma; diabetic nephropathy etc.

8. Damaged mucosal cells eg. coeliac disease, giardiasis or Crohn's disease. (There may also be impaired release of CCK-PZ and secretin.)

9. Impaired formation of chylomicra eg. abetalipoproteinaemia.

10. Decreased lymphatic uptake eg. infiltration of lamina propria with macrophages or obstruction of lymph ducts as in lymphangiectasia and lymphoma.

METHODS OF DIAGNOSING MALABSORPTION

1. Faecal Fat

Several methods have been devised for the diagnosis of malabsorption, but the test which is still considered to be the 'gold standard' is the measurement of faecal fat.

It might be expected that the amount of fat in the stool is at least partially decided by the fat intake from the diet. This subject has received considerable attention. When the dietary intake is reduced to negligible amounts in normal subjects there is still a faecal excretion of about 3g/day, which presumably represents normal endogenous losses - loss of mucosal cells and bacterial lipids. When fat intake is

varied in quantity (50, 100, and 150g/day) or type, there is little change in the amount of fat excreted (191). Even with an intake up to 200g/day there is little change in the faecal fat (192). With a normal Western diet, which contains about 50 - 100g of fat each day, the faecal fat is normally less than 6g/day and the efficiency of fat absorption is greater than 97%. However, in patients with abnormalities of fat absorption, an increase in fat intake is, not surprisingly, reflected by an increase in faecal fat (193, 194).

Consequently when fat balance studies are performed it is important to know the fat intake within fairly broad limits. A common problem in estimating fat intake is that GI patients are not infrequently anorexic, or find eating fatty foods nauseating. Feedback from the nurses and dietitians is required to correct for incomplete fat intake and such corrections are based on judged, rather than measured, estimates.

A timed stool collection is required for faecal fat determination and this poses potential problems. Because the bowels cannot be evacuated at will and individuals defaecate about once each day, it is difficult to collect an accurately timed collection over a short period of time (unless perhaps the patient has diarrhoea and requires to defaecate more than about five times per day). This problem can be solved by collecting samples for long periods of time - three to five day collections are considered to be the minimum - or by monitoring the

excretion of a non-absorbed marker such as chromium sesquioxide or cupric isothiocyanate. The former approach makes the test much less appealing for the nursing staff and patient, as well as laboratory staff. The complete co-operation of nurse and patient is needed for accurate collections and against the background of an unpleasant test this may be difficult to obtain. Personal experience has shown that the completeness of the collection (as gauged by the recovery of non-absorbed isotopic markers) varies considerably from ward to ward. Although the daytime ward sister may be convinced of the completeness of collection, she has limited control over other shifts of nursing staff.

The use of a non-absorbed marker does not necessarily solve the problems of a timed stool collection. An additional assay contributes its own degree of imprecision, measurement of a marker may not be a valid procedure in patients with diarrhoea, and new legislation limits the oral intake of non-BP substances such as these markers.

The most 'popular' technique for the laboratory determination of faecal fat is the van de Kamer method, in which the fatty acids are measured by titration after the sample has been homogenised (195). Another commonly used method is the extraction of fat from the stool into a solvent which is then evaporated leaving fat which can be measured by weight (196). The van de Kamer method does not measure non-acidic fats and so results are slightly lower than gravimetric methods. Both types of method are manual

and time-consuming and, being unpopular, are often left to junior laboratory staff. No external quality control scheme is in operation for faecal fat determination and personal experience has shown that many laboratories do not employ internal quality control schemes. This laboratory employs the van de Kamer method but sends a duplicate sample to the routine Biochemistry laboratory for measurement by gravimetry. Results commonly referred to as 'fliers' have occurred with some regularity and presumably reflect poorly controlled laboratory practices.

2. Triolein Breath Test

In 1962 an American group proposed a novel approach for the diagnosis of fat malabsorption. Their method simply involved measuring ^{14}C-carbon dioxide in the breath following the oral adminstration of a triglyceride - trioctanoate - labelled with ^{14}C in the carboxy group. After digestion and absorption the fatty acid moiety is metabolised with the production of $^{14}CO2$, which is exhaled and can easily be measured. The breath $^{14}CO2$ would be expected to be low in malabsorption and higher in normal controls and their work confirmed this theory (197)

This ingenious concept obviates the unpleasantness of stool collections and, not surprisingly, has been widely studied. The original methods for collecting and analysing breath samples were cumbersome and involved sophisticated equipment, but Abt and Schuchling simplified this by trapping CO_2 in a hyamine hydroxide solution followed by

liquid scintillation counting (198). Another important modification has been the use of other more preferable triglycerides. Trioctanoate, being a medium chain triglyceride is absorbable directly without the need for bile acid involvement, chylomicron formation, or lymphatic transport, and is less appropriate as a test fat. Tripalmitate was considered to be a more appropriate choice since it is the commonest triglyceride consumed and tests all physiological aspects of fat absorption. Although some trials have been encouraging (199, 200, 201) several have demonstrated an unacceptably high overlap of controls and malabsorbers (202, 203, 204)

In an important paper by Newcomer *et al* from Hofmann's laboratory, the three triglycerides - trioctanoate, tripalmitate, and trioleate - were used as fat probes and were compared with faecal fat analysis (203). Using faecal fat as the gold standard, the triolein breath test had a sensitivity of 100% and a specificity of 96%, while using the other two triglycerides produced excellent sensitivity but poor specificity. Since this publication there has been considerable interest in the triolein breath test, which has now been evaluated several times (205, 206, 207, 208, 209, 210, 211). These studies, however, show a considerable divergence of opinion as to the clinical usefulness of this test, as illustrated in Table 4.1.

One possible reason for these discrepancies is the design and analysis of the studies. There is no foolproof 'gold standard' test for malabsorption with which to compare the

Reference	Gold Standard	Sensitivity	Specificity	Control Group	Correlation
203	FF > 7g/day	100%	96%	Irritable Bowel	not done
205	FF > 5g/day	93%	100%	GI patients/normal FF	not done
210	FF > 7g/day	64%	100%	GI patients/normal FF	r = -0.8 (all groups)
209	FF > 5g/day	100%	100%	Healthy patients	r = -0.6*
	Clinical Acumen	85%	93%		
207	FF > 30mmol/day	89%	89%	Healthy controls	r = -0.86 (coeliacs)
208	Clinical Acumen	100%	86%	Orthopaedic	r = -0.16 (controls) r = -0.14 (Malabsorbers)
211	FF > 7g/day	42%	92%	GI patients/normal FF	excluded
206	FF > 7g/day	71%	100%	not specified	not done

Table 4.1 - Results of evaluation studies of the ^{14}C-triolein breath test.

(* calculated from published data)

results of the breath test. In the absence of a more suitable test, faecal fat measurement, despite its well known pitfalls, was mainly used for comparisons. Another alternative used was the classification of patients into those who on clinical, laboratory and radiological grounds might be expected to have malabsorption. Neither system of assessment can be considered ideal.

Most of the reports used predictive values for evaluating the data, but quoting sensitivity and specificity alone can be misleading. A small change in the reference range of the 'gold standard' (table 4.1 shows that there is a wide variation in what level of faecal fat is considered normal) can have a sizeable effect on the rate of false positives and negatives. The choice of control population also varied from healthy volunteers to irritable bowel disease and from GI patients to non-GI patients. It is unfortunate that few studies correlated the breath test with faecal fat, as this is a better way of evaluating a new test. The calculated correlation coefficients are shown in Table 4.1. (Those from Pedersons' study have been excluded for reasons quoted below.) It is interesting that there is no correlation in a study which quotes good predictive values (208) and a good correlation in a study which shows much poorer predictive values (210).

Another important factor that has varied between studies is the calorific load given with the ^{14}C-triolein. It is known that the rate of fat metabolism is dependent on the availability of other energy sources (212, 213). By

increasing the calorific load given (either as carbohydrate or fat) the peak of breath $^{14}CO_2$ is generally found to be delayed and reduced in height. For this reason Pederson's study is probably invalid, since a large fat and carbohydrate load was given and the breath was not collected for a sufficient length of time (only six hours) (211).

In general, most studies achieved good discrimination between fat absorbers and malabsorbers, however, the ability to diagnose mild cases of malabsorption has been questioned (210).

In order to calculate the breath test accurately the excretion rate of carbon dioxide must be measured. The instrumentation required for this is not widely available however, and so it is usual to assume that carbon dioxide output is normal - 9mmoles of carbon dioxide per Kg per hour. This data was derived from thirteen healthy fasting males, aged twenty-one to eighty-one, twelve of whom were aged less than twenty. (214) The average value was 8.7mmol CO_2/Kg/Hour, but there was a considerable range from 7.1 - 12.1. Interestingly, the eighty-one year old had the lowest output of CO_2 and this would be in accord with the general understanding that metabolic rate decreases with age. A more recent study has looked specifically at the effect of caloric intake, comparable to that used in breath tests, on the carbon dioxide output (215). At an intake of 200Kcal there was a small rise in carbon dioxide production, which increased to 30% with a calorie intake of 750Kcal. This

effect of calorie intake on the endogenous carbon dioxide output has a two-fold effect on ^{14}C-breath tests: $^{14}CO_2$ is diluted in a greater amount of endogenous CO_2, and the production of the $^{14}CO_2$ itself is greater. The endogenous carbon dioxide output was measured in normal fasting subjects and agreed fairly well with the earlier study (from 6 - 11 mmol/Kg/hour with an average of 8.0 - 8.2). No difference was found between healthy controls and patients with malabsorption. If an assumed value of 9mmol/kg/hour had been used in the calculation (in this case the test was the ^{14}C-glycocholic acid breath test) one of the twelve results would have been misclassified.

The excretion of $^{14}CO_2$ in the breath is affected by respiratory function as well as the rate of fat metabolism, availability of other energy sources, and basal metabolic rate. Consequently the test is considered to be unsuitable for febrile and overweight patients and those with respiratory, liver, and various metabolic illnesses (216, 217). In two studies on patients with liver disease the false positive rates were 33% and 50% (218, 219). Such exclusion criteria clearly limit the applicability of the test, and in one study on patients with suspected malabsorption 46% of the patients had at least one of these conditions (211).

A further important aspect of the test is that fat absorption is assessed over a brief period of time, usually during the morning. This may have implications in patients with a small bile acid pool. The test is usually performed

after a period of fasting during which time the bile acid pool, although diminished, is concentrated in the gall bladder. Consequently, there may be sufficient secretion of bile acids to produce a normal triolein breath test despite the possibility that under non-fasting conditions malabsorption may be occurring.

3. Dual Isotope Tests

A less common approach to the assessment of absorption involves the use of two radioisotopes which are administered orally. One radioisotope, usually ^{14}C-labelled triolein, is absorbable and the other is a non-absorbable marker. The ratio of isotopes excreted in a stool sample is compared with the ratio administered orally. If there is no ^{14}C in the stool (ie. ratio = 0) then there is 100% absorption and if the excreted ratio is the same as that administered then there is 0% absorption. Because a ratio is being measured a random stool sample alone is required.

The two non-absorbed markers which have been used for this method are ^{51}Cr-chloride which is water-soluble, and the fat-soluble ^{3}H-triether. Morgan and Hofmann, using rats with steatorrhoea induced by cholestyramine, showed a good correlation between percentage absorption by the dual isotope method (using ^{3}H-triether) and faecal fat (r = 0.89, 0.96, and 0.87 for three consecutive days) (220). The evaluation of this method in man was continued in our Gastroenterology Laboratory. Gerskowitch *et al*

demonstrated that ^{3}H-triether is almost completely recoverable from the gastrointestinal tract and does not separate from the oil phase (221). Nelson *et al* showed the dual isotope test to be reproducible with little day to day variation. Absorption in normal subjects was within a narrow range of 96.4% - 100%, while patients with steatorrhoea caused by pancreatic or small intestinal disease had percentage absorptions ranging from 0% to 90% (222). Lembcke et al used the same two fats but labelled with γ-emitting isotopes and found a good correlation with faecal fat ($r = 0.813$), with a sensitivity of 87.5% and specificity of 81.8% (223)

Pederson tested ^{51}Cr-chloride as a non-absorbable marker and, although there are theoretical objections to its use since it is water-soluble, good results were obtained, showing a significant correlation with faecal fat ($r = 0.82$) (224).

On theoretical grounds, this type of approach suffers none of the problems of the faecal fat test. As with the triolein breath test it assesses fat absorption over a brief time period, but the problems associated with this (see page 215 - 216) can be easily avoided by starting the test in the afternoon. One aesthetic objection to this test is that a stool collection is necessary, but results can be calculated using a small untimed stool which does not require homogenisation.

4. Other Tests

In addition to the tests already discusssed the following alternative techniques for diagnosing malabsorption have been used. These have not achieved widespread application, however, mainly because subsequent evaluation has shown them to be suspect.

i) Faecal Microscopy: Microscopy of the stool for fat and meat fibres is a qualitative method which is still used as the sole test for malabsorption in some centres. The test is highly operator-dependent. To achieve the optimum quality of results the test should be performed by the same individual who has a clear protocol for analysis and interpretation (225). In severe steatorrhoea the test gives good results, but when the degree of malabsorption is moderate, the sensitivity falls and has been reported to be as low as 14% (226).

ii) Serum Turbidity: After a fatty meal the turbidity of blood increases and this increase has been used as an index of the efficiency of absorption. Although Osman et al (227) showed a clear descrimination between normal controls and malabsorbers, the latter group had pancreatic insufficiency in which there is gross steatorrhoea. When the effectiveness of the technique is studied more realistically, by including patients with less severe malabsorption, there is a large overlap between normal and abnormal groups (205, 228).

iii) Urinary Oxalate: The intestinal absorption of oxalate, and consequently urinary oxalate excretion, is

increased in the presence of malabsorption. It is thought that an increased concentration of intraluminal fats results in the formation of calcium-fatty acid soaps. Consequently, there is competition between fatty acids and oxalate for calcium, resulting in the release of oxalate making it more available for colonic absorption. Hyperoxaluria is often found in subjects with malabsorption and initially the measurement of urinary oxalate following an oral load of oxalate seemed to be a promising alternative to faecal fat determination (229, 230). However, the test requires a carefully controlled intake of fat as well as oxalate and current routine methods for measuring oxalate are poor. In addition a later study has demonstrated a poor correlation ($r = 0.03$) between daily urinary oxalate and faecal fat excretion (207).

OBJECTIVES

The dual isotope fat absorption test using ^{3}H-triether as non-absorbable marker has been developed in this laboratory and is now used as a routine test of fat absorption in this hospital. However, ^{3}H-triether is not a routine item which can be readily purchased from radioisotope companies and in 1987 our stocks, which were custom-prepared by Amersham, were becoming depleted. Consequently a decision had to be made whether to reinvest in another batch of the isotope or to switch over to an alternative test of fat absorption. The main disadvantage of the former approach was the cost involved since one-off custom preparation of isotopes is an expensive procedure (1987 cost of £4,000/mCi). With regard to an alternative fat absorption method there were two which seemed to have potential, although both had possible drawbacks. The simplicity of the triolein breath test was appealing but, as has been outlined above, the results of various evaluations of this technique are conflicting. The second possibility was to continue to use a dual isotope fat absorption test but replacing ^{3}H-triether with $^{51}CrCl_3$ as the nonabsorbed marker. Our main misgiving in using $^{51}CrCl_3$ was that it is water soluble and therefore might not satisfy all the criteria required for a nonabsorbed marker. In particular we were concerned that the test isotope (^{14}C-triolein) and $^{51}CrCl3$ would move through the gut at different rates due to their different solubility properties.

We decided to solve this dilemma by comparing both of these tests with our dual isotope method which we felt could more justifiably claim to be the 'gold standard' test than the measurement of faecal fat (see pages 216 - 217). The practical aspects of the triolein breath test and the two dual isotope tests are very similar. With simple modifications it was feasible to perform the three tests simultaneously. Consequently we were in the fortuitous position of being able to use our remaining stocks of ^{3}H-triether to compare the two fat absorption tests with our existing dual isotope technique.

The individual objectives were as follows.

1. To improve our existing methods for extracting and quantitating ^{3}H and ^{14}C.

2. To modify our existing dual isotope method to ensure that ^{51}Cr could also be counted.

3. To assess the validity of using $^{51}CrCl_3$ as a nonabsorbable marker.

4. To compare the results of the triolein breath test with the dual isotope and faecal fat methods.

5. To measure the endogenous production of carbon dioxide in order to determine the validity of using an assumed value.

6. To determine the necessity of collecting specifically end-expired breath to calculate the triolein breath test

result.

7. To assess whether the triolein breath test gave valid results on the patient groups which are usually excluded, ie. febrile patients and those with metabolic and respiratory illnesses.

8. To investigate the possibility of measuring PAT-3H on single untimed stools.

MATERIALS AND METHODS

EXISTING METHOD FOR MEASURING PERCENTAGE ABSORPTION OF TRIOLEIN BY THE DUAL ISOTOPE TECHNIQUE.

The two isotopes used in the existing dual isotope fat absorption method were glycerol tri[1-^{14}C]oleate and tritiated glycerol triether (Radiochemical Centre, Amersham). The latter was prepared by catalytic reduction of 1-(9-cis-hexadecyl)2,3-didodecyl glycerol with tritium (231). The isotopes - 400kBq of ^{14}C-triolein and 2000kBq of ^{3}H-triether - were administered orally within gelatin capsules and a small aliquot was retained as standard. A 20g fat load in the form of double cream (42g) was taken at the same time.

After administration of the isotope capsules, faeces were collected over a three day period and homogenised, with the addition of water if necessary. Approximately 40ml of stool homogenate was added to a round-bottomed flask followed by a fourfold volume of chloroform:methanol (2:1) and this was refluxed for six hours. The chloroform layer was separated by overnight filtration and the radioactivity in dried aliquots (of 50, 100, and 200µl) measured by a Packard liquid scintillation counter. The window settings of the counter were adjusted to 0 - 12keV for ^{3}H and 12 - 156keV for ^{14}C. Correction for quenching was achieved using commercially available quench standards (Canberra Packard Ltd, England). The percentage absorption of triolein using ^{3}H-triether as nonabsorbable marker (PAT-^{3}H)

was calculated from the ingested and excreted isotope ratios as follows.

$$\text{Percentage Absorption} = 1 - [{}^{3}H/{}^{14}C_{ingested}] \times 100 / [{}^{3}H/{}^{14}C_{excreted}]$$

MODIFICATIONS TO THE METHOD OF QUENCH CORRECTION.

Background

Tritium and ^{14}C are β-emitting radioisotopes (ie. they decay by the release of β-particles) but the energy of these particles is not sufficient to be measured directly. The technique of liquid scintillation counting enables the quantitation of radioactivity by initially converting the energy of radiation to light energy. The substance to be measured is dissolved in a solution containing scintillant molecules. When β-particles impinge upon these molecules, photons of light are released whose intensity is proportional to the energy of the β-particle. In the case of ^{14}C, photons of energy up to 156KeV are produced whereas tritium emissions are weaker being up to 18KeV.

Any process which interferes with the production or transmission of light is referred to as quenching. In practice two types of quenching exist. With chemical quenching the efficiency of energy transfer from the β-particle to the scintillant is decreased. This can be caused by the presence of solvent molecules in the scintillation fluid. Colour quenching is the subsequent absorption of light energy that has been produced by the

scintillant. Any colour, particularly yellow, in the scintillation fluid will cause this. The result of both forms of quenching is that the intensity of light energy is decreased. The amount of quenching in each sample can be measured and appropriate corrections to the dpm made, by comparison with commercially available quench standards. When stool extracts are dissolved in scintillation fluid the solution becomes a strong yellow or golden colour making quench correction essential. During the measurement of PAT-^{3}H it was normal practice to measure the $^{3}H/^{14}C$ ratio in each of three duplicated aliquots containing different volumes of dried chloroform extract (50, 100 and 200µl). However, it was noticed that there was a frequent effect of the chloroform volume used on the measured ratio; in general, the dpm/ml (ie. radioactivity per ml of dried chloroform) of both ^{3}H and ^{14}C, decreased with increasing volumes of chloroform extract. This trend was more pronounced with highly quenched (coloured) samples. On first thought it seemed likely that the chemical quench standards did not correct accurately for samples with colour quenching. Another possibility was that there was a fault in the dual label programme in the β-counter for calculating ^{3}H and ^{14}C.

Experiment to Determine the Effect of Colour Quenching on ^{3}H and ^{14}C Counts.

In order to investigate this observation more fully, the following experiment was set up. To three sets of ten β-counting vials were added equal volumes of ^{3}H-triether

containing about 500, 5000, and 50,000dpm. Scintillation fluid was added and the ^{3}H was measured using commercially available quench standards. The window settings on the counter were adjusted to measure ^{3}H only (ie. single-label programme). There was some variation of dpm measured between the ten vials, and so eight vials whose dpm agreed best were selected from each set. The scintillation fluid was then transferred to a second set of eight glass β-counting vials containing 0, 50, 100, 250, 500, 1000, 1500, and 2000µl of dried chloroform extract from a stool sample which contained no radioactivity. The radioactivity in each vial was then counted again using both single-label and dual-label programmes (for ^{3}H in the presence of ^{14}C).

This experiment was repeated using ^{14}C-triolein which was again counted using single-label (^{14}C only) and dual-label programmes (for ^{14}C in the presence of ^{3}H).

The ^{3}H dpm results obtained were similar for the single-label and dual-label programmes. The same was true of ^{14}C dpm. This demonstrated that the dual-label programme was producing valid results.

The effect of quenching - calculated as the quench index parameter (QIP) - on the three levels of ^{3}H dpm is shown in figure 4.2. (The QIP has an inverse relationship to the degree of quenching.)

This experiment demonstrated that as the colour quenching increased (ie. QIP decreases) the ^{3}H dpm decreases. This effect was seen at the three levels of ^{3}H dpm investigated.

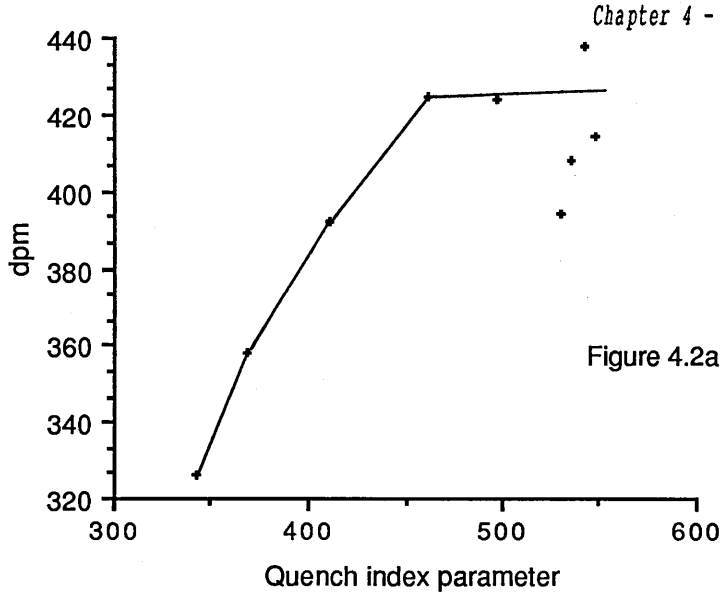


Figure 4.2a - low dpm.

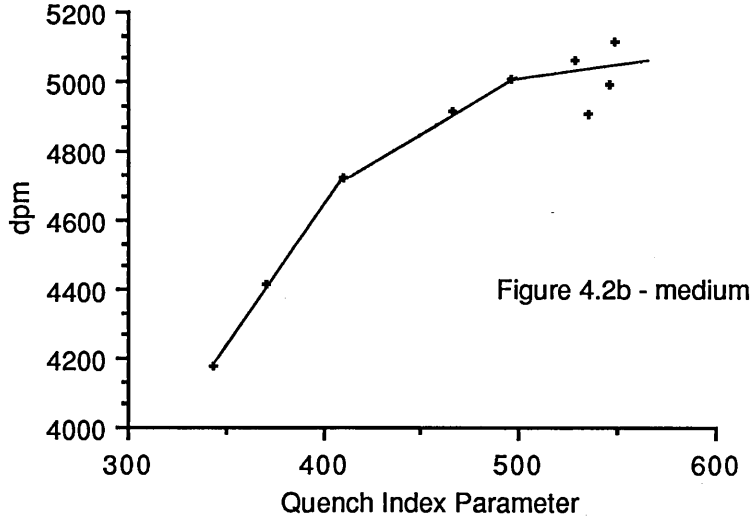


Figure 4.2b - medium dpm.

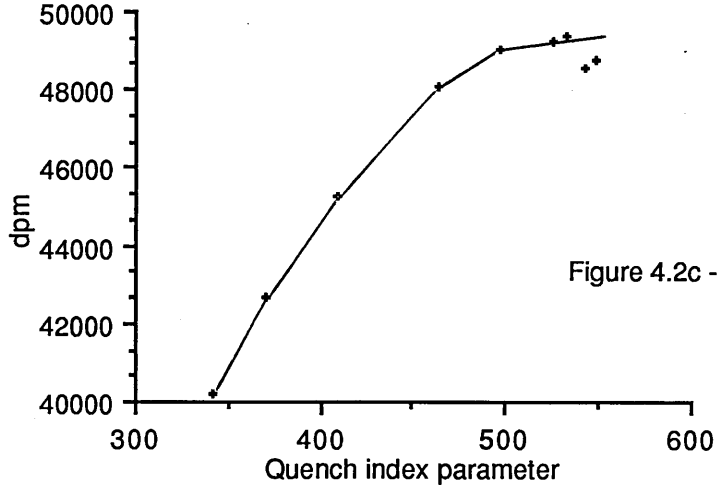


Figure 4.2c - high dpm

Figure 4.2 - Effect of quenching on 3H dpm at three different levels.

No effect of colour quenching on ^{14}C dpm was found.

It was clear that with no correction for colour quenching the results of PAT-^{3}H were inaccurate. It seemed that this problem could be solved in one of two ways; correction of quenching using colour quench standards, or decolourisation of the samples.

Use of Colour Quench Standards to Correct for Quenching

In order to correct for colour quenching a set of colour quench standards was prepared as follows. Similar amounts of ^{3}H-triether (about 100,000dpm) was added to each of fifteen glass β-counting vials. Eight vials whose dpm varied the least were chosen from the fifteen and an average dpm value was calculated. The contents of these vials were then transferred to a second set of eight β-counting vials containing differing volumes of dried chloroform extract obtained from a stool containing no radioactivity. The quench curves produced are shown in figures 4.3 - 4.6 along with quench curves obtained from chemically quenched standards. It is evident that the two ways of correcting quenching produce quite different curves. This substantiated the conclusion that a chemical quench curve is not suitable for colour quench correction.

With this lack of success of colour quench correction, attempts were then made to avoid the problem by decolourising the faecal chloroform extracts.

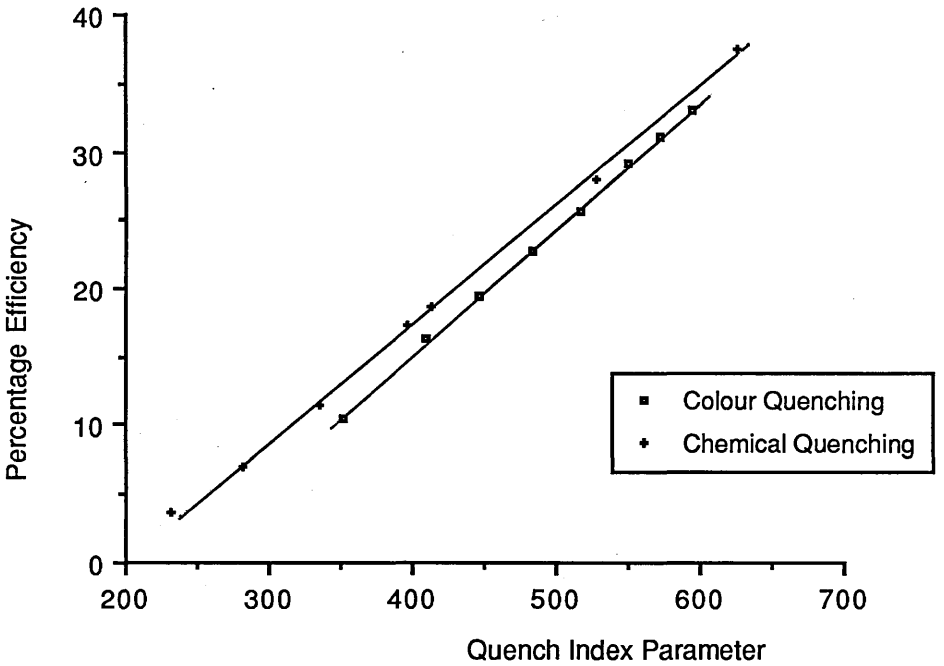


Figure 4.3- Efficiency of counting 3H in 3H channel.

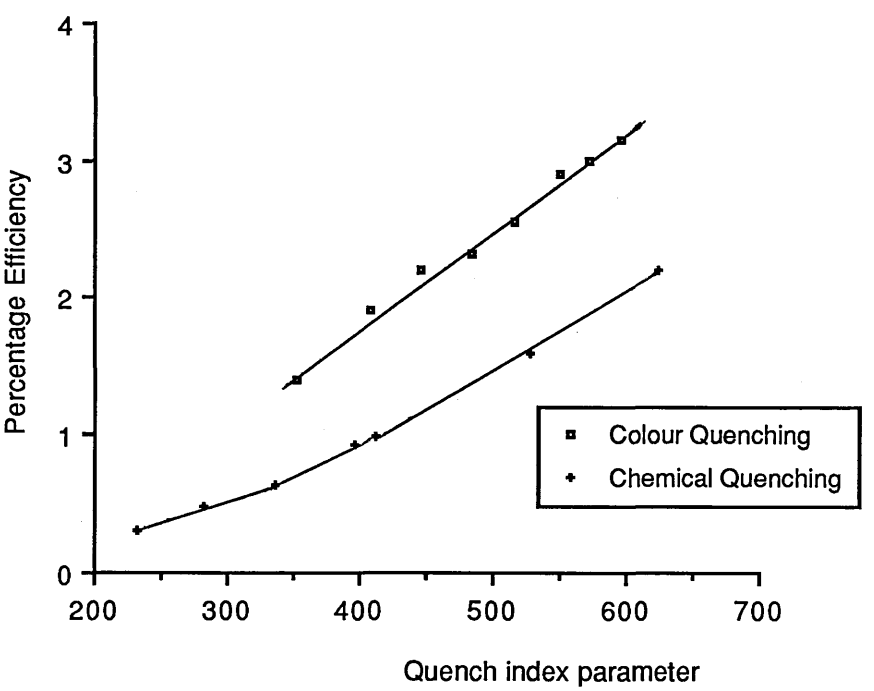


Figure 4.4- Efficiency of counting 3H in 14C channel

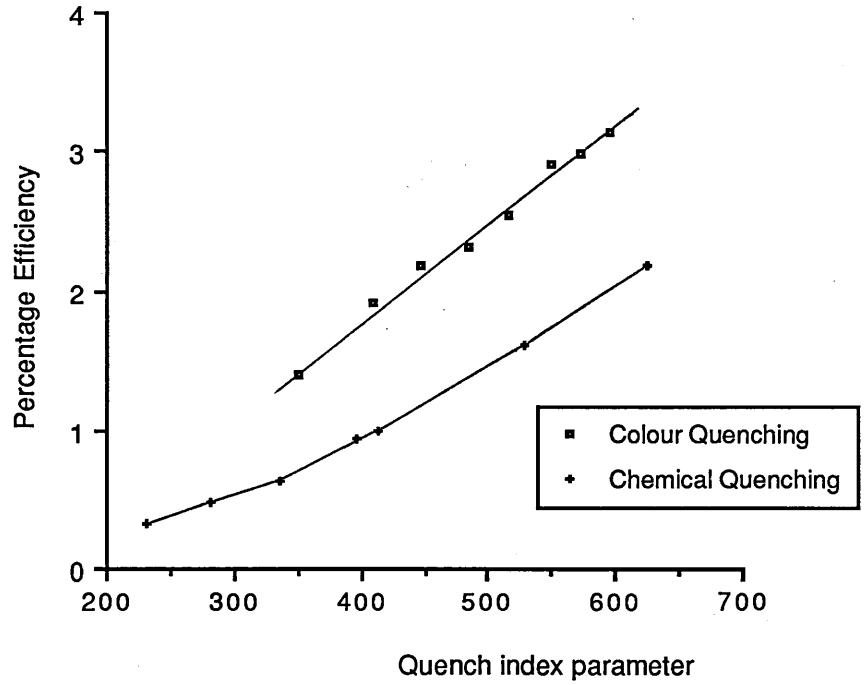


Figure 4.5 - Efficiency of counting 3H in 14C channel

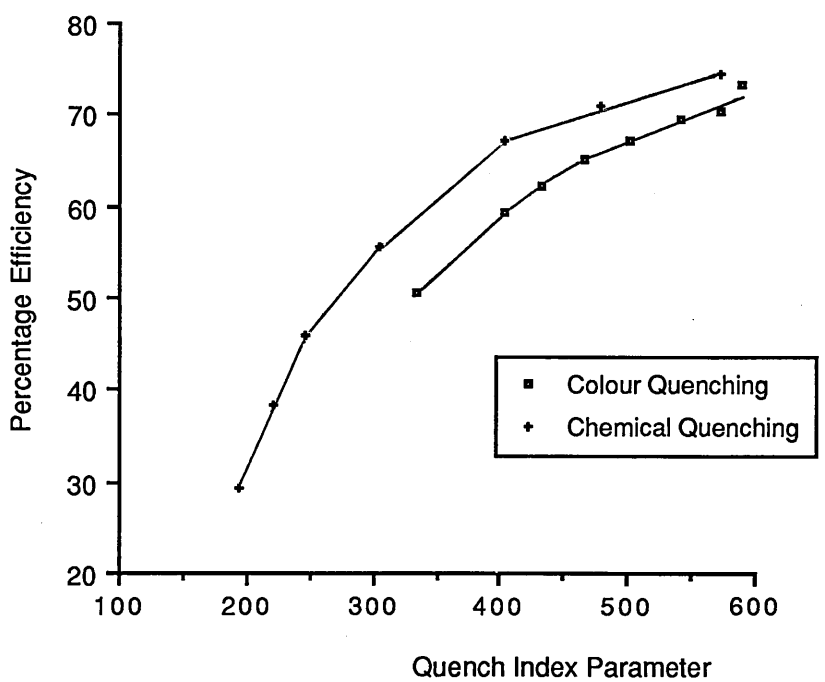


Figure 4.6 - Efficiency of counting 14C in 14C channel

Decolourisation of Chloroform Extract By Bleaching

The addition of hydrogen peroxide has been employed to decolourise samples prior to liquid scintillation counting. However, when this method was applied to decolourise faecal extracts, results were disappointing. It was found that small volumes (0.5ml) of hydrogen peroxide were ineffective in decolourising faecal chloroform extracts. By using 1ml of hydrogen peroxide decolourisation was still incomplete and in addition the ^{3}H and ^{14}C dpm increased by an average of 11.2% and 8.4% respectively.

Decolourisation of Chloroform Extract By Irradiation

The characteristic colour of stools is caused by pigments derived from bilirubin such as stercobilin and urobilin. (Hence patients with complete biliary obstruction excrete stools which are nearly white in colour.) It was speculated that these pigments could be destroyed by exposure to ultaviolet light in a manner analogous to the treatment of jaundiced neonates.

This simple procedure proved to be effective in decolourising dried chloroform extracts, with an overnight exposure being sufficient for all but the most strongly coloured samples.

To determine whether the commercially available chemical quench standards gave satisfactory results with decolourised samples the following experiment was performed.

Similar ^{3}H and ^{14}C counts (about 100,000dpm of each) were measured into seven β-counting vials, scintillation fluid was added, and the dpm measured. To a second set of seven vials were added different volumes (0, 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5ml) of chloroform extract from a stool containing no radioactivity. The samples were then evaporated and decolourised. The contents of the first set of tubes was then transferred to the second set and the counts measured. Compared with the sample containing no chloroform extract, there was no significant percentage difference for either ^{3}H (100.6%) or ^{14}C (99.4%).

With the use of chemical quench correction, ^{3}H and ^{14}C results were achieved which did not vary with the volume of chloroform extract used. It was found at a later stage that overnight exposure to light from an ordinary tungsten lamp was as effective as using a UV lamp. Longer exposure times were sometimes required to decolourise highly coloured faecal extracts or if large chloroform volumes were used. However, as long as the QIP was greater than 500, there was no effect on either ^{3}H or ^{14}C dpm.

MODIFIED METHOD FOR MEASURING PERCENTAGE TRIOLEIN ABSORPTION

It was apparent that at least one modification to the extraction procedure described above would be required. Ratios of $^{3}H/^{14}C$ can be used to calculate the PAT-^{3}H result because the triolein and triether are similarly extracted into chloroform. However, with the use of $^{51}CrCl_3$ as non-absorbable marker a ratio could no longer be used since

$^{51}CrCl_3$ - being water-soluble - does not extract into the chloroform with the ^{14}C-triolein. Consequently, to calculate the percentage absorption of triolein using $^{51}CrCl_3$ as non-absorbable marker (PAT-^{51}Cr), the absolute amount of ^{14}C-triolein excreted had to be measured. In order to measure this accurately, no losses of chloroform due to evaporation (as would occur during the filtration step) could be tolerated. It was considered that the separation of chloroform from the aqueous phase could alternatively be achieved by centrifugation. Since the refluxed stool could be centrifuged in a contained system losses due to evaporation might be minimised.

The final modified method for measuring the percentage triolein absorption is as follows. For two days prior to the test and during the three days of the stool collection, the patient ate a high fat diet containing 70 - 100g/day of fat. Dietary fat intake was calculated in those patients who were unable to tolerate this diet. After an overnight fast the patient swallowed the gelatin capsules containing ^{14}C-triolein and ^{3}H-triether. The capsules were washed down with 20g of fat in the form of double cream and 1000kBq of $^{51}CrCl_3$ dissolved in 50ml of water. Two 100µl aliquots of $^{51}CrCl_3$ solution were retained as standards. This protocol, which is broadly similar to that of Newcomer *et al* (203), was chosen so that comparative results would be available to facilitate the interpretation of the triolein breath test results.

A three day stool sample was usually collected into three

containers each covering a 24 hour period. In some cases individual stool samples were collected over the three day period. After homogenisation, an aliquot of 3 - 6g was transferred to a round-bottomed flask. A volume of 25ml of 0.9% saline and 37.5ml of chloroform:methanol (2:1) was added and the flask weighed. After refluxing for one hour (it was found that decreasing the reflux time from six hours to one hour gave similar results) the flask was cooled and reweighed. The refluxate was transferred to a stoppered test tube and centrifuged (1000rpm for five minutes) to separate the chloroform and aqueous layers. Volumes of 0.5, 1.0, and 1.5ml of the lower chloroform layer were transferred by Pasteur pipette to β-counting glass vials. When the chloroform had evaporated the dried extract was exposed to light until decolourised. The dried extract was then dissolved in 10ml of liquid scintillant (Pico Fluor 40, from Canberra Packard Ltd) and the ^{14}C and ^{3}H radioactivity was measured using the dual-label counting facility of a Packard Liquid Scintillation Counter. Quenching was measured and corrections made using commercially available chemically quenched standards. The faecal fat excretion was measured by the van de Kamer method (195) and sometimes also by a standard titrimetric method (196).

Four aliquots of homogenate of about 5g (accurately weighed) were transferred to gamma-counting vials and the dpm of ^{51}Cr was measured in a Packard gamma counter with window settings of 240 - 400keV. The PAT-^{3}H was calculated

as was described earlier (page 224) and the PAT-^{51}Cr was calculated in an analogous way as follows.

$$^{51}CrCl_3 \text{ excreted} = \frac{(dpm1 + dpm2 + dpm3 + dpm4) \times H}{(W1 + W2 + W3 + W4)}$$

where H = weight of homogenate and W = weight of aliquots counted

$$^{14}C \text{ excreted} = \frac{dpm/ml \times 25 \times H}{W}$$

(The 37.5ml of chloroform/methanol (2:1) used produced a chloroform subnatant of 25ml)

$$PAT\text{-}^{51}Cr = 1 - [^{51}Cr/^{14}C_{INGESTED}] \times 100\% / [^{51}Cr/^{14}C_{EXCRETED}]$$

ASSESSMENT OF THE MODIFIED PROCEDURE

Assessing for Completeness of Chloroform Recovery

To ensure that there was no loss of chloroform at the refluxing stage the weight of sample was measured before and after this step. The average loss of chloroform was 0.49g (sem = 0.028, n = 11). This is equivalent to a volume of 0.33ml and percentage loss of 1.3% which was considered to be acceptable.

Effect of $^{51}CrCl_3$ on the Measurement of ^{3}H and ^{14}C dpm

Although ^{51}Cr is a gamma-emitting isotope it will also produce readings on a liquid scintillation counter. It was important therefore that the $^{51}CrCl_3$ was not extracted into the chloroform layer. To assess this, two stool samples containing all three isotopes were extracted in the normal

way and aliquots of the aqueous supernatant, solid interphase, and chloroform subnatant were measured in a gamma counter.

^{51}Cr was found predominantly in the solid interphase with some in the aqueous supernatant. No ^{51}Cr was detectable in the chloroform layer. On the first twenty patient tests performed, the chloroform layer was measured in a gamma counter. Again no ^{51}Cr was measurable. it was consequently concluded that ^{3}H and ^{14}C could be confidently measured in the chloroform layer without contamination from ^{51}Cr.

Assessing the Recovery of ^{14}C and ^{3}H During Extraction

The recovery of ^{3}H and ^{14}C was assessed as follows. Aliquots of eleven stool samples were transferred to round-bottomed flasks and about 8kBq each of ^{3}H-triether and ^{14}C-triolein in ether was added. The ether was evaporated off and the samples extracted and counted normally. As standard, a similar amount of each isotope was added to a clean flask and diluted with 25ml of chloroform. The recoveries of ^{3}H-triether and ^{14}C-triolein were good at 104.7% (sem = 3.7) and 98.4% (sem = 3.1) respectively.

Reproducibility of the Extraction Procedure

The extraction procedure was performed in duplicate on the first twenty-five patient tests. Results of precision at differing ranges of PAT-^{3}H are shown in table 4.2. Because of the nature of the equation used to calculate

PAT-^{3}H the precision varies with different degrees of malabsorption. Thus, when absorption is approaching 100% large differences in the $^{3}H/^{14}C$ ratio produce relatively small changes in the percentage absorption. When the percentage absorption is low the converse is the case.

PAT-^{3}H Range	n	Mean	Standard Deviation	Coefficient of Correlation
90 - 100%	8	97.8%	0.78	0.80%
75 - 90%	12	83.3%	3.3	3.9%
< 20%	5	9.6%	2.2	23%

Table 4.2 - Precision of PAT-^{3}H at Differing Degrees of Malabsorption

In general the test showed good precision. Fortunately the poorer precision found at very low levels of absorption has no clinical implications.

Comparison with Biological Oxidation

The technique of biological oxidation enables ^{14}C and ^{3}H to be counted without prior extraction. The sample is oxidised on a metal coil at high temperature over a stream of oxygen. Tritium is trapped in an enclosed system as steam while ^{14}C isotopes are oxidised to ^{14}C-carbon dioxide which is trapped separately on Carbosorb (Canberra Packard Ltd, England). In this way ^{14}C and ^{3}H were separated from each other and collected directly into scintillation fluid in a comparatively pure form. This would have been a preferable method for counting ^{14}C and ^{3}H in the calculation of PAT-^{3}H. Unfortunately biological oxidisers

are expensive and not routinely available. However, access to one in the Department of Medicine of the Western Infirmary in Glasgow was obtained for the purposes of evaluating the above extraction procedure.

A Packard Biological Oxidiser using a platinum coil at 700°C was used. Tritium in the form of water was collected into Monophase-40 scintillation fluid and ^{14}C-carbon dioxide was trapped on Carbosorb and washed into Permafluor-5 scintillation fluid (Canberra Packard Ltd, England).

Stool samples were spiked with small quantities of ^{3}H-tri-ether and ^{14}C-triolein; the mean recovery was found to be 104.9% and 98.4% respectively but individual results varied widely. The precision in duplicate stool samples was also measured: the standard deviation was 6.9 and the coefficient of variation 10%.

A total of fifteen stool samples was analysed by both methods and their degree of correlation is shown in figure 4.7 (see Statistical Appendix, note 4). The correlation coefficient, at 0.92, was not ideal but was considered to be partially due to the performance of the biological oxidation method which in practice gave poor reproducibility (CV = 10%) and highly variable recoveries.

Accuracy of ^{3}H and ^{14}C counting

It was important that both radioisotopes, particularly ^{14}C, were accurately counted by liquid scintillation counting.

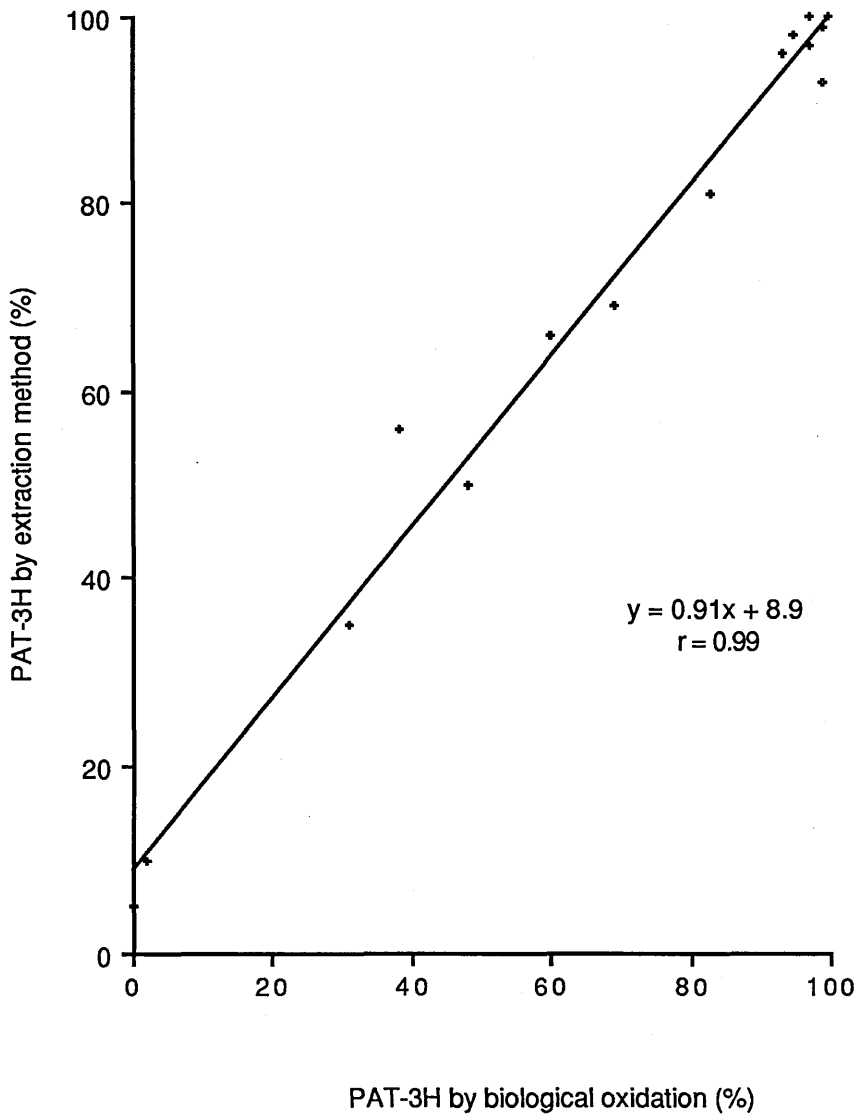


Figure 4.7 - PAT -3H results: biological oxidation vs extraction.

When PAT-^{3}H results approach 100% the ^{14}C dpm is very low therefore even a small spillover from the ^{3}H channel to the ^{14}C channel would result in the results being underestimated. This experiment was designed to study the mutual effect of the two radioisotopes on the accuracy of counting.

Known amounts of pure ^{3}H-triether and ^{14}C-triolein were added to β-counting vials to produce levels of dpm typically encountered during the measurement of PAT-^{3}H. Dpms used were 250 - 18,000 for ^{3}H and 0 - 500 for ^{14}C.

The results demonstrated that ^{3}H could be accurately measured at all levels of ^{14}C investigated. Conversely, although there was some spillover from the ^{3}H to the ^{14}C channel this was insufficient to alter the isotope ratio such that inaccurate PAT-^{3}H resulted. PAT-^{3}H results were calculated (using a value of 5 for the isotope ratio of the standard) in order to determine whether any spillover was significant. The calculated and expected PAT-^{3}H results are shown in table 4.3.

USE OF SINGLE UNTIMED STOOLS TO CALCULATE PAT-^{3}H

One of the potential benefits of dual isotope fat absorption tests is the opportunity to use a single small untimed stool sample. Although this technique still requires the use of stool samples, the test would be more like a stool occult blood test with regards to patient and staff acceptability. However, Nelson *et al* showed that results could not always be produced since the stool

^{3}H dpm	PAT-^{3}H Results					
	0dpm ^{14}C	25dpm	50dpm	100dpm	250dpm	500dpm
250	98 (100)	60 (59)				
500	100 (100)	79 (77)	56 (48)			
750	100 (100)	88 (87)	73 (71)	47 (46)		
1000	97 (100)	86 (87)	76 (74)	55 (53)		
2000	97 (100)	92 (94)	83 (88)	74 (75)	42 (38)	
4000	100 (100)	97 (97)	94 (94)	86 (87)	72 (68)	43 (37)
9000	99 (100)	98 (99)	99 (97)	94 (95)	88 (87)	74 (73)
18000	99 (100)	98 (99)	98 (99)	98 (97)	92 (93)	86 (85)

Table 4.3 - Calculated and expected PAT-^{3}H results at different levels of ^{3}H and ^{14}C. (Expected results in parentheses).

collected may not contain any isotope. They suggested therefore that the test could not be reliably carried out by collecting a single stool collection on either days one, two, three or four (222).

It was speculated that rather than collecting a stool at a specific time after the start of the test, the time of stool collection could be altered (with a knowledge of the patients' bowel habit) to ensure that isotopes were present. This was investigated on thirty consecutive patients who attended the Gastroenterology Unit for an out-patient PAT-^{3}H test. They were initially questioned about their frequency of bowel movement to decide when the stool should be collected. In 18 patients with normal bowel movements or intermittent diarrhoea the first stool passed after 24 hours was collected, in eight with troublesome

diarrhoea the first sample after 12 hours was collected, in one slightly constipated patient the first stool after two days was collected. Two patients failed to return the collection.

MEASUREMENT OF PERCENTAGE ^{14}C-TRIOLEIN IN BREATH

At 0, 1, 2, 3, 4, 5, and 6 hours after the isotope capsules had been given, end-expiratory samples of breath were collected. Factors known to affect carbon dioxide production, such as smoking and exercise (232), were minimised. Patients exhaled normally and then blew via a drying tube into a carbon dioxide trapping solution (1ml of hyamine hydroxide - methyl benzethonium hydroxide - 1ml of methanol and 50µl of thymolphthalein as pH indicator). This process was repeated until the solution changed from blue to colourless, indicating complete neutralisation of the hyamine hydroxide. Scintillation fluid was added and the sample left for four hours to allow any chemiluminescence to subside before measuring the ^{14}C by liquid scintillation counting.

Hyamine hydroxide obtained commercially is approximately 1mmol/l. The exact concentration was measured by titrating 2ml with 100mmol/l hydrochloric acid using thymolphthalein as indicator.

It soon became obvious that some patients had difficulty in collecting end-expired breath. In such patients a mixture of end-expired and normal expired breath was usually collected. To determine whether these collections had an

effect on the result, a number of patients who could collect end-expired breath in the proper way were also asked to provide normally expired breath, ie. normal exhalations rather than forced exhalations were collected.

At some point during the breath test, three or four two-minute samples of breath were collected into Douglas bags and the carbon dioxide and oxygen concentrations and total volume measured (Ventilation Monitor, PK Morgan, Chatham, Kent). It was found that some patients tended to hyperventilate when breathing through the mouthpiece and so instructions were subsequently given to encourage the patient to relax and avoid hyperventilation. Whenever possible the patient read a book or newspaper, or watched television during collection of breath so that they were less aware of their breathing rate.

The respiratory quotient (r) was calculated using the equation:

$$r = \frac{\%CO_2}{(20.9 - \%O_2)}$$

where 20.9 is the percentage of oxygen in air. High r values in these fasted and resting patients probably represented hyperventilation and so if r was found to be outwith the normal range of 0.7 - 0.9 this result was excluded from consideration.

The patients' carbon dioxide output was measured by the following equation.

$$\text{Carbon dioxide Output (mmol/hour/Kg)} = \frac{\%CO_2 \times V \times K \times 60}{22.4 \times Wt}$$

where V = volume of breath excreted per minute, K = a factor to convert volume to conditions of standard temperature and pressure (STP), and Wt = the patient's weight in kg. Dividing by 22.4 converts litres of gas at STP to moles.

The percentage ^{14}C-carbon dioxide excretion in the hourly breath samples was calculated as follows.

$$\%14C \text{ excretion in breath} = \frac{dpm_{breath} \times CO \times Wt \times 100}{C \times dpm_{standard} \times K_1 \times K_2}$$

Where CO is the carbon dioxide output in mmol/hour/Kg, Wt = weight of patient, C = molarity of hyamine hydroxide solution, K_1 = a factor (1.25) to compensate for the volume difference in β-scintillation vials between the standard solution and the 'breath solution', and K_2 = the ratio of the weight of isotope administered and that retained as standard.

Results were expressed as the percentage of the total ^{14}C isotope administered in the breath per hour. The cumulative $^{14}CO_2$ in the breath during the six hour period of the test was calculated as the sum of the average hourly outputs.

SEPARATION OF 3H-TRIETHER AND 14C-TRIOLEIN

Separation of the two radio-labelled fats was achieved by high performance thin layer chromatography (TLC) using 10cm x 10cm silica plates (LHP-K plates from Whatman Inc, England). When the method of Gerskowitch (221) was tested using the solvent system of hexane:ether (85:15), the

triether standard ran with the solvent front (ie. Rf = 1.0). Since this system would not allow the resolution of two bands with this mobility, the solvent system was changed. Triether did not move from the origin when hexane or methanol was used, but the addition of glacial acetic acid (100:5) resulted in a mobility of 0.23. The presence of ether increased the Rf further. The final solvent system of hexane:ether:glacial acetic acid (85:15:1.5) gave mobilities of 0.92 for triether (1,2,3-didodecyl triether, 0.62 for triolein (glycerol trioleate, BDH, Poole), and 0.35 for oleic acid (BDH).

Fat was detected as a yellow colour after TLC plates were sprayed with 0.05% 2',7'-dichlorofluorescein (Sigma Chemical Co, Poole) in ethanol. Radioactivity was visualised after exposing the plates to photographic film (GBX-2, Kodak, England) for several days.

To quantitate the amount of radioactivity in TLC bands, silica corresponding to the band was scraped off and the ether-soluble extract counted by β-scintillation counting.

PURIFICATION OF ^{3}H-TRIETHER

Tritiated triether was purified by a preparative thin layer chromatography method using the solvent system described above. The tritiated extract was spotted onto the TLC plate and after chromatographic separation, autoradiography was used to locate the radioactive bands (figure 4.8). The position of ^{3}H-triether was determined by comparison with a chemical standard of 1,2,3-didodecyl triether. The silica

corresponding to this was scraped from the plate and the ^{3}H-triether separated by dissolving in ether. This step was repeated, and the ether dried in a glass vial. The ^{3}H-triether was solubilised in toluene and stored at 4°C.

STABILITY OF RADIOISOTOPES TO COLONIC BACTERIA

The following experiment was performed in order to investigate the stability of ^{14}C-triolein and ^{3}H-triether isotopes to colonic bacterial enzymes. A small volume of faecal homogenate (5 - 10g) and 25ml of 0.9% saline was added to a round-bottomed flask. 50kBq of ^{14}C-triolein or ^{3}H-triether dissolved in ether was added and the solvent evaporated under a stream of nitrogen. After incubation at 37°C overnight (approximately sixteen hours) the sample was refluxed and extracted as usual into chloroform. Stool was excluded from one flask to serve as a control. A volume of 10µl of chloroform was applied to a TLC plate and after chromatography the plate was autoradiographed.

The experimental design employed could not be used to identify the presence of ^{14}C-labelled gas production such as ^{14}C-carbon dioxide or ^{14}C-methane. In order to ascertain whether either of these breakdown products was being generated, the experimental system was modified as illustrated in figure 4.9. The round-bottomed flask was made air-tight, and after incubation water was added to displace the gas. This was then bubbled through a series of tubes containing either hyamine hydroxide for thecapture of carbon dioxide, or chloroform to trap methane. Since it was not known what proportion of gas would be trapped in

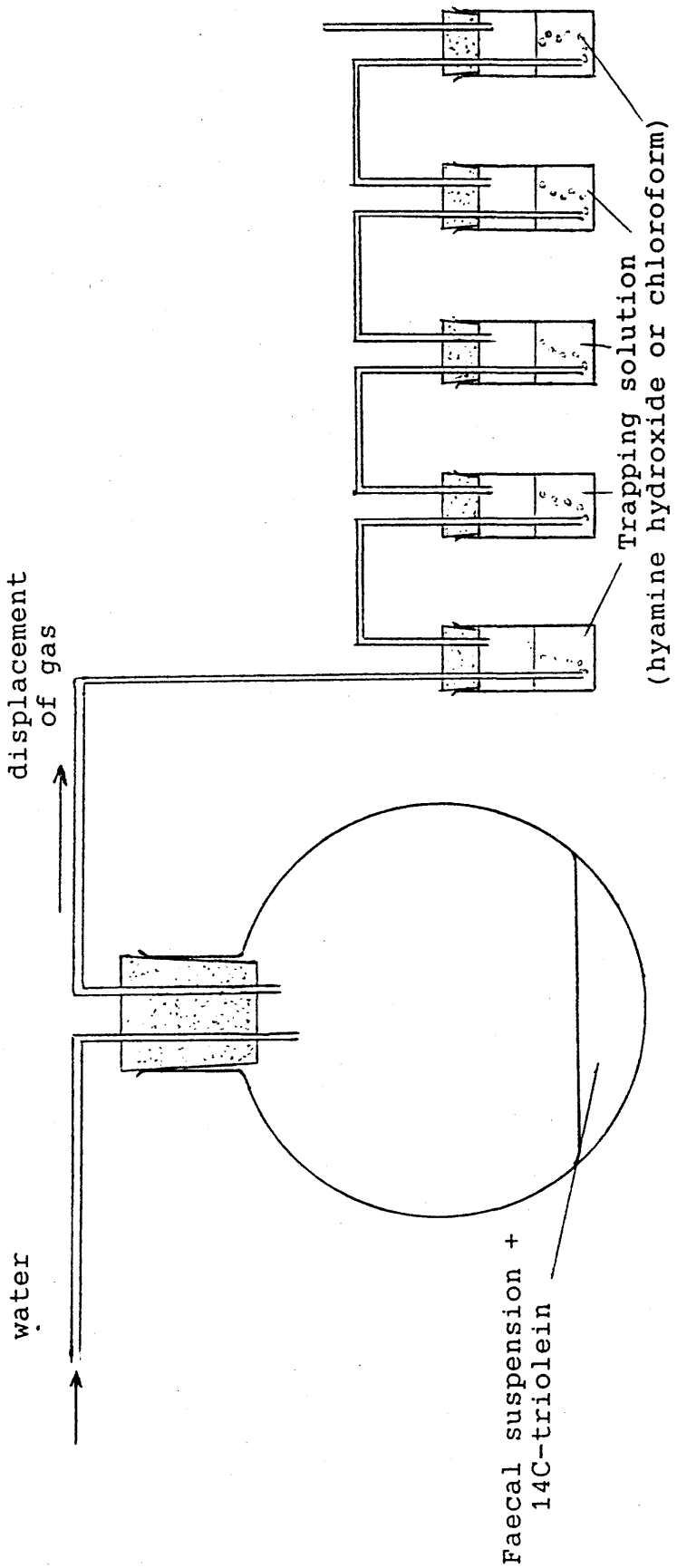


Figure 4.9 - Design of experimental system for detecting 14CO2 and 14C-methane production from 14C-triolein

solution, a series of tubes was used so that the decrease in ^{14}C could be monitored in order to gauge the recovery of ^{14}C-gas. When all the gas within the round-bottomed flask was displaced, the solutions were transferred to β-counting vials, scintillant added, and ^{14}C counted.

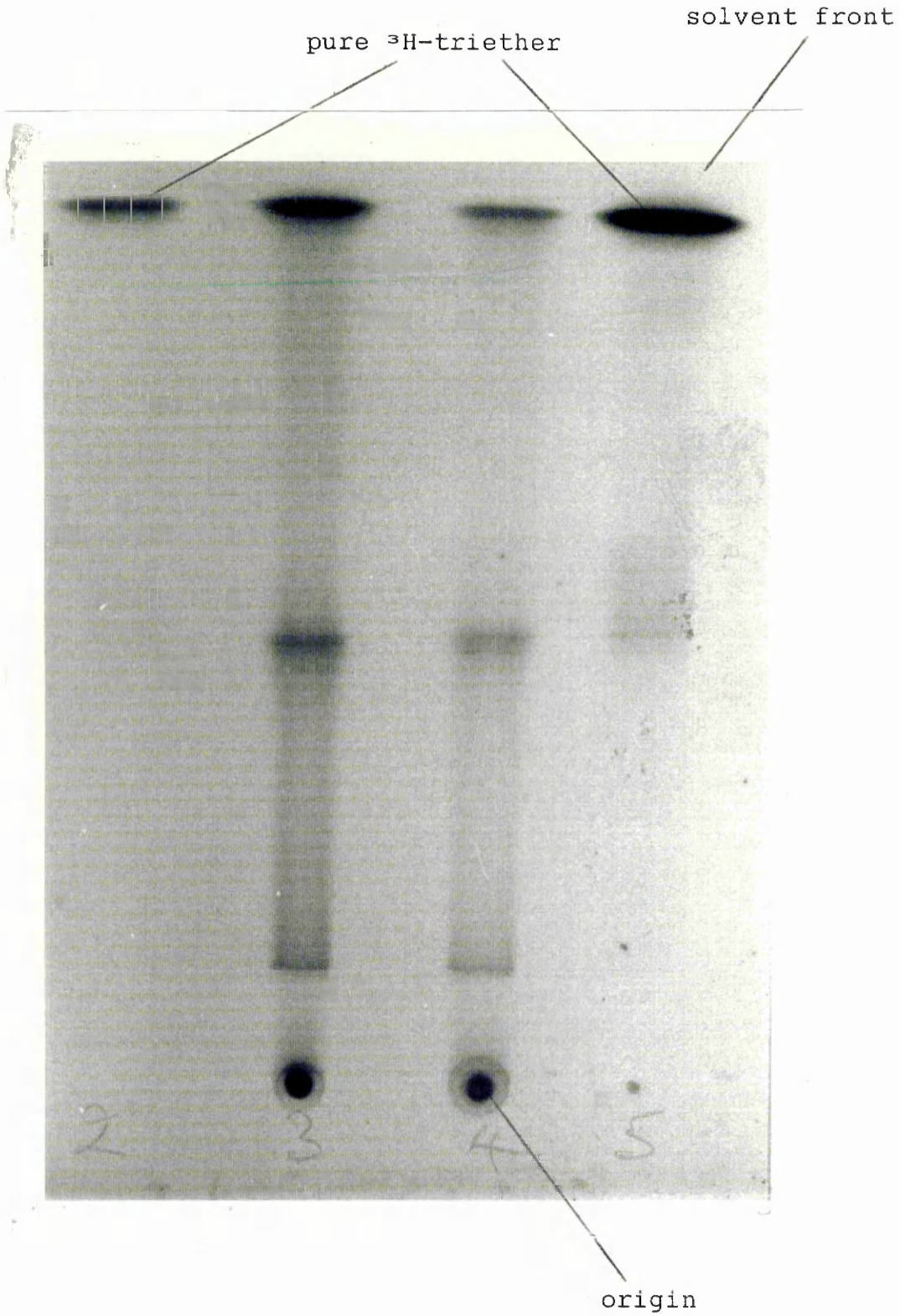


Figure 4.8 - Autoradiograph of ³H-triether before (spots 2 and 3) and after (spots 1 and 4) purification.

RESULTS AND DISCUSSION

Despite the development of a variety of techniques for detecting fat malabsorption, none stands out as the obvious routine method of choice and for assessment purposes there is no test which can be considered as definitive.

The concept of dual isotope tests, although not widely employed, has a sound theoretical basis and should provide good results provided that the following criteria for a non-absorbable marker are satisfied. i) The non-absorbable marker and absorbable test substance should be inseparable throughout the intestine except if the latter is absorbed. ii) The marker should be resistant to digestion and colonic transformation. iii) The marker should be easily and reliably measured. iv) The marker should be non-toxic. v) The marker should not be absorbed.

The present study provided an opportunity for further assessing some of these criteria.

INTRA-INDIVIDUAL VARIABILITY OF PAT-^{3}H AND PAT-^{51}CR RESULTS

The initial criterion, that non-absorbable marker and absorbable test substance should be inseparable, is one of the most difficult to ascertain. Initial *in vitro* studies have attempted to simulate the physiological situation in the gut and monitor the partitioning of test and non-absorbable probes. However, the physiology of intestinal function is so complex that any assessment is incomplete

without *in vivo* confirmation. On the other hand, since the test marker is absorbed to some extent it is difficult to assess the separability of non-absorbable marker from the test probe by *in vivo* experiments. Since neither probe is absorbed in the stomach, the potential for separation within the gastric lumen and during gastric emptying can be ascertained by *in vivo* studies. However, the only effective way of investigating the separability of the two probes in the entirety of the gastrointestinal tract is to determine if the two markers move through the intestine at the same rate. If the isotopes move at different speeds through the gut then the ratios in consecutive stools would change. This can be readily assessed by collecting individual stools from a subject who has taken the isotopes orally. If the relative movements of test and non-absorbable marker are similar then each faecal collection should give similar ratios. This investigation must be performed in subjects who have a degree of fat malabsorption so that some of the test fat (usually ^{14}C-triolein) travels through the gut and is excreted.

To assess this, stool samples from each patient were collected separately and PAT-^{3}H and PAT-^{51}Cr measured. We initially suspected that the intra-individual variability of the latter might be poor because $^{51}CrCl_3$ is water soluble. However, quite unexpectedly the initial PAT-^{3}H results, illustrated in table 4.4, showed very poor reproducibility between samples from the same patient. This imprecision was such that the calculation of

Number	Stool 1	Stool 2	Stool 3
1	97%	93%	-
2	-20%	18%	-
3	100%	100%	-
4	99%	99%	-
5	-	48%	89%
6	93%	99%	98%
7	43%	83%	-
8	100%	100%	100%

Table 4.4 - Initial PAT-^{3}H results showing intra-individual variation.

PAT-^{3}H using a random stool could have given erroneous results. In addition, one PAT-^{3}H result (patient 2) was negative, ie. the ^{3}H/^{14}C ratio in the stool was lower than the standard. Various technical reasons for this anomalous result, such as counting errors or isotopic contamination, were excluded.

These two findings, of poor intra-individual precision and a negative result, could be explained on the basis that ^{3}H-triether and ^{14}C-triolein were moving through the gut at different rates and/or that impurities of ^{3}H or ^{14}C were present. On patient number eight a urine sample was collected and this was found to contain about 5% of the ingested ^{3}H. Since previous studies have shown that ^{3}H-triether is not absorbed (220, 221) this suggested that ^{3}H impurities were present.

It was decided that more extensive investigations into the nature of this problem was required before any further patient tests were carried out.

Autoradiography of Isotopes

In order to investigate the integrity of ^{3}H-triether, TLC

was performed followed by autoradiography of the chromatogram. This revealed that '^{3}H-triether' ran not as a homogeneous band but as three predominant bands with R_f values of 0.0, 0.21, and 0.90 (figure 4.10). The bulk of the radioactivity, 61%, remained at the origin, with 20% at R_f 0.21 and only 15% at R_f 0.9 corresponding to pure triether.

This experiment clearly demonstrated that a large proportion of ^{3}H was present as other chemical forms. The ^{3}H which remained at the origin was much more polar than the tritiated form and so would be expected to a) move through the intestine at a different rate and b) be available for absorption. On both counts these initial PAT-^{3}H results were invalid.

The tritiated radioisotope was stored and dispensed by the West of Scotland Radioisotope Dispensary in the Western Infirmary, Glasgow, in two separate vials. It was subsequently found that the second vial also contained tritiated breakdown products of the triether, although relatively more of the tritiated triether was intact (about 80%).

The triether molecule itself is chemically very stable and tritium isotope has a long half-life (12.26 years). However, the presence of an isotope as an integral part of the molecule was probably the cause of its degradation, with local radiation within the molecule causing radiation autolysis. The radioactivity had been stored in concentrated form and so the decay due to irradiation was

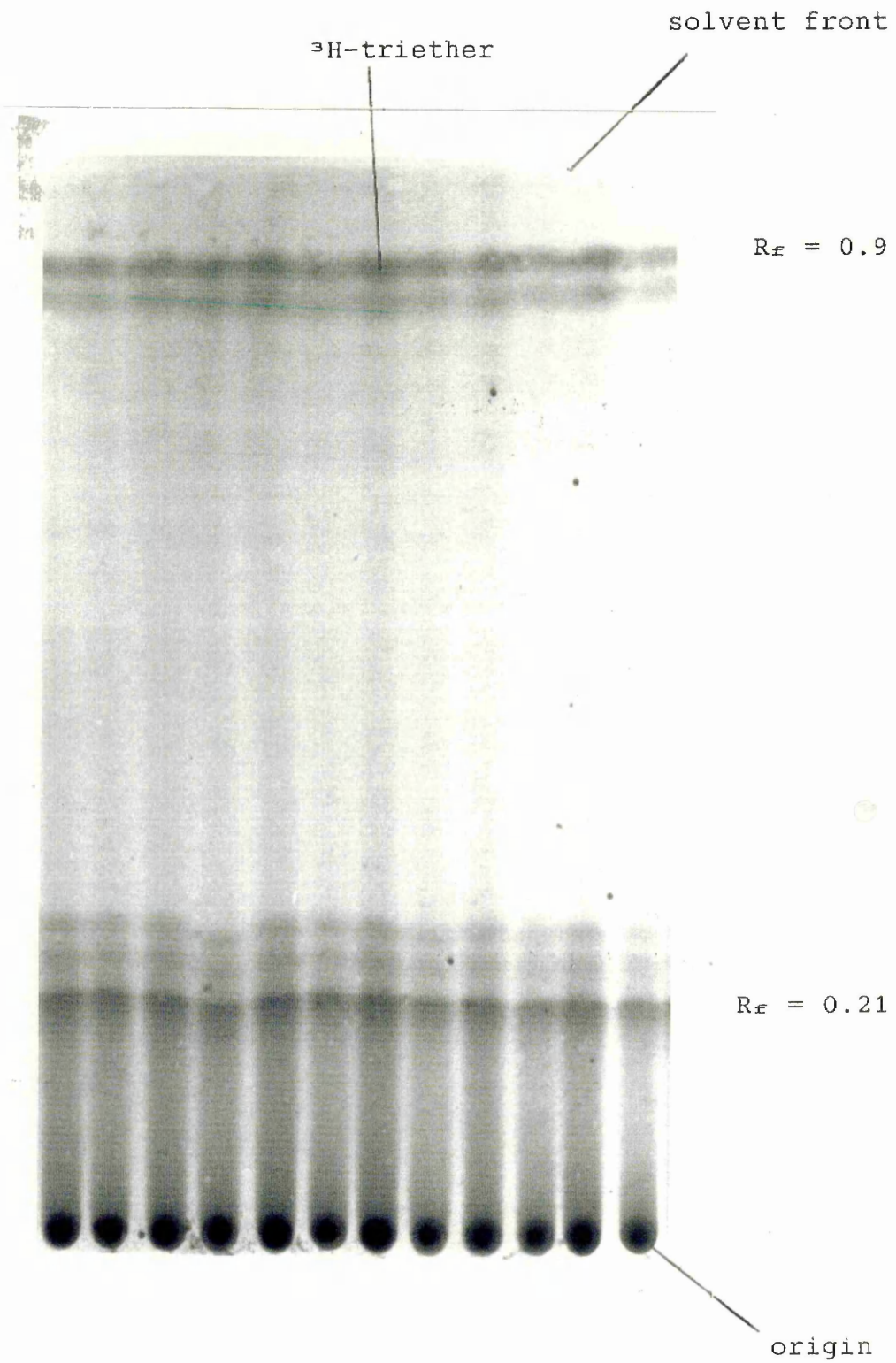


Figure 4.10 - Autoradiograph of impure ³H-triether.

accentuated. For this reason the stocks are now stored in more dilute form.

There was a salutory lesson to be learnt from this episode. Over several years the test had become accepted as a routine procedure and ^{3}H-triether was ordered when required from the West of Scotland Isotope Dispensary. The responsibility for the ^{3}H-triether was therefore shared by two distinct departments but neither was carrying out quality assessment and nor was the isotope exposed to the stringent standards of a commercial concern. A quality control procedure had not been established at the early research stage and with the responsibility for the isotope being dichotomised, none had been implemented since. Hence there was incomplete control regarding the integrity of the isotope. This oversight was probably the result of the regard of the test evolving over a number of years, from an awareness within a tightly-controlled research setting, to the abstracted approval of routine.

When the problem had been identified, the ^{3}H-triether was repurified by preparative TLC (see page 245). In addition, working practices were modified so that responsibility for the isotope was placed with only one department, the Gastroenterology Laboratory, and a quality assessment procedure was introduced to ensure that ^{3}H-treither used for future tests was pure.

* * * * * *

The stability of ^{3}H-triether was also assessed over different time intervals by TLC/autoradiography. 50kBq aliquots of purified ^{3}H-triether were stored in 5ml of toluene at -20°C, 4°C and room temperature (approximately 23°C).

Results of this experiment revealed that ^{3}H-triether appeared as a single band corresponding to pure glycerol triether at all the temperatures and times investigated. Thus the isotope was stable for up to at least two years at each of the temperatures used. Stocks of the radioisotope were subsequently stored at refrigerator temperature (4°C).

Amersham International report decomposition rates of less than 2% per annum for ^{14}C-triolein when stored in toluene under nitrogen at -20°C. This recommendation was already being followed.

* * * * * *

Once the initial problem of ^{3}H-triether impurity had been remedied the intra-individual variability was studied afresh.

Variability of PAT-^{3}H in Individual Stool Samples

PAT-^{3}H was measured (using purified ^{3}H-triether) in 108 individually collected stool samples from 37 patients. Results of 100% were found in 15 patients. These results were excluded since the stools contained no ^{14}C and so no information could be gleaned about the relative movement

of $^{51}CrCl_3$ and ^{14}C-triolein through the intestine, the main objective of this exercise. The variability of results in individually collected stools from patients with less than 100% absorption is recorded in table 4.5.

Patient Number	Stool 1	Stool 2	Stool 3	Stool 4
1	100%	100%	99%	
2	99%	99%	(99%)	
3	97%	93%	96%	(96%)
4	100%	(89%)	(90%)	
5	11%	39%	50%	
6	79%	81%		
7	(75%)	74%		
8	96%	99%		
9	11%	17%	(39%)	
10	98%	99%	97%	
11	(31%)	38%	32%	(45%)
12	23%	27%		
13	98%	100%	100%	
14	31%	32%	64%	(66%)
15	99%	(92%)		
16	(92%)	69%	86%	
17	(28%)	26%	28%	
18	97%	100%	(100%)	
19	100%	(100%)	(99%)	
20	74	(88%)	(87%)	
21	98%	100%	(100%)	
22	100%	100%	(99%)	

Table 4.5 - Intra-individual variability of PAT-^{3}H in separately collected stools. (Bracketed figures are from stools with low recovery of ^{3}H).

Inconsistent results were sometimes produced when the recovery of ingested tritium was low (<3%). When this situation arose the ^{3}H dpm were usually very low making it possible that the quantitation of ^{14}C and ^{3}H was technically imprecise. However, when small, known amounts of ^{3}H and ^{14}C were measured, the accuracy of counting was assessed revealing that both isotopes could be counted accurately even at low dpm (see pages 238 - 9).

Since the results were technically correct the cause of

intra-individual variability was presumably due to the separation of the isotopes in the intestine. This only occurred when the recovery of ^{3}H in the collection was low and so it was easily identified. Although these results are noted in the table they were excluded from subsequent consideration (see later discussion).

No overall statistical difference was found between results from consecutive stools (see Statistical Appendix, note 7). Nevertheless it was apparent that some patients showed differences when PAT-^{3}H results from individual stools were studied.

In five of the tests (numbers 5, 9, 11, 14, and 16), the absorption increased with time indicating that ^{14}C moved relatively faster through the intestine than ^{3}H-triether.

This difference in results was only found in patients with gross malabsorption and so was of no clinical importance. The remaining 17 results on patients with other degrees of absorptive capacity were comparable from stool to stool.

Hoving *et al* performed a similar study to this in which malabsorption was induced in rats by biliary fistulae or intestinal ischaemia (233). They also demonstrated a similar trend in stools collected over four separate time periods following the oral infusion of radiolabelled triolein and triether. They found that there was little difference between results except when malabsorption was more severe in which case the faecal ratio increased, again indicating a relatively faster intestinal transit

time of ^{14}C-triolein.

Our results also concurred with those of Nelson *et al* (my predecessor in the Gastroenterology Laboratory, in GRI) who found no intra-individual variation in PAT-^{3}H results except in one patient with severe malabsorption (222).

* * * * * *

When the recovery of ^{3}H-triether from a stool was very low (<3%), the isotope present probably represented either the first or last traces of label to be excreted. Stools collected between these two extremes gave reproducible results. The probable reason for this situation can be more easily explained diagrammatically. Figure 4.11 displays a representation of the relative amounts of each label in the most distal part of the intestine. Both ^{3}H and ^{14}C, despite being given as a bolus, do not traverse the intestine in such a form but disperse considerably. As a result it is quite usual to recover significant percentages of ^{3}H label in individual stools collected over a three day period. Similarly in cases of fat malabsorption ^{14}C was usually recoverable in stools over a similar time period. Hence the diagram shows a broad distribution of isotopes by the time they reach the distal colon. The extent of this dispersion is dependent on the intestinal transit time and so patients with watery diarrhoea usually excrete the label within the first 12 to 36 hours.

The distribution of triolein and triether approximately

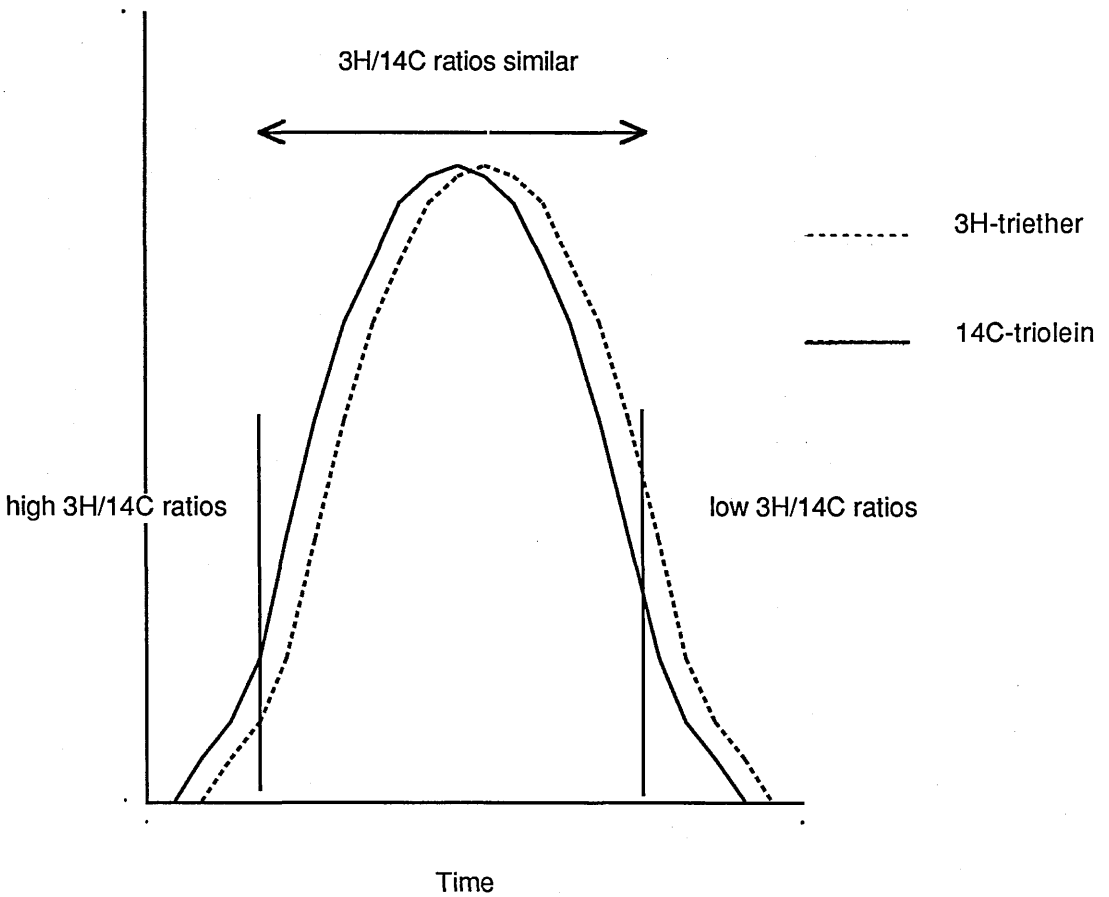


Figure 4.11 - Theoretical distribution of 3H-triether and 14C-triolein in gut.

overlap, although ^{14}C-triolein moves slightly faster. Therefore, although the $^{3}H/^{14}C$ ratio is similar at most sampling times, at the extreme ends of the distribution larger discrepancies are apparent, with low ratios (ie. high PAT-^{3}H results) at the leading end and high ratios (ie. low PAT-^{3}H results) at the trailing end.

As a result any separation of the two isotopes will be accentuated in samples at either end of the distribution of isotopes. However, on the odd occasion when a stool containing the extreme end of isotope is collected it can be readily identified because of the low ^{3}H-triether recovery and the result excluded.

* * * * * *

The inseparability of test and marker has previously been assessed indirectly by studying *in vitro* their relative solubilities in different media designed to resemble physiological situations. Thus Hofmann's group showed that when a two-phase medium was prepared - an oil phase of triolein or fatty acid and monoglyceride, and an aqueous micellar phase - the triether remained exclusively in the oil phase (234). *In vivo* work also confirmed that triether remains in the oil phase layer: Gerskowitch infused an emulsion containing ^{3}H-triether and ^{14}C-triolein into the stomach of humans, and collected gastric aspirate at regular intervals. The ratio of the two fats was similar in each of the gastric aspirates, suggesting that they remain in the same phase (221).

Some doubt was cast on these findings when Saunders *et al* found that when triether and linolenic acid were infused into the stomach the triether/linolenate ratio progressively diminished implying a more rapid gastric emptying of the triether (235). The same group also found that triethers separated from triglycerides in test meals *in vitro*.

Cortot *et al* repeated studies on gastric emptying at a later date again using an *in vitro* simulation of gastric contents and conditions, and an *in vivo* study using a duodeno-jejunal tube for aspirating luminal contents following the introduction of an homogenised meal into the stomach (236). This group concluded that triether is an ideal oil-phase marker and suggested that the conflicting results found by Saunders *et al* were due to the different triether labelling employed: the ^{3}H was incorporated in the glycerol moiety and was therefore available for exchange with hydrogen.

However, these *in vivo* studies are limited since they can only investigate the partitioning of non-absorbable and test substances in the stomach and duodenum. Because of absorption of the test substance beyond this no conclusions can be drawn about lipid partitioning distally.

Variability of PAT-^{51}Cr in Individual Stool Samples

The intra-individual variability of PAT-^{51}Cr in 104 individually collected stool samples from 38 patients was

calculated as in the previous experiment. On this occasion results of 100% were found in 49 samples from 17 patients. The results of the other twenty-one patients are tabulated as follows (table 4.6).

Number	Stool 1	Stool 2	Stool 3	Stool 4
1	97%	100%	(100%)	
2	(1%)	30%	34%	
3	(100%)	85%	92%	
4	13%	-7%		
5	63%	34%	25%	(-99%)
6	99%	99%	97%	
7	100%	99%	(100%)	
8	49%	41%	(39%)	
9	99%	100%		
10	(87%)	76%		
11	73%	77%		
12	98%	98%		
13	98%	(96%)		
14	55%	61%		
15	99%	100%		
16	(85%)	97%		
17	98%	98%	(97%)	
18	91%	(76%)		
19	100%	100%	99%	
20	76%	(85%)	(83%)	
21	100%	(87%)	(80%)	

Table 4.6 - Intra-individual variability of PAT-^{51}Cr on separately collected stools. (Bracketed figures are from stools with low recovery of $^{51}CrCl_3$).

The intra-individual results were comparable in all cases except three (patients 3,4, and 5). Two of these patients (numbers 4 and 5) had gross malabsorption and so the variability was of no clinical relevance, but in one (number 3) the variability was of clinical relevance.

Although these results suggest that the rate of flow of $^{51}CrCl_3$ through the intestine is broadly similar to ^{14}C-triolein there is a small separation of the isotopes in several samples. No overall trend in their relative movements was discernible with $^{51}CrCl_3$ sometimes having a

more rapid intestinal transit than ^{14}C-triolein.

In general, PAT-^{51}Cr gave similar results to PAT-^{3}H, ie between-stool reproducibility was good except in cases of severe malabsorption or when ^{51}Cr recovery was low. There was some evidence that the transit of ^{51}Cr through the gut was faster than for ^{14}C. Results from stools containing the last traces of ^{51}Cr to be excreted, tended to be low. This is in accord with Pederson's finding that the transit time of $^{51}CrCl_3$ (measured as the time taken from ingestion until 80% had been excreted) was less than for ^{14}C-triolein (224). They also found that the initial stool PAT-^{51}Cr results were significantly higher suggesting that the non-absorbable marker is excreted rather more quickly than ^{14}C-triolein. This would imply that the relative dispersals of ^{51}Cr and ^{14}C would correspond to the distributions illustrated (figure 4.12).

Stability of Radioisotopes to Colonic Bacteria

Some *in vitro* work was performed in this study to determine whether ^{3}H-triether was resistant to colonic bacteria, the second criteria to be met by a non-absorbable marker. This environment was simulated by incubating the isotope in a faecal suspension.

Using TLC/autoradiography it was found that the tritium was recovered as a discrete band indicating that ^{3}H-triether was resistant to metabolism by colonic bacteria. This therefore indirectly corroborated previous work which showed that triether was excreted in unchanged form

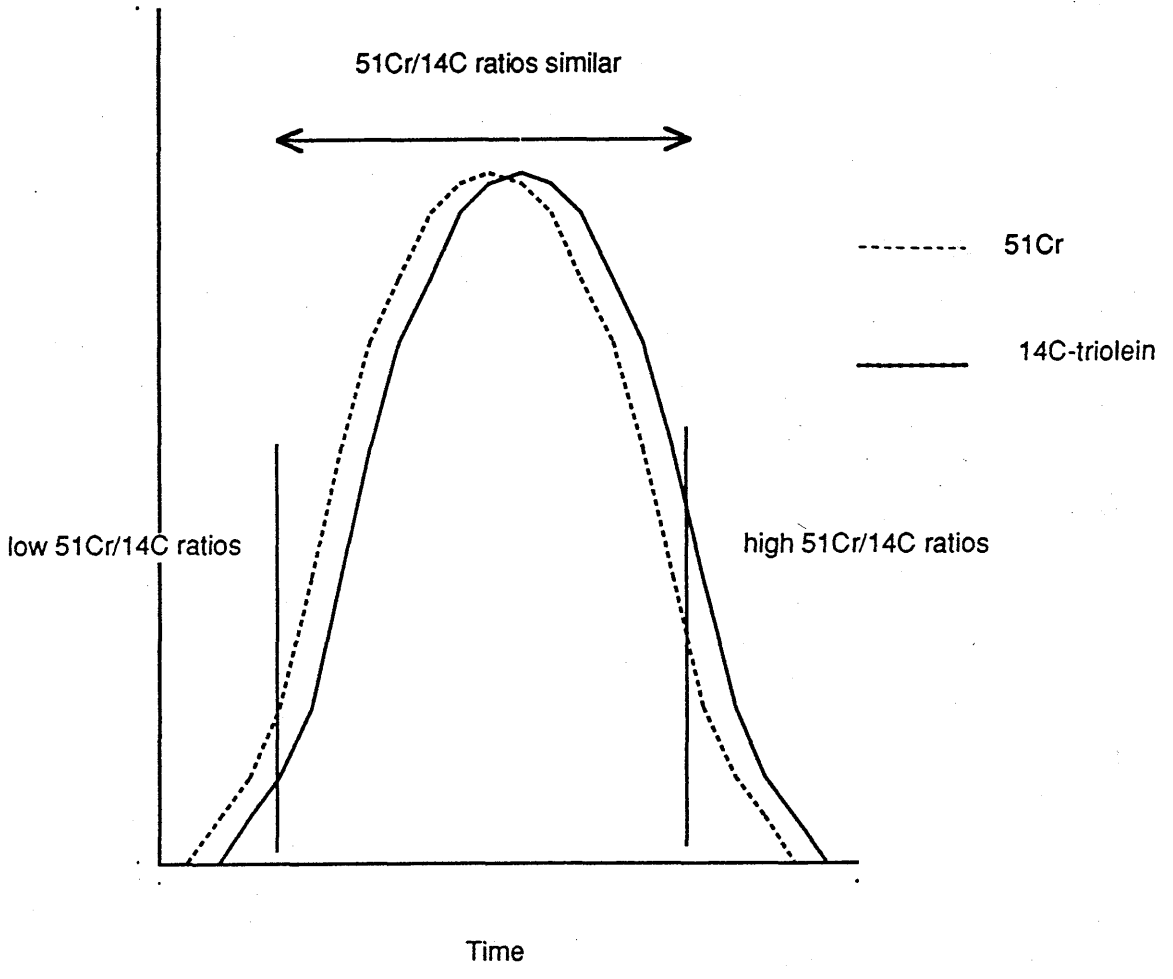


Figure 4.12 - Theoretical distribution of 51CrCl3 and 14C-triolein in gut.

following its oral administration (220, 221).

No work has been performed regarding the stability of the absorbable marker to colonic bacteria. This may be of relevance in patients with malabsorption in whom the absorbable marker may reach the colon in significant amounts.

A similar experiment was therefore performed to assess the stability of ^{14}C-triolein to colonic flora. As figure 4.13 illustrates ^{14}C-triolein was predominantly degraded to ^{14}C-oleic acid. This finding was not unexpected since it is already known that measurement of a faecal triglyceride/fatty acid ratio is not a valid means of establishing pancreatic dysfunction since the triglyceride is digested not only by pancreatic enzymes but also by colonic bacterial systems. However, an additional finding was that other more polar substances, possibly SCFAs, were also produced. It is known that SCFAs are absorbed in the colon and are an important energy source for colonic mucosa (44, 46), consequently some $^{14}CO_2$ excreted in the breath in the triolein breath test may derive from colonic absorption and metabolism of ^{14}C-triolein.

An additional experiment was performed to determine the amount of $^{14}CO_2$, and ^{14}C-methane produced by colonic bacteria. Results for $^{14}CO_2$ generation are shown in table 4.7.

The ^{14}C dpm was high in the first tube and fell to near background counts in subsequent tubes, indicating a high

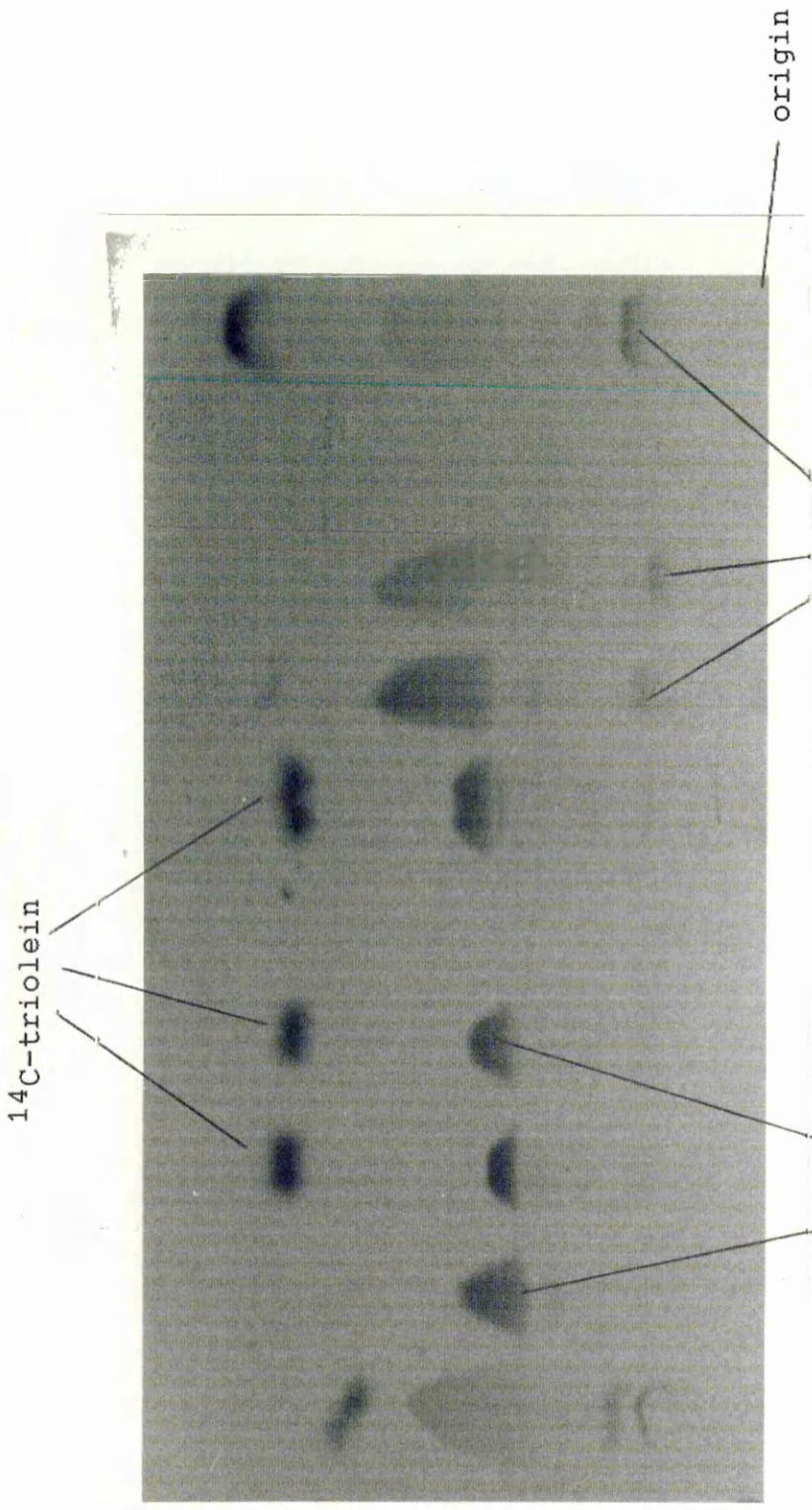


Figure 4.13 - Autoradiograph of faecal extract after incubation with ^{14}C-triolein.

recovery of ^{14}C-carbon dioxide by hyamine hydroxide. Although there was a measurable production of ^{14}C-carbon dioxide, the percentage of this compared to the total ^{14}C-triolein incubated was very low (mean = 0.01%, sem = 0.003).

Homogenate Number	^{14}C DPM in Collection Tube 1	2	3	4	5	Percentage ^{14}C
1	81	35	20	33	-	0.005%
2	108	26	22	31	23	0.006%
3	489	26	31	36	23	0.018%
4	112	36	22	22	24	0.007%
5	598	36	22	27	31	0.022%
6	167	7	4	8	8	0.006%

Table 4.7 - $^{14}CO_2$ production by faecal homogenates.

With regard to the production of methane, no measureable ^{14}C levels were detected indicating that this is not a product of bacterial metabolism of fatty acids.

The $^{51}CrCl_3$ isotope does not suffer from problems of decomposition since it is comprised of basic elements.

Quantitation of Radioistopes

The third criterion to be satisfied by the non-absorbable marker is that it should be easily and reliably measured. The reason that isotopes are employed in PAT tests is to facilitate the final measurement of the chemical probes. However, with respect to the PAT-^{3}H test several problems had to be initially overcome. The main difficulty was caused by the large amount of colour quenching present in stool extracts. A simple means of obviating this problem was eventually discovered when it was found that the chloroform extract could be decolourised by exposure to

light (see pages 231 - 2). Once colour quenching was minimised, chemical quenching was readily compensated for by means of quench correction using commercially available standards.

Stability of Radioisotopes During Refluxing

Since an isotope ratio was measured, rather than absolute concentrations of each, it was important that not only were the two isotopes inseparable in their transit through the intestine, but also during laboratory procedures prior to their measurement. This criterion was investigated, again using TLC/autoradiography methods. The refluxing stage of the analytical procedure (page 234) involves incubation of the sample at 100°C. To determine whether this condition had an effect on radioisotope stability and thus solvent partitioning, pure ^{14}C-triolein and ^{3}H-triether were exposed to typical refluxing conditions and then assessed by TLC/autoradiography. The results of this experiment demonstrated that both radioisotopes separated as discrete bands of appropriate mobility, indicating that both isotopes were stable under these refluxing conditions.

In addition the recovery of both isotopes by solvent extraction was high and had little effect on the $^{3}H/^{14}C$ ratio (see page 236).

* * * * * *

The liquid scintillation method for measuring β-emitters is not direct or specific, the energy of radioactive decay

being initially converted to photons of light energy. The discrete bursts of light produced by ^{3}H and ^{14}C during the liquid scintillation process vary only in their intensity, (^{3}H: 0 - 18meV, ^{14}C: 0 - 156meV). The quantitation of the actual dpm is achieved by complex computer calculation which incorporates information on the quenching and the spillover of counts from one channel to another.

As well as the liquid scintillation counter being able to count ^{14}C and ^{3}H simultaneously an additional requirement is that it should be capable of accurately measuring small amounts of ^{14}C in the presence of large amounts of ^{3}H. This is the situation when PAT-^{3}H results are normal or near-normal. This demand was satisfied but only when the sample and instrument had been carefully prepared (see pages 238 - 9). Consequently a great deal of caution was necessary especially when normal or slightly low results were produced.

The accurate determination of ^{14}C and ^{3}H, then, is dependent upon access to a powerful liquid scintillation counter. Fortunately such an instrument was available to us but at £250,000 each its cost clearly limits this type of fat absorption test to institutions such as teaching hospitals with this level of technology at their disposal. Consequently this test would be impracticable for most District General Hospitals.

By replacing the β-emitters by γ-isotopes a greater market potential for this test would exist. For this reason other workers have studied a similar dual isotope test,

using triolein and triether labelled with ^{131}I and ^{75}Se respectively. Following the validation of these probes in animal studies (233) the test was established in a clinical setting and judged to be satisfactory (237). Unfortunately these labels are no longer commercially available, making this approach impracticable despite its potential.

* * * * * *

If $^{51}CrCl_3$ is used as non-absorbable marker, absolute quantitation of the faecal ^{51}Cr and ^{14}C is necessary since each has to be measured separately by γ-counting and liquid scintillation counting respectively. ^{51}Cr, being a γ-emitter is easily measured and is not affected by the presence of ^{14}C. Although γ-emitters interfere with liquid scintillation counting we found that the two isotopes could be completely separated by solvent extraction, thus avoiding this potential problem (pages 235 -6).

When Pederson evaluated this method a biological oxidation technique was employed to separate ^{51}Cr and ^{14}C (224). This procedure, as well as separating 14C from 51Cr also decolourises the sample, so facilitating liquid scintillation counting of the β-isotope. The technique is relatively straightforward but, like liquid scintillation counting, is only available to centres where a biological oxidiser, another expensive item, is available.

Toxicity of Non-absorbable Marker

Perhaps the most important requirement of a non-absorbable marker is its lack of toxicity. Since ^{3}H-triether is not absorbed then there should be no systemic toxicity. Morgan and Hofmann performed preliminary toxicity experiments on rats which were injected peritoneally with ^{3}H-triether. After twenty-three days they found no histological, haematological or biochemical evidence of pathology. We have had experience with the PAT-^{3}H test on almost 1,000 patients over a twenty year period with no adverse reactions.

The radiological, rather than chemical, safety of the substance is probably of most relevance. Because ^{3}H is non-absorbable and it does not penetrate the mucosa, the exposure is confined to the intestine where we have estimated the radiation dose equivalent at 1.28mSv (690pSv/Bq).

With respect to $^{51}CrCl_3$ the possible radiological hazard is again more relevant than the chemical toxicity. Pederson calculated the intestinal radiation dose to be less than 54psV/Bq with an effective dose equivalent of less than 10psV/Bq (224).

Non-absorbablity of Marker

Finally one obvious criteria to be met by a non-absorbable marker is that it should not be absorbed. This has been assessed on several occasions previously and it is now well established that ^{3}H-triether is not absorbed. When

rats were fed ^{3}H-triether via a gastric cannula the recovery in the lymph was measured and found to be 0.14% of the administered dose and that this small proportion was probably accounted for by radioactive impurities (220). The recovery from faeces and colonic and caecal contents was found to be 93.3% which was similar to the faecal recovery of 93.9% reported in a human study, which also demonstrated the absence of marker recovery from urine and blood (221).

$^{51}CrCl_3$ has been used in tests for quantitating intestinal protein and blood loss since one of the important requirements of this test is that the labelled protein or red blood cells should not be absorbed from the intestine. It has been previously shown that when ^{51}Cr is given orally (as labelled red blood cells) an average of 98.9% was recovered in the subsequent stool collection (238). Waldmann found similar results when ^{51}Cr albumin was used (239). Although the label was protein-bound in these studies it quickly becomes detached during digestion.

PAT-^{3}H Results Using Single Stool Collections

PAT-^{3}H tests were performed in thirty-one out-patients who were asked to collect a single stool sample after a certain time period depending on their bowel habit. The time of collection was selected in order to minimise the possibility of collecting a stool which did not contain any isotope.

Twenty (65%) who had normal bowel habit or intermittent

mild diarrhoea collected a stool 24 hours after the start of the test, ten (32%) patients with severe diarrhoea were instructed to collect a single stool after twelve hours, and one patient who was slightly constipated collected a stool after 48 hours. All the stools analysed contained sufficient isotope for the result to be calculated. Two patients (6%) did not return their samples and so no results could be obtained.

EVALUATION OF THE TRIOLEIN BREATH TEST

In recent years breath tests have become more popular because of their technical simplicity and acceptability to patients and staff alike. This approach has been studied on numerous occasions but it is difficult to assess its general advantage since almost every study has employed differing protocols. Although the resulting conclusions have been diverse the overall opinion is positive.

Effect of Breath Sample Used

The triolein breath test (TBT) was certainly found to be a simple test to perform, involving only aliquotting hyamine hydroxide solution, collection of breath samples and liquid scintillation counting. Although the laboratory staff collected the hourly breath samples this could readily be performed on the ward or Gastroenterology Unit by nursing staff.

However, some patients found difficulty in following instructions for the collection of end-expired breath, the breath sample which is considered to be required for

accurate estimation of the TBT result. We were concerned that the TBT results might be invalid in such patients and so we investigated the necessity of collecting specifically end-expired breath.

In seven patients, 24 samples each of end-expired breath and normal breath samples were collected. No statistical difference between the groups was found ($p = 0.99$), and the difference between the medians was zero (95% confidence interval for this difference of -21.6 to 26) (see Statistical Appendix, note 6) . This demonstrated that normal breath samples give the same results as end-expiratory breath samples. Hence, it is not imperative that the breath samples collected are end-expiratory. Nevertheless for the purposes of this study, end-expiratory breath samples were collected whenever possible although results from patients who had difficulty in providing such samples were not excluded.

Triolein Breath Test Results

The TBT was performed on 68 consecutive patients for investigation of fat absorption. Results were expressed both as the peak $^{14}CO_2$ excretion and the cumulative $^{14}CO_2$ excretion over the six hours of the study. An assumed value of 9mmol/Kg/hour was initially used to calculate these results because of the limited number of patients on whom a calculated CO_2 output was accurately measured (see page 293).

A reference group based on the results from healthy

individuals was not available because ethical permission was not granted for performing the test on a group of healthy volunteers. Consequently, 33 patients who had no evidence of fat malabsorption on the basis of PAT-^{3}H, PAT-^{51}Cr and faecal fat results were used as an alternative means of attaining a control group. The reference ranges for these three fat absorption tests were: greater than 95% for PAT tests (based on results found in healthy volunteers) (222) and less than 25mmol/l for faecal fat.

By selecting those patients with abnormal PAT-^{3}H, PAT-^{51}Cr and faecal fat results a corresponding group of twenty patients with malabsorption was established.

Results of the TBT in controls and malabsorbers are shown in figures 4.14 and 4.15. These initial results were disappointing showing a significant overlap of the two groups. This was the case no matter which system of expressing the results, as peak or cumulative, was employed.

The reference range found by Newcomer *et al*, using a similar protocol, was greater than 3.43% dose/hour. The spread of results from malabsorbers was similar to Newcomer's findings but there were a significant number of controls who gave false positive results.

Such a degree of overlap would make the TBT test of little value but before vilifying the TBT we were keen to ensure that such unimpressive results could not have been caused by any technical errors. In particular we were concerned

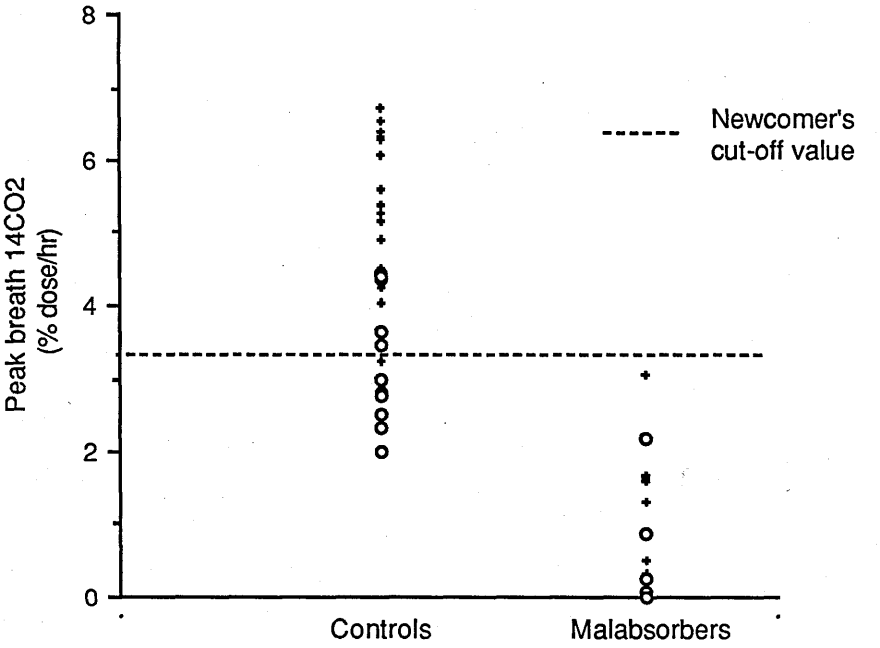


Figure 4.14 - Peak triolein breath test results compared in controls and malabsorbers (see text re symbols)

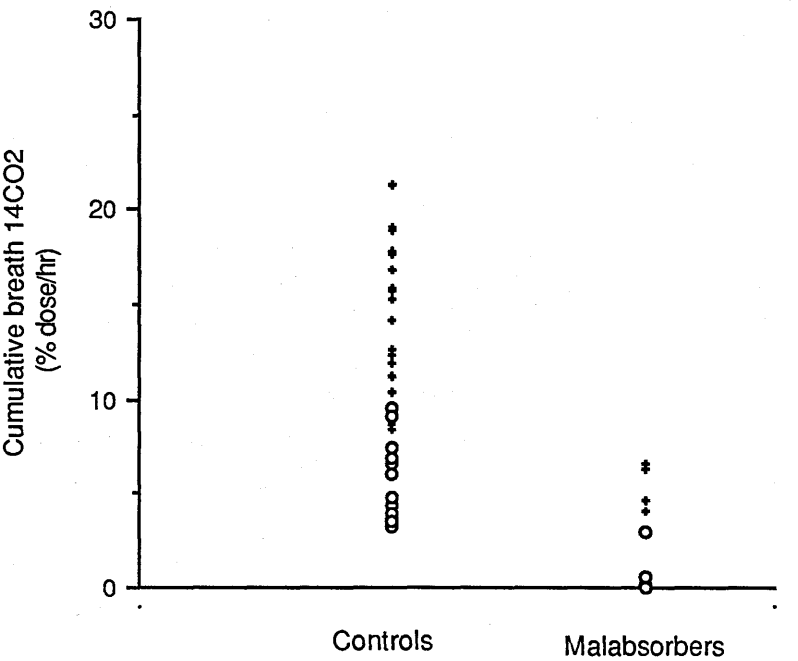


Figure 4.15 - Cumulative breath test results compared in controls and malabsorbers (see text re symbols)

that the six-hour duration of the test might be insufficient to 'catch' all the peaks.

The time of peak $^{14}CO_2$ excretion was studied and it was found that in the great majority (79%) the peak in $^{14}CO_2$ excretion came at the sixth, and last, hour of collection. (Peaks were also reached during the fourth hour in 3 (4%) and during the fifth hour in 11 (16%).) When the time of the peak $^{14}CO_2$ output is displayed in histogram form it is clear that a truncated distribution is present rather that the expected normal distribution (figure 4.16). It seemed possible therefore that in some patients the peak might have been reached during the seventh or eighth hour had these samples been collected.

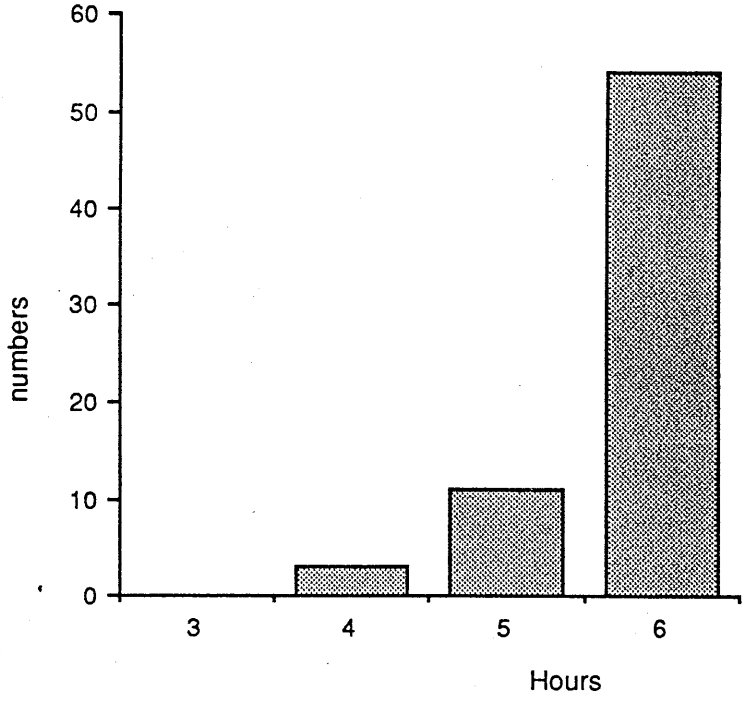


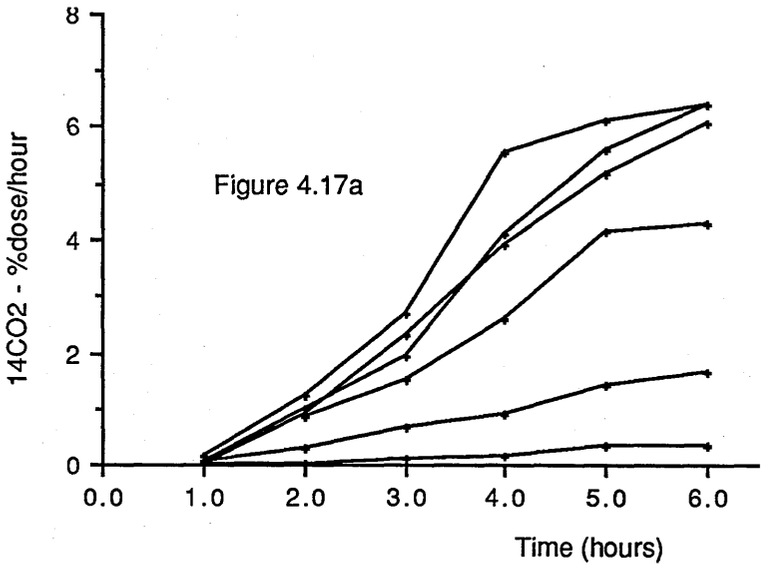
Figure 4.16 - Times of 14CO2 peaks.

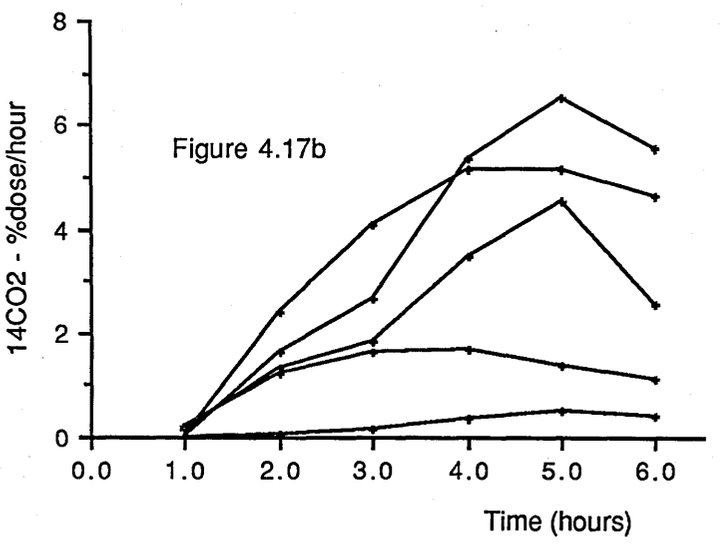
A similar distribution of the times of peak $^{14}CO_2$ excretion was found by Newcomer *et al* (203) and Einersson *et al* (206). In both cases a six hour test had been done and so some peaks may also have been missed. Nevertheless, despite this possibility both of these studies demonstrated a good discrimination between controls and malabsorbers.

The possibility of having missed late peaks prompted us to analyse the pattern of $^{14}CO_2$ excretion in more detail and so the graph of $^{14}CO_2$ with time was plotted for each test. In general, three graph types were produced; one showed a clear peak in $^{14}CO_2$ excretion (figure 4.17a) and in another the curve appeared to be plateauing at about six hours (figure 4.17b); However, in the third category a late exponential rise in breath $^{14}CO_2$ occurred (figure 4.17c). It seemed possible that in those patients whose breath $^{14}CO_2$ excretion was still apparently increasing at six hours the peak might have been missed.

In addition, with this exponential peak type the earlier $^{14}CO_2$ results, eg. at the third hour or fourth hour, were substantially lower than with the other two peak forms, suggesting that there was a delay in $^{14}CO_2$ excretion.

With the resultant suspicion that these data might be underestimates all patient data was reviewed in order to select those $^{14}CO_2$/time curves which corresponded to figure 4.17c. Of the 52 patients investigated, 18 were found to have exponential graphs of this type. In figures 4.14 and 4.15 which show the peak and cumulative $^{14}CO_2$





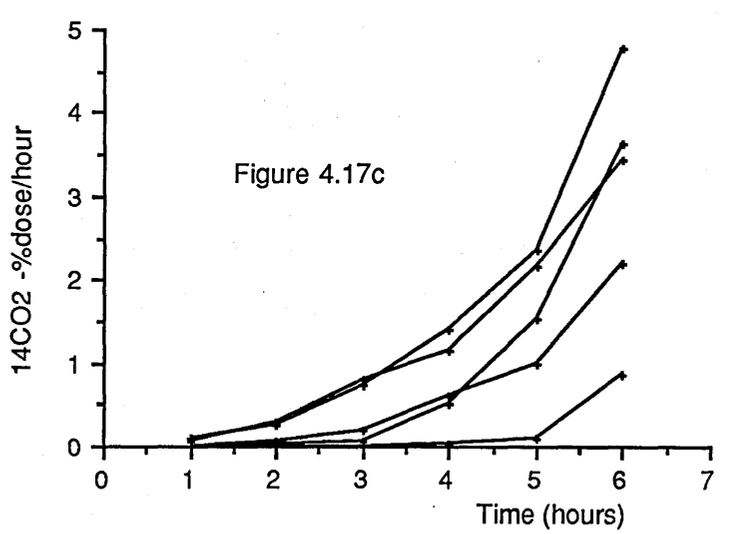


Figure 4.17 - Types of breath 14CO2 curves in triolein breath test.

results, these patients are represented by open circles.

In the control group, twelve patients gave an exponential type of $^{14}CO_2$ excretion suggestive of a late $^{14}CO_2$ peak. Of the six false positive results, ie lower than 3.43, five could be explained on the basis of a probable late $^{14}CO_2$ peak. In the malabsorption group, six graphs were increasing at six hours suggesting a late peak. In only one of these was the result high enough (2.2% of dose/hour) that it might possibly have been misclassified as a false negative had a later breath sample been collected.

The control and malabsorption groups could each be sub-dividied into two categories in which a) the $^{14}CO_2$ peak was late and had probably been missed, and b) those whose peaks were valid since they fell within the six hours of the test. The means of these data and statistical comparisons of groups (see Statistical Appendix, note 1) are shown in table 4.8.

Group	Mean	sem	p
Controls (late peaks)	5.2	0.25	
Controls (valid peaks)	3.3	0.24	< 0.0001
Malabsorbers (late peaks)	1.1	0.33	NS
Malabsorbers (valid peaks)	0.6	0.35	

Table 4.8 - Comparisons of TBT results characterised by late or true peaks in controls and malabsorbers.

In both cases the mean results from each of the subgroups of late peaks are lower, in the case of the control group significantly so. (Because the malabsorbers are a heterogeneous group the TBT result is a factor of their

degree of malabsorption as well as the lateness of the peak.) This provided further indirect evidence that patients with exponential graphs of $^{14}CO_2$ excretion vs time, produced late $^{14}CO_2$ peaks resulting in their TBT results being underestimated.

* * * * * *

In view of the probable existence of late peaks, the results of TBT tests in controls and malabsorbers were replotted omitting all results in which the peak had probably been missed. When this was done there was only a small overlap of results of controls and malabsorbers (figure 4.18) putting the potential of the breath test in a much more favourable light. By adjusting the cut-off value from 3.43% to 3.2% only one false negative result was produced. The cumulative results were also replotted (figure 4.19) but a significant overlap of controls and malabsorbers was still apparent.

Although it is scientifically suspect to exclude data retrospectively in this way, it was felt that had this not been done the breath test might have been inappropriately condemned. Although the reasoning seems valid it is clear that before this interpretation is fully justified, further tests are required using a modified protocol. However, for the next part of the discussion it is assumed that our conclusion was correct, while appreciating that it had been made retrospectively using indirect evidence rather than by the preferred studied, scientific approach.

The time at which the $^{14}CO_2$ peak occurs is dependent on

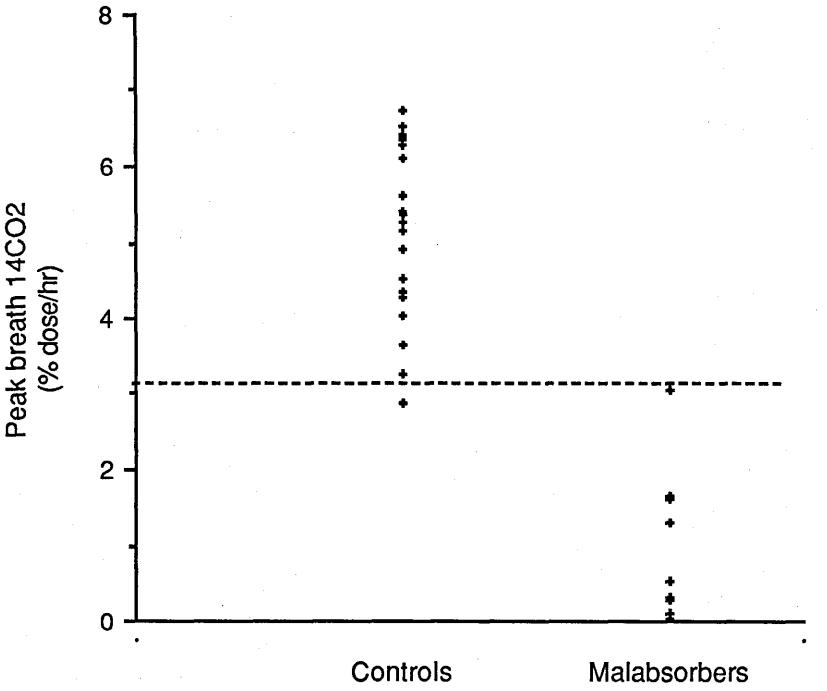


Figure 4.15 - Peak TBT results (excluding late peaks) in controls and malabsorbers.

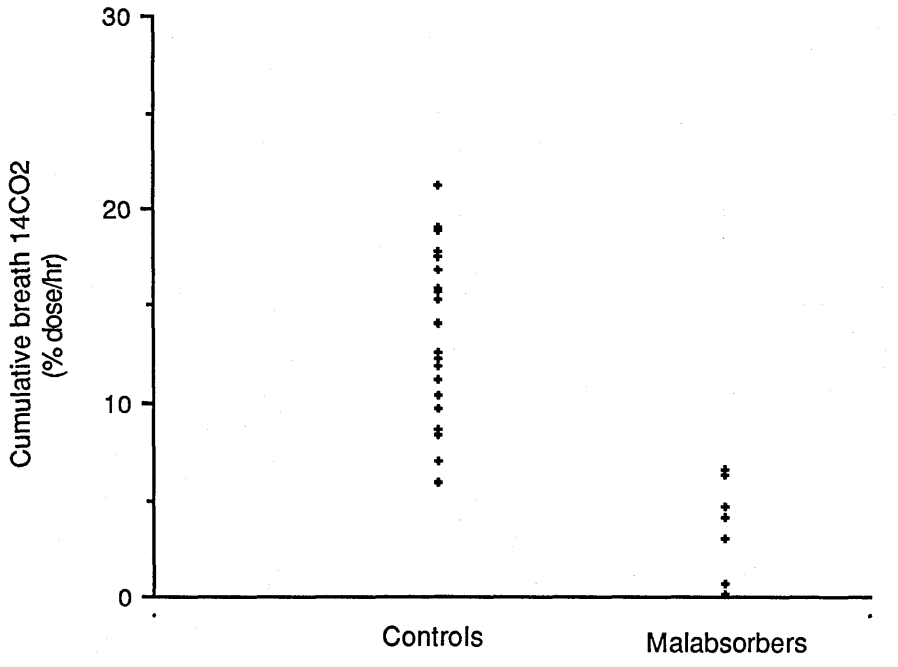


Figure 4.19 - Cumulative TBT results (excluding late peaks) in controls and malabsorbers

the rate at which the 14C-triolein is digested, absorbed and metabolised. This in turn is controlled by factors such as gastric emptying, metabolic rate and the availability of alternative energy sources. The metabolism of fatty acids such as oleic acid, is slower in the fed than the fasted state (240) because of the availability of alternative substrates. Thus if a patient is not fasted prior to the TBT then the ^{14}C-triolein is metabolised over a longer period of time causing a later and smaller $^{14}CO_2$ peak.

Similarly a delayed $^{14}CO_2$ excretion has been reported in obese patients (203) who, as well as having a lower metabolic rate, have a larger supply of endogenous fat which is available for preferential metabolism.

A small loading meal is usually given at the start of the TBT in order to provide a realistic challenge to the absorptive capacity. The size and content of this meal has varied considerably in different studies with corresponding differences in the results. Because we standardised our TBT test as far as possible with the protocol of Newcomer *et al*, like them we chose a fat meal of approximately 25g. The size of our $^{14}CO_2$ peak (lower limit of normal of 3.2%) was comparable to the 3.43% reported by Newcomer *et al* (203). In another study, when a 60g fat meal as buttered toast was given, the $^{14}CO_2$ excretion was delayed with only 42% of the subjects showing a peak by the ninth hour (205). Since a peak was often not achieved or any peaks were broad and small, the

cumulative value (at either seven or eight hours) was used and this provided a good diagnostic separation. Pederson gave a rather excessive test meal meal of 42g fat, 35g of carbohydrate and 26g of protein and found a lower limit of normal peak $^{14}CO_2$ excretion of only 1.5% (211).

Newcomer *et al* compared the effect of using 20g and 50g fat meals and showed clearly that the peak of $^{14}CO_2$ was substantially lower, by about 27%, when the higher fat meal was eaten, and that the peak occurred later, by up to three hours (203). Similarly, using the ^{14}C-xylose test it has been shown that $^{14}CO_2$ results were an average of 62% lower when the subject had consumed his normal breakfast rather than being fasted and that this effect was apparent after one hour (232).

The composition of the test meal also affects the time of $^{14}CO_2$ peak. Fat is metabolised more slowly than carbohydrate and protein and has less effect on the metabolism of ^{14}C-triolein and so has been considered the best choice as test load. The presence of carbohydrates would be expected to retard the $^{14}CO_2$ peak because they inhibit fat oxidation (240, 241). In contrast to the theory however, Turner *et al* achieved very good results (209). For reasons of palatability their diet comprised 10g of carbohydrate, 19.3g of fat and 1.4g of protein as a lemon mousse. It seemed surprising therefore that a peak of $^{14}CO_2$ excretion was achieved within six hours in most (97%) of those studied. With more calories, especially in the form of carbohydrate the peak would be expected to

be prolonged rather than expedited. Their reported lower limit of normal was only 2.8% but this might be have been due to the snack given to their subjects after the fourth hour. It is difficult to explain the reason for their relatively quick $^{14}CO_2$ excretion but it may be the result of other components in their more complex mousse menu, perhaps by increasing the rate of gastric emptying.

As far as our study is concerned, the chances of attaining a peak would be improved by increasing the times of breath collection up to seven or eight hours, or decreasing the amount or calorific content of the test meal. The former modification would immediately make the test less practicable since it could barely be performed within the working day. In addition, it would become less acceptable for the patients who must remain fasted for its duration. Indeed the six hour fast imposed in this study was vigorously objected to by several patients. On the other hand, decreasing the amount of the test meal may confer a disadvantage since an insufficient intake might not stress the gut sufficiently. Thus patients with a mild degree of malabsorption might be able to absorb a lower calorific meal quite effectively and so give a false negative result. Pederson showed that by increasing the fat diet isocalorically from 30g to 100g the faecal excretion of ^{14}C increased denoting a poorer absorption (224).

* * * * * *

As a means of determining the power of the TBT, malabsorbers were categorised into four groups according

to the severity of malabsorption; a) mild degree of malabsorption - faecal fat of 25 to 35mmol/day, and PAT of between 90% and 95%; b) moderate malabsorption - faecal fat of 35 to 60mmol/day, and PAT of 75% to 90%; c) severe malabsorption - faecal fat of 50 to 100mmol/day and PAT of 50% - 75%; and d) gross malabsorption - faecal fat over 100mmol/l and PAT less than 50% (see figure 4.20).

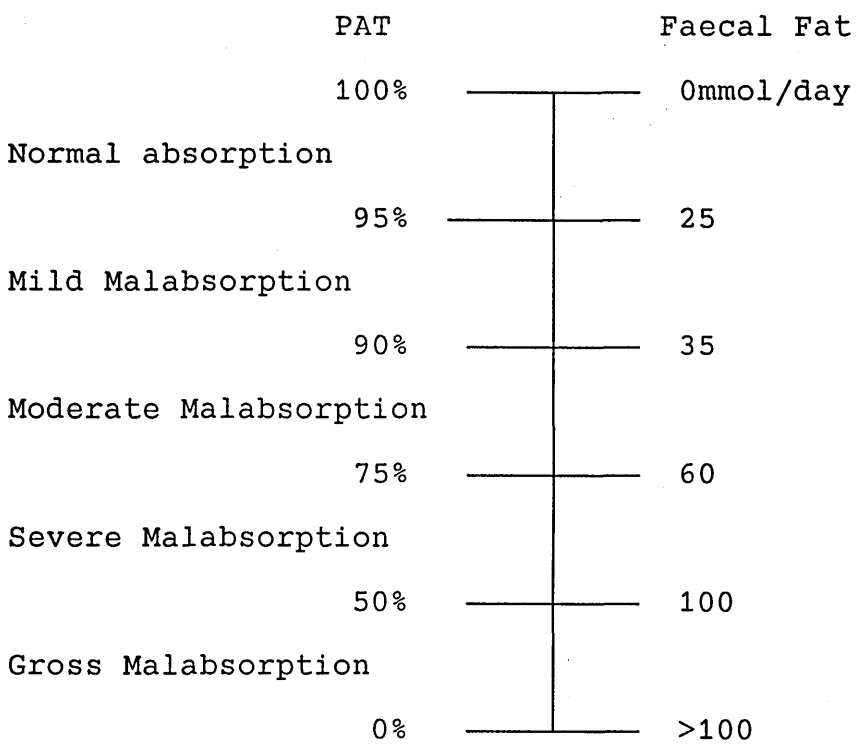


Figure 4.20 - Absorption classification system

All abnormal results could be classified using this system except for three whose faecal fat and PAT-^{3}H results were in adjacent groups. These results were not excluded but empirically placed into the less severe malabsorption group. Unfortunately with the exclusion of data showing an exponential rise in $^{14}CO_2$ at six hours no patients were allocated into the 'mild malabsorption' group.

The peak $^{14}CO_2$ results are illustrated in figure 4.21.

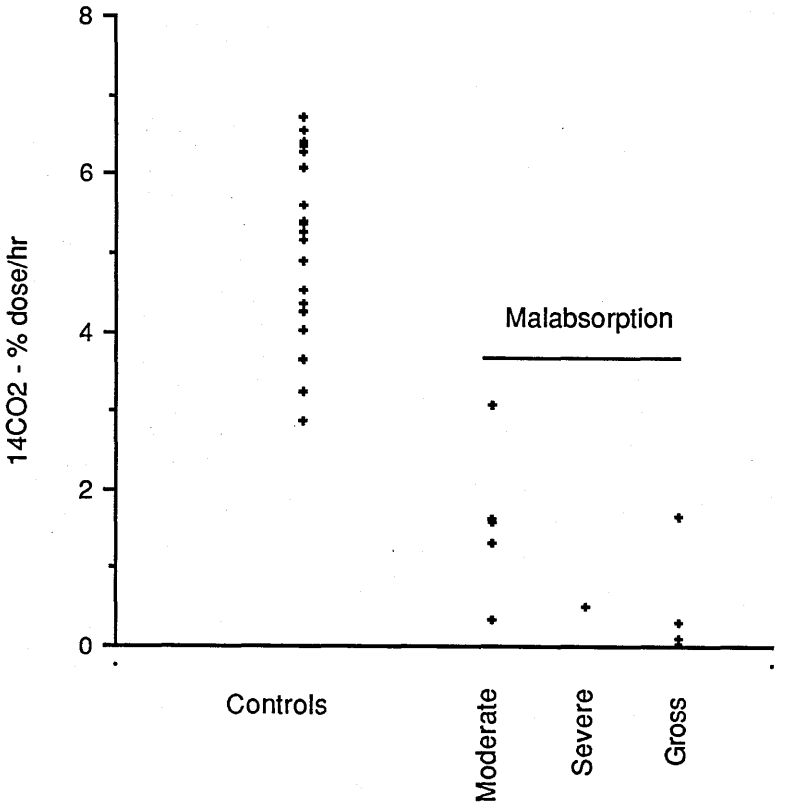


Figure 4.21 - Peak TBT results and severity of malabsorption.

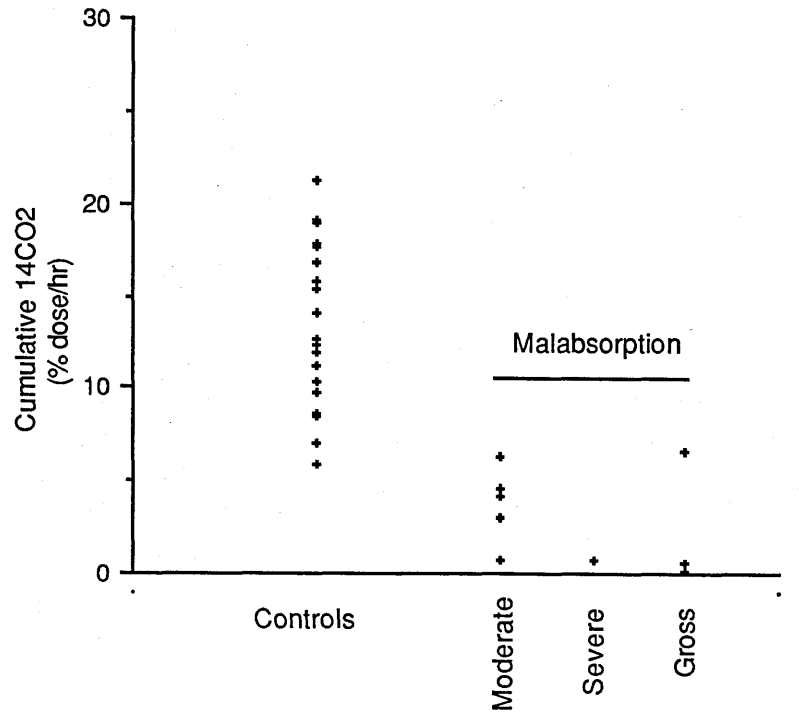


Figure 4.22 - Cumulative TBT results and severity of malabsorption.

Although most patients with a gross degree of malabsorption had very low results this was not always the case. Similarly patients with moderate malabsorption had results ranging from near normal to very low. When this data was analysed statistically (see Statistical Appendix, note 4) there was a moderate degree of correlation between the various severities of malabsorption and peak breath test result ($r_s = -0.54$). When the cumulative $^{14}CO_2$ results were plotted in the same way (figure 4.22) a similar correlation resulted ($r_s = -0.50$) but more worrying was a result which overlapped with controls in a patient with gross malabsorption.

* * * * *

Unlike many previous studies we did not exclude patients because of metabolic or respiratory illnesses despite the theoretical possibility of invalid results.

The TBT was performed in seven patients with known diabetes, two of whom were poorly controlled, five patients with varying degrees of alcoholic liver disease and one patient with a respiratory disorder (chronic obstructive airways disease). In all patients the results of the TBT agreed with the other fat absorption tests and so none of these subjects had been misclassified. However, of the seven diabetics five produced late $^{14}CO_2$ peaks (71%) as did three of the four patients with liver disease (75%). This may explain the unreliability of the TBT in patients with liver disease (242). The incidence of late peaks in the other patients studied, and who as

far as was known did not have any metabolic abnormalities, was 17%. When these data were analysed statistically, there was a significantly increased incidence of late peaks in patients with diabetes ($p = 0.007$) or liver disease ($p = 0.027$) (see Statistical Appendix, note 5).

Measurement of Carbon Dioxide Output

It was originally intended that, rather than use an assumed value of 9mmol/kg/hr for carbon dioxide excretion, this would be measured individually on each patient.

Carbon dioxide output was measured in triplicate or quadruplicate in each of 55 patients aged from 12 to 88 (mean = 53, sem = 2.8). Nineteen (35%) were excluded because the respiratory quotient (r) was higher than the reference range indicating possible hyperventilation (see page 243). Results were also discounted in a further two (4%) in whom r was abnormally low. Finally in two instances no results were possible because the Douglas bags were empty: in each case the patient was very frail and and it was thought that their exhalations were too light to shut the membrane valve completely.

The precision of CO_2 measurement was estimated and was found to be relatively poor in the thirty-two patients with normal respiratory quotient (sd = 1.05, CV = 6.7%) and worse in those with high respiratory quotient (sd = 1.94, CV = 14.7%).

The results of carbon dioxide output (in patients with normal r values) are shown in figure 4.23 along with

existing data from two previous studies. The average CO_2 excretion was calculated at 8.9mmol/Kg/hour which is in close agreement with the two other reports of 8.7 and 8.1mmol/Kg/hour. We found that the range of results at 5 to 12.4 was greater than previous studies: 7.1 to 12.1 and 6 to 11. This is perhaps explainable on the basis of the numbers investigated: we obtained results from 32 subjects in comparison to 13 and 16 from the other two studies. By increasing the numbers the mean would be expected to be similar while the range would tend to increase.

The TBT results were recalculated using the measured CO_2 output rather than an assumed value, as a possible means of improving the separation. Unfortunately calculated values were only available on a limited number because of exclusion criteria (eg. late peaks and abnormal r values) referred to earlier.

Rather than improve the distinction between controls and malabsorbers, the opposite occurred (figure 4.24). However, we were not entirely happy with the Douglas bag method of collecting samples: the precision was not good; there was a problem in collecting valid samples from patients who were anxious and so tended to overbreathe; when the force of exhalation was slight the valves did not close effectively. In addition, in the absence of a definitive means of measuring the CO_2 output we were unable to evaluate the Douglas bag system.

Consequently, we are disinclined to read too much into the

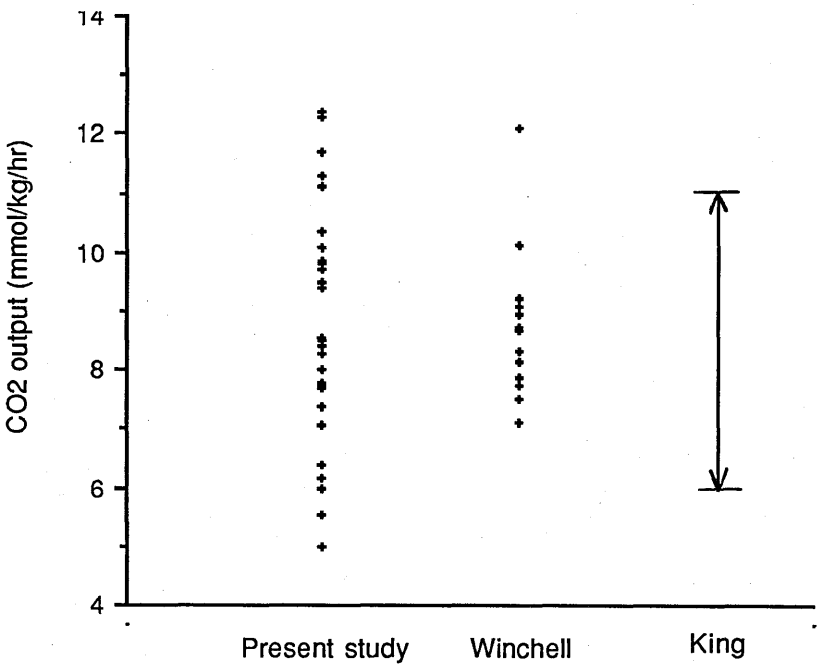


Figure 4.23 - Carbon dioxide outputs from three different studies.

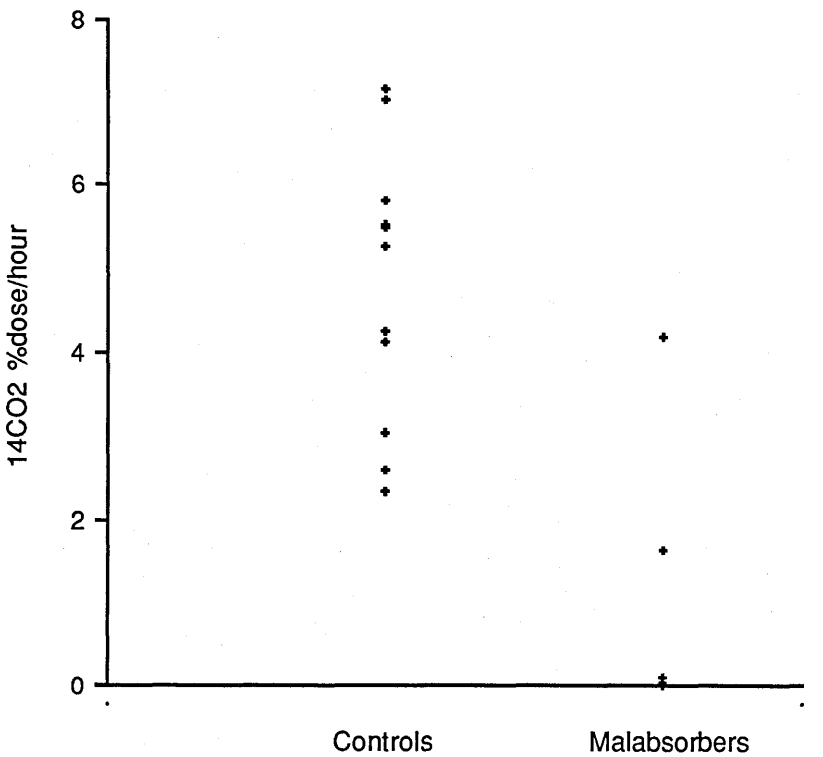


Figure 4.24 - Peak TBT results using calculated CO2 output

apparent worsening of results when calculated CO_2 results were used. Nevertheless in spite of our circumspection of the method some conclusions were possible.

If the results from the present study and the two published studies are averaged then the mean of results is approximately 9mmol/kg/hour and the results range from 6 to 12mmol/Kg/hr approximately. Consequently TBT results using an assumed value of 9mmol/Kg/hr could be overestimated or underestimated by approximately a third. This would mean that at the lower limit of normal of 3.2% there is an effective grey area of from 2.13 to 4.27. As a result there is clearly a potential for misclassification to occur. For example two possible extremes can be outlined in the theoretical situation of two patients having TBT results of 3.0 and 3.4 (ie on either side of the lower limit of normal of 3.2) based on an assumed value of 9mmol/Kg/hour. If the measured CO_2 outputs on these patients are 12 and 6mmol/kg/hour respectively, then their actual TBT results become 4 and 2.26 respectively. Therefore false positive and false negative results are bound to occur from time to time because of the variability of the actual CO_2 output.

The obvious option of measuring the CO2 output on each occasion might not be entirely practicable: as well as making the test more complex, the technology for measuring CO2 output is not always available and it is sometimes difficult to collect a representative breath collection especially in anxious subjects.

Correlation of TBT with PAT Tests and Faecal Fat

The TBT was correlated with the PAT-^{3}H and PAT-^{51}Cr tests and with the quantitative excretion of faecal fat. Good correlations were produced for each as indicated in figures 4.25, 4.26, and 4.27.

Retrospective review of Faecal Fat and PAT^{3}H Results

In the preliminary part of this study patients were categorised as controls or malabsorbers according to results of their PAT tests and faecal fat excretion. On eight occasions conflicting results made it impossible to group the patients and so these results were excluded. The analyses were repeated on these eight tests to exclude technical error but on each occasion similar results were produced. With the knowledge of the TBT results, it was possible to review these results in order to determine which test was probably valid. Although it is emphasised that this is not a scientifically controlled experiment some tentative conclusions are nevertheless possible.

The results of the various absorption tests on these patients are tabulated (table 4.9).

Slightly elevated faecal fat results were found in four patients with normal PAT tests and normal TBT results (1, 3, 4, and 8) and in three cases the TBT result was well into the normal range (4.4 or higher). This would indicate that the faecal fat excretions were falsely elevated.

In one case the PAT results were normal as was the TBT (at

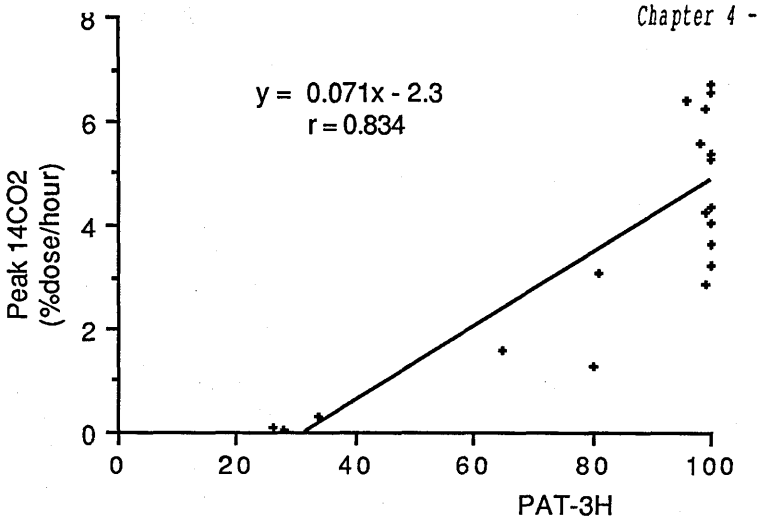


Figure 4.25 - Correlation of triolein breath test with PAT-3H

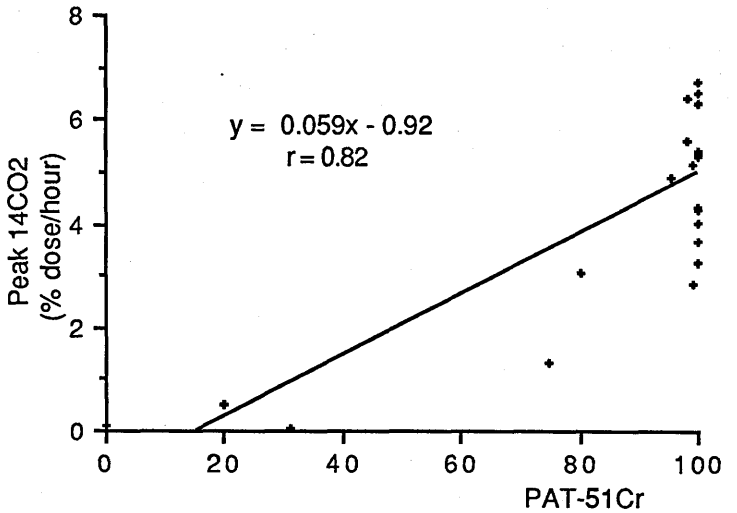


Figure 4.26 - Correlation of triolein breath test with PAT-51Cr

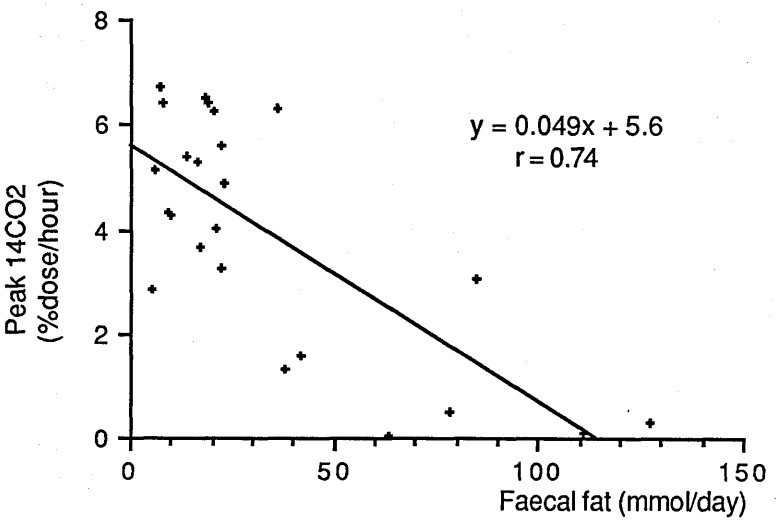


Figure 4.27 - Correlation of triolein breath test with faecal fat

4.8) but the faecal fat was very appreciably elevated, at 78mmol/day. When the patient's case-notes were reviewed there seemed no obvious reason for such a gross disparity in results. However, this patient was a diagnostic enigma. He had been admitted for investigation of leg and

Number	PAT-^{3}H	PAT-^{51}Cr	Faecal Fat (mmol/day)	Breath Test (% dose/hr)
1	-	100%	32	4.4%
2	99%	99.5%	29	2.8%
3	100%	100%	36	6.3%
4	100%	100%	31	3.2%
5	100%	100%	78	4.8%
6	-	89%	19	0.5%
7	99%	99%	39	4.7%
8	73%	87%	24	2.5%

Table 4.9 - Absorption test results where faecal fat and PAT test conflict.

arm oedema and was found to have hypoalbuminaemia and a slight protein losing enteropathy. He had no gastro-intestinal symptoms and his protein status and oedema spontaneously settled while on the ward, and after about six months he remained well.

Two patients (numbers 6 and 8) had normal faecal fat excretions while the other two tests were abnormal. Finally in one patient the PAT results were apparently falsely normal (patient 2). In this case the faecal fat and TBT results were marginally abnormal at 29mmol/day and 2.8% respectively while the PAT results were normal.

Thus of the eight occasions when the PAT and faecal fat tests did not concur, the former was the most likely to be correct in seven.

It was perhaps surprising that in only two cases were there apparently falsely normal faecal fat results. It is well known that problems of incomplete stool collection and/or poor dietary fat intake can cause such results. Conversely, the combination of an abnormal faecal fat and normal PAT and TBT is more difficult to interpret although the following three explanations are possible.

a) The simplest explanation is that the elevation in faecal fat, particularly if it is only slight, may be due to a greater dietary intake of fat .

b) The PAT-^{3}H and TBT only give information about fat absorption at a specific period of time and so if the capacity to absorb shows a diurnal variation then the faecal fat would be more likely to be correct. This situation is possible if there is a small bile acid pool which, because of overnight concentration in the gall bladder, is capable of sustaining normal micelle formation in the morning only. However, in none of the five cases of apparently falsely elevated faecal fat did the patient have evidence of bile salt abnormalities.

c) A third possibility relates to the accuracy of a three day stool collection. It has been argued that in order to obtain a representative collection longer collection periods, even up to 21 days, are required. For reasons of staff acceptability and patient compliance the collection period is usually minimised to three days. This short collection period could result in an inaccurate stool collection. For example, if a patient is passing an

average of one bowel movement each day then the situation depicted in figure 4.28 could arise.

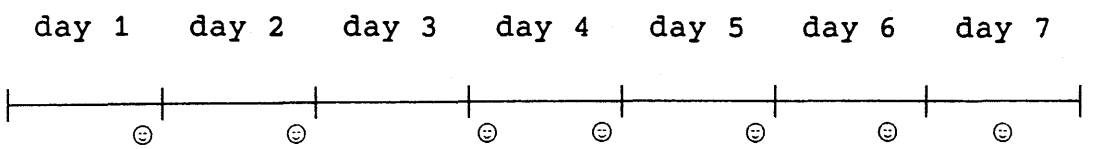


Figure 4.28 - Times of defaecation in a theoretical patient. (☺ - represents a bowel action).

A three-day collection period from day 1 - 3 would result in an underestimated result while a three-day collection over days 4 to 6 would produce an overestimate. Similar inaccuracies could occur in patients with diarrhoea when an average of more than one stool sample is passed per day. In such a circumstance the error would be a smaller, while in constipated subjects it would be greater.

Comparison of Faecal Fat, PAT-51Cr, and PAT-3H

Comparisons were made for each combination of PAT-^{3}H, PAT-^{51}Cr and faecal fat test. Results are shown in figures 4.29, 4.30, and 4.31.

PAT-^{3}H and PAT-^{51}Cr results showed high correlation ($r = 0.89$) as would be expected since both tests employ the same dual isotope principle (figure 4.29). The comparison of faecal fat excretion with each of the dual isotope tests was less favourable with low negative correlations being obtained with each test: PAT-^{3}H ($r = -0.45$) (figure 4.30) and PAT-^{51}Cr ($r = -0.39$) (figure 4.31) (see Statistical Appendix, note - 2).

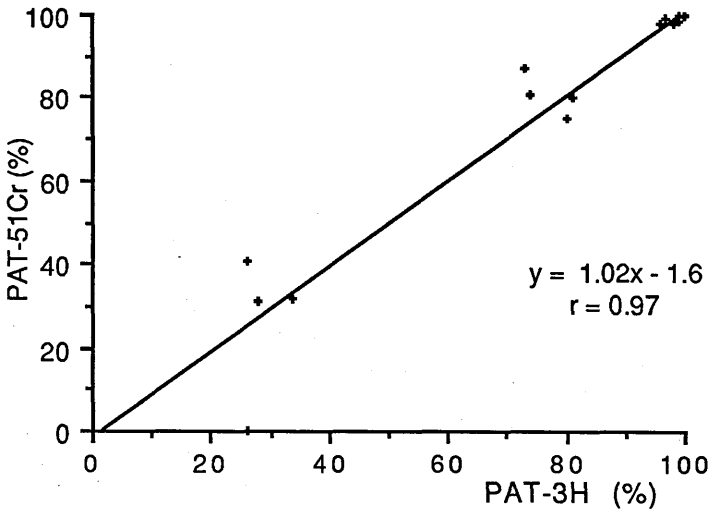


Figure 4.23 - Comparison of PAT-3H and PAT-51Cr.

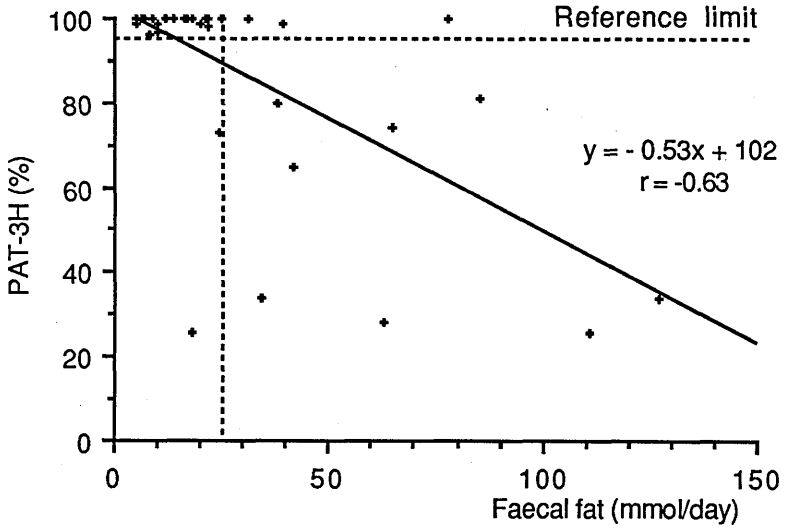


Figure 4.30 - Comparison of PAT-3H with faecal fat excretion.

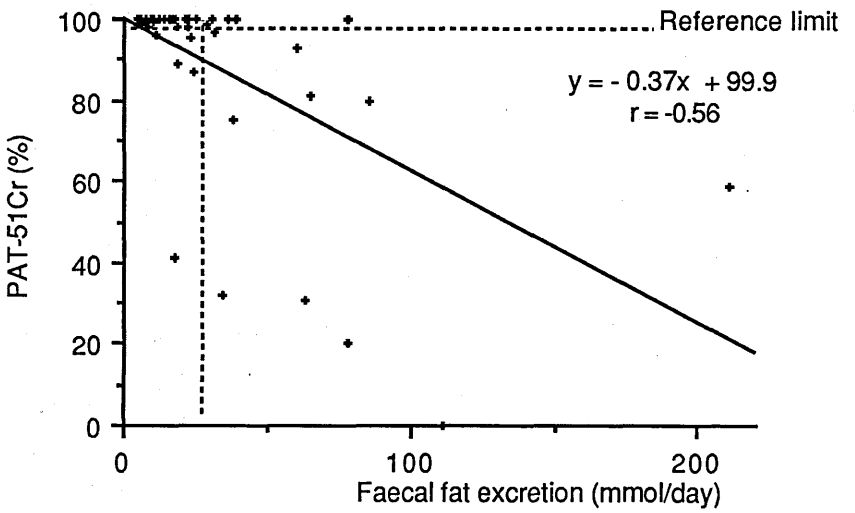


Figure 4.31 - Comparsion of PAT-51Cr with faecal fat excretion.

If the daily excretion of faecal fat is used as the reference method the PAT-^{3}H gave a sensitivity of 78% and specificity of 91%, while the PAT-^{51}Cr test produced a sensitivity of 62% and a specificity of 96%.

OVERALL CONCLUSIONS

By using a fairly simple protocol we had the oppportunity of studying four different fat absorption tests, employing three different principles, in the investigation of absorptive capacity. The relative merits of these techniques were investigated in the study in the hope of selecting the best method.

By and large, the method chosen for evaluating new techniques is the quantitation of faecal fat. Despite its well-known drawbacks in terms of acceptability and poor sensitivity, this test has certainly stood the test of time, which gives some idea of the paucity of good alternatives.

The quantitative faecal fat estimation has the benefit of being widely tested and probably still remains the commonest means of evaluating malabsorption. Its most troublesome disadvantage relates to the collection of stool samples over at least a three day period. This is an unpleasant task for all concerned and a lack of co-operation may lead to inaccuracies. The completeness of stool collection is a fundamental aspect of the test but one that may be difficult to achieve. Patients may accidentally fail to collect a sample, communication

problems between different nursing shifts may lead to missed collections etc. In addition, it is difficult to introduce any quality control on the completeness of a collection. Many patients who are being investigated for gastrointestinal complaints have a poor appetite and so their fat intake may be low. As a result, a patient with fat malabsorption may have a normal excretion of faecal fat. The possibility of falsely negative results is well-known but our results would suggest that there is also a significant false positive rate.

In our past experience of measuring faecal fat and PAT-^{3}H false positive and false negative results have both occurred. The combination of normal faecal fat and abnormal PAT-^{3}H was more common and frequently we found the discrepancy could be explained on the grounds of a falsely low faecal fat result; eg, frequently caused by anorexia, and very low stool weights or poor three-day recovery of ^{3}H-triether, implying incompleteness of collection.

In my experience, because of the unpopularity of performing faecal fat analyses, its quantitation is frequently delegated to the most inexperienced laboratory staff. During part of the study the faecal fat was being measured by two different laboratories using differing methods. In general, the two techniques agreed but it was not uncommon for marked variations to occur.

In faecal fat estimations, errors of its measurement can be minimised by paying attention to detail, eg. ensuring

adequate fat intake and completeness of stool collections, as well as reliable analysis. When evaluations of fat absorption tests are performed using faecal fat as the reference method, it is likely that such meticulous care is taken. But in the 'real world' these aspects of control are, in the main, unlikely to be achieved so reducing its practical effectiveness.

* * * * * *

In terms of simplicity and aesthetics, the TBT is the most preferable test of fat absorption. It is also relatively cheap, requires a minimum of analytical effort, and provides results the following day. The test is tolerable to patients and is easily performed on an out-patient basis, but the six hours of its duration is a slight drawback.

Although this study did not provide the extent of assessment anticipated, preliminary results, excluding those in which the peak $^{14}CO_2$ peak was probably missed, suggest that the specificity and sensitivity of the test is good. However, in the case of an abnormal result no conclusion can confidently be made regarding the severity of malabsorption.

The variability of CO_2 output and the inability to accurately and easily measure this, makes the use of an assumed value preferable. However, this introduces an additional error which could lead to overestimation or underestimation of results by up to 33%. This may be of

particular relevance in the assessment of patients with borderline results when small errors can result in normals being misclassified as mild malabsorbers and *vice versa*. Ironically, the ability of a test to detect mild degrees of malabsorption is often of particular importance, since the diagnosis of more severe malabsorption is less difficult.

As with alternative fat absorption methods, attention to detail is essential: the patient must remain relatively inactive, should not smoke, and must be and remain fasted throughout the duration of the test, eg. succumbing to the temptation of Irn-Bru or Lucozade on the bedside table may invalidate results. An additional problem is that the test is inappropriate in assessing patients with liver abnormalities.

In terms of safety the test is acceptable. The radiation exposure is modest and indeed there is probably a greater hazard from the hyamine hydroxide solution which, being caustic could burn the mouth if the patient sucks rather than blows.

* * * * * *

Unlike the quantitative faecal fat and TBT the theory behind the PAT tests is basically sound. The only possible theoretical flaw is that this approach, like the TBT, only tests fat absorption at a specific moment in time. As a result, malabsorption caused by a low pool of bile salts may be missed. However, unlike the TBT, it is

practicable to start the test in the afternoon thus obviating this potential disadvantage.

Both non-absorbable markers investigated, ^{3}H-triether and $^{51}CrCl_3$, satisfy all the necessary criteria except a slight degree of separation from the test probe. However, as long as the stool contains more than about 3% of the administered non-absorbable marker the results are valid.

There appears to be little to choose between $^{51}CrCl_3$ and ^{3}H-triether as non-absorbable marker although the latter is theoretically more acceptable and the intra-individual variability is slightly better. Although the PAT tests require a stool specimen, provided that the time of the collection is selected according to the patient's bowel habit, then a single sample will suffice.

Quantitation is relatively straightforward but there are pitfalls for the unwary in measuring small amounts of ^{14}C in the presence of large amounts of ^{3}H. As a result the technical aspects of the test must be carefully monitored and a good quality control procedure is essential.

Although more radiation is given than in the TBT test ^{3}H-triether is unabsorbed and unmetabolised and totally excreted after a few days so the effective radiation exposure is low.

The main objection to the PAT tests is that because of the instrumentation required the test is suitable only in larger institutions. Also in the case of the PAT-^{3}H test the label is not commercially available. Without

commercial interest the routine availability of the test will probably be limited to the Glasgow area where it has been developed. It will probably continue to be used as the preferred method in the Royal Infirmary although perhaps in a modified way using the TBT as a screening test. Thus if the TBT is performed after administration of the PAT-3H and if the result is inconclusive, then a stool sample could still be collected for measurement of the PAT-^{3}H.

For the reasons quoted, and in the absence of a reliable alternative, we would advocate the PAT method as being the best way of evaluating patients with possible absorption defects. However, if the facilities for measuring β-isotopes are not available so making the PAT tests impracticable, then the TBT because of its simplicity and better correlation with the PAT tests, is probably preferable.

Finally we would also suggest the PAT test has a role as the preferred method of choice in future evaluations of fat absorption tests. With this in mind it is interesting to note, that in comparison with the PAT tests, the quantitative faecal fat test produces a specificity of 85% and sensitivity of 95% while the corresponding estimates for the TBT are 95% and 100% respectively.

CHAPTER 5

DIAGNOSIS OF BILE ACID INDUCED DIARRHOEA

INTRODUCTION

'Another use that has been assigned to the bile is that it exerts a stimulating action on the intestinal walls, and this acts as a natural purgative; and in support of this view, it may be mentioned that jaundice (in which the bile does not flow into the intestine) is often accompanied by extreme constipation, and that purified ox-gall, taken in the form of pill or enema, produces an undoubted purgative action' (243).

BILE ACID PHYSIOLOGY

Bile Acid Chemistry

Bile acids act as detergents and have an important role in the digestion of fats (page 202 - 5). They are produced in the liver from the precursor cholesterol following ring-hydroxylation and cleavage of the side chain. In man, the liver produces two bile acids, the so-called primary bile acids, cholic acid (CA, 3α,7α,12α-trihydroxy-5β-cholanoic acid) and chenodeoxycholic acid (CDCA, 3α,7α-dihydroxy-5β-cholanoic acid) (figure 5.1).

The bile acids are then amidated with either glycine or taurine to produce conjugated bile acids. Conjugation, particularly with taurine, has the important physiological role of lowering the pK_a of bile acids. As such, they are stronger acids than their precursors and so are not

precipitated in acidic conditions and are poorly absorbed by passive diffusion.

Once released into the ileum, a small proportion of bile salts (about 25%) is deconjugated (244). This process is mediated by bacteria since it is inhibited by treatment with ampicillin (245).

Bile salts reaching the colon are exposed to further anaerobic bacterial metabolism and are quickly deconjugated. A second important process mediated by colonic bacteria is 7α-dehydroxylation with the consequent production of secondary bile acids; hence CA is converted to deoxycholic acid (DCA, 3α,12α-dihydroxy-5β-cholanoic acid) and CDCA to lithocholic acid (LCA, 3α-hydroxy-5β-cholanoic acid) (figure 5.1). LCA is hepatotoxic (246) and fortunately most of it is excreted, however, about 20% is delivered to the liver where it is conjugated and sulphated, rendering it effectively unabsorbable, before secretion into the bile. Consequently, the potentially harmful LCA is quickly removed from the body.

A fifth bile acid, ursodeoxycholic acid (UDCA, 3α,7β-dihydroxy-5β-cholanoic acid), is produced from CDCA by colonic bacterial epimarases and also by the liver from its 7-keto derivative (3α,7-keto-5β-cholanoic acid or 7-keto-LCA). CDCA can also be produced from this intermediate making UDCA and CDCA in effect interconvertible, with the overall equilibrium lying heavily in favour of CDCA production.

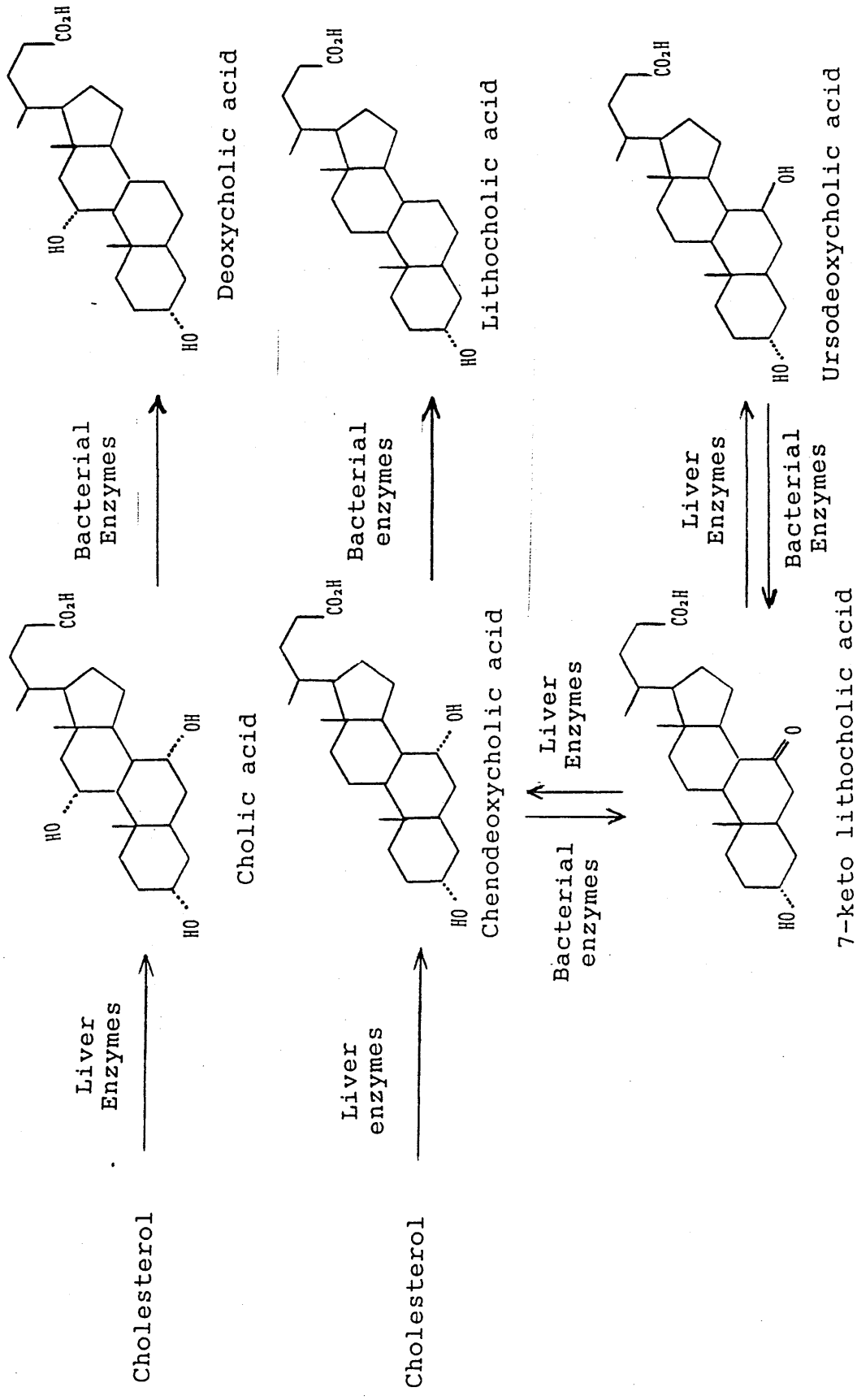


Figure 5.1 - Chemical structures and metabolism of bile acids.

Two enzymes in the bile acid pathway control the rate of synthesis: 3-hydroxy-3methyl glutaryl (HMG) CoA reductase which is the rate limiting step in cholesterol production, and 7α-hydroxylase, the rate limiting enzyme in the conversion of cholesterol to bile acids. A relative deficiency of hepatic bile acids activates the hydroxylase enzyme and the resultant increase in bile acid production stimulates HMG-CoA-reductase to produce cholesterol. In turn there is up-regulation of the hepatic LDL (low density lipoprotein) receptor and accordingly an increased hepatic uptake of cholesterol.

The Enterohepatic Circulation

Relatively large quantities of bile acids are utilised during the digestive process and rather than waste these valuable molecules, the body operates an efficient recycling process, the enterohepatic circulation. After bile acids are synthesised they are secreted into the canaliculi and stored in the gallbladder. When food is eaten the gall bladder contracts under the influence of cholecystokinin, discharging its contents into the duodenum. In fasting states, the sphincter of Oddi contracts so that most bile salts are diverted for storage in the gall bladder.

Bile salts which are deconjugated proximally are absorbed by passive processes. However, the majority of bile acids escape deconjugation and are actively absorbed in the terminal ileum, by which time they have fulfilled their

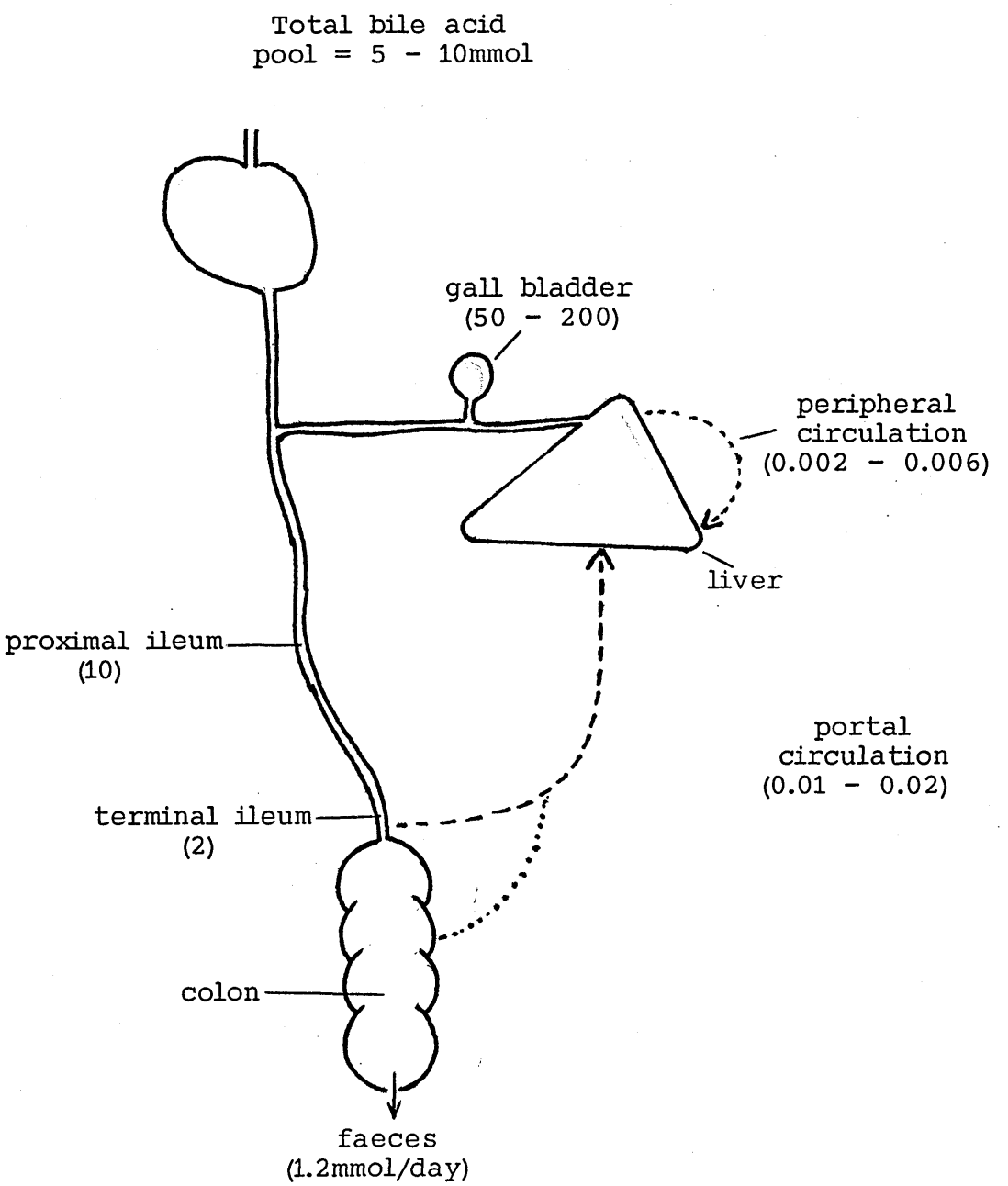


Figure 5.2 - Enterohepatic and peripheral circulation of bile acids showing millimolar concentrations (313).

physiological function in aiding fat digestion. This recovery system is highly efficient and 95% of the bile acids delivered to the terminal ileum are absorbed. The ileo-caecal valve also helps to delay the progress of luminal contents into the caecum so promoting more efficient absorption.

Once absorbed, the portal venous circulation delivers bile salts, predominantly protein-bound, back to the liver, from which they are rapidly re-secreted into the bile.

All of the bile acids, with the exception of LCA, take part in an enterohepatic circulation but at different rates, eg. DCA and CDCA conjugates have a greater passive absorption and so cycle more quickly that CA.

Colonic Handling of Bile Acids

Under normal circumstances the enterohepatic circulation cycles approximately five times per day and so about 25% of the bile acids delivered to the terminal ileum daily pass into the colon.

These bile acids are not all lost from the body because the colon also has the capacity for their absorption. This was initially demonstrated by injection of ^{14}C-cholic acid into the caecum or colon during cholecystectomy and the subsequent recovery of large amounts of radioactivity (44% - 85%, mean 59%) from the duodenal T-tube (247).

The importance of colonic absorption was re-emphasised in a perfusion experiment, which concluded that absorption

rates from a 1mmol/l bile acid solution may be as high as 11.5, 8.2, and 1.2 mmol/day for CDCA, DCA, and CA respectively (248). These figures represent a significant retrieval in cases of bile acid malabsorption (BAM) and indeed might be higher since the rate of absorption is concentration-dependent.

Most of the bile acids in the colon are not in solution but are bound to the solid phase. Although there is probably binding to bacteria (249) there is still significant association with the solid phase in germ-free animals (250). The nature of this sequestrant is probably unabsorbed dietary components especially lignin and bran 251, 252).

BILE ACID INDUCED DIARRHOEA

Pathophysiology

The integrity of the enterohepatic circulation is essential in maintaining the bile acid pool. Where this is breached, an excess of bile acids is delivered to the colon. At higher concentrations bile acids can affect colonic mucosa causing secretion and bile acid induced diarrhoea (BAID). Three types of BAID have been recognised. In type 1, disease or resection of the terminal ileum results in BAM and subsequent diarrhoea. The second is an impaired ability to absorb bile acids in an otherwise normal terminal ileum, so-called bile acid catharsis, and type 3 is associated with cholecystectomy and vagotomy (253).

Terminal Ileal Disease. Much of the recent interest in BAID was stimulated in the late sixties when bile acids, which were known to be cathartics, were implicated in the diarrhoea which often follows resection of the terminal ileum. Since the site of bile acid absorption is removed by this operation, large amounts of bile acids reach the colon where they exert their cathartic effect. Hofmann, who, along with several co-workers, has performed much of the clinical and experimental research in this area, coined the term cholerheic enteropathy. His group showed that bile acids were present in the stool of such patients in elevated concentrations and that treatment with cholestyramine, which binds bile acids, relieves this diarrhoea (254).

If the length of terminal ileum resected is relatively short, less than 100cm, there is a moderate degree of BAM with a compensating stimulation of hepatic bile acid synthesis. As a result the bile acid pool is maintained. Nevertheless, increased concentrations of bile acids still reach the colon causing cholereic diarrhoea (245).

When more than 100cm of the terminal ileum is removed the losses of bile acids into the colon and faeces cannot be adequately compensated for, despite increased hepatic synthesis. As a result the bile acid pool is low, the critical micellar concentration (page 204) cannot be attained, and fat malabsorption results. Although such patients have increased colonic concentrations of bile acids, their diarrhoea does not settle with cholestyramine

therapy (254). Instead, the diarrhoea is due to the effects of increased colonic concentrations of fatty acids, and a restricted fat intake helps to control it (255).

The amount of proximal colon removed with the terminal ileum and the degree of disruption to the ileo-caecal valve also influence the severity of BAID. Increased fluid is delivered to the colon because of the damaged ileo-caecal valve but colonic compensation (see page 43) is restricted in direct proportion to the length of missing colon (256, 257).

Primary Bile Acid Malabsorption. In the 1970s, Danish workers identified a number of patients with idiopathic BAM and diarrhoea (258). These patients had apparently normal terminal ilea with normal Schilling tests, but specific BAM identified by the glycocholic acid breath test (page 327 - 8). Although this test is not definitive for BAID, the finding that the diarrhoea was responsive to cholestyramine treatment, and that dihyroxy bile acid concentrations in faecal water were elevated, supports the supposition that bile acids were implicated. Other similar cases have been described since (259, 260) and one noted increased titres of auto-antibodies suggesting that it may be an auto-immune disorder (261).

Balistreri's group also identified a primary BAM in two neonates with diarrhoea: total stool water bile acids were high (2.11mM and 7.67mM) and the disappearance rates

of radio-labelled CA and CDCA were accelerated. They also showed a poor uptake of bile acids in biopsies from terminal ileum and jejunum. These patients developed fat malabsorption and were successfully treated with middle chain fatty acids (262).

Post-Cholecystectomy Diarrhoea. An American group has been primarily responsible for the suggestion that bile acids are implicated in post-cholecystectomy diarrhoea (PCCD). They found that three patients with PCCD had BAM and that cholestyramine treatment was successful in two of them (253). Other groups had previously shown that, following cholecystectomy, patients have an increased bacterial degradation of bile acids with an increased secondary bile acid pool (263, 264). Hutcheon also reported a series of three PCCD patients, two of whom had high faecal bile acids and abnormal glycocholic acid breath tests, and all of whom responded to cholestyramine. (265).

The association of PCCD and bile acids has since been doubted by the group who originally suggested its existence. Fromm found that of twenty-one PCCD patients only five had BAM and although their concentrations of dihydroxy bile acids in the stool water were increased, they considered these concentrations insufficient to provoke diarrhoea. In addition these patients derived no benefit from the use of cholestyramine (266). Nevertheless a brief therapeutic trial of cholestyramine may be justified (267).

Post-Vagotomy Diarrhoea. Diarrhoea occurs in some 25% of patients following truncal vagotomy, being particularly incapacitating in a small percentage (about 5%). Because a drainage procedure such as pyloroplasty is usually performed at surgery, dumping syndrome can result to varying extents and this may result in an osmotic type of diarrhoea (see page 33).

In addition, these subjects excrete larger amounts of bile acids (268, 269) and these have also been implicated in the pathogenesis of post-vagotomy diarrhoea (PVD). Although several studies have demonstrated that PVD is responsive to cholestyramine (270, 271, 272, 273), in most only a percentage of patients respond particularly those who have also had a cholecystectomy (274).

The incidence of PVD has fallen since the introduction of more selective (post-gastric) vagotomies, implying that its pathogenesis is related to deactivation of non-gastric branches of the vagus nerve. The ileo-caecal valve is innervated by the vagus and so truncal vagotomy may possibly result in poorer sphincter control and rapid emptying of ileal contents into the caecum.

The contribution of bile acids to the pathogenesis of PVD is still controversial and it is unfortunate that, of the many studies performed, dihydroxy bile acids were not measured in the aqueous phase of the stool. Nevertheless there is a considerable body of indirect evidence in support of the theory that bile acids may play a part in

the diarrhoea in some, but not all, patients following vagotomy.

Chendol Therapy. Diarrhoea is the most important side-effect of therapy with CDCA (Chendol) for the dissolution of cholesterol gall stones. It usually begins within the first few weeks, is dose related (affecting 25% of patients receiving 750mg/d and 50% of those on 1000mg/day), and subsides after a few months or if the dose is reduced. The faecal excretion of bile acids increases dramatically 7.85 to 28.07 for non-sulphated and 0.91 to 8.05 for sulphated bile acids (275). The cause of the diarrhoea is thought to be a directly related to the high concentrations of CDCA in the colon (276).

Bile Acid Malabsorption Due To Increased Small Bowel Transit Time. It is possible that an increased intestinal transit time may compromise the absorptive capacity of the terminal ileum and so may be a factor in bile acid induced diarrhoea. In order to test this theory, Meihoff and Kern measured ^{14}C-cholic acid decay rates in normal subjects who had mannitol-induced diarrhoea. They found that normal controls had an average oro-anal transit time of 26 hours and a decay constant of 0.128/day, while subjects with 'induced diarrhoea had a relative malabsorption of bile acid (decay constant of 0.253/day) and a much shorter transit time of 4.6hours. Thus, they demonstrated that diarrhoea can result in BAM, probably because of a rapid transit time. However, in terminal ileal resection the decay constant was much higher (1.43), suggesting that

decreased transit time was a minor factor in causing faecal bile acid loss (277). This work has been indirectly supported by the finding that the extent of BAM was not correlated with the small bowel transit time (278).

Which Bile Acids Cause Diarrhoea?

Following the discovery of BAID as a clinical entity, further work was performed to identify the major causative agents. Accordingly, Mekhjian *et al* demonstrated in dogs that the dihydroxy bile acids DCA and CDCA inhibit absorption of water, DCA being more potent than CDCA (279). More recent work using colonic perfusion in the rabbit has confirmed that CDCA (3α,7α dihydroxy), DCA (3α,12α dihyroxy) and the synthetic 7α,12α dihydroxy bile acids, at concentrations of 6mM, cause water secretion, increased mucosal permeability, and mucosal damage. The other bile acids tested 3α,7β (UDCA), 3α,7-keto; 3-keto,7-keto; 3α,7α,12α (CA); 3α,7α,12-keto; or 3-keto,7-keto,12-keto had no effect. They concluded that two alpha hydroxyl groups, in positions 3, 7, or 12 are necessary for BAID (280).

With improvements in GC and the introduction of capillary columns a much greater resolution of bile acids is possible and as many as 50 different types of bile acid can be found in stool (281). It is possible that some of these may also play a part in induction of diarrhoea (eg 3α,6α-dihydroxy).

Colonic Conditions Associated with Bile Acid Induced Diarrhoea

Hofmann showed that before bile acids cause catharsis they had to be in solution in the colon. His patients with short terminal ileal resections had high concentrations of total bile acids in solution (255). Although patients with large ileal resections were associated with higher daily outputs of bile acids the bile acids were mainly undissolved (248). More recently Rafter has shown that in rats, a high solubility of bile acids in the aqueous phase is required before histological or microscopic damage to the colon occurs (282).

Further work has demonstrated that intracolonic pH is a major factor governing the solubility of bile acids and that in small terminal ileal resections faecal pH was high (6.8 - 8.5), whereas in large ileal resections pH was lower (5.4 - 6.5). The solubility of CDCA and DCA is low at pHs less than 6.8 and 7.0 respectively. (283). This may partially explain the relatively small bile acid pool found in some patients with steatorrhoea (284).

The presence of unabsorbed dietary factors may also affect the availability of bile acids for colonic absorption, by increasing the bound undissolved fraction.

DIAGNOSIS OF BILE ACID INDUCED DIARRHOEA

BAID is usually responsive to treatment with cholestyramine but unfortunately this drug can be unpalatable and not without side-effects. Consequently,

rather than start this long-term therapy empirically it is considered wise to confirm the diagnosis of BAID.

With the possibility that BAID may be the cause of diarrhoea in a sub-group of IBS patients, there is also a strong research interest in having a definitive diagnostic test. The problem of not having a straightforward diagnostic method is highlighted by the observation that despite the number of studies published there is still controversy concerning the causal relationship between PVD and PCCD, and BAID.

Measurement of Faecal Water Dihydroxy-Bile Acids

Measurement of the faecal concentration of the diarrhoea-inducing bile acids (DCA and CDCA) would seem to be the obvious means of diagnosing BAID. Although the total concentration of dihydroxy bile acids in the faeces gives information on the degree of malabsorption of bile acids it gives only indirect evidence of BAID. Because bile acids are only injurious to the intestine when in solution, it is their concentration in this phase which must be measured in the form of faecal aqueous dihydroxy bile acid (FADBA) concentrations.

Measurement of faecal bile acids has, however, proved to be very difficult. Immunoassay methods have been thwarted by the difficulty in producing specific antibodies to bile acids. Although an enzymic method exists, it measures total and not individual bile acids. The methods most commonly applied are GC and HPLC, but the huge number of

published references using these techniques is a testament to their inherent problems. With HPLC the main challenge is extracting bile acids from such a heterogeneous matrix in a 'clean' enough form for injection. Although the requirements for purity are less stringent with GC, the method usually involves hydrolysis, two derivatisation procedures and several extraction steps, making this approach exceedingly time-consuming with little opportunity for automation. Despite these objections GC is the technique most frequently applied.

Once a result has been obtained there remains the problem of interpretation because of doubt about the concentrations of dihydroxy bile acids that induce diarrhoea. In colonic perfusion studies in man it has been demonstrated that DCA and CDCA at 1mmol/l do not cause secretion, but DCA at 3mmol/l and CDCA at 5mmol/l cause significant secretion of water and electrolytes (248). Despite these findings, in two studies, (248, 283) FADBA concentrations were frequently much lower in patients with apparent BAID; in the study by Mekhjian *et al* the patients had short terminal ileal resections and in McJunkin *et al's* patients, cholestyramine-treatable diarrhoea. (table 5.1).

Glycocholic Acid Breath Test.

The principle of the glycocholic acid test is that orally administered cholyl-glycine-1-^{14}C is deconjugated when it reaches the colon in individuals who are malabsorbing bile

Mekhjian et al		McJunkin et al	
DCA	CDCA	DCA	CDCA
0.9 - 2.2	0.4 - 1.4	3.55	0.22
4.1 - 5.0	0.1 - 0.13	0.79	0.75
0.2 - 1.2	0 - 0.5	0.67	0.01
0.9 - 1.9	0.4 - 0.5	1.63	0.2
		0.69	0
		0	2.25
		0	1.63
		0.02	1.19
		0	0.64

Table 5.1 - Concentrations (in mmol/l) of faecal dihydroxy bile acids in patients with BAID.

acids (285). The ^{14}C labelled glycine is then metabolised to $^{14}CO2$ which is either absorbed and exhaled or excretedin the stool. Interpretation of the results is made following the measurement of breath and faecal ^{14}C; in cases of bile acid malabsorption either or both faecal ^{14}C or breath ^{14}C is increased. The design of this test in diagnosing BAM is intrinsically poor since it follows the course of metabolised glycine rather than the molecule of interest, namely cholic acid. In addition, the test has more practical drawbacks: interpretation of the results is not straightforward, since the ratio of faecal/breath ^{14}C can vary widely depending on the location and speed of deconjugation of the cholyl glycine; it is likely that $^{14}CO2$ in the flatus is not measured; the test requires breath as well as stool analysis; and the test does not allow quantitation of the severity of BAM. The test can also produce false negatives even with severe bile acid malabsorption (271), and false positive results have also been reported (269). This test has been widely used in the investigation of BAID but it is important to

realise that it only gives information about the absorption of bile acids and not whether malabsorption is the cause of BAID. With this in mind it is not surprising that the results do not show a good correlation with the degree of diarrhoea (285).

Clinical Response to Cholestyramine Treatment

There is good evidence that diarrhoea is caused by bile acids if it resolves during a course of cholestyramine therapy. This approach has been fairly widely used and is probably a valid course of action in patients with diarrhoea following a short terminal ileal resection.

SeHCAT Test

The use of radioactively labelled bile acids to investigate BAM has been previously used mainly as a research tool (286, 287, 288, 289). These tests have not been used in routine settings, principally because they involve the use of β-emitting isotopes so making stool collections essential. The recent development of tauro-23-[^{75}Se]-selena-25-homocholic acid (SeHCAT), a bile acid attached to a γ-emitter, has simplified bile acid investigations and has particular application in the diagnósis of BAM (290). SeHCAT is given orally and after seven days its retention is measured by a whole body counter or an uncollimated γ-camera.

It is considered to be a valuable tool in assessing the role of bile acids in idiopathic chronic diarrhoea (291).

However, with respect to its use in patients with diarrhoea, the test suffers the same drawback as the glycocholic acid test, that is, it gives only indirect evidence of BAID and so cannot be considered to be diagnostic.

Although the SeHCAT test does not provide direct evidence of BAID it is possible that a modification may make it useful as a diagnostic test. In the presence of BAM a patient is likely to develop diarrhoea only if the bile acids in the colonic lumen are in solution. It is feasible that this information may be readily attained during the SeHCAT test by simply measuring the proportion of malabsorbed SeHCAT in the aqueous portion of the stool. If this were the case then diagnosis of BAID would become relatively straightforward.

This concept in diagnosing BAID would only be applicable if ^{75}Se remained attached to bile acids in the colon, and the ^{75}Se label did not affect the metabolism of the cholic acid to which it was attached.

STABILITY OF ^{75}SEHCAT

Before SeHCAT was marketed as a suitable probe for investigating bile acid absorption, several studies were performed to establish that this synthetic bile acid acted in the same way as physiological bile acids. Initial experiments in rats, following intravenous and oral administration, demonstrated that SeHCAT, was almost entirely confined to the enterohepatic circulation and so

was apparently distributed in the body in the same way as bile acids (292).

Subsequent studies in man demonstrated that SeHCAT behaves similarly to ^{14}C-labeled cholic acid in that both appear in bile at similar rates after both oral and intravenous administration, indicating that the secretion and absorption rates are similar (293).

Although these studies have confirmed that SeHCAT behaves physiologically like an endogenous bile acid, they have not considered the stability or behaviour of SeHCAT in the environment of colonic bacteria.

OBJECTIVES

1. INVESTIGATION OF THE FAECAL AQUEOUS BILE ACID CONCENTRATIONS ASSOCIATED WITH BAID

Although there are several investigations which give indirect evidence of BAID, there is no test available which can claim to be diagnostic. Perhaps the test which comes closest is the measurement of dihydroxy bile acid concentrations in stool water. The measurement itself can be performed, albeit with some difficulty; the problem lies in deciding the concentration above which BAID can be said to occur. The initial aim of this study was to determine the cut-off concentration in this respect.

2. DIAGNOSIS OF BILE ACID INDUCED DIARRHOEA USING A RANDOM STOOL COLLECTION

BAID is diagnosed by measurement of the concentration rather than the daily output of dihydroxy bile acids in stool water. Therefore it seemed likely that as long as a patient had diarrhoea, it could be investigated using a single random stool sample. To investigate this possibility, individual stool samples were collected over a 24 hour period and measured separately.

3. DEVELOPMENT OF A METHOD FOR MEASURING FAECAL CONCENTRATIONS OF BILE ACIDS

A reliable method for measuring bile acids in stool was necessary to reach the objectives of this study. In the routine functioning of the Gastroenterology Laboratory a GC method dating from 1965 had been established (294). This

has been a well-tested and widely used method but its main drawback is the time and labour required.

The measurement of faecal bile acids involves several steps: hydrolysis of conjugated bile acids, extraction, derivatisation to volatile forms, and estimation by GC. The existing method in use by the Gastroenterology Laboratory involved an initial liquid:liquid extraction step; saponification (alkaline hydrolysis); four liquid:liquid extractions to obtain neutral steroids; three liquid:liquid extractions from acidic medium to prepare bile acids; methylation of bile acids using diazomethane; three liquid:liquid extractions of the methyl esters; acetylation of methyl esters; three liquid:liquid extractions; chromatography; and finally calculation of results. Each of these steps was highly labour-intensive: extraction and derivatisation took three days followed by GC taking one to two days and culminating in a complex calculation which took several hours. The whole procedure took up to five days for processing a batch of twelve samples. With such a large number of individual procedures there were numerous possibilities for error, indeed the precision was such that duplicate samples were measured. To account for losses in the various extraction steps internal standardisation was employed.

It was intended to evaluate several modifications to this method in an attempt to simplify it and also avoid the use of hazardous chemicals such as benzene and diazomethane.

4. EVALUATION OF A MODIFICATION OF THE SEHCAT TEST TO DIAGNOSE BAID

The proportion of malabsorbed SeHCAT in the stool supernatant (PMSS) was measured in order to determine whether this information could be used, in conjunction with the result of the SeHCAT test, to identify patients with BAID.

5. MEASUREMENT OF THE STABILITY OF 75SEHCAT

Finally the stability of SeHCAT was determined after its exposure to conditions found in the colon. This was assessed by *in vitro* and *in vivo* experiments.

MATERIALS PATIENTS AND METHODS

PATIENTS

Most of the patients used were in-patients and out-patients under the care of Dr. R. I. Russell and Dr. J. F. McKenzie at Glasgow Royal Infirmary. Most were being investigated for suspected BAID with Crohn's disease being the predominant diagnosis: 23 patients had Crohn's disease with involvment of the terminal ileum or terminal ileal resection, five had diarrhoea following gastric surgery, one patient had radiation enteritis, and one had probable irritable bowel syndrome.

The faecal aqueous dihydroxy bile acid (FADBA) concentrations in patients with suspected BAID were measured and the response to cholestyramine determined in those with significant results. The FADBA concentration of 0.5mmol/l was chosen initially as the action limit above which cholestyramine was administered. Response to bile acid sequestrant therapy (cholestyramine or sucralfate) was evaluated by asking the patients to record the number and fluidity of motions passed each day over a period of two weeks. During the first week they were on their usual medication and on the second week a bile acid sequestrant was taken.

STOOL COLLECTIONS

Stool samples were collected into 2.5l plastic containers

and stored immediately after collection in a freezer (at -15°C). In the case of out-patients a portable freezer was delivered to the patients' home and stool collected individually over a 24 hour period. The faecal weight was measured and faecal supernatant obtained by centrifugation (14,00rpm) of an aliquot of the homogenate. The liquidity of the stool was expressed as a percentage (PWC, see page 75).

MEASUREMENT OF FAECAL BILE ACIDS

Faecal bile acids were measured by a modification (283) of a published method (294) which was further modified to facilitate derivatisation and minimise the potentially hazardous steps of a) methylating with the carcinogen diazomethane, and b) solubilising bile acids prior to Florisil purification into a solvent containing the carcinogen benzene.

In brief 1ml of 0.3mmol/l nor-deoxycholic acid (Maybridge Chemical Co, Tintagel, England) in methanol was added to 50ml stoppered test tubes, followed by 2ml aliquots of stool water or standard (0.3mmol/l each of LCA, CDCA, DCA, UDCA and CA in methanol) or 100mg of faecal lyophylate. Bile acid conjugates were hydrolysed using 3ml of 2N sodium hydroxide in 50% methanol at 110°C for sixteen hours. Stoppers were removed to allow any methanol to evaporate and the samples were cooled and resuspended in 35ml of distilled water making the solution 0.1mol/l in sodium hydroxide. One gram of Amberlite-XAD-7 resin was added and

the suspension mixed by rotary mixer for one hour. The aqueous portion was discarded and washed with a further 10ml of distilled water. Bile acids were solubilised by addition of a small volume (approximately 2ml) of methanol which was then transferred to separate 50ml stoppered tubes. After evaporation of methanol the residue was dissolved in a small quantity of iso-octane:-isopropanol:glacial acetic acid (70:13:17) and added to a column containing 60-100 mesh Florisil (activated by heating overnight at 200°C). The eluent was discarded and bile acids were eluted with 25ml of acetic acid:ethyl ether (1:9) into stoppered tubes. Ten millilitres of distilled water and 15ml of ethyl acetate was added and the solvents mixed. The upper solvent layer was transferred to stoppered tubes and dried. Bile acids were derivatised by methylation (200l of acetyl chloride reagent) at 55°C for 20minutes and acetylation (250ul of acetyl chloride at room temperature for 30 minutes. Ten millilitres each of ethyl ether and 20% sodium chloride was added and the ether layer transferred to separate tubes and dried. The methylated, acetylated bile acids were solubilised in a small quantity of acetone and measured by gas chromatography (Pye-Unicam 4 Series, Cambridge) with flame ionisation detection. A 3% OV-17 on Chromosorb glass column with nitrogen flow rate of 55ml/min and an oven temperature of 265°C.

The various modifications made to the method decreased the time of assay and replaced several steps in which dangerous chemicals had been used. The precision of the assay was

improved slightly but was still poor at over 10%. For this reason each sample was measured in duplicate and a mean result calculated assuming that the reproducibility was acceptable.

THIN LAYER CHROMATOGRAPHY/AUTORADIOGRAPHY OF FAECAL BILE ACIDS

Bile acids were separated by thin layer chromatography (295) and detected by autoradiography as follows. Five ml of faecal supernatant was mixed with 15ml of methanol:acetone (1:1) and heated in a waterbath at 37°C for ten minutes in order to precipitate proteins. After centrifugation (3000rpm for 10 minutes) the supernatant was transferred to another tube. The residue was washed with 5ml of methanol:acetone (1:1), centrifuged and the two supernatants were pooled and evaporated to dryness The residue was dissolved in 0.2ml of methanol and 100µl applied onto a silica gel plate (20cm x 20cm silica gel G) and dried in an unheated airstream. As standards, 50µl of a methanolic solution of 1mmol/l cholic acid and 1mmol/l taurocholic acid and 50µl of ^{75}SeHCAT were added. The plate was chromatographed in a saturated TLC chamber using two developments in the same direction of the same solvent system of iso-octane (2,2,4-trimethylpentane): ethyl acetate: glacial acetic acid (5:5:1). Development time for each run was 45 minutes. In order to separate conjugated bile acids the solvent system of butan-1-ol: water: glacial acetic acid (10:1:1) was used.

Bile acids were detected by spraying with 15%

molybdatophosphoric acid in ethanol (this was prepared on the day before use and after standing overnight was filtered and stored in a dark flask) and heating in an oven at 110°C for 6 minutes. Bile acids stain blue. The radioactivity was detected by exposing the TLC plate to photographic film for a time which was judged according to the amount of radiactivity present.

SEHCAT TEST

On day 1 the total body radioactivity was measured using a whole body radioisotope counter (in the Department of Nuclear Medicine, at Glasgow Royal Infirmary) in order to provide a basal value. A single capsule of ^{75}SeHCAT (SC.510P, from Amersham International, Little Chalfont, Bucks) containing 370kBq was then given orally. On day 7 the patient returned for measurement of the whole body retention of ^{75}Se. The SeHCAT result was expressed as a percentage of administered ^{75}Se that remained after seven days.

^{75}Se was measured in aliquots of urine, whole stool, and stool supernatant by means of a gamma counter (Canberra Packard) with the window settings at 60 - 240keV. The percentage of malabsorbed ^{75}SeHCAT in the stool supernatant (PMSS) was calculated as follows.

$$PMSS = {}^{75}Se_{supernatant} \Big/ {}^{75}Se_{whole\ stool}/PWC$$

$$= \frac{dpm/ml \times PWC}{dpm/g}$$

The form of ^{75}Se in the stool was assessed by performing

TLC/autoradiography as described above.

The pH and osmolality of the stool supernatant were measured using conventional methods.

STABILITY OF ^{75}SEHCAT TO COLONIC BACTERIA

The stability of SeHCAT to the conditions within the colon was determined in the following *in vitro* experiment. Four grams of faecal homogenate were added to screw-top vials along with 300µl of 3.3kBq/ml solution of ^{75}SeHCAT solubilised in 25mmol/l sodium bicarbonate (total of 1kBq per vial). To a similar set of vials containing faecal homogenates, 300ul of 70mmol/l taurocholic acid in 25mmol/l sodium bicarbonate was added. Control tubes consisted of either SeHCAT or TCA being added to 4ml of 0.9% saline.

The faecal suspensions were incubated overnight at 37°C prior to TLC/autoradiography.

RESULTS AND DISCUSSION

The practical application and usefulness of clinically orientated research is often evident and of direct benefit. However, in the case of bile acid induced diarrhoea (BAID) there remains several voids between the knowledge that has been uncovered and the relevance of this learning to the patient. For example it is known that an excess of DCA or CDCA in the colon can cause diarrhoea, that these bile acids must be soluble in the colon to have this effect, and the fluid and electrolyte secretion is mediated by cAMP. On the other hand tests for diagnosing BAID are poor, the FADBA concentrations which induce diarrhoea are not known, and the specific treatment of BAID is poorly tolerated and not without side-effects.

FAECAL BILE ACIDS IN PATIENTS WITH BILE ACID INDUCED DIARRHOEA

The primary objective in this study was to establish the quantitation of FADBA concentrations as a reliable method for diagnosing BAID and then utilise this method to evaluate a simpler radioisotopic approach.

It is well known that if high concentrations of DCA and CDCA are perfused through the colon of man that a secretory state is induced (248). Conversely then, it is likely that the diarrhoea in patients with very high FADBA concentrations is bile acid induced. However, when diagnosing BAID by measuring FADBA concentrations there is

an interpretative dilemma, namely deciding an appropriate cut-off concentration above which it can be stated with a reasonable degree of confidence that the diarrhoea is induced by bile acids.

To determine this value we ideally wanted stool samples from patients who had uncomplicated BAID. Although BAID occurs in several different pathologies there are only two situations in which it is uncomplicated, namely patients with the rare idiopathic bile acid catharsis, and as a side-effect of Chendol (CDCA) in the treatment of gallstones. Because of the rarity of the former we planned to induce diarrhoea in healthy patients by the use of Chendol. Unfortunately ethical permission was denied because of its documented effect in increasing liver enzymes (296, 297). Gallstone patients on Chendol were not available since it has been superseded by UDCA in the medical treatment of gallstones because of the side-effect of the former. Consequently we recruited patients, mainly with Crohn's disease, who were being routinely investigated for suspected BAID.

In order to determine the upper limit of 'normal' FADBA concentrations, we monitored the response of diarrhoea to a bile acid sequestrant in those patients with comparatively high concentrations. For this purpose we initially had to choose a realistic provisional FADBA concentration at which to start this therapy.

The concentrations of bile acids normally found in the

aqueous phase of stool supernatant has been quoted as being between 0.9 and 1.9mmol/l (248), however, of the three references quoted, in two only total faecal bile acids were measured and in the third (255) the system of preparing the supernatant (centrifugation at 100,000g for 2 - 4 hours at 25 - 40°C) may have affected the partitioning of bile acids between solid and liquid phases.

From our experience most patients with diarrhoea caused by agents other than bile acids excrete very low concentrations of FADBAs. For example, of 34 patients with diarrhoea caused by agents other than bile acids, we found that the mean FADBA concentration was 0.101mmol/l (sd = 0.08, range 0.01 to 0.3mmol/l).

A FADBA concentration of 0.5mmol/l was initially selected as a suitable provisional 'action' level. This concentration was chosen because it is higher than the concentration found in our patients with non-BAID diarrhoea, and comparable to the lowest reported concentrations (0.64, 0.69mmol/l) found in patients who were diagnosed as having BAID (see data of McJunkin *et al* table 5.1, page 327). A concentration slightly less than 0.64mmol/l was considered appropriate since it allows for some degree of error and at the same time will not result in large numbers being inappropriately treated.

During the course of the study fifteen patients were found to have 24 hour FADBA concentrations above this proposed

action limit. Eleven of the patients (numbers 1 to 11) had Crohn's disease affecting the terminal ileum and four of them had had resections of the terminal ileum (numbers 3, 5, 6, and 9). Three patients (numbers 12 to 14) had post-vagotomy diarrhoea and one patient (number 15) was suffering from radiation enteritis following radiotherapy for an ovarian malignancy.

These faecal aqueous bile acid results are listed in table 5.2.

Number	LCA	DCA	CDCA	UDCA	CA	FADBA
1a	0.01	0.02	2.1	0.11	3.24	2.12
1b	0.00	0.00	1.25	0.00	1.79	1.25
2	0.01	0.03	1.0	0.68	4.50	1.03
3	0.13	1.3	1.67	0.17	3.03	2.97
4	0.01	0.05	3.2	0.36	4.49	3.25
5	0.00	0.02	1.78	0.06	3.27	1.8
6	0.06	0.88	1.14	0.17	2.74	2.02
7	0.24	0.5	0.71	0.1	0.96	1.21
8	0.06	0.53	0.2	0.05	0.21	0.83
9	0.01	0.05	0.48	0.02	3.22	0.53
10	0.02	0.04	3.11	0.03	4.54	3.15
11	0.00	0.04	1.38	0.04	3.02	1.42
12	0.07	0.62	0.23	0.00	0.49	0.85
13	0.05	0.46	0.12	0.02	0.41	0.58
14	0.01	0.04	1.30	0.01	1.95	1.34
15	0.05	0.06	0.44	0.05	1.45	0.5
Means	0.05	0.29	1.26	0.12	2.46	1.55

Table 5.2 - Bile acid concentrations (mmol/l) in patients with elevated FADBA concentrations.

The bile acid most commonly implicated in the diarrhoea of these patients was CDCA although in two patients (number 8 and 12) DCA was the predominant diarrhoea-inducing bile acid. The cholic acid concentration closely mirrored the CDCA concentration ($r = 0.78$, see Statistical Appendix, note 2), and the other two bile acids LCA and UDCA were

usually in relatively low concentrations.

Although it was the objective of this study to assess the response of these patients' diarrhoea to cholestyramine, unfortunately for reasons outwith our control cholestyramine treatment was not initiated in seven. Of the eight patients who were started on cholestyramine, two patients (numbers 1 and 10) could not tolerate the drug and so treatment was discontinued before a response could be assessed. In the remaining six patients (numbers 2, 5, 8, 11, 12, and 14) all showed a significant response in terms of amelioration of their diarrhoea.

It was fortunate that all the patients with the lowest, and so the most pertinent, FADBA concentrations were given cholestyramine. Consequently we were able to show an improvement in the diarrhoea of those patients whose FADBA concentrations were as low as 0.58mmol/l. FADBA concentrations in the other patients who responded were 0.83, 0.85, 1.03, 1.8 and 2.02 (mean = 1.2mmol/l). Although one patient with a concentration of 0.5mmol/l responded to cholestyramine, this patient had documented fat malabsorption (PAT-^{3}H = 19%) and so the improvement in her diarrhoea could have been due to sequestration of malabsorbed fatty acids.

On the basis of these results it was concluded that BAID was associated with FADBA concentrations exceeding 0.58mmol/l. In contrast, the few studies that report FADBA concentrations suggest higher cut-off values than

this. Aldini *et al* (259) quote a figure of 3.0mmol/l but have misguidedly taken this from a study (255) in which bile acids were measured in the whole stool rather than the stool water. A figure of 1.5mmol/l, which in turn was taken from a colonic perfusion study in man, (248) has also been cited (283). However, careful scrutiny of the original paper revealed that no experiments using such a concentration had been performed. The paper in fact showed that perfusion of CDCA and DCA at respective concentrations of 5.0mmol/l and 3.0mmol/l produced secretion into the colon.

Reasons that could account for our finding of such a low cut-off FADBA concentration were considered. In the patients with BAID secondary to Crohn's disease of the terminal ileum, it is possible that co-existing Crohn's colitis could make these patients more sensitive to the presence of dihydroxy bile acids. However, to counter this possibility six patients were known to have Crohn's disease affecting the terminal ileum only. In addition, no such hypersensitivity would be expected in the patients with post-vagotomy diarrheoa who had BAID characterised by lower concentrations of FADBAs (mean of our three patients of 0.92mmol/l).

Several factors could account for this discrepancy between the perfusion experiments and our patient results. 1. Faecal bile acid concentrations may be lower than the corresponding intracolonic concentrations. The colon is known to absorb bile acids (247, 248) and this would

effectively lower the stool concentrations. In addition, faecal bile acid concentrations measured in BAID patients may be diluted by the secretion which they cause. In two colonic perfusion studies performed in Hofmann's laboratory the concentrations in the stool water were lower than those perfused (74, 248) (table 5.3). On the other hand it has been shown in four subjects that intracolonic and faecal concentrations are comparable in patients with BAID (283).

Mekhjian *et al* (74)

DCA (mmol/l)		CDCA (mmol/l)	
Concentration Perfused	Faecal Concentration	Concentration Perfused	Faecal Concentration
1	0.76	1	0.77
1	0.57	1	0.34
1	0.79	1	0.51
3	2.3	3	1.35
3	2.7	3	1.69
3	2.16	3	1.44
3	2.49	5	4.54
6	4.69	5	3.67
		5	4.09
		10	7.06

Mekhjian *et al* (248)

DCA (mmol/l)		CDCA (mmol/l)	
Concentration Perfused	Faecal Concentration	Concentration Perfused	Faecal Concentration
1 (n = 4)	0.7 ± 0.06	1 (n = 2)	0.6 ± 0.1
2 (n = 3)	1.47 ± 0.1	2 (n = 2)	1.1 ± 0.2
3 (n = 3)	2.41 ± 0.1	3 (n = 2)	1.5 ± 0.1
		5 (n = 3)	4.1 ± 0.2

Table 5.3 - Difference between colonic and faecal concentration of bile acids. (5)

2. The colonic perfusion studies were not representative

of the clinical situation. These were acute experiments performed on fit subjects, whereas our patients were exposed to chronic hyperconcentrations of bile acids. Bile acids are known to cause a morphological change in mucosa (282) and so it is possible that the colon may become sensitised to lower bile acids concentrations.

The likely reason for the discrepancy between the higher values in the perfusion study and our suggested limit of 0.58mmol/l, is probably a combination of these two factors.

It is possible that our cut-off value of 0.58mmol/l may still be an overestimate. Consequently our current policy is to suggest a trial of cholestyramine if the FADBA concentrations are as low as 0.5mmol/l. With further experience the action limit may be modified to a new and possibly lower level.

* * * * * *

The conclusions above were based on FADBA results from a stool collection that had been made over at least a one day period. It was felt that a single days' collection was adequate because these patients usually had severe diarrhoea and so passed several bowel motions per day. Therefore the inaccuracies described earlier, (pages 300 - 301) of collecting stools over a short time period, were minimised. In addition, a concentration rather than an daily output was measured and so some degree of inaccuracy of collection was therefore considered tolerable.

INTRA-INDIVIDUAL VARIATIONS IN FAECAL AQUEOUS BILE ACID CONCENTRATIONS

Initially, for reasons of practicability, some FADBA measurements were made in collections from out-patients who had provided only a single random sample of stool rather than a complete day's collection. These samples were not always delivered to us on the day of their collection and so we initially investigated the storage conditions required for stability of the bile acids. We found that the FADBA concentrations tended to decrease in stools stored at room temperature. Under such conditions, bacterial metabolism causes the stool pH to decrease (see page 92), so making bile acids less soluble in faecal fluid. We investigated the possibility of preventing this change by addition of an anti-bacterial agent but found that even when concentrated merthiolate solutions were added, bacterial processes continued. Furthermore, the addition of a solution had other potential disadvantages. The antibacterial solution would a) require to be well mixed with the stool, and b) result in a dilution of FADBAs. In order to circumvent these difficulties a portable freezer was delivered to the patient's home to enable the collection to be frozen immediately, a step that we found resulted in no measureable change in FADBA concentrations.

The rationale behind using a random stool was that, as long as a patient was suffering from diarrhoea and that this was caused by an excess of bile acids, measurement of any stool should reveal elevated FADBA concentrations.

However, to ensure that this approach was valid we measured FADBA concentrations in individual stools collected over a period of a day.

Individual stools were collected over a 24 hour period in eighteen patients and FADBA concentrations were measured in the stool supernatant. The results are listed in table 5.4.

Patient	Dihydroxy bile acid Concentrations (mmol/l)					
	Early am		Late am	Early pm	Late pm	
Patients with BAID						
1	0.6	0.66	-	1.6	6.1	-
2	1.32	-	-	0.72	1.97	2.54
3	0.84	0.77	1.99	-	-	-
4	0.42	0.44	-	3.68	1.96	-
5	1.15	3.31	-	6.62	5.36	
6	0.47	0.6	2.94	4.37	-	-
7	0.18	1.09	1.66	6.52	0.9	-
8	0.33	-	1.35	-	-	0.18
9	0.87	0.24	2.48	4.68	-	-
10	0.31	0.22	-	4.96	0.5	-
11	0.79	0.83	-	0.35	0.41	0.95
12	0.76	1.28		3.98	0.76	
Patients with non-BAID						
12	0.24	-	-	0.41	0.43	-
13	0.09	-	-	-	-	0.04
14	0.18	0.35	-	-	0.23	0.1
15	0.24	0.17	-	-	0.4	0.26
16	0.21	0.31	-	0.52	1.21	0.42
17	0.25	0.29	-	0.41	-	-
18	0.2	-	0.05	0.23	-	-
19	0.23	-	0.14	-	0.04	0.05

Table 5.4 - Aqueous faecal dihydroxy bile acid concentrations in individual stools throughout the day.

In contrast to our expectations, we found that the bile acid concentrations in separate stools varied widely during the day (table 5.4). For example in two extreme

cases, FADBA concentrations varied from 0.24 - 4.68mmol/l and 0.18 - 6.52mmol/l in stools specimens collected during the same day. Clearly our supposition that patients with BAID would always have elevated FADBA concentrations, was invalid. Indeed the concentrations varied so widely, that results based on certain samples could easily have resulted in the diagnosis of BAID being missed. For example, misleadingly low results could have been produced in some patients (numbers 9, 11, 14, 15 and 16) with BAID, particularly if a morning stool had been collected. With these large intra-individual differences it is clearly not valid to diagnose BAID using a single random stool sample. As a consequence of this discovery, subsequent measurements were made using stools collected over at least a one day period.

These results demonstrated that it was relatively common for patients with BAID to excrete stools with FADBA concentrations of less than 0.5mmol/l and still to have diarrhoea. Therefore it was considered quite likely that on occasions during the day such patients had hyposecretory concentrations of dihydroxy bile acids in the colonic lumen and yet still be in a secretory state.

This tentative conclusion of a lack of direct relationship of colonic dihydroxy bile acid concentrations with colonic fluid secretion was also given further credence. In patients with BAID the effect of FADBA concentrations on the severity of diarrhoea (as expressed by PWC, see page 75) was assessed. The results illustrated in figure 5.3

show a 'blunderbuss' pattern (r = 0.12, see Statistical Appendix, note 2)) suggesting that there was little or no relationship between these two parameters. However, this lack of correlation may have been caused by differing sensitivities of the patients to bile acids. To ascertain whether this was the case, individual linear regressions were assessed for each subject with BAID. Of the ten patients with BAID the graphs of FADBA concentration vs PWC gave positive slopes in seven (5.9, 10.9, 32.5, 0.5, 32.5, 3.6, and 3.9) and a negative slopes in three (-8.8, -0.5, and -12.7). The slope of this line gives details about the relationship between pH and FADBA concentration, a positive slope indicating a proportional association while an inversely proportional relationship was represented by a negative slope. When analysed statistically it was again revealed that even within individuals there was no statistical relationship between the severity of diarrhoea and the concentration of FADBAs (p = 0.19, 95% confidence interval -4.1 to 17.6, 90 %confidence interval -2 to 15.6, see Statistical Appendix, note 8). Thus, although patients with BAID had elevated FADBA concentrations in stools collected over a 24 hour period, there was no direct association of these concentrations with the severity of diarrhoea.

These results can be interpreted in several ways. a) The most immediately evident explanation is that the patients' diarrhoea was not mediated by bile acids. However, there was good theoretical reason to support the notion that these patients did in fact have BAID: they each had

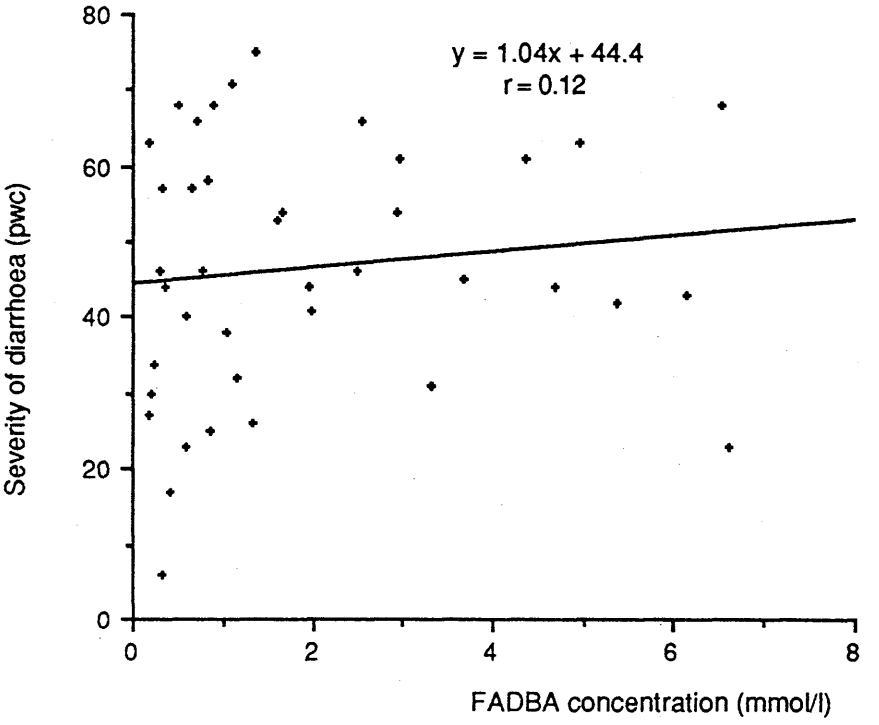


Figure 5.3 - Effect of FADBA concentration on severity of diarrhoea.

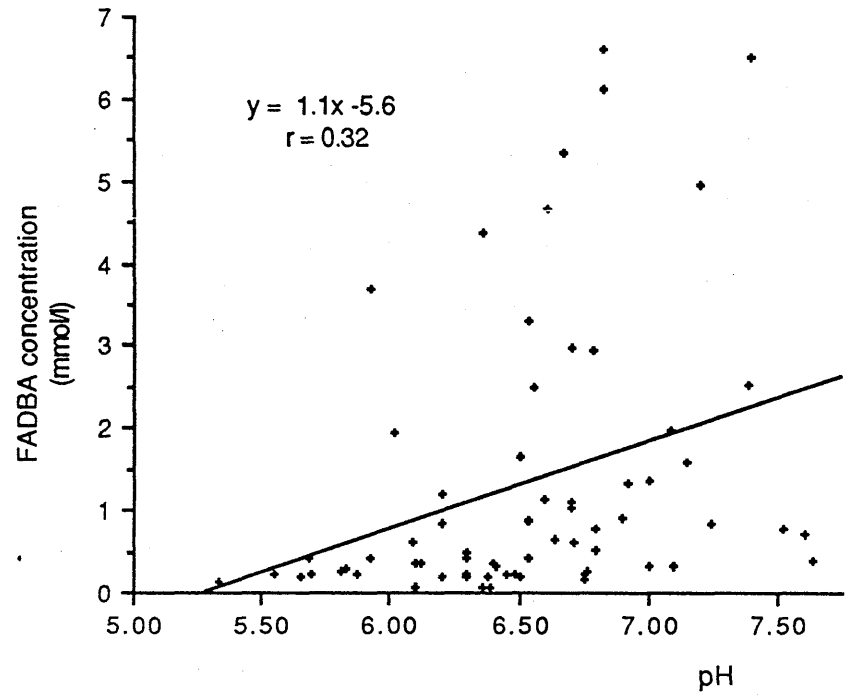


Figure 5.4- Effect of pH on bile acid concentration.

pathologies, such as terminal ileal disease or PVD, which are often associated with BAID, and in all patients tested, their diarrhoea settled or improved significantly when a bile acid sequestrant was administered. b) The mucosal sensitivity to dihydroxy bile acids is increased in patients with BAID, perhaps as a result of chronic exposure to them. To support this possibility, morphological changes to the colonic mucosal architecture can be seen when bile acids are given in higher concentrations (282). On the other hand, iatrogenic BAID (following treatment of gallstones with CDCA) often settles in time (298). This would suggest that rather than mucosal sensitisation developing, the reverse in fact occurs. c) The concentration of FADBAs in the stool do not correspond with the concentrations in the colon. This possibility seemed unlikely for the following reasons. During colonic perfusion studies (78, 248) stool concentrations were shown to be only slightly lower than those in the colon; proximal colonic contents were aspirated in a group of patients with BAID and the bile acid concentrations were similar to those found in stool water (283). d) There is not a direct temporal relationship between the dihydroxy bile acids and secretion. That is, it is possible that following exposure to bile acids the secretory effect may not be immediate and/or may be long-lived. This seemed to be the most likely explanation. For example, in some infectious diarrhoeas which, like BAID, are mediated by cAMP, a secretory state can still occur during the convalescent

stage when the toxigenic organisms have been cleared from the intestine (56).

* * * * * *

The relationship of individual stool pHs and the concentration of FADBA were analysed in the same way (Statistical Appendix, note 8). When all FADBA results were plotted against pH very poor correlation ($r = 0.32$) was seen (figure 5.4) indicating little or no apparent association. When graphs of FADBA and stool pH were plotted individually for each patient, thirteen graphs gave a positive correlation and five gave a negative correlation. The individual gradients were 1.9, 0.9, -0.5, -2.5, -1.0, 15.1,3.7, 5.2, 1.3, 3.8, 3.0, 0.6, 0.2, -0.3, 0.4, -0.4, 0.2, and 1.4. When assessed statistically (statistical note - 8) this showed a statisically significant relationship ($p = 0.04$) suggesting that there is an association, viz as pH increases bile acids become more soluble. Since the solubility of bile acids, like other chemical species, is affected by pH (283) such a relationship was not unexpected.

* * * * * *

When stools were collected individually, the time of day that the stools were passed was also recorded and so we were able to determine if there was a predictable pattern of bile acid excretion during the day.

In general, we found that the bile acid concentrations

tended to be lowest in the morning, increasing to a maximum in the afternoon and falling again in the evening (table 5.4). This diurnal variation was particularly noticeable in those patients with BAID.

Bile acid secretion into the duodenum is stimulated by food and normally mans' eating habits also follow a regular time pattern. Consequently, in retrospect, it is predictable that in patients with diarrhoea, this diurnal trend should be reflected in the stool. The body's bile acid pool concentrates in the gall bladder during the late evening and night so that, when stimulated by breakfast, there is a large outflow of bile acids into the duodenum. Therefore in patients with diarrhoea, and in particular BAID, any malabsorbed bile acids would be excreted in the stool, reaching a peak after a time period that was dependent on the duodeno-anal transit time. In our patients this corresponded approximately to six to ten hours.

A circadian fluctuation of bile acids has previously been demonstrated in the enterohepatic circulation of hamsters (299) and is present even in animals lacking a gallbladder (300, 301).

SEHCAT TEST

The recent development of the SeHCAT test has provided an effective alternative to the Schilling test in evaluating terminal ileal function (290, 302). It has also been investigated, and by and large recommended, as a way of

detecting those patients whose diarrhoea is induced by bile acids (291).

The SeHCAT test was performed on a total of twelve patients in whom BAID was suspected. The results of the SeHCAT test, FADBA concentrations, percentage of faecal aqueous ^{75}SeHCAT, and the response to cholestyramine are listed in table 5.5. Nine patients had Crohn's disease of the terminal ileum (numbers 1 -) of whom five had had resection of the terminal ileum, and one patient each with irritable bowel disease (number 10), post vagotomy diarrhoea (number 11) and terminal ileal resection for radiation enteritis (number 12). The diarrhoea in one of these patients (number 12) had resolved by the time of the test.

Patient	Faecal Dihydroxy Bile acids (mmol/l)	SeHCAT retained	SeHCAT in super-natant	Response to Bile acid binder	BAID?
1	0.1	3.2%	0.7%	none	NO
2	0.01	0%	43%	-	NO
3	0.02	14%	5%	-	NO
4	1.55	0.3%	36%	yes (c)	YES
5	-	7.2%	-	-	NO
6	0.15	8.6%	18%	-	NO
7	1.6	5.3%	16%	yes (c)	YES
8	3.47	0%	27%	not tol. (c)	YES
9	0.1	26%	0.4%	-	NO
10	0.07	39%	-	-	NO
11	0.85	30%	33%	yes (s)	YES
12	0.45	0.2%	-	yes (c)	?

Table 5.5 - Results in patients given ^{75}SeHCAT (c = cholestyramine, s = sucralfate).

The SeHCAT test was abnormally low in eight of the nine patients with Crohn's disease (numbers 1 - 8), and also in the patient with radiation enteritis (number 12). Six

patients with terminal ileal resection (numbers 1, 2, 3, 4, 5 and 12) retained an average of 4.9%, while the four with no resection retained 10.0%. Faecal fat was measured on four of the six patients (numbers 1, 2, 3, and 12) with terminal ileal resection and was grossly elevated in each. All other patients had normal results.

Hofmann originally reported that the fat malabsorption that can occur is associated with patients with a large terminal ileal resection and is caused by an inability to emulsify the fats due to a diminished bile acid pool (255). These patients frequently develop fatty acid induced diarrhoea which is treatable by replacement of dietary fats, which are mostly long chain, with medium chain fatty acids (255). It was interesting that three patients with an intact, although diseased, terminal ileum had very poor bile acid absorption but no steatorrhoea. In these patients the liver was presumably able to increase bile acid production sufficiently to compensate for the losses.

Four patients had BAID and one other may have had BAID (number 12 who is the patient referred to earlier with cholestyramine responsive diarrhoea, moderately raised FADBA, and steatorrhoea). Of the four patients who had clearly elevated FADBA concentrations, three responded to cholestyramine and the third could not tolerate it.

Five patients had very low FADBA concentrations and so were not started on cholestyramine except one lady (number

2) whose diarrhoea, not surprisingly, did not respond.

The SeHCAT test, although apparently giving a good indication of BAM was unable to identify those patients with BAID. Results of the SeHCAT test were found to be very low in three patients with BAID but were also very low in three of the five patients who did not have BAID. Thus of the six patients whose retention was abnormal only three had BAID. In addition, another patient with BAID following vagotomy had a normal SeHCAT retention. It is perhaps relevant that during the seven day course of the SeHCAT test this patient had severe diarrhoea on only two days. Since his diarrhoea was intermittent it is possible that BAM might also have been intermittent hence a normal SeHCAT result. This is consistent with the variation in bile acid excretion sometimes seen in this condition (272).

This initial result demonstrating the ineffectiveness of the SeHCAT test in detecting patients with BAID, was not unexpected. Although BAM is a pre-requisite for BAID, other conditions must also be met, most importantly the solubilisation of malabsorbed FADBAs in the colon (283). Thus in patients with large terminal ileal resections there is a considerable loss of bile acids into the colon, but because the pH tends to be low, the bile acids are not dissolved and so are not implicated in the diarrhoea. Similarly, treatment of BAID with cholestyramine causes a paradoxical increase in BAM but although the amounts of bile acids in the colon are correspondingly higher, being

bound to the resin, they are again in an inactive form

Abnormal SeHCAT retention has previously been demonstrated in individuals who were expected to have BAID, such as patients with Crohn's disease, post-vagotomy diarrhoea or post-cholecystectomy diarrhoea. However, the potential usefulness of the SeHCAT test has also been investigated in other groups of patients with diarrhoea. The specificity of the SeHCAT test in detecting patients with BAID has been claimed in two studies to be 100% (303, 304). In conflict with these conclusions, Orholm *et al* found that the SeHCAT test was frequently positive in patients whose diarrhoea was not related to bile acids; 3 of 5 with acute gastroenteritis, 5 of 6 with drug induced diarrhoea, and one each with ulcerative colitis, medullary carcinoma of the thyroid, and collagenous colitis (305). In addition, four patients with IBS gave positive results only one of whom responded, albeit transiently, with cholestyramine.

The Edinburgh group who developed the SeHCAT test, demonstrated that 6 of 43 patients with suspected IBS gave positive results and the four who were started on cholestyramine treatment all responded (291). Similarly an Italian group showed that seven of thirteen IBS patients had abnormal retentions and all responded to cholestyramine, while the seven who had normal results did not respond (306).

However, in none of these studies had BAID been

specifically diagnosed and in contrast with them, a third study reported 14 of 17 patients with idiopathic chronic diarrhoea and relative BAM, whose symptoms did not resolve with cholestyramine (307). They concluded that BAM may be a manifestation of an underlying motility or absorptive defect rather than the primary cause of diarrhoea.

Nevertheless it is becoming generally accepted that the syndrome of idiopathic bile acid catharsis, masking as IBS, may be considerably more common than realised. Such conclusions, however, should be considered with some caution. For example, in one study only patients with abnormal SeHCAT tests were given cholestyramine and so this study was not properly controlled (291).

It is also worthwhile remembering that the diarrhoea of patients with IBS often shows a significant response (35%) to placebo (308). In addition, another study found that 14 of 17 patients with idiopathic chronic diarrhoea had a decreased half life of SeHCAT (an alternative means of expressing the result), and that their symptoms did not resolve with cholestyramine (309). Their interpretation of this finding was that an underlying motility problem caused the diarrhoea and an incidental malabsorption of bile acids.

This opens up a further area of some controversy in that it is possible that an increased intestinal transit time may limit the absorptive capacity of the terminal ileum and so be result in BAID. In order to test this theory

Meihoff and Kern measured ^{14}C-cholic acid decay rates in normal subjects who had mannitol-induced diarrhoea. They found that normal controls had an average oro-anal transit time of 26 hours and a decay constant of 0.128/day, while subjects with induced diarrhoea had a relative malabsorption of bile acid (decay constant of 0.253/day) and a much shorter transit time of 4.6 hours. Thus, they demonstrated that diarrhoea can result in BAM, probably because of a rapid transit time. However, in terminal ileal resection the decay constant was much higher (1.43), suggesting that decreased transit time was a minor factor in causing faecal bile acid loss (310). This work has been indirectly supported by the finding that the extent of BAM was not correlated with the small bowel transit time (311).

Thus there is considerable evidence supporting the theory that in patients with diarrhoea there is a relative secondary malabsorption of bile acids. However, this BAM may be incidental, playing no active role in worsening the diarrhoea. We tried to determine whether this was the case. Accordingly those patients with diarrhoea and a probable rapid intestinal transit time were investigated for BAID. FADBA concentrations were measured in ten patients with diarrhoea and symptoms suggestive of a rapid intestinal transit. The diagnoses were infectious diarrhoea in two, saline laxative induced diarrhoea in 4, and one each of carcinoid syndrome, alcohol-induced diarrhoea, diabetes, dumping syndrome and unexplained

diarrhoea. The mean concentration was 0.10mmol/l (sem = 0.028) as compared with 1.70mmol/l (sem = 0.225) in nine patients with bile acid induced diarrhoea. The FADBA concentrations were significantly different in the two groups ($p < 0.001$, see Statistical Appendix, note 1). This suggests that patients with diarrhoea and rapid transit do not have BAID and that any BAM does not have any pathophysiological involvement with the diarrhoea.

* * * * * *

The considerable analytical effort required to measure dihydroxy bile acids in faecal supernatant makes this an impracticable means of routinely diagnosing BAID.

We therefore investigated the possibility of modifying the SeHCAT test so that BAID could be more readily diagnosed. We speculated that the required information regarding the colonic solubility of bile acids might be provided by collecting a stool during the SeHCAT test and determining the percentage of the radioactivity in solution. In patients with BAID we expected to find an abnormal SeHCAT retention, in the presence of increased proportion of ^{75}Se in stool water.

Although the four patients with BAID had significant percentages of ^{75}Se present in the supernatant (16%, 27%, 33% and 36%) two other patients (numbers 2 and 6, table 5.5, page 356) who did not have BAID also had high results (43% and 18%) (figure 5.5).

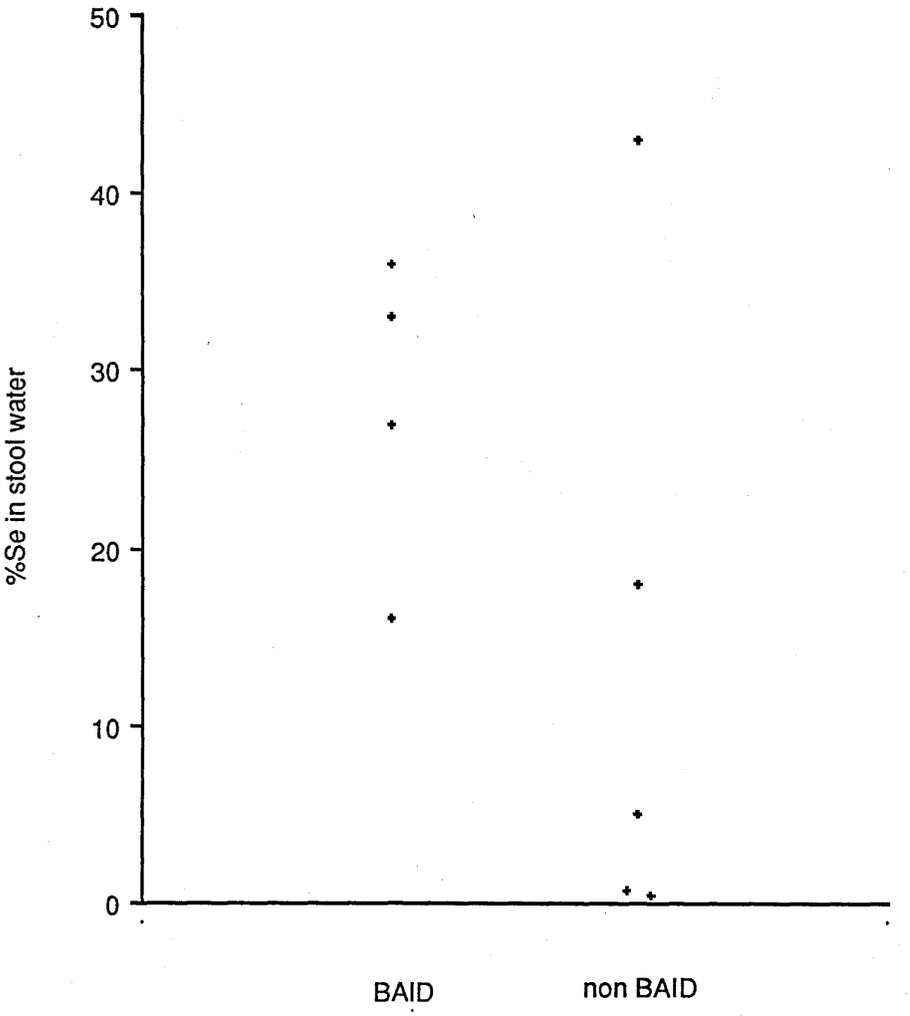


Figure 5.5 - Percentage 75Se in stool supernatant in BAID and non-BAID.

In the four patients with BAID the percentage of radioactivity in the water phase was surprisingly low (mean of 28%). In addition, this data provided little extra information regarding the diagnosis of BAID. Although non-BAID patients had a lower proportion of Se in the supernatant (mean = 13.4%) there was an overlap with BAID patients (see figure 5.5). Despite the small number of patients investigated it was obvious that this would not be a successful way of detecting BAID and so further tests were not performed.

A possible reason for this disappointing finding became apparent when the results of *in vivo* and *in vitro* SeHCAT stability experiments were analysed.

STABILITY OF SEHCAT

The rationale of measuring Se in the stool water would only be applicable if SeHCAT behaved as other endogenous bile acids. The SeHCAT molecule is structurally very similar to TCA the only difference being that the methylene group at C23 is replaced by ^{75}selenium.

In this study we assessed the *in vivo* and *in vitro* stability of the SeHCAT. The chemical form of the ^{75}Se in stool samples from patients on whom the SeHCAT test was performed was determined in 4 patients by TLC/auto-radiography. Figure 5.6 shows a typical autoradiograph: TCA has a mobility of 0 and CA has a mobility of 0.52. In each case the ^{75}Se was present predominantly as ^{75}Se-TCA and not ^{75}Se-CA as had been expected.

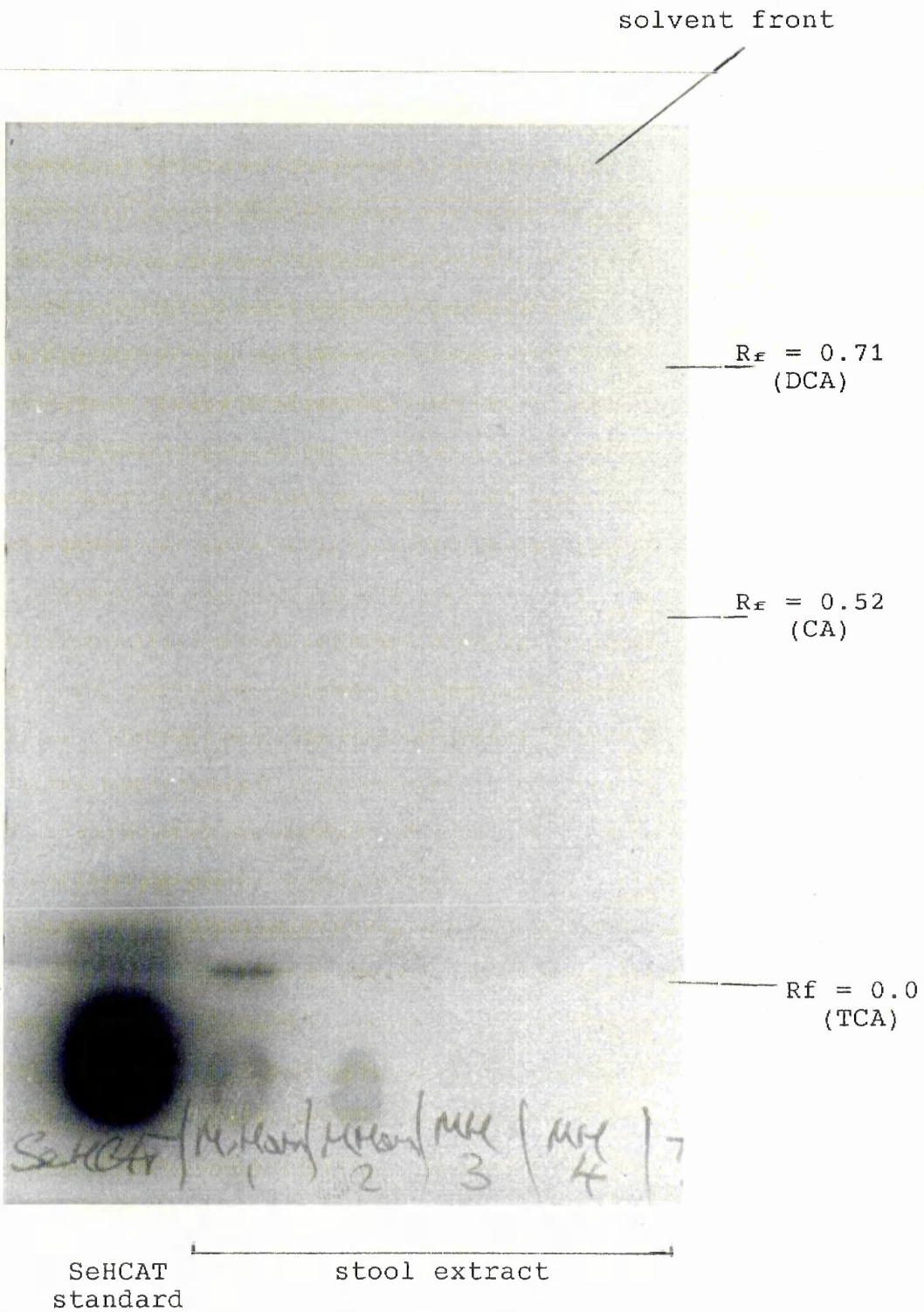


Figure 5.6 - Autoradiograph of ^{75}Se from stool after $^{75}SeHCAT$ test.

This finding was also supported by an *in vitro* experiment in which SeHCAT was exposed to faecal suspension and the resultant radioisotopic products detected by TLC/autoradiography.

The SeHCAT ran at a slightly slower mobility than the taurocholic acid standard and this was presumably due to the presence of the relatively large selenium atom within each bile acid molecule. If SeHCAT behaved similarly to its physiological counterpart, taurocholic acid, then deconjugation to cholic acid and dehydroxylation to deoxycholic acid would be expected.

The R_f values of CA and TCA after bile acid staining were found to be 0.88 and 0.28 respectively, while that of SeHCAT (by autoradiographic visualisation) was 0.08. The presence of the selenium molecule in the TCA molecule clearly retards its retention time. Since other ^{75}Se-labeled bile acids presumably are similarly affected, this made definitive identification of the isotope bands difficult. Unfortunately we did not have access to standard ^{75}Se-bile acids such as ^{75}Se-deoxycholic acid, ^{75}Se-cholic acid or ^{75}Se-taurodeoxycholic acid. However, isotope bands which probably corresponded to these substances, ie that moved slower than their unlabelled counterparts, were detected. The approximate percentages of each breakdown product was analysed and is shown in table 5.6.

In comparison when TCA was incubated with the same five

faecal samples there was almost complete deconjugation to cholic acid.

Bile acid	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Standard
SeHCAT	30%	25%	45%	30%	55%	100%
Se-CA	20%	15%	15%	25%	5%	0%
Se-DCA	15%	5%	10%	25%	0%	0%
Se-TCA	30%	45%	25%	20%	35%	0%

Table 5.6 - Percentage of ^{75}Se-labeled substances after faecal incubation of ^{75}SeHCAT.

Our *in vivo* and *in vitro* studies demonstrated that SeHCAT is not as readily deconjugated to cholic acid and consequently, unlike physiological bile acids, it is excreted in the faeces as a conjugate. This concurs with a previous report which showed that when exposed to cholylglycine hydrolase, there was 58% deconjugation of TCA in two hours but only 8% deconjugation of SeHCAT, with a further 5% being converted to an unknown product, probably selenium labeled tauro-deoxycholic acid. Anaerobic incubation with three bacteria normally resident in the colon, resulted in considerable deconjugation, 7α-dehydroxylation and dehydrogenation (312).

* * * * * *

These results demonstrate that ^{75}SeHCAT does not behave like its physiological counterpart TCA. The presence of a large additional atom, in ^{75}Se in the bile acid molecule affects the way that it is metabolised by colonic bacteria, perhaps as a result of steric hindrance in its association with enzymes. The result is that colonic

deconjugation of SeHCAT is limited.

This could explain the poor result of faecal water ^{75}Se concentration in discriminating between patients with and without BAID. For this concept to work $^{75}SeHCAT$ (or ^{75}Se-bile acid) would have to behave like physiological bile acids expecially with respect to solubility, the most important factor in controlling the proportion of $^{75}SeHCAT$ in solution. By remaining as a conjugate the solubility of $^{75}SeHCAT$ is considerably affected. For example CA has a pKa of around 6 while the pKa of TCA, and presumably $^{75}SeHCAT$, is much lower at 2.5.

OVERALL CONCLUSIONS AND FURTHER WORK

Gastroenterology is a discipline in which simple tests of function or integrity are at a premium. Some of the difficulties in evaluating gut function were illustrated in this study. The intestine is more difficult to investigate compared to other organs. For example, in contrast to the investigation of organs such as liver, kidney and heart there are no simple tests which reflect intestinal function or integrity in the same way as liver function tests, urea and cardiac enzymes. Absorptive capacity is a good example of a fundamental gut function test which is surprisingly difficult to measure.

Because of the problems in assessing intestinal function directly, many indirect function tests have been developed; eg. the triolein breath test for malabsorption, ^{14}C-xylose test for small bowel overgrowth, the pancreolauryl test of pancreatic function and hydrogen breath test for carbohydrate intolerance. The development of such indirect tests all tend to follow a similar evolution: an initial study demonstrates encouraging results and considerable promise for the test, while subsequent reports reveal practical difficulties and highlight problems of interpretation and limitations with regard to the predictive value. Ultimately, each test has its protagonists and so it usually survives in a small number of individual laboratories. The differing, and at times conflicting, conclusions of published assessments

also confuse the issue. These variations are often the result of differing protocols employed, choice of control subjects and/or different methodologies.

The triolein breath test has achieved more success than most, but this is probably due more to the severe limitations of alternatives. Following the present evaluation of this test, albeit incomplete, our conclusions are that it is the 'best of a bad lot'. The design of the dual isotope fat absorption test is theoretically more sound and in practice it is more reliable as a test. Nevertheless we also appreciate that in its present form it has a poor market potential. However, we would strongly advocate it as the definitive approach for assessing other fat absorption tests. Even in this role the test would prove invaluable; one of the greatest difficulties in evaluating a new approach is that there is usually no adequate 'reference method'.

For the same reason the potential use of the osmotic gap has not been fully evaluated. Despite the fact that only one (very ineffective) evaluation of the osmotic gap has been published (118), it is recommended in almost all major Gastroenterology textbooks as a useful test in descriminating between osmotic and secretory diarrheoa. Its reputation as a worthwhile test has even resulted in its inclusion as a topic in a current Mastership in Clinical Biochemistry examination. Our conclusion with regard to the value of the osmotic gap (and also potassium/sodium ratios) is that it has little place in

the investigation of diarrhoea. Its only possible (and at present unevaluated) use is to exclude secretory or osmotic diarrhoea. Because of the rarity of pure forms of these two types it would take a lengthy study to fully determine its predictive value in this respect.

* * * * * *

The time limitations of this study resulted in some aspects not being fully explored. For this reason, various aspects of the work presented here are being continued or extended.

1. Measurement of cAMP in stool water. During the course of this study aliquots of stool samples were collected in such a way as to enable subsequent measurement of cyclic AMP. Consequently, an opportunistic study is possible to determine its role in identifying those causes of diarrhoea in which cyclic AMP acts as an intracellular mediator. It may also be useful in determining whether cAMP is incriminated in the pathogenesis of some diarrhoeal illnesses.

Only one group have measured faecal cAMP concentrations and found them to be considerably elevated in patients with cholera (1248 ± 344pmol/l), rotavirus (353 ± 183pmol/l) and *E. coli* (heat labile toxin producing) diarrhoea (870 ± 250pmol/l) compared with lavage fluid from normal subjects (33 ± 4pmol/l) (314).

2. Treatment of laxative induced diarrhoea. Most studies

of this condition have concentrated on aspects other than its treatment. Since the Gastroenterology Laboratory provides a diagnostic service by performing laxative screens we are in a position to co-ordinate further prospective studies regarding its treatment.

Psychiatrists are aware of the reluctance of the patient being referred to them, because of the refusal to accept that a mental problem exists. Rather than the patient going to a psychiatrist the reverse is increasingly done as a way of avoiding the stigma of classing the patient as a'psychiatric case'.

In the present study little information was gleaned about possible therapeutic approaches since only three patients admitted taking laxatives, and only one subsequently received psychiatric treatment. As a way of circumventing the issue of psychiatric referral we are now liaising with a psychiatrist, who will attend the Gastroenterology clinic to review the patient in the guise of a physician.

3. Full evaluation of the triolein breath test. Although several conclusions regarding the triolein breath test were made, some were based on circumstantial evidence. We have altered the design of the test (reducing the fat load from 25 to 18g, and increasing the breath sampling time from 6 to 7 hours) to maximise the possibility of catching the peak of $^{14}CO_2$ excretion. An animal study may also be performed to determine the necessity of administering a fat load when performing fat absorption tests.

4. Evaluation of Treatment of BAID. The treatment of BAID remains a difficult medical challenge and the present approach of using cholestyramine is unsatisfactory because of side-effects. By binding bile acids, cholestyramine not only reduces the risk of BAID but also decreases their absorption and so leads to further bile acid malabsorption and sometimes to steatorrhoea. The drug also has to be taken in large doses to be effective in treating diarrhoea. These side-effects are the result of bile salts being sequestered in the small bowel. By administering cholestyramine in an enteric coated form to enable its delivery direct to the colon it may be possible to circumvent such problems. This approach had been successfully investigated in the past but in the absence of a diagnostic test of BAID the patient group was poorly defined (315). The measurement of FADBA as a means of diagnoising BAID has enabled us to investigate enteric coated cholestyramine more fully.

Since bile acids are also bound by other exogenous susbtances in the diet (251) it is possible that its treatment may be achieved by dietary manipulation. This possibility will also be studied.

Depending on the success of enteric coated cholestyramine in treating BAID further studies of a more speculative nature may be possible with respect to its potential in treating some patients with IBS. Excess colonic bile acids cause diarrhoea (245) while in their absence severe constipation results (316). Although the diarrhoea in

such patients is not related to bile acid abnormalities, an agent which can control the 'activity' of colonic bile acids may also control bowel movements.

5. Use of TLC to diagnose BAID. With the failure of the SeHCAT modification in diagnosing BAID, TLC has been considered as a simpler, alternative methodology to GC for the measurement of dihydroxy bile acids. Although TLC only provides a semi-quantitative result, it is likely that this will be adequate. BAID is normally associated with gross elevations of FADBA concentrations while patients with diarrhoea caused by other agents have very low concentrations.

With this in mind we are at present evaluating the possibility of semi-quantitation of bile acids by this means. Figure 6.1 shows a TLC plate of faecal water bile acids in various concentrations as well as a set of standards. The patients with BAID (numbers 4, 13, 14, 16, and 17) can be readily identified while those with non-BAID show absent or very faint bile acid bands. Only at concentrations of less than 1.0mmol/l is there likely to be any difficulty in deciding whether diarrhoea is caused by bile acids.

Other improvements can be made to TLC techniques to render them more quantitative; eg. scraping off the bile acids spots and quantitating the relevant dihydroxy bile acids enzymatically (295) or densitometrically (317). These will be investigated as part of future trials.

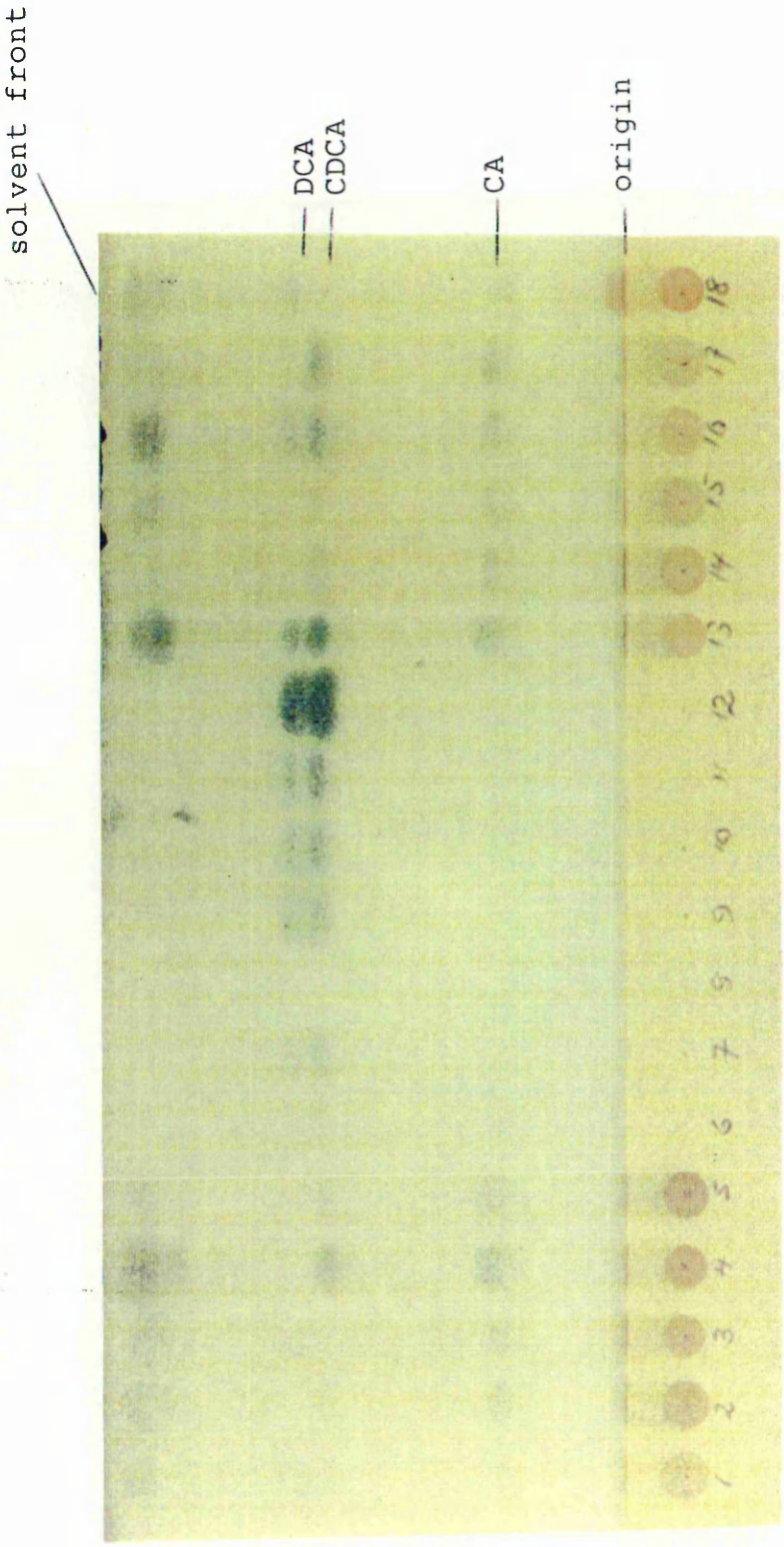


Figure 6.1 - Photograph of TLC plate used for semi-quantitative measurement of dihydroxy bile acids.

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The main objective of this project was to perform a thorough evaluation of several methods of relevance to the investigation of patients with diarrhoea. The methods studied have already been in routine use to a greater or lesser extent but from a scrutiny of the literature it was felt that none had been either effectively or comprehensively evaluated. For example, despite the concept of the faecal osmotic gap being formulated over ten years ago, the first full evaluation was performed in the present study; the potential value of the K/Na ratio was suggested in 1984 but until now had not been assessed; the role of bile acids in inducing diarrhoea was appreciated in the late 1960s and yet little work has been performed since to establish a diagnostic test.

Most gastrointestinal assays are performed relatively infrequently in the Manual Laboratory of Biochemistry Departments and quality control and standardisation tend to be poor compared with the more automated tests. Working in a laboratory dedicated to Gastroenterology investigations provided the opportunity to perform this project and also supported the notion that there is merit in centralising Gastrointestinal Laboratory facilities in order to provide a better diagnostic service, increase technical and interpretative expertise, and enable further development of diagnostic tests.

STATISTICAL APPENDIX

Note 1 - Mann-Whitney U-test.

Note 2 - Pearson product moment correlation.

Note 3 - The comparison of the variabilities of OG1 and OG2 in stools collected consecutively from the same patient, presented a statistical dilemma. At first thought, the variation in results were calculable simply by comparing the standard deviation or coefficient of variation. However, it was inappropriate to compare the standard deviations for each group of data since those from the OG1 group were higher simply because the OG1 results were higher than OG2 results. Similarly the coefficients of variation (sd/mean expressed as a percentage) could not be used because very low means sometimes arose because of negative OG results, so making the cv erroneously high. Comparison of inter-quartile ranges was also impossible since in each group of results there were usually only three or four data points. For these reasons the ranges of OG results were compared using the Mann-Whitney U-test.

Note 4 - Spearman rank order correlation.

Note 5 - Fischer's exact probability test.

Note 6 - Student's *t*-ratio two-sample test.

Note 7 - Wilcoxon matched-pairs signed-rank test.

Note 8 - The line of regression was calculated for each

individual and then the difference of these gradients from 0 were assessed using a one-sample Student's *t*-test with the Null hypothesis that the sample mean did not differ significantly from zero.

Note 9 - The intra-individual differences for each analyte were measured and these were then used to determine the statistical correlations by the Spearman rank moment correlation.

BIBLIOGRAPHY

1. Anon. (1973): Policy of the journal and instructions to authors. Biochem J; 131, 1.

2. LA Turnberg (1987): Pathophysiology of diarrhoea, page 41. In: JJ Misiewicz, RE Pounder, and CW Vanables, eds. Diseases of the Gut and Pancreas. Blackwell Scientific.

3. RJ Leigh and LA Turnberg (1982): Faecal incontinence: the unvoiced symptom. Lancet i; 1349.

4. LR Schiller, RB Hogan, SG Morawski, SG Santa, CA Bern, RP Norgaard, and JS Fordtran (1987): The incidence and significance of bile acid malabsorption in patients with chronic idiopathic diarrhea. Gastroenterol; 92, 151.

5. Goy (1976): Faecal characteristics contrasted in the irritable bowel syndrome and diverticular disease. Am J Clin Nutr; 29, 1480.

6. DM Tucker, HH Sandstead, GM Logan *et al* (1981): Dietary fiber and personality factors as determinants of stool output. Gastroenterol; 81, 879.

7. AM Connell, C Hilton, G Irvine, JE Lennard-Jones and JJ Misiewicz (1965): Variation of bowel habit in two populations. Brit Med J; ii, 1095.

8. J Park PhD Thesis (Cambridge).

9. AHG Love, TG Mitchell and RA Phillips (1968): Water and sodium absorption in the human intestine. J Physiol; 195, 133.

10. SF Phillips (1972): Diarrhoea: A current view of the pathophysiology. Gastroenterol; 63, 495.

11. JS Fordtan, FC Rector, TW Locklear and MF Ewton (1967): Water and solute movement in the small intestine of patients with sprue. Gastroenterol; 46, 287.

12. H Murer, H Lincke, R Kinne (1980): Isolated brush border vesicles as a tool to study disturbances in intestinal solute transport. In: M Field JF Fordtran and SG Schultz, eds. Secretory Diarrhoea. Am Physiol Soc, Bethesda, Maryland.

13. JS Fordtran, FC Rector, TW Locklear and MF Ewton (1967): Water and solute movement in the small intestine of patients with sprue. J Clin Invest; 46, 287.

14. SG Schultz and R Zalusky (1964): Ion transport in isolated rabbit ileum. 1. Short-curcuit current and sodium flux. J Gen Physiol; 47, 567.

15. HJ Binder and CL Rawlins (1973): Electrolyte transport across isolated large intestinal mucosa. Am J Physiol; 225, 1232.

16. JS Fordtran, FC Rector, MF Ewton (1965): Permeability characteristics of human small intestine. J Clin Invest; 44, 1935.

17. CO Billich and R Levitan (1969): Effects of sodium concentration and osmolality on water and electrolyte absorption from the intact human colon. J Clin Invest; 48, 1336.

18. SG Schultz 1984): A cellualr model for active sodium absorption by mammalian colon. Ann Rev Physiol; 46, 435-51.

19. KA Spring and AC Ericson (1982): Epithelial cell volume regulation. J Memb Biol; 69, 167.

20. DG Levitt, AA Hakim and N Lifson (1969): Evaluation of components of transport of sugars by dog jejunum *in vivo*. Am J Physiol; 217, 777.

21. K Loeschke, CJ Bentzel and TZ Csaky (1970): Asymmetry of osmotic flow in frog intestine: functional and structural correlation. Am J Physiol; 218, 1723.

22. JS Fordtran, FC Rector and NW Carter (1968): The mechanism of sodium absorption in the human small intestine. J Clin Invest; 47, 884.

23. NI McNeil (1983): Differences in electrolyte handling through the human large intestine. In: E Skadhauge and K Heintze eds. Intestinal Absorption and Secretion (Falk Symposium 36), MTP Press, Lancaster.

24. S Nundy, M Chir, D Malamud, H Obertop, J Sczerban and RA Malt (1972): Onset of cell proliferation in the shortened gut. Colonic hyperplasia after ileal resection in the dog. Gastroenterol; 72, 263.

25. E Urban, PE Starr and AM Michel (1983): Morphologic and functional adaptations of large bowel after small bowel resection in the rat. Dig Dis Sci; 28, 265.

26. GL Hill (1976): Normal ileostomy physiology. In: Ileostomy, Surgery, Physiology and Management. New York: Grune and Stratton.

27. K Wright, T Poskitt, JC Cleveland and T Herkovic (1969): The effect of total colectomy on morphology and absorptive capacity of ileum in the rat. J Surg Res; 9, 301.

28. G Flemstrom and A Garner (1982): Gastroduodenal bicarbonate transport: characteristics and proposed role in acidity regulation and mucosal protection. Am J Physiol; 242, G183.

29. U Hopfer and CM Liedtke (1987): Proton and bicarbonate transport mechanisms in the intestine. Ann Rev Physiol; 49, 51.

30. L Smeeton, B Hurst, A Allen and A Garner (1983): Gastric and duodenal bicarbonate transport *in vivo*: influence of prostaglandins. Am J Physiol; 245, G751.

31. JS Fordtan and TW Locklear (1966): Ionic constituents and osmolality of gastric juice and small intestinal fluids after eating. Am J Dig Dis; 11, 503.

32. J-R Melagdelada, VLW Go and WHJ Summerskill (1979): Different gastric, pancreatic and biliary responses to solid-liquid or homogenised meals. Dig Dis Sci; 24, 101.

33. Conference report (1985): Brit Med J; 291, 1247.

34. LA Turnberg, JS Fordtran, NW Carter, and FC Rector (1970): Mechanism of bicarbonate absorption and its relationship to sodium transport in the human jejunum. J Clin Invest; 49, 548.

35. LA Turnberg, FA Bieberdorf, SG Morawski and JS Fordtran (1970): Inter-relationships of chloride, bicarbonate, sodium, and hydrogen transport in the human ileum. J Clin Invest; 49, 557.

36. JM Evanson and SW Stanbury (1965): Congenital chloridorrhoea or so-called congenital alkalosis with diarrhoea. Gut; 6, 29.

37. K Dharmsathaphorn, KG Mandel, H Masui, and JA McRoberts (1985): Vasoactive intestinal polypeptide-induced secretion by a colonic epithelial cell line. J Clin Invest 75; 461.

38. HJ Kennedy, EAS Al-Dujaili, CRW Edwards, and SC Truelove (1983): Water and ileostomy balance in subjects with a permanent ileostomy. Gut; 24, 702.

39. SF Phillips and J Giller (1973): The contribution of the colon to electolyte and water conservation in man. J Clin Lab Med; 81, 733.

40. G Nell, W Forth, W Rummel and R Wanitschke (1976): Pathway of sodium moving from blood to the intestinal lumen under the influence of oxiphenisatin and deoxycholate. Arch Pharmacol; 293, 31.

41. CJ Edmonds and P Richards (1970): Measurement of rectal electrical potential difference as an instant screening test for hyperaldosteronism. Lancet; ii, 624.

42. ES Foster, JP Hayslett and HJ Binder (1984): Mechanism of active potassium absorption and secretion in the rat colon. Am J Physiol; 246, G611.

43. ES Foster, GI Sandle, JP Hayslett and HJ Binder (1986): Dietary potassium modulates active potassium absorption and secretion in rat colon. Am J Physiol; 251, G619.

44. NI McNeil, JH Cummings and WPT James (1978): Short chain fatty acid absorption by the human large intestine. Gut; 19, 819.

45. JH Bond, BE Currier, H Buchwald and MD Levitt (1980): Colonic conservation of malabsorbed carbohydrate. Gastroenterol; 78, 444.

46. H Ruppin, S Bar-Meir, KH Soergal, CM Wood, and MG Schmitt (1980): Absorption of short chain fatty acids by the colon. Gastroenterol; 78, 1500.

47. JC Debongnie and SF Phillips (1978): Capacity of the human colon to absorb fluid. Gastroenterol; 74, 698.

48. M Field, D From, Q AL-Awqati and WB Greenough III (1972): Effect of cholera enterotoxin on ion transport across isolated ileal mucosa. J Clin Invest; 51, 796.

49. JG Banwell, NF Pierce, RC Mitra et al (1970): Intestinal fluid and electrolyte transport in human cholera. J Clin Invest; 49, 183.

50. HJ Binder (1989): Absorption and secretion of water and electrolytes by small and large intestine. Page 1022. In: MH Sleisenger and JS Fordtran eds. Gastrointestinal Disease - Pathophysiology, Diagnosis, Management. WS Saunders Co.

51. JV Verner and AB Morrison (1958): Islet cell tumour and a syndrome of refractory watery diarrhoea and hypokalaemia. Am J Med; 25, 374.

52. RG Long, MG Bryant, SJ Mitchell, TE Adrian, JM Polak and SR Bloom (1981): Clinicopathological study of pancreatic and ganglioneuroblastoma tumours secreting vasoactive intestinal peptide (VIPomas). Br Med J; 282, 1767.

53. CJ Schwartz, DV Kimberg, HE Sheerin, M Field and SI Said (1974): Vasoactive intestinal peptide stimulation of adenylate cyclase and active electrolyte secretion in intestinal mucosa. J Clin Invest; 54, 536.

54. M Field (1981): In: J Johnson, ed, Physiology of the Gastrointestinal Tract. Raven Press, New York.

55. M Field, LH Graf Jnr, WJ Laird, and PL Smith (1978): Heat-stable enterotoxin of *Escherichia coli*: *in vitro* evvects oj guanylate cyclase stability, cyclic GMP concentration, and ion transport in small intestine. Proc Natl Acad Sci; 75, 2800.

56. JG Banwell, SL Gorbach, NF Pierce, R Mitra and A Mondal (1971): Acute undifferentiated human diarrhoea in the tropics II. Alteration in intestinal fluid and electrolyte absorption. J Clin Invest; 50, 890.

57. M Donowitz, N Asarkof and G Pike (1980): Serotonin-induced changes in rabbit ileal active electrolyte transport are calcium dependent and associated with increased ileal calcium uptake. J Clin Invest; 66, 341.

58. S Hughes, G Warhurst, NB Higgs, LG Guigliano, and BS Drasar (1983): *Clostridium difficile* cytotoxin induced intestinal secretion in rabbit ileum *in vitro*. Gut; 24, 94.

59. M Donowitz, J Wicks, G Pike, L Battisti and R DeLellis (1984): Effect of Senokot on rat intestinal electrolyte transport. Evidence of Ca^{++} dependance. Gastro; 87, 503.

60. DG Gall, D Chapman, M Kelly and JR Hamilton (1975): Sodium transport in jejunal crypt cells. Gastroenterol; 72, 452.

61. M Field (1974): Secretory Diarrhoea. Gastroenterol; 66, 1063.

62. GM Roggin, JG Banwell, JH Yardley and TR Hendrix (1972): Unimpaired response of rabbit jejunum to cholera toxin after selective damage to villus epithelium. Gastroenterol; 63, 981.

63 HA Serebro, FL Iber, JH Yardley and TR Hendrix (1969): Inhibition of cholera toxin action in the rabbit by cycloheximide. Gastroenterol; 56, 506.

64. M Field, PL Smith and JE Boulton (1980): Ion tranport across thge isolated intestinal mucosa of the winter flounder, *Pseudopleuronectes americans*: II Effects of cyclic AMP. J Membr Biol; 55, 157.

65. N Hirschhorn and HS Frazier (1973): The electrical profile of stripped, isolated rabbit ileum. John Hopkins Med J; 132, 271.

66. HC Elliott, CCJ Carpenter and RB Sack (1970): Small bowel morphology in experimental canine cholera. A light and electron microscopic study. Lab Invest; 22, 112.

67. MJ Welsh, PL Smith, M Fromm and RA Frizzell (1982): Crypts are the site of intestinal fluid and electrolyte secretion. Science; 218, 1219.

68 D-A Hallback, M Joelal, A Sjoquist and O Lundgren (1982): Evidence for cholera secretion emanating from the crypts. Gastroenterol; 83, 1051.

69. JS Fordtran , FC Rector, TW Locklear and MF Ewton (1967): Water and solute movement in the small intestine of patients with sprue. J Clin Invest; 46, 287.

70. B Kerzner, HJ McLung, M Kelly, D Butler, G Gall and JR Hamilton (1975): Intestinal secretion in acute viral enteritis: a function of crypt-like enterocytes. (abstr.) Gastroenterol; 68, 909.

71. AF Hofmann (1969): The syndrome of ileal disease and the broken enterohepatic circulation: cholerheic enteropathy. Gastroenterol; 52, 752.

72. AF Hofmann and JR Poley (1969): Cholestyramine treatment of diarrhoea associated with ileal resection. N Engl J Med; 281, 397.

73 WJ Forth, J Baldauf and W Rummel (1963): Ein Beitrag zur Klarung des Wirkungsmechanismus einiger Laxantien. Naunyn-Schmiedebergs Archs Pharmakol Exp Pathol; 246, 91.

74. HS Mekhjian, SF Phillips and AF Hofmann (1971): Colonic secretion of water and electrolytes induced by bile acids: perfusion studies in man. J Clin Invest; 50, 1569.

75. JW Dobbins and HJ Binder (1976): Effect of bile salts and fatty acids on the colonic absorption of oxalate. Gastroenterol; 70, 1096.

76. HJ Binder, C Filburn and BT Volpe (1975): Bile salt alteration of colonic electrolyte transport: role of cyclic adenosine monophosphate. Gastroenterol; 68, 503.

77. HJ Binder, JW Dobbins, LC Racusen and DS Whiting (1978): Effect of propranolol on ricinoleic acid- and deoxycholic acid-induced changes of intestinal electrolyte movement and mucosal permeability. Gastroenterol; 75, 668.

78. AF Hofmann, JR Poley, HS Mekhjian and SF Phillips (1970): Hydroxy fatty acid - an apparent cause of diarrhoea in patients with ileal resection and steatorrhoea. (abstr.) J Clin Invest; 49, 44A.

79. CS Soong, JB Thompson, JR Poley and DR Hess (1972): Hydroxy-fatty acids in human diarrhoea. Gastroenterol; 63, 748.

80. CM Bliss, DM Small and RM Robertson (1973): Water phase fatty acid excretion in diarrhoea. (Abstr.) Gastroenterol; 64, 701A.

81. HV Ammon and SF Phillips (1973): Inhibition of colonic water and electrolyte absorption by fatty acids in man. Gastroenterol; 65, 744.

82. HJ Binder (1974): Cyclic adenosine monophosphate controls bile salt and hydroxy-fatty acid induced colonic electrolyte secretion. J Clin Invest; 53, 7A.

83. TS Gaginella, VS Chadwick and JC Debongnie (1977): Perfusion of the rabbit colon with ricinoleic acid: dose related mucosal injury, fluid secretion and increased permeability. Gastroenterol; 73, 95.

84. HJ Binder (1973): Faecal fatty acids - mediators of diarrhoea? Gastroenterol; 65, 847.

85. NF Pierce, CCJ Carpenter, HL Elliott and WB Greeenough (1971): Effect of prostaglandins, theophylline, and cholera exotoxin upon transmucosal water and electrolyte movement in the canine jejunum. Gastroenterol; 60, 22.

86. LC Racusen and HJ Binder (1980): Effect of prostaglandins on ion transport across colonic mucosa. Dig Dis Sci; 25, 900.

87. P Sharon M Ligumsky D Rachmilowitz and V Zor (1978): Role of prostaglandins in ulcerative colitis. Enhanced production during active disease and inhibition by sulphasalazine. Gastroenterol; 75, 638.

88.4 K Steven, P Lange, K Bukhave and J Rask-Madsen (1981): Diarrhoea in villous adenoma of rectum: effect of treatment with indomethacin. Gastroenterol; 80, 1562.

89. K Launiala (1968): The effect of unabsorbed sucrose and mannitol on the small intestinal flow rate and mean transit time. Scand J Gastroenterol; 3, 665.

90. DR Saunders and HS Wiggins (1981): Conservation of mannitol, lactulose and raffinose by the human colon. Am J Physiol; 241, G397.

91. Z Shehadeh, RM Grantham, GA Brecher and ED Jacobson (1969): The effects of infusion rate and osmolality on volumogenic diarrea. Gastroenterol; 57, 24.

92. RL Dillard, H Eastman and JS Fordtran (1965): Volume-flow relationships during the transport of fluid through the human small intestine. Gastroenterol; 49, 58.

93. RM Grantham, GA Brecher and ED Jacobson (1970): Fluid dynamics in volumogenic diarrhea. Digestion; 3, 1.

94. CG Clark, D Karamanolis and MWN Ward (1984): Preference for proximal gastric vagotomy combined with cholecystectomy. Br J Surg; 71, 185.

95. JMS Dixon (1960): The fate of bacteria in the small intestine. J Pathol Bacteriol; 79, 131.

96. WJ Snape, GM Cartson and S Cohen (1976): Colonic myoelectric activity in the irritable bowel syndrome. Gastroenterol; 70, 326.

97. JW Dobbins and HJ Binder (1981): Pathophysiology of diarrhoea: alterations in fluid and electrolyte transport. Clin Gastroenterol; 10, 605.

98. LB Fernandez, E Gonzales, A Mazi, and MI Ledesma de Paolo (1971): Fecal acidorrhoea. N Engl J Med; 284, 295.

99. R Schilli, RI Breuer, F Klein *et al* (1982): Comparison of the composition of faecal fluid in Crohn's disease and ulcerative colitis. Gut; 23, 326.

100. R Goiffon, B Goiffon and G Fron (1961): Contribution a l'etude des electrolytes des selles. II. Mesure des cations Na, K, Ca, Mg, NH_4. Gastroenterologia (Basel); 69, 223.

101. O Wrong, A Metcalfe-Gibson, RBI Morrison, ST Ng, and AV Howard (1965): *In vivo* dialysis of faeces as a method of stool analysis. 1. Technique and results in normal subjects. Clin Sci; 28, 357.

102. O Wrong and A Metcalfe-Gibson (1965): The electrolyte composition of faeces. Proc Roy Soc Med; 58, 1007.

103. MJ Tarlow and H Thom (1974): A comparison of stool fluid and stool dialysate obtained *in vivo*. Gut; 15, 608.

104. P Vernia, RI Breuer, A Gnaedinger, G Latella and ML Santoro (1984): Composition of fecal water. Comparison of '*in vitro*' dialysis with ultrafiltration. Gastroenterol; 86, 1557.

105.TJ Bjork, KH Soergal and CM Wood (1976): The composition of 'free' stool water. Gastroenterol; 70, 864.

106. GMPJ Costongs, LP Bos, LGJB Engels and PCW Janson (1985): A new method for chemical analysis of faeces. Clin Chim Acta; 150, 197.

107. JS Fordtran (1967): Speculations on the pathogenesis of diarrhoea. Fed Proc; 26, 1405.

108. PF Down, L Agostini, J Murison and OM Wrong (1972): The interrelationships of faecal ammonia, pH and bicarbonate: evidence of colonic absorption of ammonia by non-ionic diffusion. Clin Sci; 43, 101.

109. Y-F Shiau, GM Feldman, MA Resnick and PM Coff (1985): Stool electrolyte and osmolality measurements in the evaluation of diarrhoeal disorders. Ann Int Med; 102, 773.

110. GJ Krejs, RS Handler and JS Fordtran (1980): Diagnostic and pathophysiological studies in patients with chronic diarrhoea. Pps 141-151. In: M Field, JS Fordtran and SG Schultz, eds. Secretory Diarrhoea. Am Physiol Soc, Bethesda, Maryland.

111. NW Read, GJ Krejs, MG Read, CA Santa Ana, SG Morawski and JS Fordtran (1980): Chronic diarrhoea of unknown origin. Gastroenterol; 78, 264.

112. GJ Krejs and JS Fordtran (1983): Diarrhoea. Pps 257-280.In: MH Sleisenger and JS Fordtran, eds. Gastrointestinal Disease. Philadelphia, London, Toronto, WB Saunders Co.

113. O Wrong and A Metcalfe-Gibson (1965): The electrolyte content of faeces. Proc Roy Soc Med; 58, 1007.

114. GJ Krejs, JH Walsh, SG Morawski, and JS Fordtran (1977): Intractable diarrhea. Intestinal perfusion studies and plasma VIP concentrations in patients with pancreatic cholera syndrome and surreptitious ingestion of laxatives and diuretics. Am J Dig Dis; 22, 280.

115. DA Johnson, and EL Cattau (1986): Stool chemistries in patients with unexplained diarrhea. AFP; 33, 131.

116. JR Mathias (1983): Diarrhoea: acute and chronic. Page 101. In: S Cohen, ed. Clinical Gastroenterology. New York, J Wiley and Sons.

117. VS Chadwick (1984): Clinical investigation of patients with malabsorption and diarrhoea. Page 428. In, IAD Bouchier, RN Allan, HJF Hodgson and MRB Keighley, eds. Textbook of Gastroenterology. Bailliere Tindall, England.

118. K Ladefoged, OB Schaffalitzky, de Muckadell, and S Jarnum (1987): Faecal osmolality and electrolyte concentrations in chronic diarrhoea: Do they provide diagnostic clues? Scand J Gastroenterol; 22, 813.

119. JE Mitchell, RI Breuer, L Zuckerman, J Berlin, R Schilli and K Dunn (1980): The colon influences ileal resection diarrhoea. Dig Dis Sci; 25, 33.

120. SL Gorbach (1983): Infectitious diarrhoea. Page 956. In: eds MH Sleisenger and JS Fordtran, eds. Gastrointestinal disease. Philadelphia, London Toronto, WB Saunders Co.

121. A Mondzac, GE Ehrlich and JE Seegmiller (1965): An enzymatic determination of ammonia in biological fluids. J Lab Clin Med; 66, 526.

122. DR Wilson TS Ing, A Metcalfe-Gibson and OM Wrong (1968): In vivo dialysis of feces as a method of stool analysis: III. The effect of intestinal antibiotics. Clin Sci; 34, 211.

123. Y-F Shiau (1987): Clinical and laboratory approaches to evaluate diarrheal disorders. CRC Critical Reviews in Clinical Laboratory Sciences; 25, 43.

124. MG Kane (1983): Production of secretory diarrhoea by IV infusion of VIP. New Engl J Med; 309, 1482.

125. JR Mathias and MH Clench (1985): Review: Pathophysiology of diarrhoea caused by bacterial overgrowth of the small intestine. Am J Med Sci; 289, 243.

126. AS Kliger, HJ Binder, C Bastl, and JP Hayslett (1981): Demonstration of active potassium transport in the mammalian colon. J Clin Invest; 67, 1189.

127. JP Hayslett, J Halevy, PE Pace, and HJ Binder (1982): Demonstration of net potassium absorption in mammalial colon. Am J Physiol; 242, G209.

128. GT Keusch and M Donowitz (1983): Pathophysiological mechanisms of diarrhoeal diseases: diverse aetiologies and common mechanisms. Scand J Gastroenterol; 18, (suppl 84), 33.

129. LJ Witts (1937): Ritual purgation in modern medicine. Lancet; i, 427.

130. FW Price (1921): A Textbook of the Practice of Medicine. 1st edition. Oxford Med.

131. WG Thompson (1980): Laxatives: Clinical pharmacology and rational use. Drugs; 19, 49.

132. H Binder and M Donowitz (1975): A new look at laxative action. Gastroenterol; 69, 1001.

133. Personal communication. Proprietary Assocation of Great Britain (1989):

134. AM Connell, C. Hilton, G Irvine, JE Lennard-Jones, JJ Misiewicz (1965): Variation of bowel habit in two populations. Brit Med J; ii, 1095.

135. P Neeser and UC Dubach (1983): Laxative abuse in patients of an outpatient clinic. Praxis; 72, 124.

136. WG Thompson and KW Heaton (1980): Functional bowel disorders in apparently healthy people. Gastroenterol 79, 283.

137. JD Killen, CB Taylor, MJ Telch, KE Saylor, DJ Maron and TN Robinson (1986): Self-induced vomiting and laxative and diuretic use among teenagers. Precursors on the binge-purge syndrome? J Am Med Assoc; 255, 1447.

138. HW Soper (1938): Phenolphthalein. Am J Dig Dis; 5, 297.

139. JH Wittoesch, RJ Jackman and JR McDonald (1958): Melanosis coli: general review and study of 887 cases. Dis Colon Rectum; 1, 172.

140. WB Schwartz and AS Relman (1953): Metabolic and renal studies in chronic potassium depletion resulting from overuse of laxatives. J Clin Invest; 32, 258.

141. BJ Houghton and MA Pears (1958): Chronic potassium depletion due to purgation with cascara. Brit Med J; i, 1328.

142. NF Coghill, PM Allen, and PF Edwards. (1959): Electrolyte losses associated with the taking of purges investigated with the aid of sodium and potassium radioisotopes. Brit Med J; 1, 14.

143. JA Litchfield (1959): Low potassium syndrome resulting from the use of purgative drugs. Gastroenterol 37, 483.

144. JS Staffurth and EN Allott (1962): Paralysis and tetany due to simultaneous hypokalaemia and hypocalcaemia with other metabolic changes. Am J Med; 33, 800.

145. MD Rawson (1966): Cathartic colon. Lancet; i, 1121.

146. R Upadhyay (1989). Personal communication.

147. WD Heizer, AL Warhshaw, TA Waldman and L Loster (1968): Protein-losing gastroenteropathy and malabsorption syndrome with factitious diarrhoea. Ann Int Med; 18, 839.

148. B Frame, HL Guiang, HM Frost and WA Reynolds (1971): Osteomalacia induced by laxative ingestion. Arch Int Med; 128, 794.

149. JH Cummings, GE Sladen, OF James, M Sarner and JJ Misiewicz (1974): Laxative-induced diarrhoea: a continuing clinical problem. Br Med J; i, 537.

150. PH Slugg and WD Carey (1984): Clinical features and follow-up of surreptitious laxative users. Cleve Clin Q; 51, 167.

151. GJ Krejs, JH Walsh, SG Morawski and JS Fordtran. (1977): Intractable diarrhoea. Intestinal perfusion studies and plasma VIP concentrations in patients with pancreatic cholera syndrome and surreptitious ingestion of laxatives and diuretics. Am J Dig Dis; 22, 280.

152. NF La Russo and DB McGill (1975): Surreptitious laxative abuse. Delayed recognition of a serious condition: a case report. Mayo Clin Proc; 50, 706.

153. HP Wolff, P Vecsie, H Kruck *et al.* (1968): Psychiatric disturbances leading to potassium depletion, sodium depletion, raised plasma renin concentration and secondary hyperaldosteronism. Lancet; i, 257.

154. RJ van Rooyen and F Ziandy (1972): Hypokalaemic alkalosis following the abuse of purgatives. S Afr Med J; 46, 998.

155. H Miller (1989). Personal communication.

156. D Fleischer and ME Ament (1977): Diarrhoea, red diapers and child abuse. Clin Pediat; 17, 820.

157. JM French, R Gaddie, and N Smith. (1956): Diarrhoea due to phenolphthalein. Lancet; i, 551.

158. CD Devore, MH Ulshen, and RE Cross (1982): Phenolphthalein laxatives and factitious diarrhoea. Clin Pediatr; 21, 573.

159. JH Cummings (1974): Laxative abuse. Gut; 15, 758.

160. S Mondo, LM Meier (1988): Legal implications of drug testing in the workplace. Am Pharm; 28, 30.

161. FA de Wolff, EJM de Haas, and M Verweij (1981): A screening method for establishing laxative abuse. Clin Chem; 27, 914.

162. J Morton (1987): The detection of laxative abuse. Ann Clin Biochem; 24, 107.

163. AI Morris and LA Turnberg (1979): Surreptitious laxative abuse. Gastroenterol; 77, 780.

164. P Bytzer, M Stokholm, I Andersen, NA Klitgaard and OBS de Muckadell (1989): Prevalence of surreptitious laxative abuse in patients with diarrhoea of uncertain origin: a cost benefit analysis of a screening procedure. Gut; 30, 1379.

165. K Ewe and U Karbach (1986): Factitious diarrhoea. Clin Gastroenterol; 15, 723.

166. JW Fairbairn (1949): The active constituents of the vegetable purgatives containing anthracene derivatives. J Pharm Pharmacol; 1, 683.

167. JD Hardcastle and JL Wilkins (1970): The action of sennosides and related compounds on human colon and rectum. Gut; 11, 1038.

168. D Rachmilewitz and F Karmali (1979): Effect of bisacodyl and dioctyl sodium succinate on rat intestine prostaglandin E2 content, Na-K-ATPase and adenyl cyclase. Gastroenterol; 76, 1221A.

169. K Ewe (1980): Effect of rhein on the transport of electrolytes, water, and carbohydrates in the human jejunum and colon. Pharmacol; 20 (Suppl. 1), 27.

170. L Lemmens and E Borja (1976): The influence of dihydroxy anthracene derivatives on water and electrolyte movement in rat colon. J Pharm Pharmacol; 28, 498.

171. RF Harvey and AE Read (1973): Saline purgatives act by releasing cholecystokinin. Lancet; ii, 185.

172. A Vyth and PE Kamp (1979): Detection of anthraquinone laxatives in the urine. Pharm Weekblad; 1, 84.

173. PA Perrone and JR Gant (1984): Advances in anion analysis: Reversed-phase ion-pair chromatography with indirect photometric detection. Perkin-Elmer Liquid Chromatography Applications, LCTB-1, Perkin elmer Corporation, Norwalk, CT.

174. RJ Bertolacini and JE Barney (1957): Colourimetric determination of sulphate with barium chloroanilate. Analyt Chem; 29, 281.

175. TT Terho and K Hartiala (1971): Method for the determination of the sulphate content of glycosaminoglycans. Anal Biochem; 41, 471.

176. M Dick (1969): Use of cuprous thiocyanate as a short-term continuous marker for faeces. Gut; 10, 408.

177. Scottish Development Department (1987): Population and vital statistics.

178. P Bytzer and NA Klitgaard (1986): Chromatographic demonstration of anthaquinone derivatives in the urine. A method of proving surreptitious laxative abuse. Ugeskr Laeger; 148, 1390.

179. HJ Seelig (1964): The requirement of magnesium by the normal adult. Summary and analysis of published data. Am J Clin Nutr 14, 342.

180. GJ Krejs and JS Fordtran (1983): Diarrhoea. Page 257. In: MH Sleisenger and JS Fordtran, eds. Gastrointestinal Disease: Pathophysiology, Diagnosis and Management 3rd edition. WB Saunders Philadelphia.

181. RF Harvey, SY Salih, and AE Read (1983): Organic and functional disroders in 2000 Gastroenterology outpatients. Lancet; i, 632.

182. FA de Wolff, PM Edelbroek, EJM de Haas and P Vermeij (1983): Experience with a screening method for laxative abuse. Human Toxicol; 2, 385.

183. B Frame, HL Guiang, HM Frost, and WA Reynolds (1971): Osteomalacia induced by laxative ingestion. Arch Int Med; 128, 794.

184. JS Stafforth and EN Allot (1962): Paralysis and tetany due to simultaneous hypockalaemia and hypocalcaemia with other metabolic changes. Am J Med; 33, 800.

185. Goldfinger (1969): Hypokalaemia, metabolic acidosis and hypocalcaemic tetany in a patient taking laxatives. J Mount Sinai Hosp; 36, 113.

186. MD Rawson (1966): Cathartic colon. Lancet; i, 1121.

187. DJ Gross, E ben Chetrit, P Stein, A Rosler, and M Eliakim (1980): Oedema associated with laxative abuse and excessive diuretic therapy. Isr J Med Sci; 16, 787.

188. I Ullrich and G Lizarralde (1978): Amenorrhoea and oedema. Am J Med; 64, 1080.

189. WD Heizer, AL Warshaw, TA Waldmann anbd L Laster (1968): Protein-losing gastroenteropathy and malabsorption syndrome with factitious diarrhoea. Ann Int Med; 68, 839.

190. BWD Bradley, CM Murphy, and IAD Bouchier (1969): Intraluminal bile salt deficiency in the pathogenesis of steatorrhoea. Lancet; ii, 400.

191. JH Annegers, JH Boutwell, and AC Ivy (1948): The effect of dietary fat on faecal fat excretion and subjective symptoms in man. Gastroenterol; 10, 486.

192. A Luthra, SK Mehta, RN Chakravarti and PN Chhuttani (1970): Fat studies on normal Indian adults: effect of dietary fat on excretion of fat in faeces. Ind J Med Res; 58, 714.

193. BE Walker, J Kelleher, T Davies, CL Smith, and MS Losowsky (1973): Influence of dietary fat on faecal fat. Gastroenterol; 64, 233.

194. SK Mehta and RN Chakravarti (1971): Influence of dietary intake of fat on faecal fat excretion in tropical sprue. Ind J Med Res; 59, 1435.

195. JH Van de Kamer, HTB Huinink and HA Weijers (1949): Rapid method for the determination of fat in faeces. J Biol Chem; 177, 347.

196. C Sobel (1964): In: RJ Henry, ed. Clinical Chemistry. Principles and techniques. Harper and Row, New York.

197. AD Schwabe, FJ Cozzetto, LR Bennett and SM Mellinkoff (1962): Estimation of fat absorption by monitoring of expired radioactive carbon dioxide after feeding a radioactive fat. Gastroenterol; 42, 285.

198. AF Abt and SL von Schuchling (1966): Fat utilisation test in disorders of fat metabolism. A new diagnostic method applied to patients suffering with malabsorption syndrome, chronic pancreatitis, and arteriosclerotic cardiovascular disease. Bull John Hopkin's Hosp; 119, 316.

199. SK Bhatia, TK Bell, AHG Love and DAD Montgomery (1969): An evaluation of a test using ^{14}C labelled triglyceride in the evaluation of steatorrhoea. Ir J Med Sci; 2, 545.

200. PJ Burrows, JS Fleming, ES Garnet DM Ackery, DE Colin-Jones and J Bamforth (1974): Clinical evaluation of the ^{14}C fat absorption test. Gut; 15, 147.

201. PR Mills, PW Horton and G Watkinson (1979): The value of the ^{14}C breath test in the assessment of fat absorption. Scand J Gastroenterol; 14, 913.

202. GC Strange, J Reid, D Holton, NP Jewell, and IW Percy-Robb (1980): The glyceryl ^{14}C-tripalmitate breath test: a reassessment. Clin Chim Acta; 103, 317.

203. AD Newcomer, AF Hofmann, EP DiMagno, PJ Thomas and GL Carlson (1979): Triolein breath test. A sensitive and specific test for fat malabsorption. Gastroenterol; 76, 6.

204. J Adlung and H Grazikowske (1979): Diagnosis of fat absorption with ^{14}C-tripalmitate and ^{3}H-palmitic acid. Scand J Gastroenterol; 14, 587.

205. PS West, GE Levin, GE Griffin and JD Maxwell (1981): Comparison of simple screening tests for fat malabsorption. Br J Med; 282, 1501.

206. K Einersson, I Bjorkhem, R Eklof and R Blomstrand (1983): ^{14}C-Triolein breath test as a rapid and convenient screening test for fat malabsorption. Scand J Gastroenterol; 18, 9.

207. B Akesson and C-H Floren (1984): Use of the triolein breath test for the demonstration of fat malabsorption in coeliac disease. Scand J Gastroenterol; 19, 307.

208. RN Butler, MJ Lawson, NJ Gehling and AK Grant (1984): Clinical evaluation of the ^{14}C-triolein breath test: a critical analysis. Aust New Zea J Med; 14, 113.

209. JM Turner, S Lawrence, IW Fellows, I Johnson, PG Hill, and GKT Holmes (1987): ^{14}C-Triolein absorption: a useful test in the diagnosis of malabsorption. Gut; 28, 694.

210. L Benino, LA Scuro, E Menini *et al* (1984): Is the ^{14}C-triolein test useful in the assessment of malabsorption in clinical practice? Dig; 29, 91.

211. NT Pederson (1982): Estimation of ^{14}C-triolein assimilation as a test of lipid assimilation. Breath test of measurement of serum radioactivity. Scand J Gastroenterol; 17, 309.

212. JH Bragdon (1958): ^{14}Carbon dioxide excretion after the intravenous administration of labelled chylomicrons in the rat. Arch Biochem; 75, 528.

213. J Brown (1960): Fat and carbohydrate in humans: a study of nutritional and hormonal effects. Calif Med; 93, 132.

214. HS Winchell, H Stehelin, N Kusubov, B Slanger, M Fish, M Pollycove, and JH Lawrence (1970): Kinetics of carbon dioxide-bicarbonate in normal healthy males. J Nucl Med; 11, 711.

215. CE King and PP Toskes (1981): Alteration of carbon dioxide production during nonfasting isotopic carbon dioxide breath tests: concise communication. J Nucl Med; 22, 955.

216. WF Caspary (1978): Breath tests. Clin Gastroenterol; 7, 351.

217. GH Tomkin, TK Bell, and DR Hadden (1971): Evaluation of malabsorption test using ^{14}C-triglyceride. Ir J Med Sci; 140, 449.

218. SR Absalom, SH Saverymuttu, JH Maxwell, and GE Levin (1988): Triolein breath test of fat absorption in patients with chronic liver disease. Dig Dis Sci; 33, 565.

219. MA Korsten, MB Klapholz, MA Leaf, and CS Lieber (1987): Use of the triolein breath test in alcoholics with liver damage. J Lab Clin Med; 109, 62.

220. RGH Morgan and AF Hofmann (1970): Use of ^{3}H-labelled triether, a nonabsorbable oil-phase marker, to estimate fat absorption in rats with cholestyramine-induced steatorrhea. J Lip Res; 11, 231.

221. VP Gerskowitch and RI Russell (1974): Tritiated glycerol triether as an oil-phase marker in man. J Lip Res; 15, 432.

222. LM Nelson, JF McKenzie, and RI Russell (1980): Measurement of fat absorption using [^{3}H]glycerol triether and [^{14}C]glycerol trioleate in man. Clin Chim Acta; 103, 325.

223. B Lembcke, A Losler, WF Caspary, P Schurnbrand, D Emrich, and W Creutzfeldt (1986): Clinical value of dual isotope fat absorption test system (FATS) using glycerol [^{125}I]-trioleate and glycerol [^{75}Se]-triether. Dig Dis Sci; 31, 822.

224. NT Pederson (1983): Estimation of lipid assimilation from faecal samples using ^{14}C-triolein as tracer and $^{51}CrCl_3$ as non-absorbable marker. Scand J Clin Lab Invest; 43, 323.

225. GD Drummey, JA Benson and CM Jones (1961): Microscopical examination of the stool for steatorrhoea. New Engl J Med; 264, 85.

226. JG Moore, E Englert, AH Bigler and RW Clark (1971): Simple faecal tests of absorption. A prospective study and critique. Am J Dig Dis; 16, 97.

227. KL Osman, WJ Zinna and GK Wharton (1957): Simplified test of fat absorption: comparison of serum turbidity, chylomicronaemia, and total lipid values after fat test meals. J Am Med Assoc; 164, 633.

228. SJ Bentley, RD Eastham and RF Lane (1975): Oral butter fat test meal with serum nephelometry in suspected fat malabsorption. J Clin Pathol; 28, 80.

229. H Andersson and R Gillberg (1977): Urinary oxalate on a high-oxalate diet as a clinical test of malabsorption. Lancet; ii, 677.

230. DS Rampton, GP Kasidas, GA Rose, and M Sarner (1979): Oxalate loading test: a screening test of steatorrhoea. Gut; 20, 1089.

231. WJ Baumann and HK Mangold (1966): Purification of triethers. J Org Chem 31, 498.

232. RL Kachroo, MM Rehani, N Kachroo, MS Khuroo, and SA Zarger (1987): Optimising the value of C-14 breath test. Indian J Med Res; 86, 119.

233. J Hoving, JHP Wilson, A Valkema and MG Woldring (1977): Estimation of fat absorption from single faecal specimens using ^{131}I-triolein and ^{75}Se-triether. A study in rats with and without induced steatorhea. Gastroenterol; 72, 406.

234. RGH Morgan and AF Hofmann (1970): Synthesis and metabolism of glycerol-^{3}H triether, a nonabsorbable oil-phase marker for lipid absorption studies. J Lip Res; 11, 223 - 30.

235. DR Saunders and TK O'Brien (1972): Disappointment with triethers as markers for measuring triglyceride absorption in man. Gut; 13, 867 - 70.

236. A Cortot, SF Phillips, and J-R Melagdelada (1979): Gastric emptying of lipids after ingestion of an homogenised meal. Gastroenterol; 76, 939.

237. B Lembcke, A Losler, WF Caspary, P Schurnbrand, D Emrich and W Creutzfeldt (1986): Clinical value of dual-isotope fat absorption test system (FATS) using glycerol [^{125}I]-trioleate and glycerol [^{75}]-triether. Dig Dis Sci; 31, 822 - 8.

238. FG Ebaugh, T Clemens, G Rodnan, and RE Peterson (1958): Quantitative measurement of gastrointestinal blood loss. 1. The use of radioactive ^{51}Cr in patients with gastrointestinal haemorrhage. Am J Med; 25, 169.

239. TA Waldmann (1961): Gastrointestinal protein loss demonstrated by ^{51}Cr-labelled albumin. Lancet; ii, 121.

240. WJ Lossow and IL Chaikoff (1955): Carbohydrate sparing of fatty acid oxidation. I The relation of fatty acid chain length to the degree of sparing. II The mechanism by which carbohydrate spares. Arch Biochem; 57, 23.

241. JH Bragdon (1958): $^{14}CO2$ excretion after the intravenous administration of labeled chylomicrons in the rat. Arch Biochem; 75, 528.

242. SR Absalom, SH Saverymuttu, JD Maxwell, and GE Levin (1988): Triolein breath test of fat absorption in patients with chronic liver disease. Dig Dis Sci; 33, 565.

243. R Chambers (1874): Digestion. Page 564. In: Chambers's Encyclopaedia, Vol 5 Revised edition. London W and R Chambers.

244. R Lewis (1972): Modification of bile acids by intestinal bacteria. Arch Intern Med; 130, 545.

245. AF Hofmann (1967A): The syndrome of ileal disease and the broken enterohepatic circulation; cholerheic enteropathy. Gastroenterol; 52, 752.

246. RH Palmer (1972): Bile acids, liver injury and liver disease. Arch Intern Med; 130, 606.

247. PG Samuel, M Saypol, E Meilman, EH Mosbach, and M Chafizadeh (1968): Absorption of bile acids from the large bowel in man. J Clin Invest; 47, 2070.

248. HS Mekhjian, SF Phillips, and AF Hofmann (1979): Colonic absorption of unconjugated bile acids. Perfusion studies in man. Dig Dis Sci; 24, 545.

249. A Norman (1964): Faecal excretion products of cholic acid in man. Br J Nutr; 18, 173.

250. BE Gustafsson and A Norman (1968): Physical state of bile acids in intestinal contents of germfree and conventional rats. Scand J Gastroenterol; 3, 625.

251. MA Eastwood and D Hamilton (1968): Studies on the absorption of bile salts to non-absorbed components of diet. Biochim Biophys Acta; 152, 165.

252. G Salvioli (1981): Cholesterol, bile acid and bile salt adsorption to bran *in vitro*. Pharmacol Res Comm; 13, 413.

253. H Fromm, S Farivar, and B McJunkin (1977): Type 3 bile acid malabsorption and diarrhoea. Evidence for a new clinical entity. Gastroenterol; 72, 1060A.

254. AF Hofmann and Poley (1969): Cholestyramine treatment of diarrhoea associated with ileal resection. New Engl J Med; 281, 397.

255. AF Hofmann and JR Poley (1972): Role of bile acid malabsorption in pathogenesis of diarrhoea and steatorrhoea in patients with ileal resection. I. Response to cholestyramine or replacement of dietery long chain triglyceride by medium chain triglyceride. Gastroenterol; 62, 918.

256. JH Cummings, WPT James, and HS Wiggins (1973): Role of the colon in ileal resection diarrhoea. Lancet; i, 344.

257. JE Mitchell, RI Breuer, L Zuckerman, J Berlin, R Schilli, and JK Dunn (1980): The colon influences ileal resection diarrhoea. Dig Dis Sci; 25, 33.

258. EH Thaysen and L Pedersen (1976): Idiopathic bile acid catharsis. Gut; 17, 965.

259. R Aldini, A Roda, F Bazzoli *et al* (1980): Primary bile acid diarrhoea. Ital J Gastroenterol; 12, 251.

260. EH Thaysen (1985): Idiopathic bile acid diarrhoea reconsidered. Scand J Gastroenterol; 20, 452.

261. L Popovic (1987): Primary bile acid malabsorption. Gastroenterol; 92, 1851.

262. JE Heubi, WF Balistreri, JD Fondacaro, JC Partin, and WK Schubert (1982): Primary bile acid malabsorption: defective *in vitro* ileal active bile acid transport. Gastroenterol; 83, 804.

263. GW Hepner and AF Hofmann (1974): Increased bacterial degradation of bile acids in cholecystectomised patients. Gastroenterol; 66, 556.

264. EW Pomare and KW Heaton (1973): The effect of cholecystectomy on bile salt metabolism. Gut; 14, 753.

265. DF Hutcheon, TM Bayliss, and TR Gadacz (1979): Post-cholecystectomy diarrhoea. J Am Med Assoc; 241, 823.

266. H Fromm, AK Tunuguntla, AK Malavolti, M Sherman and S Ceryak (1987): Absence of a significant role of bile acids in diarrhoea of the heterogeneous group of post-cholecystectomy patients. Dig Dis Sci; 32, 33.

267. AF Hofmann (1988): The enterohepatic circulation of bile acids in health and disease. In: MH Sleisenger and JS Fortran, eds. Gastrointestinal Disease. WB Saunders.

268. JG Allan, VP Gerskowitch and RI Russell (1974): The role of bile acids in the pathogenesis of post-vagotomy diarrhoea. Br J Surg; 61, 516.

269. VM Duncombe, TD Bolin, and AE Daves (1977): Double blind trial of cholestyramine in post-vagotomy diarrhoea. Gut; 18, 531.

270. JA Ayulo (1972): Cholestyramine in post-vagotomy syndrome. Am J Gastroenterol 57, 207.

271. TV Taylor, ME Lambert, and HB Torrance (1978): Value of bile acid binding agents in post-vagotomy diarrhoea. Lancet; i, 635.

272. G Blake, TL Kennedy, and STD McKelvey (1983): Bile acids and post-vagotomy diarrhoea. Br J Surg; 70, 177.

273. JG Allan and RI Russell (1977): Cholestyramine in treatment of post-vagotomy diarrhoea - a double-blind controlled trial. Br Med J; i, 674.

274. SD Ladas, PET Isaacs, Y Quereslin, and G Sladen (1983): Role of the small intestine in post-vagotomy diarrhoea. Gastroenterol; 85, 1088.

275. G Salvioli and R Salati (1979): Faecal bile acid loss and bile acid pool size during short term treatment with ursodeoxycholate and chenodeoxycholate in patients with radiolucent gallstones. Gut; 20, 698.

276. RG Danzinger, AF Hofmann, LJ Schoenfield, and JL Thistle (1972): Dissolution of cholesterol gallstones by chenodeoxycholic acid. New Engl J Med; 286, 1.

277. Meihoff and Kern (1968): Bile salt malabsorption in regional ileitis, ileal resection and mannitol induced diarrhoea. J Clin Invest; 47, 261.

278. G Sciarretta, G Fagioli, A Furno *et al* (1987): ^{75}SeHCAT test in the detection of bile acid malabsorption in functional diarrhoea and its correlation with small bowel transit. Gut; 28, 970.

279. HS Mekhjian and SF Phillips (1970): Perfusion of the canine colon with unconjugated bile acids. Gastroenterol; 59, 120.

280. VS Chadwick (1977): Structure activity relationships of bile acids. Eur J Clin Invest; 7, 241A.

281. KDR Setchell, JA Ives, GC Cashwin, and AM Lawson (1988): On the homogeneity of stools with respect to bile acid composition and normal day to day variations. Clin Chim Acta; 162, 257.

282. JJ Rafter, VW Eng, R Furrer, A Medline, and WR Bruce (1986): Effects of calcium and pH on the mucosal damage produced by deoxycholic acid in the rat colon. Gut; 27, 1320.

283. B McJunkin, H Fromm, RP Sarva, and P Amin (1981): Factors in the mechanism of diarrhoea in bile acid malabsorption: faecal pH - a key determinant. Gastroenterol; 80, 1454.

284. A Jonas and A Diver-Haber (1988): Bile acid sequestration by the solid phase of stools in cystic fibrosis patients. Role of pancreatic enzymes. Dig Dis Sci; 33, 724.

285. H Fromm, PJ Thomas, and AF Hofmann (1977): Sensitivity and specificity in test of distal ileal function: prospective comparison of bile acid and vitamin B_{12} absorption in ileal resection patients. Gastroenterol; 64, 1077.

286. MM Stanley and B Nemchausky (1967): Faecal ^{14}C-bile acid excretion in normal subjects and patients with steroid-wasting syndromes secondary to ileal dysfunction. J Lab Clin Med; 70, 627.

287. WE Meihoff and F Kern Jr (1968): Bile salt malabsorption in regional ileitis, ileal resection, and mannitol-induced diarrhea. J Clin Invest; 47, 261.

288. JF Woodbury and F Kern Jr (1971): Fecal excretion of bile acids: a new technique for studying bile acid kinetics in patients with ileal resection. J. Clin. Invest; 50, 2531.

289. M van Blankenstein, T Hoyset, P Horchner, M Frenkel, and JHP Wilson (1977): Faecal bile acid radioactivity, a sensitive and relatively simple test for ileal dysfunction. Neth J Med; 20, 248.

290. H Nyhlin, MV Merrick, MA Eastwood, and WG Brydon (1983): Evaluation of ileal function using 23-selena-25-homotaurocholate, a -labelled conjugated bile acid. Initial clinical assessment. Gastroenterol; 84, 63.

291. MV Merrick, MA Eastwood, and MJ Ford (1985): Is bile acid malabsorption underdiagnosed? An evaluation of accuracy of diagnosis by measurement of SeHCAT retention. B Med J; 290, 665.

292. GS Boyd, MV Merrick, R Monks and IL Thomas (1981): Se-75-labeled bile acid analogs, new radiopharmaceuticals for investigating the enterohepatic circulation. J Nucl Med; 22, 720.

293. MV Merrick, MA Eastwood, JR Anderson and H McL Ross (1982): Enterohepatic circulation in man of a gamma-emitting bile-acid conjugate, 23-selena-25-homotaurocholic acid (SeHCAT). J Nucl Med; 23, 126.

294. SM Grundy, EH Ahrens, and TA Miettinen (1965): Quantitative isolation and gas-liquid chromatographic analysis of total fecal bile acids. J Lip Res; 6, 397.

295. A Bruusgaard (1970): Quantitative determination of the major 3-hydroxy bile acids in biological material after thin layer chromatographic separation. Clin Chim Acta; 28, 495.

296. Anon (1981): National co-operative gallstone study. Chenodiol for dissolution of gallstone. Ann Int Med; 95, 257.

297. I Bouchier (1980): The medical treatment of gallstones. Ann Rev Med; 31, 59.

298. HYI Mok, GD Bell, and RH Dowling (1974): Effect of different doses of chenodeoxycholic acid on bile-lipid composition and on frequency of side-effects in patients with gallstones. Lancet; ii, 253.

299. K-J Ho (1976): Circadian distribution of bile acid in the enterohepatic circulatory system in hamsters. J Lip Res; 17, 600.

300. K-J Ho (1975): Circadian rhythm of biliary excretion and its control mechanisms in rats with chronic biliary drainage. Am J Physiol; 229, 1427.

301. KM Botham, ME Lawson, GJ Beckett, IA Nimmo, IW Percy-Robb, and GS Boyd (1981): Portal blood concentrations of conjugated cholic and chenodeoxycholic acids. Relationship to bile salt synthesis in liver cells. Biochim Biophys Acta; 665, 814.

302. R Ferraris, R Jazrawi, C Bridges, and TC Northfield (1986): Use of a -labeled bile acid [^{75}SeHCAT] as a test of ileal function. Gastroenterol; 90, 1129.

303. G Sciarretta, G Vicini, G Fagioli, A Verri, A Ginevra and P Malaguti (1986): Use of 23-selena-25-homocholyltaurine to detect bile acid malabsorption in patients with ileal dysfunction or diarrhoea. Gastroenterol; 91, 1.

304. C Schleurlen, W Kruis, U Bull, F Stellaard, P Lang, and G Paumgarter (1986): Comparison of ^{75}SeHCAT retention half-life and faecal content of individual bile acids in patients with chronic diarrhoeal disorders. Digestion; 35, 102.

305. M Orholm, JO Pederson, T Arnfred, P Rodbro, and EH Thaysen (1988): Evaluation of the applicability of the SeHCAT test in the investigation of patients with diarrhoea. Scand J Gastroenterol; 23, 113.

306. G Sciarretta, G Vicini, G Fagioli, A Verri, A Ginevra, and P Malaguti (1986): Use of 23-selena-25-homocholyltaurine to detect bile acid malabsorption in patients with ileal dysfunction or diarrhoea. Gastroenterol; 91, 1.

307. LR Schiller, RB Hogan, SG Morawski *et al* (1987): Studies of the prevalence and significance of radiolabeled bile acid malabsorption in a group of patients with idiopathic chronic diarrhea. Gastroenterol; 92, 151.

308. WG Thompson (1984): The irritable bowel. Gut; 25, 305.

309. LR Schiller, RB Hogan, SG Morawski, CA Santa Ana, MJ Bern, RP Norgaard, GW Bo-Linn, and JS Fordtran (1987): Studies of the prevalence and significance of radiolabeled bile acid malabsorption in a group of patients with idiopathic chronic diarrhea. Gastroenterol; 92, 151.

310. Meihoff and Kern (1968): Bile salt malabsorption in regional ileitis, ileal resection and mannitol induced diarrhoea. J Clin Invest; 47, 261.

311. G Sciarretta, G Fagioli, A Furno *et al* (1987): ^{75}SeHCAT test in the detection of bile acid malabsorption in functional diarrhoea and its correlation with small bowel transit. Gut; 28, 970.

312. R Monks and GS Boyd (1988): Biologic stability of tauro-23-[^{75}Se] selena-25-homocholic acid. J Nucl Med; 29, 1411.

313. Northfield and McColl (1973): Postprandial concentrations of free and conjugated bile acids down the length of the normal human small intestine. Gut; 14, 513.

314. A Molla, I Lonnroth, F Jahan, PK Bardhan, AM Molla and J Holmgren (1985): Stool cyclic AMP in diarrhoea due to different causative organisms. J Diarrhoea Dis Res 3, 199-204.

315. O Jacobsen, L Hojgaard, EH Moller, TO Wielandt, M Thale, S Jarnum, and E Krag (1985): Effect of enterocoated cholestyramine on bowel habit after ileal resection: a double blind crossover study. Br Med J; 290, 1315.

316. RH Dowling (1983): Bile acids in constipation and diarrhoea. In: Bile acids in Gastorenterology. MTP Press.

317. RR O'Moore and IW Percy-Robb (1973): Analysis of bile acids and their conjugates in jejunal juice by thin layer chromatography and direct densitometry. Clin Chim Acta 43, 39 - 47.