Mechanisms Of Post-Operative Sepsis And Renal Impairment In Obstructive Jaundice

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Abstract

After open operations to relieve obstructive jaundice, several complications are excessively common. These include renal impairment, infections and sepsis. Under some circumstances, bile components can injure the bowel wall.

The hypothesis underlying this thesis was that after such operations, bile returning to the intestinal lumen increases bowel-wall permeability, allowing potentially harmful bowel contents to enter the tissues. These contents may include agents known to be harmful, such as bacteria and endotoxin, as well as agents whose toxicity is unknown, such as urobilinogen. A second hypothesis was that urobilinogen is nephrotoxic.

The aims were firstly, to develop a method of reversible obstructive jaundice in the rat. Secondly, to use the model to measure alterations in bowel-wall permeability to labelled bacteria, endotoxin and ethylenediaminetetraacetic acid (EDTA) after the relief of obstructive jaundice. Thirdly, to assess the toxicity of urobilinogen to renal cells cultured in vitro. Finally, to measure urobilinogen in various body fluids (including serum and urine) in rats and in human patients undergoing operations to relieve obstructive jaundice.

Various difficulties were encountered with the model of reversible obstructive jaundice, which limited its usefulness in measuring bacterial translocation from the bowel to the tissues. However, modification of the model allowed it to be used to assess alterations in bowel-wall permeability after bile was returned to the intestinal lumen. No such alterations were seen with normal bile or bile from subjects with obstructive jaundice, even when the bile was infected.

Urobilinogen was found to be toxic to cultured renal cells in concentrations which may reasonably be expected in disease, although caveats apply to this conclusion.

A urobilinogen assay, previously described by other workers, was found to be unsuitable for assay of specimens from jaundiced subjects, because interference from an unknown substance or substances occurred.

In summary, bile returning to the intestinal lumen of rats with obstructive jaundice did not alter bowel-wall permeability to endotoxin or EDTA. Urobilinogen was toxic to in vitro cultured renal cells in concentrations that may be expected to occur in disease. A previously-described assay for urobilinogen was found not to be useful for assaying specimens from jaundiced subjects.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

1/9/1998

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1. INTRODUCTION AND LITERATURE REVIEW

1.1 Note on Definitions

Sepsis and renal failure have long been recognised as being excessively common after operations on patients with obstructive jaundice (OJ). However, the terms 'renal failure', 'renal impairment' and especially 'sepsis' have been used rather loosely in the past, although sepsis has recently been more precisely defined (as discussed in more detail on p. 45). This often means that when comparing papers that report these complications, only limited conclusions can be drawn. With these limitations in mind, the definitions used by previous workers have been accepted, but where necessary, comment is made on whether those definitions may have affected either the interpretation of the author's own results, or their comparison to other work.

The aim of this thesis is to investigate some of the possible mechanisms underlying these common complications of operations to relieve obstructive jaundice. It refers specifically to conventional 'open' operations, i.e. those involving laparotomy, because it is after these procedures that these problems have been most frequently reported by previous workers. However, other means of treating obstructive jaundice, such as endoscopic and percutaneous approaches, are also discussed where relevant.

Many earlier papers using the term 'jaundice' do not precisely define it (e.g. Robson 1903, Heuer 1934, Thompson et al 1940, Aird 1953, Williams et al 1960, Hadjis et al 1986). Presumably, this is at least partly because of the limited laboratory tests available to the authors of many of these papers.

To some extent it is a subjective term, for the simple reason that it describes the discolouration of the skin occurring in hyperbilirubinaemia, and the ease with which this discolouration can be seen varies between individuals. Jaundice is not usually visible until the serum bilirubin is about three times the normal level, or about 50 μmol/L (Gollan and Schmid 1979).

This problem of subjectivity is seldom encountered in more recent papers dealing with the subject, because most such papers use a biochemical definition based on serum bilirubin levels. However, it is replaced by a lesser problem, namely a lack of uniformity in this definition. Commonly, the (arbitrary) level of a total serum bilirubin of more than 100 micromoles per litre (μmol/L) is used (e.g. Armstrong et al 1984a, Thompson et al 1986 and 1989, Pain et al 1991, Parks et al 1994), but others have used different definitions, such as a total serum bilirubin of more than 50 μmol/L (Gillen and Peel 1986), more than 136 μmol/L (Semeraro et al 1989), or more than 200 μmol/L (Smith et al 1985). This lack of uniformity is clearly important when comparing results from different studies, because patients with higher pre-operative serum bilirubin levels may be at greater risk of post-operative complications (Hunt et al 1980, Greig et al 1988).

1.2. Overview of the clinical problem

Modern surgical texts and recent reviews describe a number of post-operative complications as being excessively common after operations to relieve OJ (Williamson 1994, Cobb and Chapman 1994, Fogarty et al 1995, Green and Better 1995, O'Connor 1985, Dixon et al 1984, Armstrong et al 1984b). These include acute renal failure (ARF), infections or 'sepsis', gastrointestinal bleeding and impaired wound healing. Some of these complications, and the excessive mortality associated with them, were recognised more than eighty years ago (see below, p.31).

Large patient series confirming these early observations were published more recently. Glenn and McSherry (1963) compared these operations against operations on the biliary tract in non-jaundiced patients, while Dawson (1965b) compared them against other operations of comparable size, such as oversewing of a perforated peptic ulcer.

Much of the pathophysiology underlying these complications is poorly understood. Unfortunately, contradictory findings between different research groups are common, and reliance on data from older papers with flawed methodology is not infrequent. This thesis attempts to discuss and clarify the pathophysiology of two groups of these post-operative complications. They are firstly infection and sepsis, and secondly renal impairment or failure.

Clues to possible causes of these post-operative complications are suggested by comparing them to problems occurring in other patient groups. In particular, these groups include patients with jaundice from non-obstructive causes, and patients with OJ prior to release of the obstruction. Surprisingly, few authors make such comparisons. ARF in patients with jaundice from non-obstructive causes is often discussed with little or no

reference to patients with OJ, and *vice versa.* Similarly, most authors discussing ARF in patients with OJ, group pre-operative and post-operative ARF together, seemingly attaching little significance to the important physiological differences between the two groups.

1.3. Complications of jaundice

1.3.1. Complications of non-obstructive jaundice

1.3.1.1. Sepsis and infection in non-obstructive jaundice

In a broad sense, an association between liver failure and remote infection has been recognized for at least 160 years. Gimson (1987) describes a report in 1836 of "pneumonia biliosa", meaning a pneumonia in association with hepatic failure.

However, this description was clearly not of a post-operative problem, because operations to relieve obstructive jaundice are not known to have been performed until more than fifty years later. Also, the patients in this report may have had jaundice from non-obstructive causes, such as hepatocellular disease. Lastly, it is unclear whether the patients developed jaundice or pneumonia first.

[NOTE: Gimson cites as a reference, a paper published in 1896 (Garvin, I. 1896. Remarks on pneumonia biliosa. South Med Surg 15: 36). The present author was unable to obtain a copy of this paper. It is not available in Australia, and neither the British Library nor any of its affiliated libraries holds a copy.]

Nevertheless, infections are now known to occur more commonly in patients with jaundice from non-obstructive causes (Pinzani and Zipser 1987). In a retrospective review of 149 sucessive cirrhotic patients admitted to an intensive care unit with upper gastrointestinal haemorrhage, Bleichner et al (1986) found that bacterial infection was present in 22% on admission. These infections included 'septicaemia', spontaneous bacterial peritonitis, urinary tract infections and pneumonia.

1.3.1.2. Renal impairment in non-obstructive jaundice

ARF is a well-recognised complication of jaundice from non-obstructive causes (such as haemolysis or hepatocellular failure). There is, unsurprisingly, a variety of known causes (such as nephrotoxic drugs and dehdration) which are not specific to jaundiced patients (Baker and Tomson 1994).

There is also a well-described but poorly understood syndrome of renal failure occurring in the setting of hepatic failure, known as the hepatorenal syndrome. This term has been used loosely in the past to describe a variety of conditions that were probably unrelated (see below, p.35). However, it has recently been more precisely defined as "unexplained renal failure occurring in patients with liver disease in the absence of clinical, laboratory, or anatomic evidence of other known causes of renal failure" (Epstein [1994]). Confusingly, this definition appears to include unexplained renal failure occurring in patients with obstructive jaundice, either pre- or post-operatively. Yet most recent authors writing on hepatorenal syndrome, do not discuss such patients (Epstein [1994], Green and Better [1994], Amend [1993], Badalamenti et al [1993], Ginès et al [1993], Pinzani and Zipser [1987], Wilkinson [1987]). The reason for this is unclear; possibly, these authors consider that ARF after relief of OJ is simply caused by acute tubular necrosis secondary to hypovolaemia. Amend, for example, states:

"Although an association between oliguric renal failure and liver disease was described ... following biliary tract surgeries, many of these ... cases appear to have had acute renal tubular necrosis".

Oddly, Amend seems to be referring to reports from the 1930s, when peri-operative fluid management was less well understood than today. Yet more recent reports clearly

describe the occurrence of post-operative ARF in apparently well-hydrated, normotensive patients with OJ (Aird 1953, Dawson 1968a, Armstrong et al 1984a).

Conversely, many recent reviewers discussing renal failure after operations to relieve obstructive jaundice allude to the hepatorenal syndrome of non-obstructive jaundice only very briefly (Fogarty et al 1995, Green and Better 1995, Wait and Kahng 1989).

Despite a large amount of research work, the aetiology of hepatorenal syndrome remains unclear, although a wide range of hormonal, neural and haemodynamic mechanisms have been suggested (Epstein 1994).

Histological findings are often useful in determining the cause of disease. However, this is not so in the hepatorenal syndrome, where the literature on the histological findings is inconsistent. For example, reviewers generally accept that the renal histology in patients dying of hepatorenal syndrome is normal or near-normal (Ginès et al 1993, Badalamenti et al 1993, Amend 1993, Pinzani and Zipser 1987), but this assumption does not seem well-founded. For example, there are few published papers on the subject, and most reviewers refer to papers from the 1950s and 1960s, particularly those of Hecker and Sherlock (1956), Papper et al (1959), Baldus et al (1964) and Shear et al (1965). Some of these papers include patients with other known causes of ARF such as gastrointestinal haemorrhage (Papper et al 1959, Hecker and Sherlock 1956), and they frequently report a variety of histological changes, although these changes are often inconsistent. For example, Baldus et al (1964) found 'areas of focal interstitial nephritis, hydropic change in the tubular epithelium, occasional hyalinized glomeruli, and slight tubular dilatation. In two jaundiced patients, the tubules contained bile pigment.'

Shear et al (1965) provide almost no details of their patients' renal histology, other than to write:

'Deterioration of tubular function sometimes occurred preterminally, and histologic evidence of tubular damage was found in most of the kidneys examined. These changes are likely the results of preterminal hypotension and sepsis, and cannot be incriminated in the etiology of the initial abnormalities in renal function'.

Papper et al (1959) are similarly brief, reporting simply that 'except for bile staining and occasional minimal tubular cell flattening, the kidneys examined histologically (18 patients) were essentially normal.'

Hecker and Sherlock (1956) reported 9 patients dying of liver failure, of whom only 7 underwent autopsy, and in 4 of whom there was evidence of gastrointestinal haemorrhage. Furthermore, the renal histology is reported in only one patient, in whom it was abnormal. In the other 6 patients who underwent autopsy, the kidneys are described simply as "normal" or "essentially normal", but it is not stated, (and it is unclear from the wording), whether renal histology, or simply macroscopic inspection was performed. The latter possibility is raised both by the frequent lack of a histological report of other organs, and by the report in one patient that "The kidneys were macroscopically normal."

Furthermore, papers published since those cited above, have detected renal tubular injury using more sophisticated tests, such as measurement of glomerular tubular reflux (Kanel and Peters 1984) or urinary lysozyme assay (Wilkinson et al 1978). The latter authors also found histological evidence of acute tubular necrosis in 17 of the 34 patients with acute renal failure complicating cirrhosis or fulminant hepatic failure. Although they avoided using the term 'hepatorenal syndrome', they concluded:

"...renal failure complicating hepatocellular disease may occur both without and with tubular necrosis."

Finally, the claim that renal histology is normal in patients with hepatorenal syndrome, does not sit well with the more recent conclusion by Eknoyan (1983) that 95% of patients with cirrhosis have lesions visible with electron microscopy. That author reported that significant glomerular abnormalities (such as increased mesangial matrix and thickening of the glomerular basement membrane) were found in over 95% of 116 renal biopsies from patients with cirrhosis (Eknoyan 1983). However, as he commented:

"the glomerular lesions are accompanied neither by clinical manifestations nor biochemical abnormalities indicating renal dysfunction." (Eknoyan 1983 ibid)

In summary, the histological findings are unhelpful in hepatorenal syndrome. The literature is confused as to whether histological abnormalities are present or not, and where abnormalities are described, they are non-specific.

The hepatorenal syndrome appears to share some similarities with ARF developing after operations to relieve OJ (as well as an elusive aetiology). This raises the possibility that a common cause may be responsible. For example, there is probably an element of renal vasoconstriction in both conditions (Baum et al 1969, Fletcher et al 1982, Bomzon and Kew 1983, Amend 1993, Badalamenti et al 1993), Platt et al 1994). Furthermore, since the renal histopathology is believed to be unremarkable, it is often proposed that vasoconstriction is the primary lesion in hepatorenal syndrome (Platt et al op. cit., Amend op. cit., Epstein 1994). However, as Goldstein and Schnellmann (1996) observed:

"The pathogenesis of ARF is complex... in fact, in most instances, ARF is associated with increased renal vascular resistance and/or tubular damage."

These authors also pointed out that renal vasoconstriction occurs secondarily to a variety of nephrotoxins such as cyclosporin, amphotericin B, cisplatin and radiocontrast agents. Goldstein and Schnellmann also observed that although the mechanisms by which these agents induce vasoconstriction are not known, there are several plausible explanations. For example, they may cause an imbalance in the release of vasoconstrictor and vasodilator substances, or cause direct vascular damage. Alternatively, they may act on the tubules, either by impairing tubular absorption (resulting in afferent arteriolar constriction via tubuloglomerular feedback), or by causing sufficient tubular damage to increase tubular permeability, allowing backleak of the filtrate out of the tubules and into the circulation (Goldstein and Schnellmann 1996).

Examination of the urine is often helpful in determining the cause for ARF. Unfortunately, as with histopathology, the literature is somewhat unclear on the subject. Although the urine is frequently low in sodium but of high osmolality (a finding that suggests intact tubular function), there is considerable variation from patient to patient. Unfortunately, there appears to be no recent literature describing the urinary findings in jaundiced patients with post-operative ARF.

1.3.2. Complications of obstructive jaundice

1.3.2.1. Complications of unrelieved obstructive jaundice

1.3.2.1.1. Renal impairment in unrelieved obstructive jaundice

Meade (1968) credits the French physician Jean Fernel (1497 - 1558) with the earliest known, adequate clinical account of common bile duct obstruction. He wrote: "the bowels are obstinate and sluggish, faeces whitish, the urine reddish and thick so that it frequently becomes dark, the bile diffused with blood throughout the whole body disfigures the skin with jaundice."

(NOTE: Meade dates the above quotation by Fernel to 1581, but this is almost certainly an error, because it contradicts the statement earlier in Meade's book, that Fernel lived from 1497 to 1558. These latter dates are correct according to other authorities [Walton et al 1994]).

Prior to the development of operations to relieve OJ, jaundice of any kind was treated by physicians. Differentiation between jaundice due to extrahepatic obstruction, and that due to other causes, was often not undertaken; indeed it was often not possible because of the limited technology available.

In 1852 George Budd, (later Sir George), Professor of Medicine at the King's College Hospital, London, wrote of jaundice:

"The colouring matter of the bile is eliminated by the kidneys, at least in part, as in the liver itself, through the agency of the secreting cells. If the urine be examined under the microscope, cells from the convoluted tubules of the kidney may often be seen deeply

coloured with bile." He further stated of obstructive jaundice: "In the jaundice that results from long-continued closure of the common duct... there is little more to be done than to regulate the diet... and to take care to do nothing likely to disorder the action of the kidney, through which the bile finds its way out of the system." (Budd 1852).

These statements suggest that Budd was aware of an association between OJ and renal impairment, although he does not describe such an association in further detail.

In 1911, von Beck and Simon of Mannheim, discussing Steinthal's case (see below, p.34), briefly reported that they had observed cases of death from uraemia in jaundiced patients upon whom no operation had been performed.

By 1933 Elsom in Philadelphia was able to write:

"Obstructive jaundice produces anatomic changes in the kidneys consisting chiefly of the degeneration of the tubular epithelium, long familiar to pathologists under the name cholemic nephrosis."

This author studied the renal function of sixteen patients with obstructive jaundice, and observed a number of abnormalities. The most consistent was a large number of both hyaline and granular casts in the urine (up to 44,000 times the normal number), plus a lesser excretion of white cells and epithelial cells (Elsom 1933). The significance of these findings is uncertain, because hyaline (but not granular) casts may sometimes be found in normal urine (Baker and Tomson 1994). Moreover, Wilkinson et al (1978) examined the urine of patients with acute renal failure secondary to cirrhosis and fulminant hepatic failure, and although they also frequently found casts, they bluntly stated:

"... the findings in the sediment were of no value at all."

Elsom also found that urea clearance was decreased in around half the patients, but the significance of this too is somewhat doubtful, because by his own admission the "standards for normal have not been fully worked out..." [for the urea clearance test]. Furthermore, all the patients had normal or near-normal serum biochemistry (although this is a very insensitive index of renal function because of the large functional reserve of the kidneys) [Baker and Tomson 1994]. As Elsom himself commented:

"In no one of the patients described here was the disturbance in renal function of serious clinical significance."

Elsom provided no information about the post-operative course of the patients; indeed surgery itself is only implied, and not specifically mentioned. However, perhaps the most significant of his observations is that "As the jaundice subsided the evidence of renal injury entirely disappeared." This statement hints at the complexity of the problem: although operation may appear to precipitate renal injury in some patients, in other patients, a pre-existing renal injury may seem to be improved by operation. This implies that more than one cause exists for renal injury in these patients; which is almost certainly the case, as discussed further below.

Elsom's findings were later confirmed by Thompson et al (1940) who studied the renal function of 32 patients with OJ. They too found casts in the urine, although not in such large numbers. They also ligated the bile ducts of 6 dogs and found similar although less prominent, changes in the urine. The dogs had a variety of histological abnormalities in their kidneys, such as dilatation of the tubules with desquamation of the epithelium. However, as discussed below (see p.41), there are several important physiological

differences between the jaundiced dog and man, which limit its use as an experimental model.

Furthermore, more recent authors regard "cholemic nephrosis" (as described above by Elsom, p.21) as of little or no significance. For example, Papper (1983) stated:

" 'Biliary or cholemic nephrosis' has no functional significance, because azotemia can exist without the lesion and the latter may be observed in the presence of normal renal function."

More recent papers also report renal impairment or ARF in non-operated patients with obstructive jaundice. However, the exact incidence is unclear, and it is frequently unclear whether the ARF is directly attributable to the OJ itself, or to some other co-existing cause. There are several reasons for this. Firstly, multiple possible causes for renal injury are sometimes present in the same patient with OJ. This makes it difficult to be sure whether renal impairment is due to the OJ itself, or to some other cause accompanying it.

For example, infection of the biliary tract even in the abscence of OJ, is associated with renal impairment and acute renal failure. This has been recognised since at least 1933, when Bartlett in St. Louis described 6 cases of "nephritis with suppression of urine" which complicated a variety of biliary tract infections, including cholangitis and cholecystitis in the abscence of jaundice.

Burden et al (1975) reported 16 patients admitted to hospital with either acute cholecystitis (14 patients) or cholangitis (2 patients), in whom bile duct obstruction was excluded by oral cholecystography or laparotomy, or both. Only 6 patients had normal renal function; 6 others had acute reversible renal failure, of whom 3 required dialysis.

Renal function returned to normal in all patients after resolution of the infection, implying that it had previously been normal.

As in OJ, the mechanism of renal failure in this setting is unclear. However, Burden et al found evidence of intravascular coagulation, and suggested this as the cause. They further suggested that the coagulation was initiated by bacteraemia. This may explain the otherwise contradictory finding by Gubern et al (1988), that no association existed between positive bile culture and pre-operative renal function; Gubern et al specifically excluded patients with cholangitis from their study.

Burden's suggestion that bacteraemia, rather than OJ per se, was the cause of ARF concurs with other articles by Dawson (1968b), Sørensen et al (1971), Andersen et al (1971), and Bismuth et al (1975). All four articles describe patients with ARF occurring in patients with cholangitis and OJ. However, many (although by no means all) of these patients were also hypotensive, which adds a further confounding factor, as hypotension is itself a well-recognised cause of ARF (Brady and Brenner 1994). In surviving patients, the renal impairment resolved after operation, in contrast to appearing after it or being worsened by it, and most of the authors even recommended surgery as an effective from of treatment (Sørensen et al op.cit., Andersen et al op.cit., Bismuth et al op.cit.). This suggests that in many of these patients, infection and hypotension were major factors in the development of ARF.

Burden's suggestion is also in keeping with the recent theory that renal impairment in the setting of OJ is due to endotoxaemia. Nevertheless, although systemic infection is a well-recognised cause of ARF, the mechanism is believed to be via decreased systemic vascular resistance causing renal hypoperfusion and pre-renal failure (Baker 1994). Yet, in Burden's series, dehydration or hypotension was present in only one patient, and even the

three patients who required dialysis "were remarkable for their healthy appearance at the height of their illnesses."

The issue is further confused by the fact that most of Burden's patients had elevated serum bilirubin levels (despite proven patency of the bile ducts), although only mildly so (median serum bilirubin 3.6 mg/mL [61.4 μmol/L]). However, this may have been due to non-specific reactive hepatitis, which is common even in simple cholecystitis: Geraghty and Goldin (1994) found that it was present on liver biopsy in 18 of 53 patients (34%) with cholecystitis alone. They further observed that non-specific reactive hepatitis was associated with an obstructive pattern of liver function tests in some patients.

Nevertheless, there seems to be a minor conflict between the relatively high median serum bilirubin in Burden's patients (61.4 μmol/L) and Geraghty and Goldin's finding that only 34% of patients with simple cholecystitis have non-specific reactive hepatitis, many of whom have normal liver function tests. Another possibility to account for this apparent conflict, is that a comparatively high proportion of Burden's patients had transient OJ prior to presentation. Alternatively, it may be explained by the fact that Geraghty and Goldin included patients with either acute or chronic cholecystitis, while Burden et al omitted patients with chronic cholecystitis.

Still, unless patients with cholangitis are specifically excluded from studies, it is difficult to be certain that renal impairment in unoperated patients with OJ, is directly attributable to OJ and not coexistent infection, hypotension, or both. Unfortunately, there are very few reports examining renal function of patients with OJ, that specifically exclude patients with cholangitis.

A second reason that it is difficult to establish how frequently renal impairment occurs in non-operated patients with OJ, is simply that there is wide variation in the reported incidence. For example, at least one study found that pre-operative creatinine clearance was *higher* in patients with obstructive jaundice than in non-jaundiced control patients ($p < 0.02$) [Dawson (1965a]. On the other hand, Gubern et al (1988) found that renal impairment (defined as a creatinine clearance <70 mL/min/1.73 m^2) was present in 65% of patients presenting with OJ; patients with cholangitis were specifically excluded from this trial. Since the purpose of Gubern's trial was to examine the effect of mannitol in jaundiced patients, no non-jaundiced patients were included for comparison. Nevertheless, the finding strongly suggests that renal impairment occurs commonly in unoperated patients with OJ, and may be caused by the OJ itself rather than a complication of it.

However, the issue is further confused by the fact that OJ is often partial or intermittent, or both (Benjamin 1988). This is because the occurrence of renal impairment in a non-operated patient with OJ, does not necessarily exclude the possibility that the act of releasing the obstruction causes renal impairment. That is, patients with intermittent obstruction must obviously undergo periodic spontaneous relief of the obstruction.

Other studies which have *not* specifically excluded patients with cholangitis, have found the pre-operative incidence of renal impairment in OJ to fall between the two extremes provided by Dawson and Gubern et al.

For example, Hunt (1980), defining renal impairment as a creatinine clearance < 80 mL/min/1.73 m^2, found the incidence to be 54%, while Parks et al (1994) found the incidence of renal impairment (by at least one biochemical index [serum urea or creatinine, creatinine clearance rate]) to be between 17 and 22%. Allison et al (1979) measured sodium and potassium excretion and glomerular filtration rate (GFR) [creatinine], and

found no significant difference between jaundiced patients and non-jaundiced control patients.

A retrospective review by Mairiang et al (1990) found that 64 of 130 patients (49%) with OJ due to cholangiocarcinoma, had renal *failure* (defined as per Miller et al [1978]) on admission to hospital. This article is unusual because it describes patients from an area of Thailand where the parasitic liver fluke *Opisthorcis viverrini* is endemic; infestation by this parasite is associated with cholangiocarcinoma. However, the article is important because it describes a large series of patients with relatively advanced obstructive jaundice; the onset of jaundice occurred a mean of 40.1 days (range 12 days - 5 months) prior to admission, and the patients had a high mean serum bilirubin of 478.6 μmol/L.

One possible conclusion to be drawn from from this study is that ARF eventually occurs in at least half of patients with untreated OJ. Unfortunately, a number of potential confounding factors were present. For example, caution must be exercised in comparing this series of patients with patients in western countries, where a variety of different causes of OJ exist, and *Opisthorcis* infestation is extremely rare. For example, it is possible that *Opisthorcis* infestation itself, or drugs used to treat it, may be associated with renal impairment even in the absence of OJ. Since infestation by this parasite was found in 61 of the 64 patients (95%) with renal failure, such an association could substantially influence the results. Unfortunately, a flaw in this paper is that Mairiang et al give little information about the 66 patients without ARF, and the frequency of opisthorciasis is not reported in this group.

Also, the mean body temperature on admission of patients in this series was 38 °C. It is therefore possible that renal failure was associated with underlying systemic infections (which were proven to exist by blood culture in 27 [42%]) rather than because of OJ itself.

Regrettably, the paper does not report the frequency of infection in the group of patients without ARF.

One possible reason for the wide variation in reported incidence of renal impairment in pre-operative patients is simply that patients with OJ are usually treated once the condition is recognised. Therefore papers reporting patients with untreated OJ, are almost exclusively describing pre-operative patients. If OJ alone does cause renal impairment, then it might be expected that the proportion of patients with renal impairment would rise with the duration of untreated OJ. The reported incidence would therefore vary according to the duration of untreated disease.

Numerous studies have been performed, using experimental animal models to examine the effect of OJ on renal function. Unfortunately, as Green and Better (1995) observed:

"Interpretation of the various studies related to changes in GFR or RBF [renal blood flow] in patients and animals with obstructive jaundice is fraught with major difficulties because of conflicting results. Aside from interspecies variability, discrepancies between the different studies may be because of the use of anesthetics that are known to affect both RBF and GFR."

This accords with Allison (1995), who wrote:

"Unfortunately there is great variability in the effects of chronic bile duct ligation in different species making direct comparison difficult."

For example, some studies have demonstrated that rats with common bile duct ligation (CBDL) retain salt and water, while others have not. This difference has later been attributed to variations in rat strain and dietary salt intake (Green and Better 1995). Also, rats may have a decreased blood volume soon after CBDL, but become hypervolaemic 8 to 10 days post-operatively (Better 1983).

Other studies have demonstrated a variety of histopathological changes in the kidney, visible with the electron microscope. These include degenerative change in the proximal convoluted tubule, and glomerular abnormalities such as appearance of granules in the gomerular basement membrane (Allison et al 1978).

However, because of the inter-species variability highlighted by Allison, and Green and Better (above) and also because of difficulties in correlating structural and functional abnormalities in the kidney, the significance of many of these findings is uncertain.

Most recent authors attach more significance to changes in extra-renal physiological variables, that may theoretically affect the kidney peri-operatively. For example, extracellular water volume, and to a lesser extent plasma volume, are reduced in human patients with OJ (Sitges-Serra et al 1992). It has been postulated that this predisposes these patients to pre-renal failure and acute tubular necrosis (ATN) (ibid.).

In summary, renal impairment and even ARF clearly occur in patients with obstructive jaundice *prior* to operations to relieve the obstruction. However, it is difficult to establish to what extent the renal defect is due to OJ itself, rather than to other complications which commonly co-exist, and which may themselves cause renal impairment or ARF. These include systemic infection (especially cholangitis) and hypotension.

1.3.2.1.2 Sepsis and infection in unrelieved obstructive jaundice

As described above, infection is often present in OJ, in the form of cholangitis. Of itself, this does not necessarily imply vulnerability to infection attributable to systemic effects of OJ. Rather, it is adequately explained by the simple fact that stasis in a hollow viscus, such as the bile duct, predisposes to infection. However, there is evidence that other bacterial infections, such as wound infections, occur more frequently in patients with obstructive jaundice (Greig et al 1988, Armstrong et al 1984a). Furthermore, there is considerable experimental evidence to suggest impaired immune function occurs in obstructive jaundice (Roughneen et al 1986, Vane et al 1988, Katz et al 1984 and 1991a, Andy et al 1992, Swain et al 1995, Haga et al 1996, Tjandra et al 1997), although some aspects of this impairment remain unclear (Whalan et al 1998).

1.3.2.2. Complications of operations to relieve obstructive jaundice

1.3.2.2.1. Historical overview

Operations to relieve OJ first appeared in the late nineteenth century. According to Meade (1968), early procedures included milking bile duct stones into the duodenum (Handfield Jones, 1878) or the gallbladder (Musser, Keen); various types of cholecystenterostomy (von Winiwarter, 1884; Gaston; Terrier; Kappeler; Courvoisier; Robson); and crushing the stones with forceps, without opening the bile duct (JK Thornton 1889).

It is unclear which surgeon performed the first successful choledochotomy. Meade (1968) states that they were probably performed in 1889, with several different surgeons

(Thornton, Abbe in New York, Heussner and Marcy in Boston) apparently originating the operation independently. However, Mayo Robson, a prominent contemporary of these surgeons, described choledochotomy in 1897, and emphatically declared:

"Courvoisier performed the first successful operation on January 22, 1890, and ... it has since been performed by Langenbech, Thornton ... and others."

(NOTE: Mayo Robson was at that time, the Hunterian Professor of Surgery and Pathology, Royal College of Surgeons of England, and Senior Surgeon to the General Infirmary at Leeds).

The excess post-operative mortality in jaundiced patients was recognised early in the history of these operations. In 1897 Robson reported a series of 170 patients upon whom he had operated for a variety of biliary tract diseases. Seventy-seven of those patients were jaundiced. Robson was able to boast:

"...I have never lost a single patient after any operation for gall-stones in the absence of malignant disease, deep jaundice, or infective cholangitis..." (Robson 1897)

Only 10 of his 77 jaundiced patients died post-operatively, giving an overall mortality rate of just under 13%. This compares favourably with modern surgery: papers published since 1960 were recently reviewed both by Fogarty et al (1995) and by Wait and Kahng (1989). Each review therefore included over 2,000 patients, and found mean overall mortality rates of 14% and 16% respectively.

Although such comparisons are interesting, it is clearly not possible to draw many meaningful conclusions from them, because important dissimilarities in patient characteristics are likely. These include the fact that Robson frequently operated on

patients with relatively simple problems (such as a single, small impacted gallstone), which did not need complex procedures to fix them. Such simpler cases appear less frequently in modern patient series of open operations, because they are often treated by endoscopic or percutaneous means.

Even 70 to 100 years after Robson's text was published, studies examining risk factors for post-operative death in jaundiced patients, continue to identify factors very similar to those stated by him. Such studies consistently report very high serum bilirubin (over 200 or 300 μmol/L) and malignancy as major risk factors (Dawson 1968a, Hunt 1980, Pitt et al 1981, Blamey et al 1983, Dixon et al 1983, Armstrong et al 1984a, Keighley et al 1984, Shirahatti et al 1997). Cholangitis is notably absent as a major risk factor; this presumably reflects the development of antibiotics since Robson's time.

Robson reported the cause of death in his patients only in brief note form. For example, "Ninth day, haemorrhage and exhaustion" or "Patient extremely exhausted at time of operation, which probably did not much hasten death." Like many medical reports from that era, these accounts can seem rather vague and subjective if judged by today's standards. It is therefore not possible to be sure of the exact cause of death in most of his cases.

1.3.2.2.2. Renal impairment after operations to relieve obstructive jaundice

The earliest report of post-operative acute renal failure (ARF) in patients with obstructive jaundice is generally credited to Clairmont and von Haberer in Vienna. In 1911, they described a series of 5 patients who underwent operations to relieve obstructive jaundice. Three developed anuria within 24 hours of operation, and died within 4 days.

One became oliguric immediately post-operatively (passing only 200 mL of very concentrated urine in the first 24 hours), but eventually made a full recovery. In the fifth patient "there was never any threat of anuria or uraemic symptoms"; she remained perfectly well 6 months post-operatively. The authors felt that the latter two patients survived because of early operation, and recommended that "we should operate on cases of gallbladder disease before severe liver damage occurs."

In the same paper, the authors reported that, inspired by their clinical observations, they had ligated the bile ducts of 23 dogs to create OJ, which they later released. They reported that "the clinical findings could be mimicked in the dogs". Unfortunately, they did not provide further details of these experiments.

[NOTE: Possibly because it is in German, Clairmont and von Haberer's paper is often wrongly quoted in the English-language literature. For example, several authors (Dawson 1968a, Allison et al 1979, Wait and Kahng 1989, Fogarty et al 1995, Green and Better 1995, Allison 1995) state that all five patients died, when in fact two made a full recovery. Furthermore, one author states, in two different papers, that the patients had merely cholecystectomies (Allison et al 1979, Allison 1995).

The literature citing this report contains several other errors which are less important, in the sense that they do not substantially change the conclusions to be drawn. For example, some of the above papers describe the patients as being "five women" (Allison et al 1979), or "five young women" (Allison 1995) which is wrong; there were four women and one man (who incidentally was described as a "Herr aus Persien" [man from Persia]). Whether the word 'young' is accurate is a matter of opinion, but the patients' ages ranged up to 61 years, with a median of 48. Also, the same two articles describe the authors as 'two German surgeons'. The accuracy of the word 'German' is contestable because, although the report does not state the original nationality of the authors, it does state that they were both working at the "1

Universitätsklinik in Wien". Wien (Vienna) is of course in Austria, not Germany. Finally, at least one recent paper erroneously gives the year of publication as 1910 (Fogarty et al 1995)].

In October of the same year, Steinthal of Stuttgart reported a case of a patient who died of anuria 4 days after an operation to relieve OJ. Steinthal commented that it was "exactly the same as those of Clairmont and von Haberer". He felt that the chloroform anaesthetic, interacting in some way with the liver injury caused by the OJ, was responsible.

Later authors, especially in the United States, wrote extensively on associations between renal failure and hepatic disease. However, not until 1933 did reports in the English language literature acknowledge the prior work published in German (Bartlett 1933). It is therefore unclear whether the authors of English-language reports published prior to that date were aware of the earlier work of their German-speaking colleagues; quite possibly their observations were made independently.

In 1922, Walters and Parham of the Mayo Clinic described cases of uraemia occurring in patients with OJ, noting that it could occur both pre- and post-operatively. However, the authors did not report the incidence of these events, nor speculate on the cause.

In 1924, Heyd in New York described "a series of gall-bladder cases that have exhibited unusual and peculiar post-operative complications." He classified these into three groups, of which groups II and III were both jaundiced. Group II experienced a "pronounced vasomotor collapse" about 48 hours post-operatively, while Group III experienced "coma, occurring [5 or 6 days after operation] in a patient with a diminishing obstructive jaundice." Heyd did not mention the renal function in great detail. Although

he wrote of Group II "there has been ample renal function", it is possible this alluded to a form of high-output renal failure, because he further stated "there is a change in the percentage of the rest nitrogen to urea nitrogen". Unfortunately, it is not made clear whether this "change" was an increase or decrease.

Wilensky and Colp in New York (1927) observed cases of "nephritis (renal epithelium degeneration)" in patients with OJ, and commented that in these patients, renal function could occur improve, deteriorate, or stay the same after operations to relieve OJ. In a later paper, Wilensky (1939) expressed the opinion that "the more protracted form of disease of the liver and kidney, the so-called hepatorenal sydrome" were due to "a poisonous body of some kind, which passes through the liver and kidney."

In 1932 Helwig and Schutz in Missouri described "a liver kidney syndrome". They described six patients with a variety of hepatobiliary disorders, who died of apparent uraemia (defined as a low urine output and high 'blood non-protein nitrogen'). However, the range of hepatobiliary disorders was extraordinarily wide (including a previously-healthy boy aged 16, with motor-vehicle accident trauma to the liver), and only two of the patients had obstructive jaundice; the other four were not jaundiced at all.

Boyce and McFetridge in New Orleans, published several papers on the subject over the period 1934-6. They classified what were, by that time, referred to as "liver deaths" into only two distinct groups. Group I consisted of patients who died "shortly after operation with hyperpyrexia." In patients in Group II, however "death is deferred for 10 to 14 days when uremic symptoms predominate, and post-mortem examination reveals ... degenerative changes in the convoluted tubules of the kidneys." (Boyce and McFetridge 1935). They believed that the renal injury was triggered by the release of biliary

obstruction, which allowed the escape of a substance that was toxic to the kidneys, although they did not elaborate on what that substance may have been.

In 1934, Heuer retrospectively reviewed deaths occurring after various biliary tract operations at the New York Hospital. He compared his findings to 21 published articles on the same subject, originating from hospitals in both the United States and Europe. Although he prudently warned that, owing to incomplete records (some reports included operations dating back to before 1900) his data were only approximate, his review therefore included 36,623 patients. He found an overall mortality rate of 6.6%, and concluded that the major causes of death were, in decreasing order of frequency:

(1) advanced gall-bladder disease with gangrenous perforation and/or intra-abdominal abscess;

(2) technical errors by the surgeon;

(3) pulmonary complications including both pneumonia and pulmonary embolism;

(4) "cardiorenal disturbance" which he did not precisely define, but which appears to include both renal failure and complications of ischaemic heart disease;

(5) "liver deaths" as previously defined by Heyd, although Heuer himself commented that Heyd's Type II (vasomotor collapse occurring about 48 hours post-operatively) was the only type he had personally observed frequently.

As later authors such as Garlock and Klein (1938), Dunphy (1950) and Dawson (1968a) were to point out, early descriptions of the 'hepatorenal syndrome' often included a wide variety of seemingly different pathophysiological events. Garlock and Klein (1938) of the Mt. Sinai Hospital, New York, observed:

"Many of the reported instances of "hepatorenal" syndrome are based on clinical grounds only and lack corroborative autopsy findings. Those reports that include postmortem examinations present a curious lack of uniformity of the pathologic picture."

Dunphy (1950) was no less critical. He wrote:

"the pathologic physiology of the so-called 'hepatorenal syndrome' following operations on the gallbladder and bile ducts varies widely ... and can be produced by sepsis, by accidental ligation of the hepatic artery, and by renal failure associated with pre-existing renal disease."

However, this insight did not lead to the abandonment of the view that renal impairment was excessively common following operations to relieve obstructive jaundice. Rather, the problem was recognised to be a clinical entity, distinct from the variety of miscellaneous problems with which it was formerly grouped. In 1953 Aird (Professor of Surgery at the University of London) wrote:

"The most perplexing case (sic) of death after extensive operations on the biliary passages, on the pancreas, and on the liver itself, is renal failure. Renal failure, of course, has to be feared even in extremely jaundiced patients who are not submitted to operation, but there seems no question that renal failure may be precipitated in a jaundiced patient by the operation itself."

In 1963, Glenn and McSherry reviewed the previous 11 years experience of the New York Hospital-Cornell Medical Centre, in operations for non-malignant biliary disease. This series of 2,358 patients is therefore quite heterogeneous but, perhaps surprisingly, it does not make specific mention of jaundice. The overall mortality in this

series was only 1.7%. Consequently, several later authors have referred to this article as proving the rarity of ARF in non-jaundiced patients undergoing biliary tract operations (Dawson 1968a, Pain et al 1985 Green and Better 1995). This is probably a valid conclusion to draw, because most of the patients it describes underwent operations where jaundice is uncommon (e.g. simple cholecystectomy for chronic cholecystitis). Nevertheless, the article does contain a subgroup of 388 patients who "underwent cholecystectomy plus choledochotomy for either chronic or acute cholecystitis and evidence of common duct obstruction", plus a futher 139 who underwent choledochotomy alone (having had a previous cholecystectomy). Unfortunately, Glenn and McSherry do not state specifically what that 'evidence of common duct obstruction' was.

There is also another subgroup of 73 "miscellaneous procedures", which includes drainage of the common bile duct; mortality in this group was 12.3%. It is therefore unclear how many, if any, of these patients were jaundiced, but it seems unwise to state, as Dawson, Pain and Green and Better (above) did, that none was jaundiced.

[NOTE: Green and Better's paper also claims that "In another series, the incidence of postoperative renal failure was 6.8% in 103 jaundiced patients, contrasted with an incidence of renal failure of only 0.1% in 2,353 emergency partial gastrectomies for perforated peptic ulcer."(Green and Better 1995) This is very inaccurate. The paper Green cites (Dawson (1968a) did indeed report a post-operative incidence of renal failure of 6.8% in 103 patients with obstructive jaundice. But Dawson in fact contrasted this with two papers by other authors. One was that by Glenn and McSherry 1963 (cited above), and the other was by Desmond and Seargeant (1957) who reported only 62 patients (not 160 as claimed by Dawson, and certainly not 2,353 as claimed by Green) undergoing emergency partial gastrectomies for perforated peptic ulcer. As Dawson correctly stated, none of Desmond's patients developed post-operative renal failure (not an incidence of 0.1% as claimed by Green).]

A low proportion of jaundiced patients may explain the low post-operative mortality of 2.8% in the first subgroup, and 2.2% in the second. The causes of death in these patients are not given separately from those of the main patient group.

However, only 39 deaths (1.7%) occurred in Glenn and McSherry's series, of which only 3 (0.13%) were due to "renal insufficiency". All 3 of these patients had pre-existing renal disease. Moreover, only 6 patients (0.25%) died of 'infection' (including liver abscesses). Importantly, Glenn and McSherry drew these figures only from the 22 patients who underwent post-mortem examination. Since 39 patients died post-operatively, the true rate of death from renal failure or infection, or both, may have been slightly higher. Furthermore, the paper's stated subject matter is restricted to post-operative death, so no mention is made of post-operative morbidity such as renal impairment or failure not resulting in death.

In 1964, Dawson of King's College Hospital in London published the first of several highly influential articles investigating post-operative renal failure in patients undergoing laparotomy for OJ (Dawson 1964, 1965a, 1965b, 1966, 1968a, 1968b; Baum, Stirling and Dawson (1969)]. In one of the earliest reports to examine the incidence of the problem, he performed "a retrospective analysis of the records of 103 consecutive patients with obstructive jaundice undergoing laparotomy from 1956 to 1963". He noted:

(1) an overall mortality of 28 (27.2%) of whom 7 (6.8%) died of ARF;

(2) the contrast between this figure and the complete absence of ARF in 160 patients undergoing emergency partial gastrectomy for perforated chronic peptic ulcer; (although as stated above, Dawson's figure of 160 is wrong: the true figure is 62);

(3) that deaths, and especially deaths from ARF, were proportionately more frequent in patients with deeper jaundice.

Then-recent previous work by Williams et al (1960) had shown that jaundiced dogs were more easily rendered hypotensive by haemorrhage than were normal dogs, while Barry et al (1960) and Mueller (1965) had shown that ischaemic renal injury could apparently be prevented by osmotic diuresis.

Dawson reasoned that peri-operative haemorrhage may have contributed to renal ischaemia and ARF in jaundiced patients. He therefore ligated the bile-ducts of rats which had previously undergone a right nephrectomy (Dawson 1964). After allowing OJ to develop for 7 days, he subjected the rats to 60 min. of left renal ischaemia. This resulted in a rise in serum urea and occurrence of renal lesions histopathologically more severe than those seen in similar animals not given renal ischaemia, or non-jaundiced animals given renal ischaemia. Dawson then treated another group of rats with mannitol (an osmotic diuretic), reporting that this reduced the post-operative rise in urea and the severity of the renal injury seen on histology.

In further experiments, Dawson acutely bled (20mL/kg body weight) 17 dogs prior to, and three to four weeks after bile duct ligation, and measured the immediate effect on blood pressure, estimated renal bood flow and glomerular filtration rate (GFR) (Dawson 1966). He found that a significant decrease in renal blood flow and GFR occurred after the haemorrhage, but these changes lasted only about 30 mins, and there was no significant difference between the dogs' responses before and after bile duct ligation.

However, the fact that peri-operative administration of mannitol abolished the transient decrease in GFR encouraged Dawson to a series of clinical investigations

(Dawson 1968a). He examined the peri-operative renal function of 15 patients with obstructive jaundice, using as a control group 12 patients undergoing either cholecystectomy or partial gastrectomy. He detected a post-operative fall in creatinine in all patients, except two in the control group. However, this fall was proportionately greater in the jaundiced patients ($p < 0.02$), and was not seen in a further seven jaundiced patients given mannitol peri-operatively.

Nevertheless, some aspects of Dawson's methodology warrant caution in interpreting the results. For example, mannitol was given to the rats as an aqueous solution, the volume of which was not controlled for. That is, each rat given mannitol received 15 mL of intra-peritoneal mannitol solution peri-operatively, plus a further 15 mL at 24 and 48 hours post-operatively. Assuming (from Dawson's data) a mean rat body weight of 390 g, this is equivalent by body weight, to administering to a 75 kg man, a total of 8.7 litres of fluid over slightly more than 48 hours. Yet rats not given mannitol received no extra fluid at all.

Furthermore, as Dawson himself pointed out, "the significance of the results of experiments using jaundiced dogs is difficult to assess because there is a considerable pathological difference between jaundiced man and the jaundiced dog." (Dawson 1966) For example, jaundiced dogs always become hypotensive, while human patients do not. Also, even after 3 to 4 weeks of biliary obstruction, dogs do not develop a deep jaundice; Dawson's dogs had a mean bilirubin level of only 4.4 mg/100mL (74.8 μmol/L), in comparison to their pre-operative mean of 0.5 mg/100mL (8.5 μmol/L).

Moreover, as Dawson noted, dogs with OJ develop near-total anorexia. Consequently, the dogs in his experiments lost a mean 15% body mass (Dawson ibid). This may have influenced the results, for example by altering the volumes in body-water

compartments. Also, the dogs' decreased oral intake may have included decreased water intake. If so, then the dogs would have been relatively dehydrated, introducing another confounding factor.

Furthermore, in Dawson's first clinical study (comparing renal function and non-jaundiced patients), the non-jaundiced group was significantly different to the jaundiced group in several important respects. They had worse pre-operative renal function (mean creatinine clearance of 64 mL/min c.f. jaundiced 93, $p < 0.02$); but were younger (mean age of 54 years c.f. 67 in the jaundiced group, $p < 0.05$); and less hypertensive (mean systolic blood pressure of 150 mmHg c.f. jaundiced 170). (Dawson 1968a)

Finally, Dawson's second clinical study (involving administration of mannitol) was performed on only seven jaundiced patients. Again, they were significantly younger than the control group (56 years c.f. 67 years, $p < 0.01$). The study was not a randomised controlled trial; it was a case-control study with the controls being simply the jaundiced patients of the earlier work. Also, it appears that peri-operative fluid management differed between the two groups; in the patients given mannitol "One bottle (540 mL) of mannitol (10%) was infused [pre-operatively, ... and] after the operation, one or two bottles of mannitol (5%) were infused in each 24-hour period to maintain the urine output at over 1 mL/min." Neither an equivalent volume of fluid, nor the intention to maintain urine output at a particular level is described for the patients not given mannitol. (Dawson 1968a)

These flaws in Dawson's experimental design may explain why, despite the subsequent widespread adoption of the practice of giving peri-operative mannitol in this clinical setting, the only randomised controlled trial to examine the efficacy of this practice, found it to be of no benefit (Gubern et al 1988). Disturbingly, this study also found that in

patients given mannitol, post-operative creatinine clearance was significantly worse than pre-operatively ($p < 0.05$), while it remained unchanged in the no-mannitol control group.

The present incidence of post-operative renal failure after laparotomy for OJ, is somewhat in dispute. Some reviewers state that the incidence of around 8 to 10% has changed little since the 1960s, (Fogarty et al 1995, Green and Better 1995, Wait and Kahng 1989), but there are anecdotal reports that the incidence is decreasing, and one recent prospective study describes a post-operative incidence of renal impairment of only 22%, with no patients suffering ARF (Parks et al 1994). However, this study includes only 23 patients. .

A retrospective review by the same author, of 120 patients undergoing biliary bypass, recently reported that only 5 (4%) developed post-operative renal impairment, of whom only 1 (<1%) had ARF (Parks et al 1997). However, the term 'renal impairment' is not defined in this paper, and it includes 60 patients with benign disease who had a median serum bilirubin level of only 27.5 μmol/L. Also, the patient with ARF died of it. Nevertheless, despite these limitations, the figures do appear considerably better than those from earlier reports.

Moreover, the reported mortality for ARF in this setting appears to be decreasing, from 100% in reports dating from the 1960s (Dawson 1965b, Williams et al 1960) to around 60-70% in more recent reports (Dixon 1983, Keighley et al 1984).

However it is difficult to draw many conclusions from such data, as patient factors vary between papers. These factors include the underlying cause of jaundice, (and its duration and related factors such as malnutrition), co-morbidity, age, previous surgery, and presence or absence of cholangitis.

Just as in the hepatorenal syndrome, the histological findings in the kidneys of OJ patients dying of post-op ARF do not help to clarify the cause. There is very little recent literature, and the literature that does exist does not always support its claims with sound experimental evidence. For example, Wait and Kahng (1989) state:

"Renal failure which develops in jaundiced patients after biliary tract surgery ... is characterised by acute tubular necrosis with all of the accompanying pathologic and physiologic changes."

However, they provide no references to support this claim. Similarly, Fogarty et al (1995) state:

"The majority of patients who develop acute renal failure in the presence of obstructive jaundice have widespread histological changes, with marked tubular necrosis and peritubular fibrin deposition."

These authors give two references in support of their claim. One is Wait and Kahng (1989), cited above. The other is Papper (1983), which describes patients with cirrhosis rather than obstructive jaundice, and furthermore states:

"In our experience, ATN [Acute Tubular Necrosis] is rare..."

In any case, the histopathological findings of the kidneys may be of only limited help to suggest causes of renal impairment in these patients. This is because the histopathological picture of the kidneys in ARF from other (unrelated) causes, often has a variety of non-specific findings (Brady and Brenner 1994). This is probably because acute

tubular necrosis is the 'final common pathway' for many different causes of ARF (Baker and Tomson 1994).

1.3.2.2.3. Sepsis and infection after operations to relieve obstructive jaundice

Papers reporting causes of mortality and morbidity after operations to relieve OJ often describe 'sepsis' or 'infection' as a common cause (Holman et al 1979, Hadjis et al 1986, Greig et al 1988. However, these terms have largely been used without clear definition, to describe a subjective clinical impression. This is not necessarily a criticism of the papers concerned; rather, it reflects the fact that the very complex nature of sepsis was not fully understood at the time those papers were written. Nevertheless, this lack of a uniform definition of sepsis, especially (but not only) in the literature published prior to 1992, causes difficulties in drawing comparisons between reports.

As a result of advances in immunology, especially in the 1980s, the complexity referred to above is now better (but by no means fully) understood. For example, sepsis was once defined simply as "overwhelming infection, with division of bacteria in the blood" (Basmajian et al 1982) whereas it is now believed that 'sepsis' is the systemic inflammatory response to an infection (American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference 1992, Giroir 1993). Also, the response can be triggered by a number of non-infective causes, such as trauma, and may continue once the trigger has been removed (ibid.). Furthermore, the inflammatory response is believed to be beneficial if it is localised to the site of infection or injury and of an appropriate degree, but harmful if it is not (ibid.). The complex cytokine pathways which appear to underlie sepsis are also being elucidated.

These advances have led to the term 'sepsis' being more precisely defined. In 1992, the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference referred to above, published the following definition of sepsis, which has gained widespread acceptance:

'The systemic response to infection. This systemic response is manifested by two or more of the following conditions as a result of infection : Temperature >38 °C or <36 °C; Heart rate >90 beats/min; Respiratory rate >20 breaths/min or $PaCO_2$ <32 torr; WBC [White blood cell count] >12,000 cells/mm^3, or >10% immature (band) forms.'(ibid.)

This new body of knowledge has also led to the introduction of the acronym SIRS (Systemic Inflammatory Response Syndrome), to describe the response (inappropriate in degree or duration) to infection, or to other insults such as trauma or pancreatitis, independent of its cause (ibid.). The natural history of patients with SIRS is that they may recover, or may progress to develop dysfunction or failure of multiple organs such as lungs, kidneys and liver. Organ dysfunction in this setting has also been more precisely defined, and with these definitions have come the acronyms MOF (multiple organ failure) and MODS (multiple organ dysfunction syndrome) (ibid.). Regardless of their initial cause, these clinical entities frequently represent the final common pathway to death for patients in intensive care units. In the interests of clarity, some authors have recommended that the word 'sepsis' be reserved to describe SIRS arising in response to infection (ibid.), while at least one has recommended that the word be abandoned altogether (Christou 1996).

Much of the literature describing infection or sepsis in OJ pre-dates these advances. This means that interpretation of such reports has become more complex. Some describe

complications which are clearly infectious (e.g. intra-abdominal abscess [Thompson et al 1987, Al Fallouji and Collins 1985, Greig et al 1988], wound infection [Al Fallouji and Collins op. cit., Greig et al op. cit., Armstrong et al 1984b], cholangitis [Al Fallouji and Collins op. cit.]), while others report 'septic' complications occurring in the abscence of detectable infection (Greig et al op. cit.). In still others, not enough information is provided to enable the present-day reader to know whether disease was due to infection, SIRS or both (Holman et al 1979, Keighley et al 1984, Smith et al 1985, Rothschild et al 1989).

Despite this confusion, sepsis and/or infection are clearly especially common in these patients, occurring in over 20% of patients in some series (Al Fallouji and Collins op.cit.). Furthermore, there is overlap between the two, as infection is obviously an important cause of SIRS.

1.3.2.3. Complications following non-operative treatment of obstructive jaundice

1.3.2.3.1. Introduction

Further clues to the possible causes of post-operative complications in jaundiced patients are suggested by examining the incidence of complications following procedures to relieve OJ, but not involving laparotomy. These procedures include percutaneous and endoscopic stent insertion. Because they do not involve laparotomy, they presumably also avoid many of the peri-operative fluid disturbances which inevitably accompany laparotomy. These include the effects of general anaesthetic on cardiovascular function, and the effects of fasting and paralytic ileus (Sear and Rosewarne 1994). Therefore, the

likely relative contribution to renal injury, of peri-operative fluid disturbances can be assessed.

1.3.2.3.2. Percutaneous drainage of obstructive jaundice

Furthermore, percutaneous techniques may, in theory, suggest the relative contribution to renal injury of the return of bile to the intestinal lumen, because they frequently use external biliary drainage. Unfortunately, assessment of that contribution is not as simple as it may first seem, because several confounding factors are present.

For example, studies of percutaneous stent insertion usually report infective complications such as cholangitis or 'septicaemia' (Speer et al 1987, Koven et al 1981, Rothschild et al 1989). However, these are complications both of surgery, and of the drainage procedures themselves, because the presence of an externally draining catheter allows direct introduction of infection via the skin (Koven et al ibid, Mcpherson et al 1982). In one study where external biliary drainage was used as definitive treatment of OJ, "practically every patient with drainage periods of more than four months had at least one attack of cholangitis" (Hansson et al 1979). This makes it difficult to establish the effect (if any), of pre-operative external biliary drainage on post-operative infectious complication rates.

Furthermore, because of the association between cholangitis and renal impairment (described above), comparisons of the rate of renal impairment are also clouded.

Another confounding factor is that percutaneous drainage procedures may provide combinations of internal and external drainage of bile; this confuses analysis of the effect of bile returning to the intestinal lumen. For example, Dooley et al (1984) inserted biliary

endoprostheses via the percutaneous transhepatic route in 62 patients, but drained the bile externally for the first 12 to 48 hours. Smith et al (1985) performed a similar procedure pre-operatively, but bile was drained externally for 24 to 72 hours prior to conversion to internal drainage.

Finally, mainly because of the hazards of the procedure itself (such as haemorrhage from hepatic vessels), pre-operative percutaneous drainage has largely fallen into disfavour (Clements et al 1993a). Therefore, there are few randomised controlled trials describing it, and those that exist contain relatively small numbers of patients.

Despite these caveats, the literature in existence does suggest that pre-operative external drainage may decrease the rate of both post-operative infection and renal impairment. In a randomised prospective trial, Gundry et al (1984) examined the incidence of post-operative complications in patients with OJ undergoing pre-operative percutaneous transhepatic cholangiography (PTHC), followed by 9 days of external biliary drainage prior to operation. A control group of patients underwent PTHC followed immediately by operation. The control group had significantly more infective complications and 'sepsis' than did the patients with 9 days of external drainage. They also had a higher incidence of renal failure (3/25 c.f. 1/25), but this was not significant.

This concurs with the findings of Hansson et al (1979) who reported a series of 105 patients with OJ, treated by percutaneous transhepatic biliary drainage either as definitive treatment, or pre-operatively for a median of 15 days, or immediately following surgery. All drainage was external only; no patient developed renal failure.

1.3.2.3.3. Endoscopic drainage of obstructive jaundice

However, few of the confounding factors described above, apply to patients undergoing endoscopic insertion of stents to relieve OJ. Most studies examining endoscopic drainage of OJ report a lower total incidence of short-term post-procedure complications than for open operations (Shepherd et al 1988, Kiil et al 1988, (Cheung and Lai 1995), although this is offset by an increased rate of long-term complications requiring hospital re-admission, and overall survival in the two groups is similar (Shepherd et al ibid).

Smith et al (1994) in a randomised prospective trial, compared endoscopic stenting with open surgical bypass in over 204 patients with malignant low bile duct obstruction. Patients undergoing open operation had an incidence of "severe acute renal failure" (although this was not defined) of 9%, compared to an incidence in stented patients of 4%. This difference was not significant ($p = 0.25$, Fisher's exact test). Furthermore, the use of mannitol was not specifically precluded from patients undergoing open operations; mannitol may actually impair renal function (Gubern et al 1988).

These findings lend support to the hypothesis that the act of returning bile to the intestinal lumen plays an important part in development of renal impairment, and that peri-operative fluid disturbances do not, by themselves, adequately explain this complication.

As mentioned above, Smith et al (1994) found a trend towards ARF being more common in patients undergoing open operations than after endoscopic relief of OJ (although the difference was not significant). From a statistical view-point, discussing non-significant trends is a somewhat dubious exercise. However, this trend raises another relevant topic for discussion. The theory that post-operative complications in OJ are due to

excessive absorption of intestinal contents, does not necessarily demand that complications occur with equal frequency following endoscopic an open procedures. This is because of the effects of paralytic ileus, which almost invariably follows laparotomy and manipulation of the bowel, but is most unusual after endoscopic procedures (Shellito 1994, Britton 1994). That is, in paralytic ileus there is often overgrowth of colonic bacteria in the small bowel (Simon and Gorbach 1986, King and Toskes 1979). Consequently, increased quantities of bacteria and bacterial products are theoretically available for absorption. Furthermore, in conditions of intestinal stasis, bile flowing into the intestine will tend to 'pool' in the proximal intestine. If bile increases bowel-wall permeability, then these conditions theoretically favour increased absorption of luminal contents.

1.4. Proposed mechanisms of post-operative complications after operations to relieve obstructive jaundice

1.4.1. Possible increased bowel-wall permeability after operations to relieve obstructive jaundice

1.4.1.1. Introduction

It is possible that after operations to relieve obstructive jaundice, both sepsis and ARF may have a common underlying cause. That cause may be a post-operative increase in bowel-wall permeability, allowing entry into the tissues of potentially harmful luminal contents. These may include bacteria and bacterial products, like endotoxin, which are known to be harmful. It may also include other substances whose toxicity is unknown, such as urobilinogen. (The possible role of urobilinogen is discussed in further detail below, p.58). Indeed, the theory that ARF in patients with OJ is due to endotoxin, (first proposed by Wardle and Wright in 1970), has widespread current suppport (Fogarty et al 1995, Green and Better 1995, Wait and Kahng 1989).

Similarly, there is experimental evidence to support the theory that impaired gut barrier function in untreated OJ allows bacterial translocation to take place, which in turn may perpetuate systemic sepsis (Clements et al 1996b). This theory is analagous to the recent popular theory that multi-system organ failure, which occurs in many patients (including those with OJ), in intensive care units is 'driven' by increased bowel-wall permeability (Border 1988, Marshall et al 1993).

There are several pieces of circumstantial evidence to support the theory that a post-operative increase in bowel-wall permeability may occur in patients with OJ, and allow

access to the tissues of luminal contents. These include overgrowth of gut flora, impaired ability of the liver to clear the portal blood of potentially harmful contents, and evidence from *in vitro* studies that bile and components of bile can injure intestinal epithelium under some circumstances. These are discussed below.

1.4.1.2. Alterations in gut flora in obstructive jaundice

Firstly, in OJ there may be both quantitative and qualitative alterations in the bowel flora within the bowel lumen, with relative overgrowth of some bacterial species. Deitch et al (1990a) compared caecal bacterial populations in bile-duct-ligated, sham-ligated and unoperated control mice. They found a 12-fold increase in the number of gram-negative bacilli and a 6-fold increase in lactobacilli.

These findings were corroborated by Kalambaheti et al (1994), who demonstrated bacterial overgrowth in the rat gut (especially distally) only 48 hours after exclusion of bile from the lumen. Similarly, Clements et al (1996b) demonstrated overgrowth of Gram negative aerobes in the rat caecum after one week of biliary exclusion.

Burke et al (1977) found an increase in Proteus sp. in the stomach, mid-jejunum and caecum of rats with choledochovesical fistulae. They also found an increase in the numbers of coliform bacilli in the stomach and mid-jejunum, and of lacto-bacilli in the stomach. There was no change in numbers of anaerobic organisms. Nonetheless, they also found that similar changes in bacterial flora occurred in pair-fed control rats (i.e sham-operated rats whose food intake was restricted to the same amount eaten by treated rats). They therefore concluded that the increase in bacterial numbers may have been due simply to restricted food intake. They further speculated that hunger may have led to more coprophagia by the rats (and therefore more bacteria in the upper gastrointestinal tract),

although the animals were caged on wire mesh to minimise this behaviour. Also, this explanation does not adequately account for the high levels of gut bacteria in rats with biliary diversion, because they had unlimited access to food (unless after undergoing this procedure, rats for some reason prefer eating faeces to rat food). Finally, because it used rats with choledochovesical fistulae, this study only tested the effect of excluding bile from the intestinal lumen, and not the effects of jaundice.

However, it is unclear whether bacterial overgrowth also occurs in the intestinal lumen of human patients with obstructive jaundice. Cahill (1983) aspirated intestinal contents from the duodenojejunal junction, mid small bowel and terminal ileum of jaundiced and non-jaundiced patients undergoing laparotomy. He found no significant difference between the two groups at any site. However, for ethical reasons it is obviously impossible to perform laparotomies on, and aspirate intestinal contents from, normal human subjects. Therefore, it is possible that the diseases (such as gastric cancer) afflicting Cahill's non-jaundiced patients may also have caused intestinal bacterial overgrowth.

Another possible explanation for the apparent difference between human subjects and rats and mice is that most of the secretory immunoglobulin A (sIgA) in the rat gut lumen comes from the bile (Jackson et al 1992). In contrast, most of the intraluminal sIgA in man is secreted by the intestinal epithelium (Deitch et al 1990a). Therefore, absence of bile in the intestine may theoretically allow bacterial overgrowth to a greater extent in rats than in human subjects.

There does not appear to be any published work describing the effect of OJ on the gut population of Clostridium ramosum (the organism primarily responsible for synthesis of urobilinogen; see below, p.58).

1.4.1.3. Increased bowel-wall permeability in obstructive jaundice

Secondly, increased bowel-wall permeability is known to occur pre-operatively in OJ, in both rats (Parks et al 1996, Reynolds et al 1996) and human patients (Welsh et al 1996, Parks et al 1996). This increase probably accounts for the bacterial translocation which is observed in OJ in rats, (Parks et al 1996, Reynolds et al 1996) and mice (Deitch et al 1990a).

Studies by Jackson and Dai (1994) and Matovelo et al (1989, 1990) support the suggestion that return of bile to the intestinal lumen may further increase bowel-wall permeability. Jackson and Dai (1994) gave lethal doses of endotoxin to rats and found haemorrhagic lesions in the intestinal mucosa after only 4 hours; these lesions were not seen when bile was excluded from the intestinal lumen. However, this work was published only in abstract form, so further details are unavailable.

Matovelo et al (1989) incubated loops of rat jejunum for 30 minutes, with isotonic solutions of unconjugated deoxycholic acid in varying concentrations. They then examined the loops with electron microscopy, either immediately, or after 15 or 150 minutes 'recovery period'. They found epithelial lesions including swelling and degeneration of epithelial cells, and ranging up to full-thickness necrosis of the underlying lamina propria. The severity and reversibility of these lesions were concentration-dependent. However, all but the most severe lesions had reverted to near-normality after 150 minutes of recovery. Furthermore, this injury was associated with an increase in permeability of the loops to (the permeability marker) polyethylene glycol. In a subsequent paper, they showed that this remarkably rapid recovery time was probably due to migration and flattening of nearby epithelial cells (Matovelo et al 1990).

Fasano et al (1990) reported that chenodeoxycholic acid increased the permeability of rabbit jejunal and ileal mucosa to lactulose. These studies concur with others reporting injury by bile salts to the colon (Freel et al 1983) and stomach (Karlqvist et al 1986).

Despite the above in vitro studies, there is almost no literature describing the short-term effect (minutes to hours) of the return of bile to the intestinal lumen of jaundiced subjects. However, one study recently reported an unexplained increase in bowel-wall permeability occurring in the first 24 hours following operation to relieve OJ (Parks et al 1996). These authors studied the bowel-wall permeability of 33 patients undergoing such operations, and compared the results to those obtained from 11 normal volunteers, and from 6 non-jaundiced patients undergoing either laparotomy or endoscopic retrograde cholangiopancreatography. They found a pre-operative increase in the bowel-wall permeability of patients with OJ, which returned to normal within 28 days of relief of OJ. This pre-operative change was not found in the non-jaundiced patients. Interestingly, they also found that an unexplained increase in bowel-wall permeability occurred on the first post-operative day, in both the jaundiced and non-jaundiced patients. Permeability returned to pre-operative levels by the seventh post-operative day.

1.4.1.4. Impaired ability of the liver to clear portal blood in obstructive jaundice

Thirdly, it is known that in OJ, the ability of the Kupffer cells of the liver to clear potentially harmful agents from the portal blood is impaired. These agents include both bacteria (Cardoso et al 1982, Katz et al 1984, Clements et al 1996a) and endotoxin (Clements et al 1996a).

There is almost no literature describing clearance of urobilinogen by the liver in obstructive jaundice, but the little information available suggests that its clearance is also impaired. Bernstein (1971) administered ^{3}H-labelled urobilinogen intravenously to normal human volunteers, and to six patients with a variety of hepatobiliary disorders, including one patient with obstructive jaundice. He found that while the control patients excreted a median of 10% of the injected dose in the urine, patients with liver disease excreted a median of 50% in the urine, and the patient with obstructive jaundice excreted 65% in the urine. Moreover, 80% of the urobilinogen had been cleared from the serum after 15 minutes in the control group, while in the patient with OJ only 75% had been cleared after 6 hours. This obviously implies that the ability of the liver to clear urobilinogen from the blood was greatly impaired in that patient.

1.4.1.5. Summary

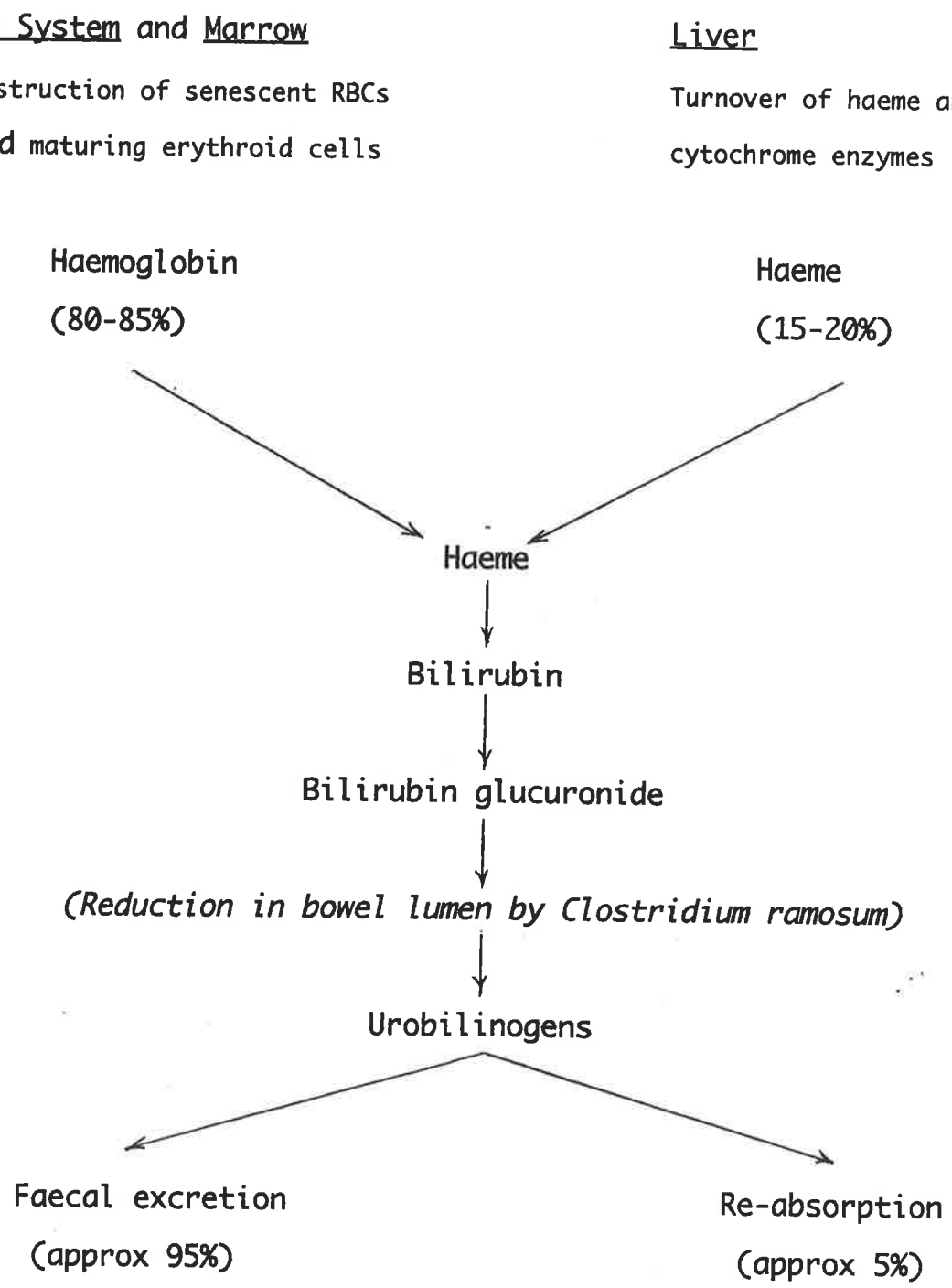
In summary, evidence already exists that in OJ, there is impairment of some of the barriers (relatively low numbers of gut bacteria, a relatively impermeable bowel-wall, and intact Kupffer cells) which normally limit (or prevent) potentially harmful bowel contents such as bacteria, endotoxin and urobilinogen reaching the systemic circulation. There is also evidence that in some settings, bile and bile contents can have an injurious effect on the bowel wall. However, very little is known about the short-term (hours to days) effects on bowel-wall permeability, of the relief of OJ. This thesis aims to investigate those effects.

Only about 1% of total daily production reaches the systemic circulation, to be excreted in the urine (Bourke et al op. cit., Billing 1986).

(NOTE: It is this fraction in the urine, where it was first detected, which led to it being given the name urobilinogen, which is now realised to be something of a misnomer).

In the circulation, about 80% is bound to plasma proteins, but this proportion probably falls when plasma urobilinogen levels are high (Bourke et al op cit). The remaining 20% probably circulates in the free state. If entry of bilirubin into the gut is prevented, as occurs in OJ, synthesis of urobilinogen is also prevented, unless there is infection of the bile ducts (Winkelman et al 1974).

METABOLIC PATHWAY OF UROBILINOGEN FORMATION



1.4.2.2. Evidence to support the 'urobilinogen theory'

1.4.2.2.1. Evidence to suggest that increased renal exposure to urobilinogen occurs after operations to relieve obstructive jaundice

It is hypothesised that in patients undergoing operations to relieve OJ, some or all of the following known or suspected pathophysiological disturbances may act together, to increase renal exposure to urobilinogen post-operatively:

(a) The net amount of bilirubin excreted post-operatively is thought to be high (Benjamin 1988). Although the concentration of bilirubin in the bile excreted immediately after operation may be low, the volume of bile produced is sufficiently large to outbalance its low concentration (ibid.). It is hypothesised that increased bilirubin excretion into the intestine may result in increased post-operative urobilinogen synthesis.

(b) Bowel-wall permeability to a variety of molecules such as polyethylene glycol (Parks et al 1996) and lactulose (Welsh et al 1996), is increased up to 30-fold in patients with OJ. Whether or not urobilinogen is also affected in this way is not known. Nevertheless, it may reasonably be expected to be, because the increase in permeability appears to be non-specific, and the molecules named above have similar or larger molecular weight (m.w.) than urobilinogen. (NOTE: Urobilinogen has a m.w. of 592 (Moscowitz et al 1970), while that of lactulose is 342.3. The m.w. of polyethylene glycol [a synthetic polymer] varies between 150 and around 20,000 (Swinyard and Pathak 1980); that used by Parks et al (1996) had a m.w. of 4000).

(c) In OJ, the liver's ability to 'filter' the portal blood is often impaired, thus allowing 'spill-over' into the systemic circulation of a variety of gut-derived substances that in health are largely or completely removed. As discussed above, the small amount of evidence available suggests that urobilinogen is included in this list of gut-derived substances (Bernstein 1971; see p. 57).

(d) The glomerulus selectively filters molecules on the basis of their size and charge. Molecules such as inulin (with a molecular weight [m.w.] of 5,200) are freely filtered, while macromolecules such as albumin (m.w. 69,000) are restricted (Guyton 1991). Therefore, urobilinogen free in the circulation is freely filtered at the glomerulus (Bourke et al 1965), and so theoretically is potentially able to injure the renal tubules distally.

(e) As mentioned above, there is overgrowth of the intestinal flora in patients with intestinal stasis following laparotomy, and possibly also in patients with OJ. There does not seem to be any published work specifically describing the behaviour of C. ramosum in these circumstances. However, such overgrowth may include that organism. Furthermore, it is possible that in some patients, conditions may tend to favour preferential growth of C. ramosum over other enteric organisms. This is because C. ramosum is inherently resistant to many antibiotics, including those routinely used prophylactically in patients undergoing biliary tract operations (Saxerholt et al 1986, Midtvedt et al 1986).

Some of the factors listed above, may allow urobilinogen to be either formed in, or absorbed from (or both), the small bowel of the post-operative jaundiced patient to a greater extent than in the small bowel with relatively normal motility which follows endoscopic procedures. There are also other theoretical reasons why this may be so. For example, the normal site of synthesis of urobilinogen is the distal ileum and colon, as this is the site of

normal colonic flora of which C. ramosum is a member (Moscowitz et al 1970, Bloomer et al 1970, Saxerholt et al 1984). However, in intestinal stasis, colonic flora frequently invade and overgrow in the proximal small bowel (Simon and Gorbach 1986). Urobilinogen is absorbed to a much greater degree from the duodenum than from the terminal ileum (Lester et al 1965). Therefore, if the concentration of urobilinogen in the small bowel were increased (as may theoretically occur if C. ramosum were to overgrow in the proximal small bowel), absorption of urobilinogen may be expected to increase.

There is almost no published data describing behaviour of urobilinogen peri-operatively, although Watson (1937a) stated:

"There is little doubt that reabsorption of urobilinogen from the bowel is in considerable measure dependent on the rate at which the feces move through the lower portion of the intestinal tract. In the presence of injury to the liver the associated urobilinuria is much more marked if constipation, intestinal obstruction or ileus exist."

To illustrate his point, he reported the case of an 18-year-old female patient with "recurrent attacks of mild jaundice" (although a definitive diagnosis is not stated) who underwent two laparotomies; the first for removal of an ovarian cyst, and the second for splenectomy. Both operations were followed by ileus. After the first operation, the urinary urobilinogen excretion rose from 2.5 mg/day to 380.8 mg/day, while after the second operation, it rose from 26.2 mg/day to 138 mg/day (Watson's normal reference range 0 - 4.0 mg/day) [Watson 1937b].

Watson's claim was given further (although still tenuous) support by Lester et al (1965), who administered ^{14}C-labelled urobilinogen to six patients with a variety of hepatobiliary conditions. Alluding to Watson's comments (above), Lester described one

patient in whom "the rate of absorption ... of [urobilinogen] isotope increased abruptly twelve hours [after infusion into the terminal ileum]. It is therefore of interest that this patient had received 50 mg of meperidine [an opiate with inhibitory effects on bowel motility] ... shortly before the upward deflection in the curve began."

1.4.2.2.2. Evidence to suggest that urobilinogen may be nephrotoxic

There is some circumstantial evidence to support the hypothesis that urobilinogen may have a nephrotoxic effect. This includes the following:

(a) Other disease states in which serum urobilinogen is markedly elevated (e.g. hepatocellular failure, haemolysis, myoglobinaemia), are commonly complicated by ARF (Kaplan and Issellbacher 1994). In haemolysis and myoglobinaemia, production of bilirubin is greatly increased, while in hepatocellular disease, liver uptake and excretion of urobilinogen is impaired (ibid.). Just as in post-operative patients with OJ, the mechanisms underlying the renal failure are poorly understood and no single aetiological agent has been identified. Indeed the highly lethal hepatorenal syndrome (discussed above, p.15) which has recently been defined as acute renal failure with no apparent cause, occurs in disease states characterised by high serum urobilinogen levels (Papper 1983, Billing 1986, Epstein 1994, Kaplan and Issellbacher op. cit). These include cirrhosis, acute hepatitis and fulminant hepatic failure (ibid.).

(b) If urobilinogen is responsible for post-operative decline in renal function after relief of OJ, this would explain an apparent paradox surrounding previous experimental treatments aimed at protecting renal function in patients undergoing operations to relieve OJ. These trials were driven by the hypothesis that renal impairment is caused by endotoxin, entering the body from the gut. The treatment consisted of emptying the gut

using either large-bowel irrigation (Hunt et al 1982) or lactulose, a powerful laxative (Pain et al 1991). They were modestly successful in preventing renal failure, yet paradoxically, more specific anti-endotoxin agents such as polymixin B have been shown to have no effect (Ingoldby et al 1984) (see further discussion on p.209).

In summary, there is evidence to suggest that urobilinogen may be nephrotoxic in some circumstances, including in patients undergoing operations to relieve obstructive jaundice. However, almost nothing is known about either the toxicity of urobilinogen, or its quantities in the body at the time of such operations. This thesis aims to investigate those effects.

1.4.2.3. Note on the chemistry of the urobilinoid compounds

These compounds were first discovered in the 19th century. Jaffe in 1868 is credited with being the first to describe, and name, the closely related compound urobilin (Watson 1963, With 1968). In 1871, Maly performed an amalgam reduction of bilirubin to produce a substance which he called "hydrobilirubin" but which was probably a mixture of compounds, including urobilinogen (Watson 1953) while Fischer in 1911 was probably the first to prepare pure urobilinogen (Watson 1953, op. cit.). The molecular structure was subsequently worked out by several individuals, including Gray and Nicholson and colleagues [1949, 1958a and 1958b], and Watson and colleagues (1944, 1953).

Much of the literature examining the chemical structure and biological behaviour of urobilinogen was published prior to 1970, and there is surprisingly little recent literature referring to it. [NOTE: The present author speculates that interest in urobilinogen has apparently waned, largely because of the widespread use of ultrasound imaging. Prior to the introduction of ultrasound, clinicians wishing to differentiate obstructive from non-obstructive jaundice put great emphasis on the level of urobilinogen in their patients' blood and urine (Klatskin 1958). Ultrasound imaging is both more sensitive and more specific than these tests, and from a clinical viewpoint has made them largely redundant.]

The nomenclature of this chemical family has arisen over more than a century, in a rather haphazard way. Consequently, as Lester et al (1965) wrote:

"The nomenclature of these compounds is hopelessly confused, and the only systematic formulation related to structure is understandably unpopular".

However, the bile pigments and related compounds all share a similar basic molecular structure. They are all composed of four molecular structures named pyrroles;

and are therefore known as tetrapyrroles. Various forms occur, with different side-chains such as vinyl or ethyl groups; these forms are given Roman-numeral designations (such as IX) (Lemberg and Legge 1949).

In molecules such as haem, the four pyrroles are linked together to form a ring; these molecules are thus known as cyclic tetrapyrroles. The four links between the pyrrole molecules are named α, β, χ and δ. As haem is metabolised, the ring is broken at one of these links, forming a linear tetrapyrrole, of which bilirubin is an example (Lemberg and Legge op. cit.). The name of this molecule is therefore determined by its side-chains (e.g. IX) and by the point at which the ring is broken (e.g. α). It is believed that only molecules of the form IXα occur in nature (Gray et al 1958a, Gray and Nicholson 1958b).

Further modifications to the side-chains and to the links between the pyrrole groups, yield a subset of linear tetrapyrrole molecules known as urobilinoids. The urobilinoids can be classified basically into the coloured compounds (pigments), and the colourless compounds (chromogens) which are their precursors. The urobilinogens are chromogens. By name they are *i*-urobilinogen (also known as mesobilirubinogen), *d*-urobilinogen, and *l* -urobilinogen (also called stercobilinogen). (The urobilins have names which correspond, but are suffixed -obilin). Although the prefixes, which refer to their optical activity, [viz. levo, dextro and optically inactive] might suggest that these compounds are isomers, in fact they are not (With 1968). Any one of the three may be the predominant urobilinogen in urine and faeces, but it is usually l-urobilinogen (Winkelman et al 1974). So far as is known, they all have the same diagnostic significance (Winkelman op cit).

1.5. Experimental models of reversible obstructive jaundice

Although a large amount of work has been done examining the effects of obstructive jaundice itself on various body systems, until recently very little experimental work has examined the effects of reversal of obstructive jaundice. There may be at least two reasons for this:

(1) Firstly, although several different models of reversible obstructive jaundice have been reported in the literature, none is entirely satisfactory. Such models include choledochovesical fistula with silastic tubing (Burke et al 1977, Diamond et al 1990a), and choledochoduodenostomy by side-to-side anastomosis (Ding et al 1992), or by tube placement (Saitoh et al 1995), but they all have the disadvantage of needing two laparotomies; the first to cause biliary obstruction and the second to relieve it. This second laparotomy is undesirable because it is a major physiological insult to a jaundiced animal and it is time-consuming. Furthermore, it is reported to be technically difficult due to friable and inflamed tissues (Ryan et al 1977, Yu et al 1993).

In 1990, Diamond and Rowlands described a rat model in which a length of Silastic tubing was inserted into the bile duct and diverted to the bladder. A similar model had been described previously by Burke et al (1976). Although Diamond and Rowlands' main aim was to provide sterile 'external' biliary drainage, they observed that the model could be easily adapted to include a period of obstruction. The obstruction could be created by simply ligating the tubing at the initial operation. However, although this model overcame the technical difficulties associated with handling a dilated, friable bile duct, it still required a second laparotomy to reverse the obstruction (Diamond and Rowlands 1990a).

(2) Secondly, previous authors may have assumed that reversing obstructive jaundice simply results in reversal of the pathophysiological changes associated with it.

In experimental animals, obstructive jaundice results in increased levels of bacterial translocation (Deitch et al 1990a) and both portal and peripheral endotoxaemia (Van Bossuyt et al 1990). Whether this is due to the systemic effects of obstructive jaundice (such as decreased Kupffer cell phagocytosis) or the luminal effects (such as bacterial overgrowth), or a combination of them, remains unclear. Several authors have tried to answer this question, with conflicting results.

In 1990, Diamond et al compared frequency of endotoxaemia in rats undergoing biliary obstruction and subsequent relief of obstruction (internal and external), and non-obstructed biliary diversion to the bladder. They found that endotoxaemia was significantly more frequent in biliary obstructed rats than in non-obstructed rats, but that relieving this obstruction either externally or internally, reversed the endotoxaemia. When rats in the same experimental groups were given lead acetate (to eliminate Kupffer cells), prior to administration of intravenous endotoxin, most of those with simple biliary obstruction died, but most of those in the other experimental groups survived. Diamond et al concluded that "...biliary obstruction is a more important factor than is gastrointestinal bile flow in the development and reversal of endotoxaemia." (Diamond et al 1990b).

In 1992, Slocum et al performing a similar experiment, concluded almost exactly the reverse. They compared rates of bacterial translocation between groups of rats with (i) common bile duct ligation, (ii) choledochovesical fistula, (iii) sham operation and (iv) no operation. They found that there was no significant difference between groups (i) and (ii) in rates of bacterial translocation to mesenteric lymph nodes, liver or spleen, but that both groups had significantly greater bacterial translocation to these organs than groups (iii) and (iv). They also found that subepithelial oedema was present in the ileal mucosa of both the bile duct-cannulated and the bile duct-ligated rats. This group had previously described a

similar type of ileal mucosal injury in other settings where bacterial translocation was also seen, such as thermal injury and hypovolaemic shock.

They therefore concluded that it was "... primarily the absence of bile in the intestine that promotes mucosal injury and bacterial translocation and not biliary obstruction." The different conclusions drawn by these two research groups performing similar experiments are somewhat difficult to reconcile. In particular, there appears to be a conflict between Slocum's observation that choledochovesical fistula alone causes bacterial translocation to the same extent as common bile duct ligation, and Diamond's findings that choledochovesical fistula (either alone or after a period of OJ) is not associated with either excess mortality or endotoxaemia. The conflict may be partly resolved by the fact that the two reports measured different things; bacterial translocation is clearly not the same as endotoxaemia or death. Another possible explanation for these seemingly contradictory findings may be the potentially confounding factor of an intra-peritoneal foreign body (i.e. a silicone cannula) in animals with choledochovesical fistulae. Guo et al (1993) found that some intraperitoneal prosthetic materials, including silicone, significantly alter reticuloendothelial function and rates of bacterial translocation to both systemic organs and gut-associated lymphoid tissues.

The effects of restoring bile flow to the intestine are even less clearly understood. Almost all published work describing the changes occurring in this setting describes studies performed after drainage of at least seven days (Gouma et al 1987, Ding et al 1993a, 1993c, Saitoh et al 1995) or several weeks (Roughneen et al 1986, Diamond et al 1990b, Katz et al 1991b, Megison et al 1991, Pace et al 1991, Ding et al 1992, 1993b and 1993c, Clements et al 1996a). There is almost no published literature reporting the immediate effects (minutes to days) of return of bile to the intestinal lumen of subjects with obstructive

jaundice. This is perhaps surprising, because the literature in existence shows an injurious immediate effect of bile, or bile components, in these circumstances.

In summary, translocation of bacteria from the gut lumen into the tissues is thought to play a role in the excessive frequency of septic complications following operations on patients with obstructive jaundice. However, it is unclear whether the systemic or local effects of obstructive jaundice are more important in causing bacterial translocation.

Also, there is evidence to suggest that bile returning to the intestinal lumen may, under some circumstances, injure the bowel wall and allow entry into the tissues of luminal contents. However, almost nothing is known about the short-term (minutes to days) effect on bowel-wall permeability of the return of bile to the intestinal lumen. This thesis aims to investigate those effects.

Lastly, most animal models of reversible obstructive jaundice presently in existence need a laparotomy to relieve the obstruction. This is a major insult to animals which are already compromised by being jaundiced. A model with a less invasive means of relieving the obstruction is desirable. This thesis aims to develop such a model.

2. AIMS

The aims of this thesis are:

2.1. To develop a reversible model of obstructive jaundice which does not require a laparotomy for relief of the obstruction.

2.2. To use the model to examine the short-term effect (minutes to days) of relieving obstructive jaundice:

2.2.1. on bacterial translocation from the gut lumen to the tissues. Firstly, to establish if there is an alteration in the amount of bacterial translocation from the gut shortly after reversal of obstructive jaundice. Secondly, to design the experiment in such a way that factors which may be responsible for such an alteration can be identified. Specifically, to discover whether the returning bile has a local effect on the gut wall, or whether the act of releasing obstructive jaundice has a systemic effect which acts on the gut to increase bacterial translocation.

2.2.2. on bowel-wall permeability, using endotoxin and ethylenediaminetetra-acetic acid (EDTA) as the permeability probes.

2.3. To synthesise urobilinogen and examine its toxic effect, if any, on a cultured renal cell line.

2.4. To measure urobilinogen concentration in the bile, serum, urine and intestinal contents of rats before and after operations to relieve obstructive jaundice.

2.5. To measure urobilinogen concentration in the serum and urine of human patients before and after operations to relieve obstructive jaundice.

3. MATERIALS & METHODS

3.1. Sepsis and infection

3.1.1. Effect on bacterial translocation of the return of bile to the intestinal lumen

Aim

The aim of this experiment was to establish if the act of relieving obstructive jaundice caused an increase in bacterial translocation to the mesenteric lymph nodes, liver, spleen, cardiac or portal blood. An isolated loop of small bowel (the Thiry-Vella loop, or TVL) was used, as described below, p.77. In theory, this allows the relative importance of the systemic effects of the relief of OJ (e.g. decompression of the liver, return of serum bilirubin levels to normal), to be assessed in comparison to the local effects of the relief of OJ (e.g. effects of bile on bowel wall permeability and intra-luminal bacterial numbers).

Experimental design

The design of the experiment is shown in the flow chart overleaf.

Thiry-Vella Loop and biliary obstruction
biliary diversion to:

small bowel	bladder	colon	Thiry-Vella loop
(Group A)	Group B)	(Group C)	(Group D)

7 days post-operatively: release of obstruction

24 hours after release of obstruction:
blood and internal organs taken for quantitative microbiological assessment

Group A (control)

Under general anaesthetic (G.A.) and sterile conditions, 8 rats underwent laparotomy, formation of Thiry-Vella loop (as described below, p.77) and choledochotomy. The distal bile duct was ligated, and the proximal bile duct cannulated with the specially prepared cannula described below. The free end of the cannula was inserted into the small intestine (SI), 12 cm from the ileo-caecal valve, using 5-0 silk with a purse-string technique. The cannula thus formed a duct between bile duct and small bowel.

[NOTE: It could be argued that it would more closely resemble the normal state, to insert the cannula into the duodenum rather than the ileum. However, the aim was to use the same region of bowel as that in the Thiry-Vella loop (see below p.77), to avoid introducing a confounding factor. Because the duodenum absorbs most of the body's nutritional requirements, to include it in a Thiry-Vella loop would be very likely to produce a severe catabolic state which, even if the animals were to survive it, would confound the results.]

A loop of the cannula was brought to the skin surface in the posterior cervical region and kinked by compression inside a length of vinyl tubing, as described below, so

causing obstructive jaundice. Seven days post-operatively, under G.A. this length of vinyl tubing was removed, thus allowing bile to flow via the cannula into the S.I. The Thiry-Vella loop was instilled with 200 μL of the standardised suspension of labelled E.coli described below, p.80.

Rats often lick their abdomens, and especially wound sites (personal observation), which constitutes a potential pathway for organisms in the TVL to reach the rest of the gut. To prevent this, both stoma sites were painted with povidone-iodine solution ('Betadine', Faulding Pharmaceuticals, F.H. Faulding and Co. Ltd., 1538 Main North Rd., Salisbury South, South Australia 5108) and then oversewn.

Twenty-four hours after the above procedure, the rats were killed and their organs harvested. Under G.A, and sterile conditions, the laparotomy wound was re-opened. Blood was taken from the portal vein and from the heart by needle puncture through the diaphragm. The animals were killed by intracardiac injection of 1-molar potassium chloride solution, and the liver, spleen, mesenteric lymph nodes and lungs harvested for microbiological analysis. A specimen of Thiry-Vella loop and of distal ileum was then taken for histological examination, and a sample of caecal contents and Thiry-Vella loop contents taken for serial dilution and culture.

Group B

A further 8 rats were treated as per the control group, with the exception that the distal end of the cannula was diverted to the bladder and ligated. Thus when the cannula was unkinked, bile flowed to the bladder to be excreted.

Group C

Another 8 rats underwent identical treatment to that of the control group except that the bile was re-diverted to colon instead of small bowel.

Group D

A final group of 8 rats differed in treatment from the control group only in that the bile was re-diverted to the Thiry-Vella loop.

3.1.1.2. Experimental techniques

3.1.1.2.1. Anaesthesia

General anaesthesia was by inhalation of a mixture of nitrous oxide, oxygen and halothane ('Fluothane', ICI Pharmaceuticals, Great Britain).

3.1.1.2.2. Thiry-Vella loop

Under G.A., the rats were placed supine on a specially-designed heated plate maintained at 37 °C. The abdomen was shaved, painted with povidone-iodine ('Betadine', Faulding Pharmaceuticals, F.H. Faulding and Co. Ltd., 1538 Main North Rd., Salisbury South, South Australia 5108) and opened in the midline. A point on the ileum 2 cm from the ileo-caecal valve was identified. The bowel was divided at this point and also at a point 10 cm more proximally, without dividing the mesentery, i.e. without disrupting the neurovascular supply or lymphatic drainage. The proximal and distal ends of the loop so created were each brought to the anterior abdominal wall as an enterostomy, and secured with 5-0 non-absorbable suture material. Continuity of the remaining bowel was restored by end-to-end anastomosis with 6.0 non-absorbable suture material. At the conclusion of

the experiment, the Thiry-Vella loops were stained with haematoxylin and eosin and examined under light microscopy by Dr. Bing Wei, of the Department of Pathology, The Queen Elizabeth Hospital, Woodville Rd., Woodville, South Australia 5011.

3.1.1.2.3. Bile duct ligation

At laparotomy under G.A. and sterile conditions, the hepatoduodenal ligament was mobilized and two ligatures of 3.0 non-absorbable suture material tied tightly around the bile duct, taking care not to include pancreatic tissue. The intervening segment of bile duct was then divided.

3.1.1.2.4. Biliary diversion method

To investigate the effects of relieving obstructive jaundice, a reversible model of biliary obstruction is needed. As discussed in the introduction, several such models already exist in small animals, but they have the disadvantage of needing two laparotomies; the first to cause biliary obstruction and the second to relieve it. An improved method that avoids this second laparotomy was therefore developed. It is essentially the model previously described by Diamond and Rowlands (1990a) with minor modifications.

A 25-cm length of 'Silastic' silicone rubber tubing, (Dow Corning Corporation Medical Products, Midland, Michigan, U.S.A.) with an external diameter of 0.94 mm, and an internal diameter of 0.51 mm was prepared by modifying each end. The modifications consisted of placing cuffs around each end. Each cuff was simply a 2-mm length of the same silastic tubing. One end of the tubing had one cuff placed around it, 3 mm from the end. The other end had two cuffs; the first 3 mm from the end, and the second 2 mm more proximally. The cannula was then sterilised before use. In most experiments, this was by

ethylene oxide gas. However, use of this gas was discontinued at the Royal Adelaide Hospital during the course of the experiments. Autoclaving was therefore substituted.

At laparotomy, the bile duct was identified and a transverse choledechotomy made at a point 1 cm below the confluence of the hepatic ducts. The single-cuffed end of the tubing was inserted into the choledochotomy and secured in place with two 5-0 silk ligatures around the outside of the bile duct. One ligature was placed distal to the cuff (which could be easily seen and palpated in the duct), and one was placed proximally. The ligatures were placed sufficiently tightly that the cuff could not become dislodged, nor bile leak, but not so tight as to prevent flow of bile through the tubing. This second point was checked by observing free flow of bile from the distal end of the tubing.

The distal end of the bile duct was ligated with non-absorbable suture material to ensure that no reflux of enteric contents could occur. Care was taken to ensure that pancreatic tissue was not included in the ligature. The free (i.e. double-cuffed) end of the cannula was diverted to the chosen site and secured in place with a purse-string suture of 5-0 or 6-0 silk. This was done by making 3 full-thickness, continuous 'bites' on the anterior wall of the intestine (or apex of the bladder), in the shape of an equilateral triangle. A 1-mm incision was made in the tissue enclosed by the triangle, and the tubing inserted into the lumen, so that the more distal cuff was inside the lumen, and the more proximal cuff outside. The tubing was then secured in position, and the incision closed, by passing the silk suture between the proximal cuff and the adjoining tubing.

A loop of the intervening length of tubing was then brought through the anterior abdominal muscle layers in the right upper quadrant, 1.5 cm lateral to the laparotomy incision. This loop was tunnelled subcutaneously to the posterior cervical region, and brought out through the skin which had previously been prepared by local shaving and

painting with povidone-iodine. The apex of this loop was snared with 5-0 silk, and drawn inside a 1.5 cm length of vinyl tubing with external diameter of 0.96 mm and internal diameter 0.58 mm (Dural Plastics and Engineering, Dural, N.S.W., Australia). This resulted in kinking and compression of the silastic tubing sufficient to cause complete obstruction. (This fact was checked in pilot experiments, by connecting one end of the tubing to a 30 mL syringe filled with water, and attempting to force the water through the obstruction. Sufficient force to burst the silastic tubing could be generated without any leakage occurring from the distal end.) The cannula was then returned beneath the skin and sutured loosely in place, and the skin wound closed.

To relieve the obstruction, the animal was placed under G.A. The posterior cervical region was painted with povidone-iodine, and the previous incision in that area re-opened. A short length of the cannula was delivered to the skin surface and the occluding vinyl tubing was removed, so unkinking the cannula. The cannula was returned subcutaneously, and the wound sutured closed.

Other modifications of Diamond and Rowlands' method were also tried but not found to be useful. These included inserting a 1.5-cm length of firm polyethylene tubing into the end of the cannula to be inserted into the bile duct. This was done in an attempt to prevent occlusion of the silastic tubing (which is soft), by the ligatures. However, it was found that the polyethylene tubing tended to become dislodged from the bile duct. This was probably because it was firmer than the silastic, so the ligatures did not tend to 'bite' into it.

3.1.1.2.5. Preparation of standard Escherichia coli suspension

Streptomycin-resistant Escherichia coli 0111 were cultured in a standard nutrient broth (Oxoid Nutrient Broth, Unipath Ltd., Basingstoke, Hampshire, England) at 37 °C. The broth was centrifuged at 2500 r.p.m. for 10 min. and the resultant pellet of organisms resuspended in normal saline. The optical density of the solution was measured spectrophotometrically (Varian DMS-80 UV-visible spectrophotometer). The optical density was titrated with normal saline to 1.0 at a wavelength of 620 nanometres, which corresponds to 1 x 10^7 organisms per mL.

3.1.1.2.6. Harvest procedures

This operation occurred 24 hours after the Thiry-Vella loop was injected with labelled E. coli. Under G.A. and sterile conditions, the laparotomy wound was re-opened. Using a 21-gauge needle, 1 mL of blood was withdrawn from the portal vein, and then from the heart by direct cardiac puncture. The rat was killed by intra-cardiac injection of 1-molar potassium chloride.

The systemic and portal venous blood specimens were plated onto MacConkey and streptomycin-nutrient agar plates. After 24 and 48 hours of aerobic incubation, the number of organisms was counted and recorded as described below.

Internal organs were harvested for counting in the following order:

(I) Liver: A segment of liver weighing approximately 1g was removed, rinsed thoroughly with normal saline to remove adherent remnants of portal blood, placed in a pre-weighed specimen container already containing 2 mL of sterile normal saline, and weighed. It was then processed for quantitative microbiological analysis as described below (see 'Assessment of bacterial translocation' this page).

(ii) Spleen: As for liver, except that the entire spleen was removed.

(iii) Mesenteric lymph nodes: As for spleen.

(iv) Lungs: As for spleen.

(v) Gut: Approximately 200 mg of gut contents were taken from each of the caecum and the Thiry-Vella loop, placed in sterile pre-weighed containers and weighed. 10 mL of sterile normal saline were then added, and the resultant mixture vigorously agitated by a 'vortex' mechanical agitation device. Exactly one mL of the resulting suspension was added to a second sterile tube containing 10 mL of sterile normal saline. This second tube was then vigorously agitated, and the process of serial dilution 1:10 repeated to a total of 10 times. 0.2 mL aliquots from each tube were then plated onto MacConkey and streptomycin-nutrient agar plates. After 24 and 48 hours of aerobic incubation, the number of organisms was counted and recorded as described below.

3.1.1.2.7. Assessment of bacterial translocation

After the MLN, liver, spleen and lungs had been harvested as described, they were weighed and homogenised in 2 mL of sterile 0.9% NaCl solution. 0.2 mL aliquots of homogenate were plated onto nutrient and McConkey's agar and the plates read after 24 and 48 hours of aerobic incubation. In keeping with other studies examining bacterial

translocation (e.g. Deitch 1989, Reed et al 1991, LaRocco et al 1993, Nieuwenhuijzen et al 1993, Fukushima et al 1994), cultures for strict anaerobic bacteria were not performed, because obligate anaerobic bacteria rarely translocate from the gut (Steffen et al 1988). Growth of organisms, as colony-forming units (C.F.U.s) was recorded as follows:

growth of < 110 C.F.U.s

growth of > 110, but < 1000 C.F.U.s

growth of > 1000, but < 5000 C.F.U.s

growth of > 5000 C.F.U.s.

3.1.1.2.8. Comparative histopathology

The Thiry-Vella loop and a specimen of adjacent distal ileum were collected for standard haematoxylin and eosin preparation followed by histopathological examination under light microscopy.

3.1.2. Effect on bowel-wall permeability of the return of bile to the intestinal lumen

Aim

The aim of this series of experiments was to return bile to the intestinal lumen of rats with obstructive jaundice, and measure the effect on bowel-wall permeability to a radio-labelled endotoxin and ethylenediaminetetraacetic acid (EDTA). The effect of jaundiced rat bile, jaundiced and infected rat bile, non-jaundiced human bile, and jaundiced

human bile were all assessed. For simplicity, the design of each experiment is described more fully in the 'Results' section.

Briefly, obstructive jaundice was created in rats by inserting a silastic cannula int othe common bile duct, and occluding the cannula. Seven days later, the obstruction was released, and the flowing bile collected and injected into the intestinal lumen, together with radio-labelled EDTA and endotoxin. In some rats, the bile was infected with E. coli and incubated for 24 hours prior to injection. In other rats, human bile from 'normal' patients or patients with obstructive jaundice was used. In these rats, it was not necessary to collect the bile, so at the initial operation the bile duct was simply ligated.

3.1.2.1. Experimental methods

3.1.2.1.1. Method of labelling endotoxin with Iodine-125

The method used was essentially that described by Ulevitch (1978), with minor modifications. Numbers in square brackets [] refer to the explanatory notes following the description below.

(a) Preparation of endotoxin for iodination (p-OH methylbenzimate-endotoxin derivative)[1]

Preparation of 50 mM p-OH MBI in 0.05M borate buffer, pH 8.0: [2]

(i)Sodium tetraborate, hydrated ($Na_2B_4O_7.10H_2O$), 1.905g was dissolved in 100mL of de-ionised water and the pH adjusted to 8.0 with HCl.

(ii) 46.9mg of methyl p-hydroxybenzimidate,HCl (Sigma, M8384) was suspended in 10 drops of 0.1 N HCl [3], vortexed; most dissolved. 4.5 mL borate buffer and 10 drops of 0.1 N NaOH, and all dissolved. [4] ie 50 mM p-OH-MBI solution made.

To 5mL of p-OH-MBI in borate buffer, was added 5 mg endotoxin (E.coli 0111:B4 lipopolysaccharide, phenol-extract, chromatographically purified, Sigma L-3012) . The endotoxin crystallized out after initally dissolving, but after being mixed on a rotator for 18 hrs at 40 °C, no precipitate remained. The solution was dialysed against 4 x 5L de-ionised water at 4 °C for 2 days [5], then freeze-dried and stored at -20 °C.[6]

(b) Iodination of modified lipopolysaccharide (M-endotoxin)

To 300 μL of M-endotoxin (as 1 mg/mL in PBS) [7] was added 5 μL of $Na^{125}I$ (1 mCi per 10 μL, i.e. 0.5 mCi), washed down with 10 m μL PBS and mixed.[8]

10 μL Chloramine-T (5 mg/mL in PBS) was then added.[9] This was mixed and incubated for 10 minutes. To this solution was added 100 μL of 1.2 mg/mL sodium metabisulphite in PBS, and the solution mixed for 1 minute.[10] To this was added 300 μL of 2 micrograms/mL KI in PBS, and the solution mixed and incubated for 10 mins. [11]

The solution was then applied to a PBS-equilibrated G-25 medium column, and collected in 0.5 mL fractions.[12]. The fractions were counted by gamma-counter.

Explanatory notes

(1) The basic aim is to alter the endotoxin slightly; specifically, to bind an OH^- group to it, which can then be replaced by I^- in a permanent (irreversible) manner. Therefore, in a harsh, chemically reactive environment such as the gut, the I^- will not be lost.

(2) Borate buffer: This is simply a convenient buffer to keep the solution at a pH where the reaction will take place optimally.

(3) 0.1 N HCl: Initially, the p-OH -MBI when added to the borate buffer did not completely dissolve, so that a small crystalline pellet settled to the bottom of the 5ml Reacti-vial. Basically, because p-OH MBI has an HCl group on it, and such substances dissolve best in an acid solution, the HCl was added to help it dissolve.

(4) The NaOH was added to neutralize the HCl.

(5) Dialysis: This involves placing the solution in a porous bag, which does not allow the passage of molecules with a molecular weight greater than approximately 20,000. It serves to get rid of the impurities (which are small molecules, namely free MBI, borate, Cl-, Na+ etc.) leaving behind the modified endotoxin (which is a large molecule).

(6) Freeze-drying: There are two reasons for freeze-drying. Firstly, like many powdered substances the endotoxin simply stores better when stored without water. Secondly, because the freeze-dried powder is 'purer', that is, not 'contaminated' by water, the concentration of any solutions subsequently made from it will be more precisely known.

(7) PBS: *P*hosphate *B*uffered *S*aline. This is a standard, commonly used buffer which is near-isotonic and therefore considered approximately physiological (see note 1).

(8) 'Washed down:' Because 5 microlitres is a very small volume, any tiny droplets of it which may remain adherent to the reaction tube are important. They are therefore washed down into the reaction tube to ensure that they participate in the reaction.

(9) Chloramine-T is an oxidizing agent, which serves to remove the OH- ions, therefore leaving a space which is then occupied by the I-.

(10) Sodium metabisulphite: This is a reducing agent. The addition of 100 microlitres of it swamps the solution, therefore stopping the reaction started by the Chloramine-T.

(11) Potassium Iodide: This performs two functions.

Firstly, prior to this solution being added, there will be ^{125}I adherent to the endotoxin, but which is not strongly (ie covalently) bound, and which can therefore fall off later and so potentially confound any experiments in which it may be used. Adding a large amount of non-radioactive I^- dilutes the ^{125}I molecules to the point where they are no longer a significant contaminant.

Secondly, the addition of a large amount of I^- provides a 'mass' which is important for subsequent chromatography. The reason is complicated, but essentially, if there is only a very small amount of I^- with a large amount of endotoxin, the I^- will not behave as it should when attempts are made to separate the two substances chromatographically. Under these circumstances, the I^- tends to adhere to the endotoxin.

(12) Separation Method: The G-25 medium column is a type of chromatography column. It consists of a tube approximately 10 cm in height, containing microscopic dextran spheres, each of which has a hole in

it. In solution, larger molecules pass rapidly through such a column, because they flow around the outside of the spheres, being too large to enter the holes. Small molecules, on the other hand, pass slowly through such a column because they tend to pass through the holes. Consequently, when a solution containing both large (e.g. endotoxin) and small (e.g. free I^-) molecules is passed through this column, the large molecules tend to emerge first. Collecting the emerging solution in 0.5 ml aliquots, that is, serially in twenty tubes, allows identification of the tubes containing each solute. This is because, progressing from the first to the last tube, two separate 'peaks' of radioactivity can be identified, the first representing the endotoxin and the second, the free I^-.

3.1.2.1.2. Method of checking proportion of bound to unbound I-125

Introduction

It was necessary to ensure that the technique used to attach the radio-label ^{125}Iodine to the endotoxin (described in the 'Methods' chapter), was successful. Otherwise, when measuring the amount of radio-label in a particular specimen, it is uncertain whether the result indicates the presence of endotoxin, or simply free iodine.

Methods

A strip of silica gel impregnated glass fibre sheet ITLC SG 1-cm wide and approx 15cm long was cut, and two marks, 10 cm apart, made on it with an ink pen. A small droplet of solution was placed on one mark, and the strip stood upright, with the wetted mark lowermost, in a glass vessel containing a layer of acetone approximately 1 cm deep. When the acetone had risen up the strip by capillary action and reached the second mark (after about 5 minutes), the strip was removed and air-dried. It was then cut into segments at 1-cm intervals, and the segments were serially assayed for radio-activity. Because the free I-125 moves easily with the acetone, while the bound I-125 moves much more slowly, this results in most of the counts being in the lower segments. A typical analysis is shown overleaf (as assayed on 03/06/96):

Segment	Counts per minute
1	1272273
2	646280
3	224689
4	74260
5	18927
6	11850
7	8762
8	23197
9	36430
10	21749
11	6550
12	483
13	130

Assuming the first three segments contain the bound I-125, and the remaining segments contain free I-125, the percentage of bound I-125 can be calculated:

$$\frac{\Sigma\ 1\text{–}3}{\Sigma 1\text{–}3 + \Sigma\ 4\text{–}13} = \frac{2{,}143{,}242}{2{,}143{,}242 + 202{,}338}$$

$$= 91.3736\ \%.$$

3.1.2.1.3. Preparation of ^{125}I-labelled endotoxin solution

Because synthesis of the ^{125}I-labelled endotoxin is a complex and time-consuming process, it was not practical to make a fresh batch each day. Therefore, a large batch was made up and stored, and a final 'working' solution made up fresh each day. This was made up to a concentration of approximately 10^5 counts per minute/millilitre (cpm/mL); usually, 10 mL was made up at a time. 1 mg/mL of 'cold' (i.e. non-radioactive) endotoxin was then added. This was done to avoid falsely-low doses of endotoxin being used. That is, small amounts of endotoxin tend to adhere to the walls of the container it is placed in. When only weak solutions of endotoxin are used, the loss of any of it may severely confound the results. Adding relatively large amounts of 'cold' endotoxin avoids this problem.

The exact number of cpm administered to each rat (as a 0.5 mL dose) was calculated by measuring the cpm in the syringe before and after injection.

3.1.2.1.4. Preparation of ^{51}Cr-EDTA solution

^{51}Cr-EDTA is readily available commercially. It was simply necessary to make up a new solution each day, containing approximately 10^5 cpm/mL. The exact dose was calculated as per that of the ^{125}I-endotoxin described above.

3.1.2.1.5. Technique for measuring permeability of the bowel wall

Under G.A. and sterile conditions, rats underwent laparotomy while lying supine on a specially-designed plate maintained at 37 °C. A point on the ileum 1cm from the

ileocaecal valve was identified and an occlusive ligature placed at that point.. A second point, 20 cm more proximal, was identified and a second occlusive ligature placed, so creating a closed loop of ileum. Using a 25-gauge needle, 500 μL of ^{51}Cr-EDTA solution was injected into a 'knuckle' of bowel at the proximal end of the loop, followed by 500 μL of the ^{125}I-endotoxin solution, and then 500 μL of bile. (The dose of radioactivity in each aliquot of radioactive solution was measured immediately prior to use). The knuckle of bowel was then ligated with 5-0 silk to prevent leakage through the needle-hole. The loop of bowel was returned to the abdominal cavity and the abdomen closed.

The rats were kept under anaesthesia for 60 minutes, after which the abdomen was re-opened. 1 mL of blood was taken from the portal vein and then from the heart by direct cardiac puncture, placed in pre-weighed tubes and weighed. The rats were then killed by intra-cardiac injection of 1-molar potassium chloride, and the following internal organs collected into pre-weighed tubes:

Liver, mesenteric lymph nodes, spleen, lungs, thyroid, kidney and bladder, loop of gut. The thyroid was collected to measure the amount of ^{125}I (if any) which became detached from the endotoxin; the thyroid selectively takes up free iodine, but not that bound to endotoxin. Each specimen was then weighed and placed in a gamma-counter (together with the tubes, needles and syringes used to inject the radio-labelled solutions) to measure the contained radioactivity, as counts per minute (c.p.m.).

3.2. Renal impairment

3.2.1. Effect of urobilinogen on in vitro cell cultures

Experimental design

Using the techniques described below, urobilinogen was synthesised and made into solutions of varying concentration. These were then incubated with known numbers of 'Vero' cultured renal cells for 24, 48 and 72 hours. The number of surviving cells was then counted using a Neutral Red stain.

3.2.1.1. Experimental techniques

3.2.1.1.1. Synthesis of urobilinogen

Urobilinogen is not commercially available. A method of synthesis was therefore needed. The method used was that of Henry et al (1961) with minor modifications. Essentially, this is an oxidation-reduction reaction, in which the closely-related compound urobilin is reduced to urobilinogen.

All reagents used were analytical grade. To exclude light as much as possible, all experiments took place in a dimly lit room and reaction vials were wrapped in aluminium foil. The amount of urobilin was varied from experiment to experiment; in some cases 0.5 mg was used, while in others 1.0 mg was used.

Urobilin IX (Sigma, St. Louis, Mo., U.S.A.) was dissolved in 3.0 mL of 31/3% weight/vol NaOH in a 10 mL polypropylene reaction vial (Laboratory Supplies [S.A.], 37

Woodlands Tce., Edwardstown, S.A.). One mL of 20% weight/vol $FeSO_4$ was added, and the tube placed on a mechanical agitation device and vigorously shaken by hand once every 30 min. After 3 hours, the tube was centrifuged at 1500 r.p.m. for 10 mins (MSE Mistral 3000 bench-top centrifuge). The supernatant was decanted into a second vial containing 40 mg ascorbic acid, and filtered through Whatman 540 hardened ashless filter paper, using vacuum suction apparatus.

In some later experiments, where the urobilinogen was used immediately, ascorbic acid was omitted from both the urobilinogen solution and the corresponding control solution (see below, section 3.2.1.1.2). Where urobilinogen was not used immediately, it was stored under argon in liquid nitrogen and used after no more than 3 days.

Synthesis of urobilinogen was verified by spectrophotometric assay as described below.

3.2.1.1.2. Synthesis of control or 'blank' solution

A control or 'blank' solution was made at the same time as the urobilinogen solution. It was identical except that no urobilin was added to the NaOH.

Before being placed on cells, the urobilinogen and control solutions were neutralised to physiological pH by titration with hydrochloric acid (HCl) solutions. The pH was lowered to approximately 11 with 1 molar HCl, and the remainder of the titration was done with physiologically isotonic HCl (300 mosm). This was done to avoid excessive dilution of the urobilinogen solution. The volumes of 1 molar solution used were identical for the urobilinogen and blank solutions, while the volumes of isotonic HCl

were similar. Any differences between the two were eliminated by addition of an appropriate volume of normal saline to the solution which required the less HCl.

3.2.1.1.3. Urobilinogen assay

The method used was that of Kotal & Fevery (1991) with minor modifications. It relies on two facts. Firstly, that iodine readily oxidises urobilinogen to urobilin, and secondly that urobilin combines with zinc to form a complex with a characteristic green colour. This green colour can be measured spectrophotometrically.

Four hundred μL of specimen solution was placed in a 10 mL polypropylene reaction vial, and 2.4 mL of 54 mmol/L zinc acetate in dimethylsulphoxide (DMSO) added. Two hundred μL of 25 mmol/L iodine in 120 mmol/L in water were added and the vial shaken vigorously on a bench-top vortex for 60 sec. One hundred μL of 82 mmol/L cysteine in water were added and the solution was centrifuged at 7,250 rpm (= approx. 4,500 x g) at 0 °C for 3 min (Beckman model J2-21M centrifuge, Beckman U.S.A.). The resultant supernatant was labelled U+I+. Its optical density was measured spectrophotometrically (Varian DMS-80 UV-visible spectrophotometer) against a second solution (U+I-) which was identical except that de-ionised water was substituted for iodine solution. Kotal and Fevery recommended measuring the optical density between the wavelengths 540 to 440 nm, because the urobilin-Zn complex has an absorbancy band with a peak at 508.5 nm.

Because not all the urobilin was necessarily converted to urobilinogen, the amount of residual urobilin was calculated. The U+I- was measured against a 'double blank' solution ('U-I-'; i.e containing neither urobilin nor iodine). This was prepared from the 'blank' solution described above under the heading 'synthesis of urobilinogen'.

By analysing one specimen against another, the concentration of urobilinogen and urobilin could be calculated. That is, solution U+I+ compared against U+I- yielded the concentration of urobilinogen, while solution U+I- compared against U-I- yielded the concentration of urobilin. Comparing U+I+ against U-I- measured the total amount of urobilinogen plus urobilin; this could be used to check the precision of the other assays.

3.2.1.1.4. Cell culture methods

Vero cells

These cells were a kind gift of Mr. Tuck Weng Kock from the Virology Laboratory, Institute of Medical and Veterinary Science, Frome Rd., Adelaide, South Australia. According to the American Type Culture Collection, the Vero cell line was initiated from the kidney of a normal adult African Green Monkey (Cercopithecus aethiops) by Yasumura and Kawakita, of Chiba University, Japan, in 1962. The precise cell of origin is not recorded. This cell line was chosen because of its renal origin; no other renal cell lines were readily available.

The cells were cultured in HEPES-buffered Dulbecco's modification of Eagle's medium (HDMEM) (Sigma, St. Louis, Mo., U.S.A.) with 10% foetal calf serum (FCS). They were kept in an incubator maintained at 37 °C, with 95% humidity and an atmosphere of 5% CO_2 in air. Once confluence occurred, the cells were either used immediately, or the culture medium was changed to maintenance medium (identical to culture medium except containing only 1% FCS), until use.

Prior to use, they were twice washed with 15 mL phosphate-buffered saline (PBS) for 10 min. After discarding the second wash, 3 mL of trypsin (Sigma, St. Louis, Mo., U.S.A.) were added and the flask gently agitated until the cells were seen to be dislodged from its floor. Ten mL of maintenance medium were added, and the cells were placed in a sterile 20-mL polypropylene tube (Laboratory Supplies [S.A.], 37 Woodlands Tce., Edwardstown, S.A.) and centrifuged at 1000 rpm for 5 min. The supernatant was aspirated, the cells resuspended in 10 mL maintenance medium, and the centrifugation repeated. The supernatant was aspirated, 2 mL of maintenance medium added, and the pellet of cells resuspended by gentle agitation with a disposable 1 mL pipette, avoiding creation of a froth.

Ten μL of the resultant cell suspension was added to 90 μL of 0.0025% Trypan Blue stain and the cells counted on a haemocytometer. The cell count determined the amount of maintenance medium needed to dilute the cell suspension to a concentration of 1×10^6 cells per mL. One hundred μL of diluted cell suspension were added to each microwell of culture plate, i.e. 1×10^5 cells per microwell.

T47D cells

This cell line had been in storage in the Department of Surgery since June 1991, having been donated to the Department by a research laboratory at Flinders Medical Centre, Bedford Park, South Australia, in 1987. It is an adherent epithelial cell line derived from a human breast carcinoma. It was chosen for several reasons. These include the following:

(1) It is a 'well known' cell line, in the sense that it is widely used by research groups in many parts of the world, for many different purposes;

(2) many published research papers describe its use for the specific purpose of measuring toxic effects of various compounds (eg. Coradini et al 1995,Manni et al 1995 Ashagbley et al 1996);

(3) it is a human cell line.

The culture methods used were similar to those for Vero cells, as described above, except that a different culture medium (RPMI, Sigma, St. Louis, Mo., U.S.A.) was used.

3.2.1.1.5. Neutral Red assay

The method used was that of Löwik et al (1993) with minor modifications. It relies on the fact that the supravital stain Neutral Red is actively taken up by viable cells, but not by dead cells. Specifically, it is incorporated into the lysosomes of viable cells (Borenfreund and Puerner, 1984). This assay, or modifications of it, has been widely used to demonstrate toxicity to a variety of cell lines, by a variety of substances, such as acetaldehyde to human epithelial cells (Grafstrom et al 1994), N-alkyl-sulphate to keratinocytes (Wilheilm et al 1994), organotins to astrocytes (Richter-Landsberg and Besser 1994), glucocorticoids to keratinocytes and fibroblasts (Korting et al 1995), and inorganic mercury to rat renal cortical epithelial cells (Endo and Sakata 1995).

A solution of 0.5 mg/mL Neutral Red in normal saline was prepared. Fifty μL were added to each microwell, and the plate returned to the incubator. After 90 min., the supernatant was removed by gently inverting and blotting the plate. The cells were washed twice by gentle addition of PBS (pH adjusted to 7.4) at 250 μL per well. After the second

wash was removed by inversion and blotting, 100 μL of 0.05 M NaH_2PO_4 in 50% ethanol were added to each well to extract the dye from the cells. The optical density of each well was measured at a wavelength of 540 nm using a reference of 650 nm (BIO-RAD model 3550-UV spectrophotometric microplate reader).

3.2.1.1.6. Effect of urobilinogen on Vero cells

The urobilinogen solution and a corresponding 'blank' solution described above, were neutralised to physiological pH by titration with iso-osmotic (300 milliosmoles) hydrochloric acid. Because of the small volumes (approx 3 mL) of solution involved, a fine-diameter pH probe was used (paediatric monocrystant antimony pH catheter, Synectics Medical, Stockholm, Sweden). It was read with a Digitrapper Mk II Gold ambulatory pH recorder (Synectics, as above).

The resultant solutions were sterilised by filtration through a 0.2 μm pore cellulose acetate filter (Sartorius Minisart SM 17597, Sartorius AG, Goettingen, Germany), and serially diluted 1 mL: 1mL with sterile de-ionised water, i.e. producing solutions of concentration 1, 1/2, 1/4, 1/8 etc.

A flat-bottomed 96-well cell culture plate was prepared in the following way:

To prevent possible 'edge effect' errors due to dehydration, the outermost 36 wells were filled with PBS (200 μL/well). One hundred μL of cell suspension containing 1 x 10^5 cells, were added to each of the innermost 60 wells, so forming ten columns of six wells each. To each column was added 50 μL/well of urobilinogen solution, with its corresponding 'blank' occupying the next column as shown in FIG. The last two columns contained negative and positive controls. The negative control was de-ionised water alone.

Several different positive control solutions were tried in preliminary experiments. These included solutions of 8 μg/mL zinc chloride, zinc sulphate, or zinc acetate, which were used by the donors of the cells. Sodium hypochlorite ('Milton's solution'), which is commonly used as a positive control in cytocidal experiments (Neville De Young B.Sc., Department of Surgery, University of Adelaide, South Australia, [personal communication]), was also tested. However, even when made up to four times recommended strength, these solutions all gave surprisingly high optical density readings with the Neutral Red assay, implying the presence of large numbers of viable cells. Typical optical density readings were around 0.3 for wells containing 10^5 cells (supposedly all dead). This compares with readings of around 1.2 from wells containing 10^5 viable cells, and less than 0.01 from wells which had been stained but contained no cells. The reason for these high readings is unclear. Possibly it may have been an artefact caused by the solutions leaving chemical residues in the wells; these residues may then have been stained by Neutral Red. Therefore, hypertonic saline (nine percent) was used as a positive control, and found to result in reliably very low readings (less than 0.03) with the assay.

3.2.1.1.7. Effect of urobilinogen on T47D cells

A series of experiments was attempted with this cell line, using exactly the same experimental design as for the Vero cells. However, as discussed in the results section, difficulties were encountered with this cell line which made results inaccurate, so the experiments were not pursued.

3.2.1.1.8. Control experiments

The urobilinogen solution manufactured above, unavoidably differed in several ways from the control solution, in addition to the presence of urobilinogen. These differences included:

(1) The presence of urobilin. This was present because the synthesis reaction did not proceed to completion. Assays showed that only approx 76% of the urobilin was converted to urobilinogen.

(2) A higher osmotic concentration, due to the presence of urobilin and urobilinogen.

(3) The presence of differing concentrations of Fe^{2+} and Fe^{3+} ions. Because the synthesis of urobilinogen from urobilin is a reduction reaction, it must be balanced by an oxidation reaction, namely the oxidation of Fe^{2+} to Fe^{3+}. Therefore, if Fe^{3+} were to be more toxic than Fe^{2+}, it could influence the results. However, the number of moles of Fe^{3+} was extremely small compared to the number of moles of Fe^{2+}. This is because of the very small number of moles of urobilin in solution.

That is, the concentration of the $FeSO_4$ solution was 0.71934 M. One mL therefore contained 7.1934×10^{-4} moles Fe^{2+}.

On the other hand, the 3 mL of solution containing 1mg of urobilin (m.w. = 627.2) contained 1.5943×10^{-6} moles.

Assuming:

(1) for every 1 mol of urobilin reduced to urobilinogen, 2 mol of Fe^{2+} were oxidised to Fe^{3+} (because 2 H^+ ions were added to the urobilin), and

(2) a conversion rate of 76%, therefore the number of moles of Fe^{3+} formed was:

$1.5943 \times 10^{-6} \times 2 \times 76/100 = 2.42 \times 10^{-6}$.

3.2.1.1.8.1. Role of urobilin in toxicity

Serial dilutions of urobilin were made up in phosphate-buffered saline (pH of resultant solution = 7.41) and their toxicity assayed using the same methods as that in the assessment of the toxicity of urobilinogen.

3.2.1.1.8.2. Role of osmolarity in toxicity

Undiluted (and therefore maximally toxic) 'blank' solution was made up in the usual way. It was then diluted 1:1 with solutions of sodium chloride. These varied in concentration from 10 x normal saline to pure de-ionised water. Toxicity of these solutions was assayed using the same methods as that in the assessment of the toxicity of urobilinogen.

3.2.1.1.8.3. Role of Fe^{2+} versus Fe^{3+} in toxicity

The 'blank' solution was made up in the usual way. An equivalent solution was also made up, containing an equivalent concentration of Fe^{3+} ions. Serial dilutions of these solutions in normal saline were then made up, and their toxicity assayed using the same methods as that in the assessment of the toxicity of urobilinogen.

3.2.2. Urobilinogen levels after relief of obstructive jaundice in rats

This series of experiments did not progress beyond the pilot stage, because unforeseen (and previously undescribed) problems were encountered with the urobilinogen assay of Kotal and Fevery (1991). This is described more fully in the discussion and conclusions chapter.

Experimental design

Four rats underwent upper midline laparotomy as previously described. Using a 25-gauge needle, 2 mL of systemic blood were taken from the inferior vena cava (IVC). Haemostasis was ensured by gentle finger pressure for 2 min. A sterile silastic cannula was inserted into the bile duct as described above, and the distal end of the bile duct was ligated. Bile was collected for 10 minutes. An occluder was placed around the distal part of the cannula, as previously described. Urine was collected by gentle compression of the bladder from within the abdominal cavity, and the laparotomy wound was closed. All specimens were collected into sterile 10 mL polypropylene containers (Laboratory Supplies [S.A.], 37 Woodlands Tce., Edwardstown, S.A.), and all except blood were immediately frozen at -80 deg C. Prior to freezing, blood was allowed to clot, then centrifuged at 1500 rpm for 10 min. and the supernatant (serum) aspirated and frozen. The pellet of cell debris was discarded. Specimens were processed for urobilinogen assay within 24 hours of collection.

The rats were left for 3 weeks to develop OJ. Under G.A., the laparotomy wound was then re-opened. 2 mL of blood were taken from the IVC. A subcutaneous injection of 3 mL normal saline was given into the left flank, to offset the loss of fluid as blood and bile. The occluder was removed from the cannula, allowing free flow of bile. The bile

was collected for 10 min; this was usually sufficient time to collect 800 uL (the minimum volume required for the urobilnogen assay). Where the total volume collected was less than 800 μL, it was supplemented with de-ionised water to a total of 800 μL (e.g. if there were 240 mL of bile, 560 μL of water were added). The volumes involved were recorded and the assay reading corrected for this amount.

The distal end of the cannula was sutured into the duodenum, using the technique of equilateral triangulation described previously, above. Systemic blood, urine and faeces were then collected as described above. The rats were allowed to awaken, and returned to their cages. Four hours later, under G.A., the same specimens were again collected, together with portal blood and small-bowel contents taken from the mid-point of the small bowel. (Portal blood and small-bowel contents were not collected at the initial operation in order to minimise the surgical insult to the animals). The rats were then killed, and all specimens were assayed for urobilinogen.

3.2.3. Urobilinogen levels after relief of obstructive jaundice in human patients

Like the similar experiments performed with rats, this planned series of experiments did not progress beyond the pilot stage, because as described more fully in the discussion and conclusions chapter, unforeseen (and previously undescribed) problems were encountered with the urobilinogen assay of Kotal and Fevery (1991).

Ethical approval was obtained from the Ethics Committee of The Queen Elizabeth Hospital prior to the experiments. Specimens of urine and serum were obtained from 3 patients with obstructive jaundice prior to, and at intervals after open operation to relieve

obstructive jaundice. These intervals were 6 hours, 24 hours, 48 hours and 96 hours post-operatively. The specimens were then assayed for urobilinogen.

4. RESULTS

4.1. Sepsis and infection

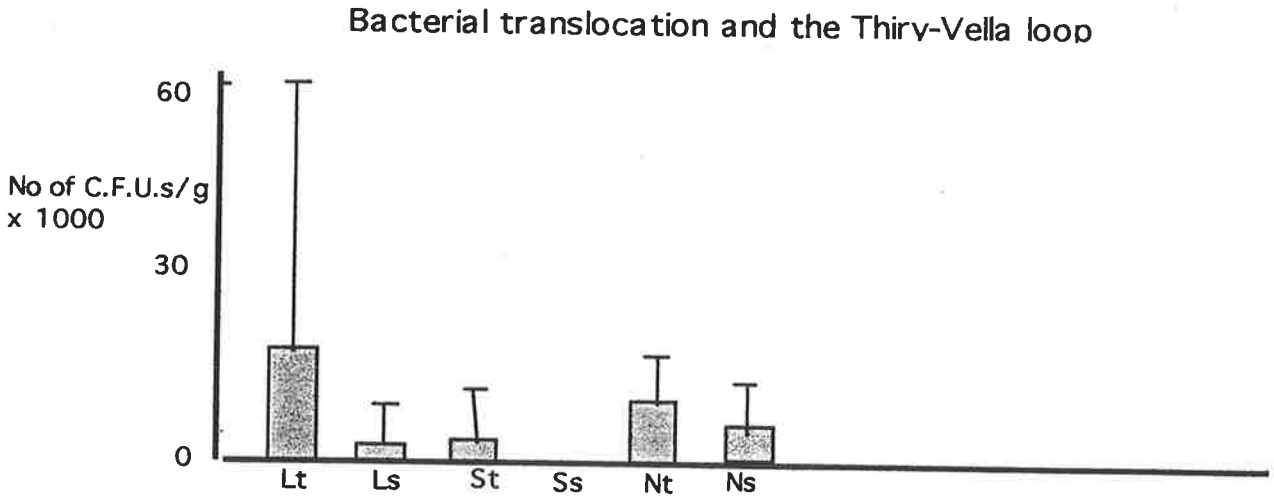
4.1.1. Bacterial translocation

4.1.1.1. Verification of experimental models

Several problems with the experimental models were encountered in this series of experiments. These meant the results could not be interpreted to answer the question the experiment set out to answer. These can be summarised as follows:

(a) The Thiry-Vella loop itself was found to cause bacterial translocation. This translocation probably occurred from both the TVL itself and from the remainder of the gut. However, because of potential contamination of the gut by animals licking their ileostomy sites (see Discussion and Conclusions, p. 197) the site from which translocation occurred is not certain.

Animal	Liver total cfu/g	Liver strep res cfu/g	Spleen total cfu/g	Spleen strep res cfu/g	MLN total cfu/g	MLN strep res cfu/g
1	10.5×10^4	16.6×10^3	20.5×10^3	1.5×10^3	20.6×10^3	15.2×10^3
2	7.4	0	110	18	5.7×10^3	57
3	0	0	103	392	500	500
4	10	0	43	0	18.2×10^3	16×10^3
5	0	0	0	0	6.0×10^3	533
6	647	0	915	0	6.4×10^3	545



LEGEND:

Lt =	total	no.	of	all	organisms	translocating	to	liver	(cfu/g)
Ls =	"	"	"	labelled	"	"	"	"	(")
St =	"	"	"	all	"	"	"	spleen	(")
Ss =	"	"	"	labelled	"	"	"	"	(")
Nt =	"	"	"	all	"	"	"	MLN	(")
Ns =	"	"	"	labelled	"	"	"	"	(")

The above data were analysed with a two-tailed Mann-Whitney U-test (InStat package). The differences between the total number of translocating organisms; and the number of labelled (streptomycin-resistant) organisms were not significant for any organ. [p = 0.2403 (liver), p = 0.3095 (spleen), p = 0.2403 (MLN)].

Histology of Thiry-Vella Loops

An increased diameter was seen in the Thiry-Vella loops to which bile had been diverted, compared to segments of distal ileum from the contiguous bowel. This is consistent with distension in a closed loop of bowel. Otherwise, no histological abnormalities were seen in the Thiry-Vella loops.

(b) The model of reversible obstructive jaundice was found to be unreliable. Although it successfully achieved obstructive jaundice (as shown by significantly elevated serum bilirubin and liver enzymes), reversal of the obstruction apparently occurred in only about half the animals. Since bile was seen to be flowing through the cannula immediately after the moment of release of obstruction, the model appeared to be satisfactory. However, it was felt that definitive confirmation was needed, that the obstruction had truly been relieved. In keeping with published literature on the subject, this was by assay of serum bilirubin and liver enzymes at appropriate time points.

As the graphs below show, it was found that in some animals relief of the obstruction was unsatisfactory. Further animals in this series were therefore autopsied after release of OJ, specifically to clarify this problem, by careful examination of the distal end of the cannula. It was found that particles of debris had collected in the distal end of the cannula and caused obstruction. This problem with the model does not seem to have been described in the literature. This is simply because there appears to be no published work describing insertion of the cannula into the gut. The original descriptions of the model [Burke et al 1977, Diamond and Rowlands 1990(a)] and subsequent published experimental work using the model (e.g. Diamond et al 1990(b), Slocum et al 1992, Clements et al 1996a), only report insertion of the cannula into the bladder. Obviously, the lack of solid matter in the bladder makes the problem of blockage by debris unlikely.

This problem has been reported in human patients with biliary stents, in whom the blockage is frequently due to an adherent mixture of bacterial microcolonies and amorphous material (Dowidar et al 1991). Although the problem usually takes several months to occur in human patients, suggesting a different mechanism of occlusion, the lumen of the rats' cannulae is obviously much narrower than that of human stents. Therefore, it seems reasonable to assume that only a thin layer of debris was needed to block the rat cannulae.

The laboratory that performed the assays of bilirubin, alkaline phosphatase and gamma-glutamyl transferase ('Vetlab', 33 Flemington Rd., Glenside, South Australia, 5065), was unable to provide a reference range for normal rat serum. Specimens were therefore collected from 5 normal rats and assayed for those substances. The results were as follows:

Total serum bilirubin:

Median 1 μmol/L (range 0 - 2)

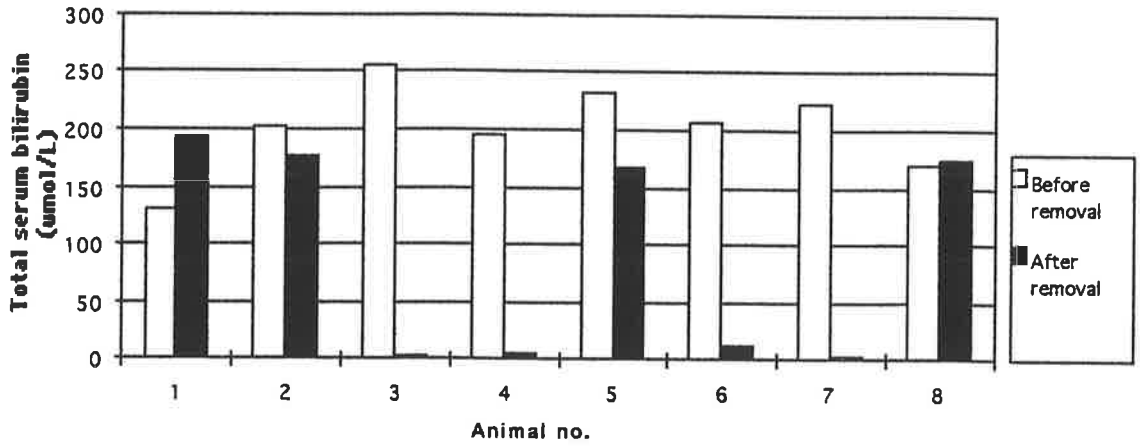
Serum alkaline phosphatase:

Median 116 units/L (range 102 - 219)

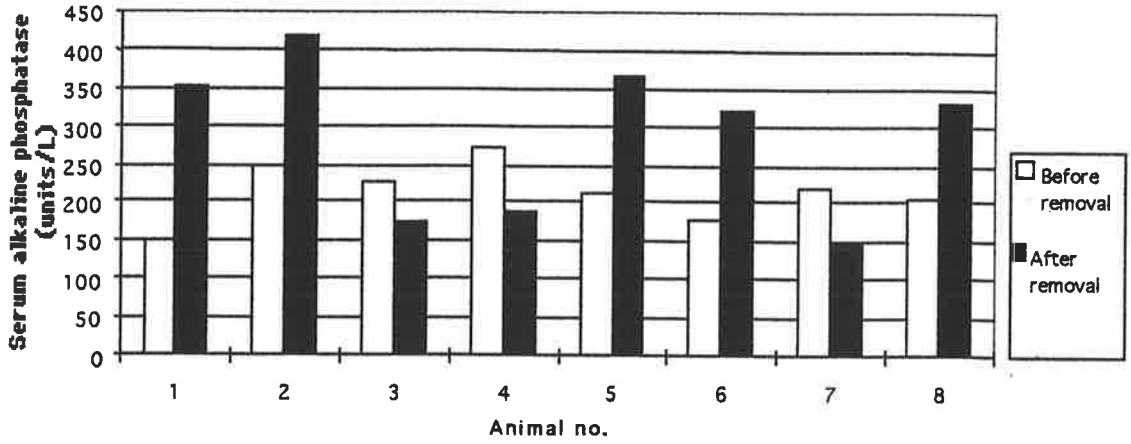
Serum gamma-glutamyl transferase:

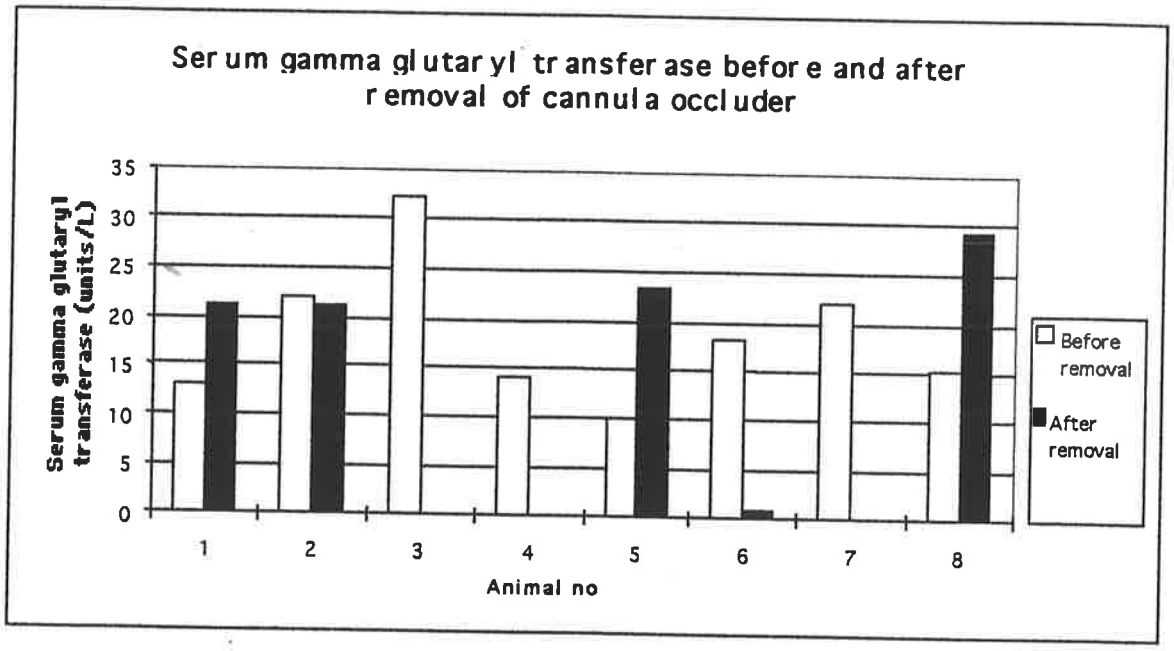
Median 2 units/L (range 0 - 9).

Serum bilirubin before and after release of cannula obstruction



Serum alkaline phosphatase before and after removal of cannula occluder





(c) The combination of TVL, insertion of silastic tubing into the gut or bladder, together with OJ, caused excessive mortality in the rats, not seen when each operation was performed alone. As required by the ethics governing animal experimentation, many of these animals did not actually die of the complications described below. Rather, they were killed because they looked ill, and the complications were discovered at autopsy.

There were many different causes of death (see following pages). The mortality rate in rats with simple OJ is accepted to be around 35% (Allison et al 1978). It is therefore unsurprising that when OJ is combined with other procedures with their own hazards, the mortality rate is high. That is, each individual animal underwent five procedures (TVL, creation of OJ by insertion of obstructed silastic tubing into the bile duct and insertion of the distal end into either the gut or bladder, plus removal of blood via the portal vein and at cardiac puncture), at the initial operation. This was followed by several further procedures (release of the obstruction in the silastic catheter, instillation of labelled bacteria into the gut, and oversewing of the TVL stomas) at the second operation. Finally, a third procedure (the

harvest) was performed. Each individual procedure has its own technical difficulties. Although the probability of a technical failure in any one of these procedures was reasonably low, when all three procedures were combined in a single animal, the probability that at least one error occurred was increased. Also, since the animals were jaundiced they were less likely than healthy animals to tolerate relatively minor errors.

The causes of death were:

(1) Technical failure of the silastic tubing.

This included:

slippage of tubing from bile duct ..... [16]

" " " " site of diversion ..... [6]

bile leak around (apparently intact and enlocated) silastic tubing..... [5]

rat disrupting abdominal wound and pulling tubing through..... [4]

Subtotal ..... [31]

(2) Complication of TVL.

This included:

bowel obstruction ..... [6]

TVL retraction caused by rat chewing through sutures holding stoma in place...[3]

gastric dilatation ..... [2]

parastomal hernia ..... [1]

anastomotic breakdown ..... [1]

Subtotal ..... [13]

(3) Death under anaesthetic.

The cause for this was unclear in most cases, but was presumed to be excessive depth of anaesthesia. In some cases, it occurred shortly after blood was collected via cardiac puncture, and complications of this procedure (e.g. cardiac dysrhythmia, haemopericardium with pericardial tamponade) were suspected as the cause. This method of blood collection was necessary because of the relatively large volume of blood required. Other methods, such as collection from a tail vein, cannot be relied upon to yield such volumes.

Subtotal ..... **[8]**

(4) Other miscellaneous causes.

These included intra-abdominal or wound abscess, inability to release obstruction due to loss of cervical ligature, and exclusion from results because of the presence of intra-intestinal parasites.

Subtotal ..... **[6]**

(5) Unknown (no cause found at autopsy).

Subtotal ..... **[11]**

Total unexpected deaths ..... **[69]**

4.2. Bowel-wall permeability

4.2.1. Rat bile

4.2.1.1. Rat bile: jaundiced, non-infected

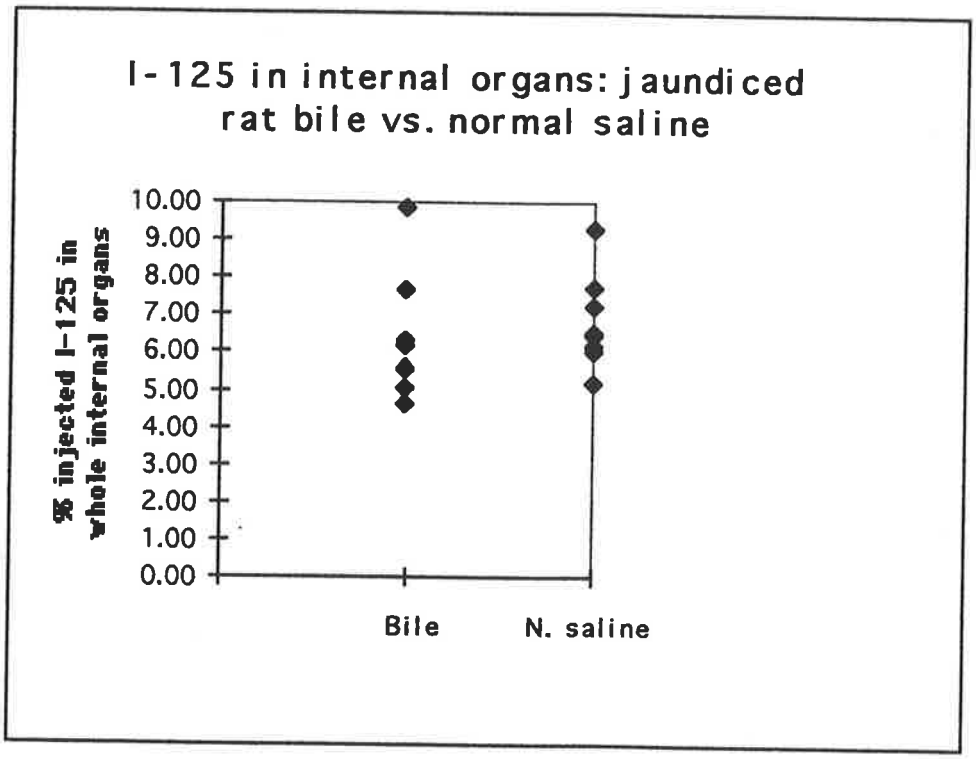
Introduction and aim

The aim of this experiment was to assess the effect on bowel-wall permeability to EDTA and endotoxin, of the return of bile, for 60 minutes, to the intestinal lumen of rats with 7 days of OJ.

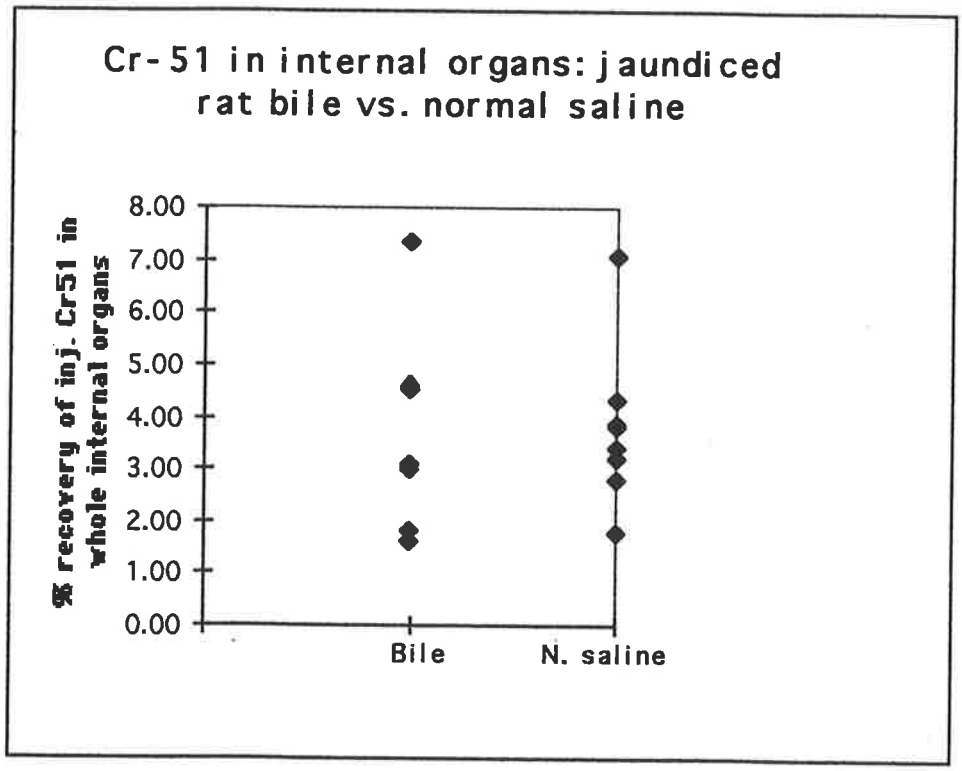
Methods

Using the techniques previously described in the 'Methods' chapter, a silastic cannula was inserted into rats' (n = 7) common bile ducts and blocked. Seven days later, the blockage was released and the resultant flow of bile collected. The bile was then injected into the small-bowel lumen together with radio-labelled endotoxin (I-125) and EDTA (Cr-51). After 60 minutes, the amount of radio-label in the portal and cardiac blood, and in the internal organs (liver, spleen, mesenteric lymph nodes, lungs, kidney and bladder), was counted. A control group of rats (n = 8) received the same treatment except that the collected bile was discarded, and normal saline used in its place.

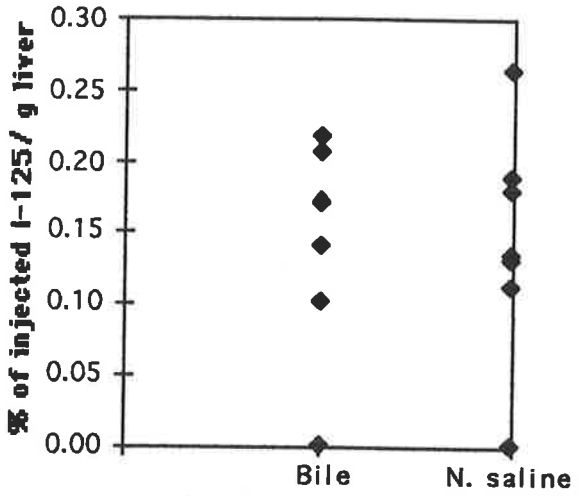
Results



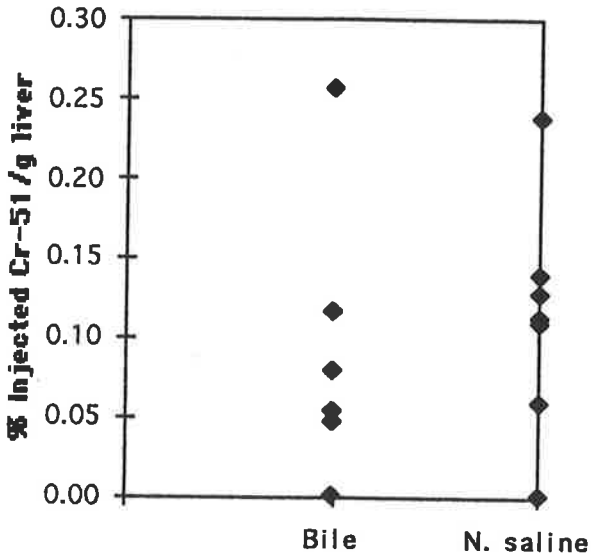
The above data were analysed using a 2-tailed Mann-Whitney U test (InStat statistical package). There was no statistically-significant difference between the two groups ($p = 0.3969$).

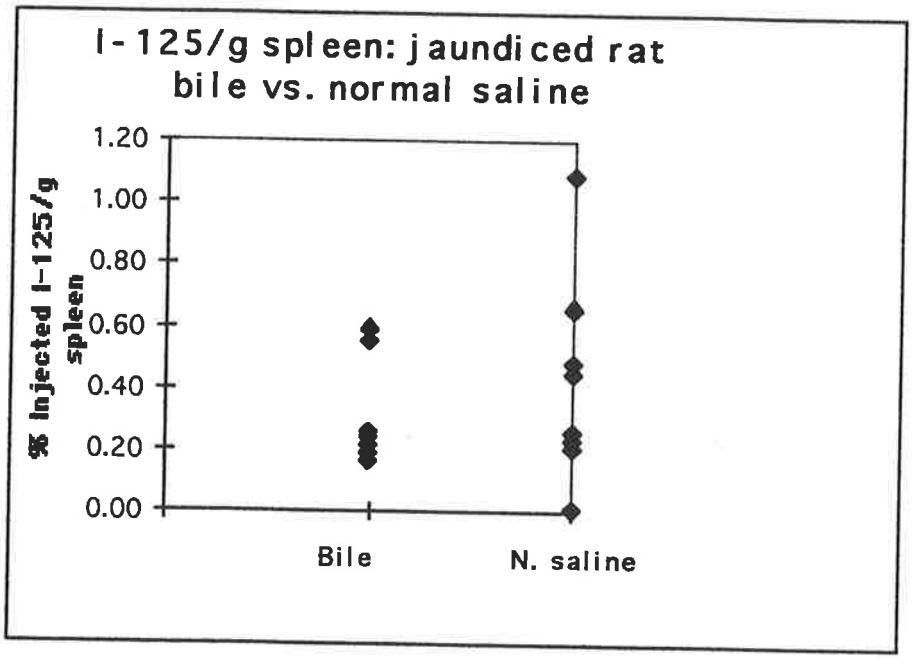

Cr-51 in internal organs: jaundiced rat bile vs. normal saline
% recovery of inj. Cr51 in whole internal organs
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Bile
N. saline

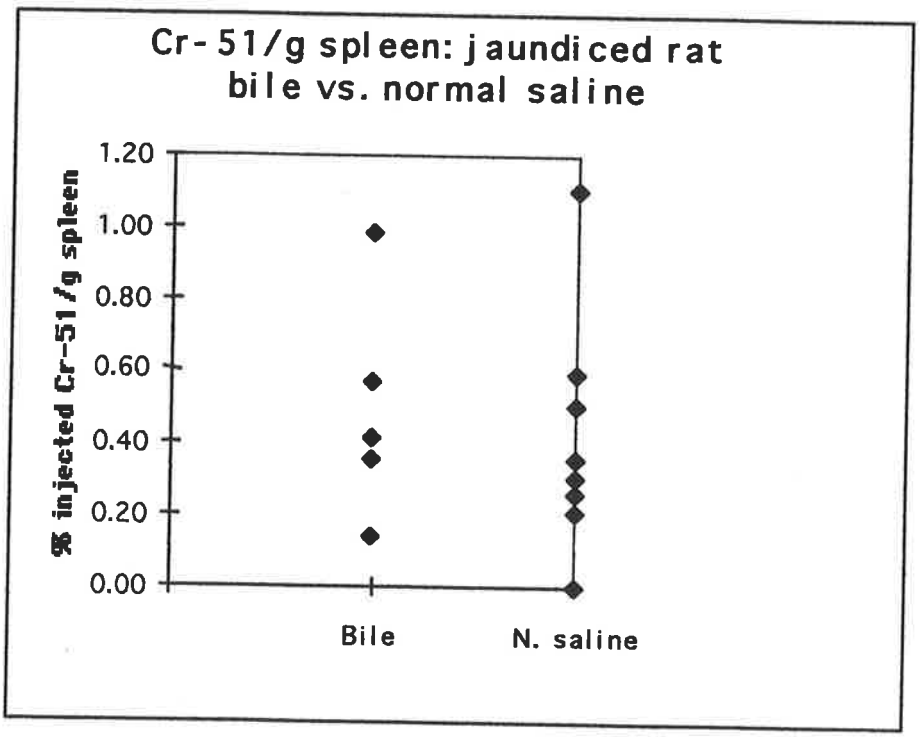
I-125/g liver: jaundiced rat bile vs. normal saline

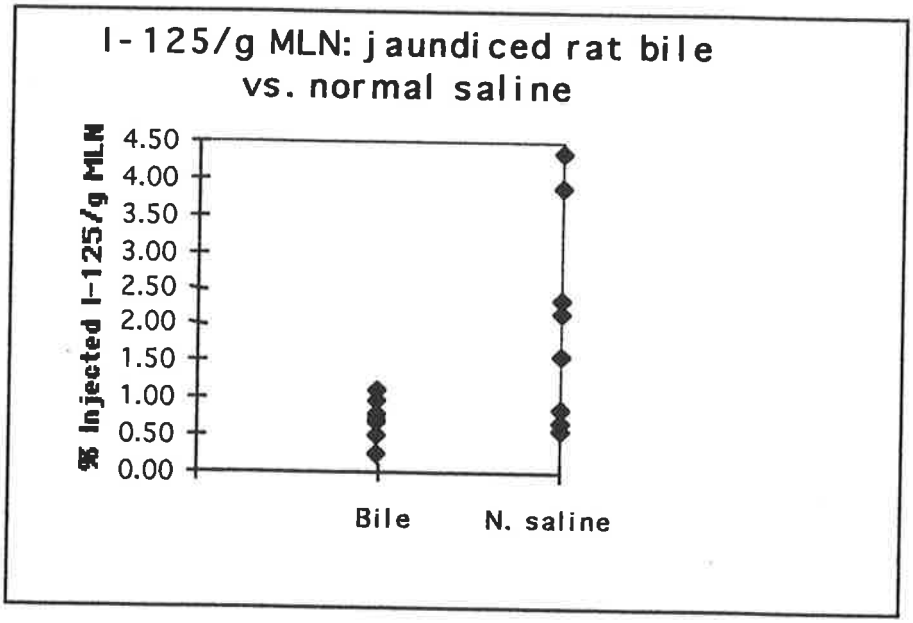


Cr-51/g liver: jaundiced rat bile vs. normal saline

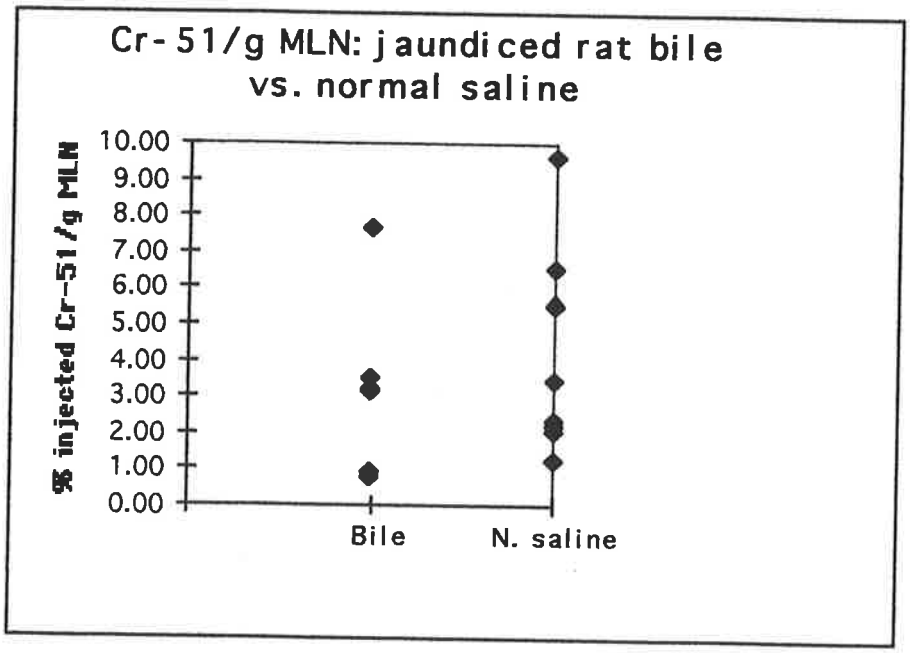


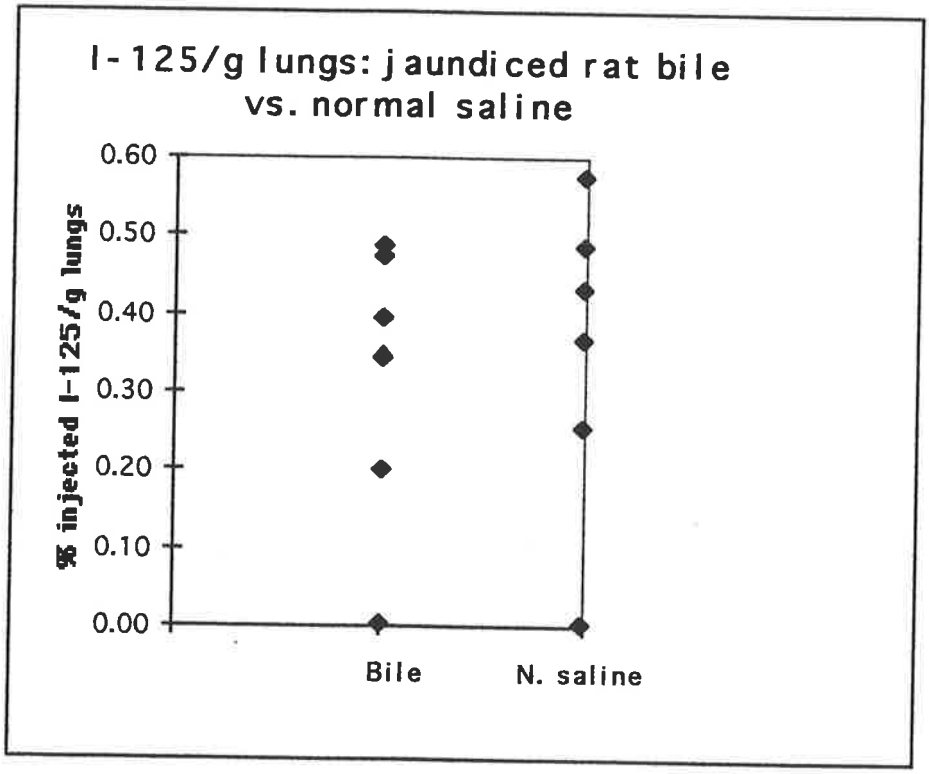

I-125/g spleen: jaundiced rat
bile vs. normal saline
% injected I-125/g spleen
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Bile
N. saline

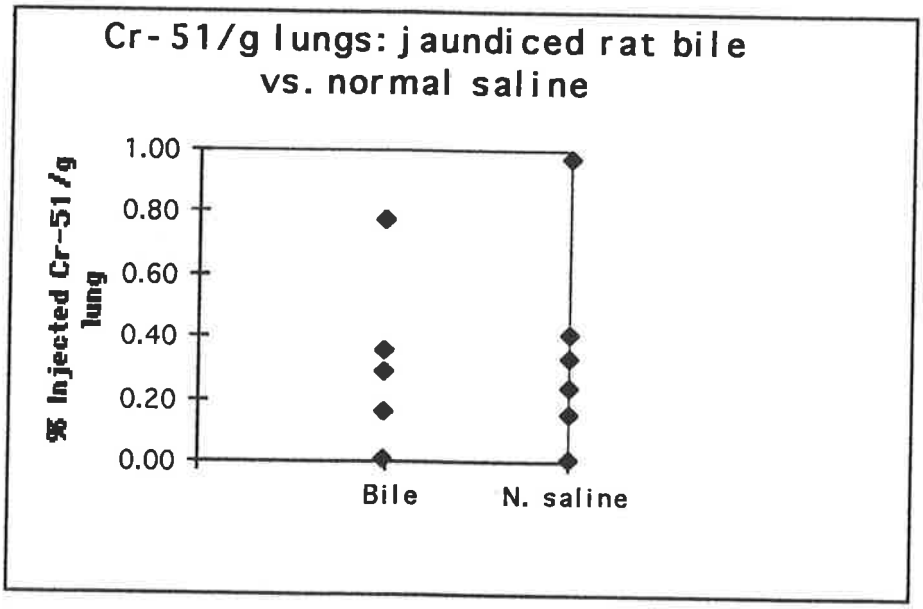

Cr-51/g spleen: jaundiced rat
bile vs. normal saline
% injected Cr-51/g spleen
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Bile
N. saline

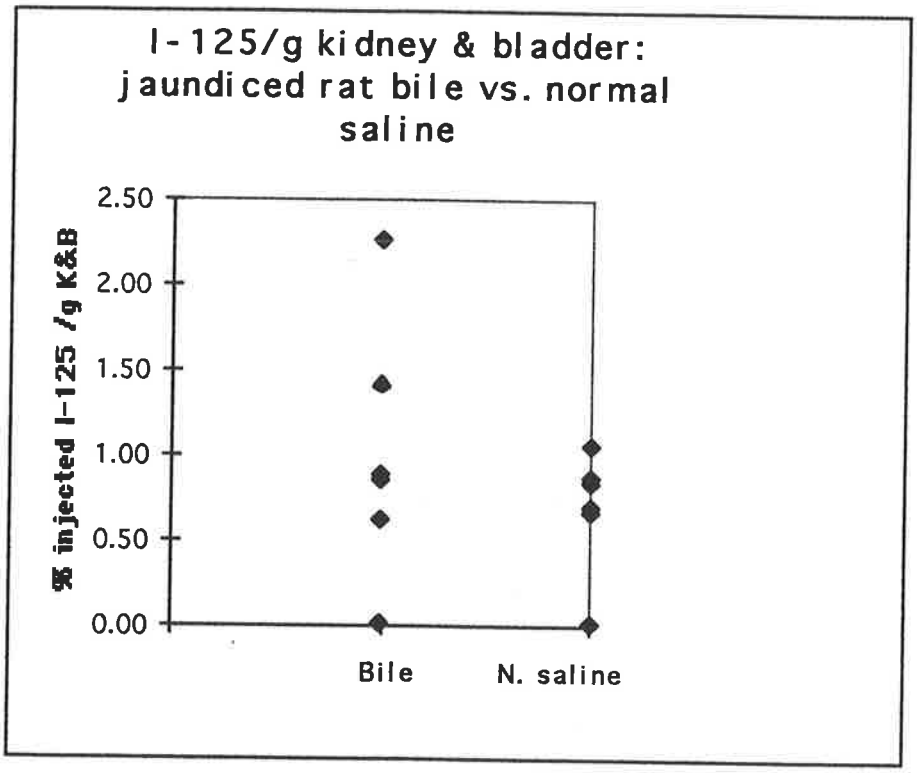


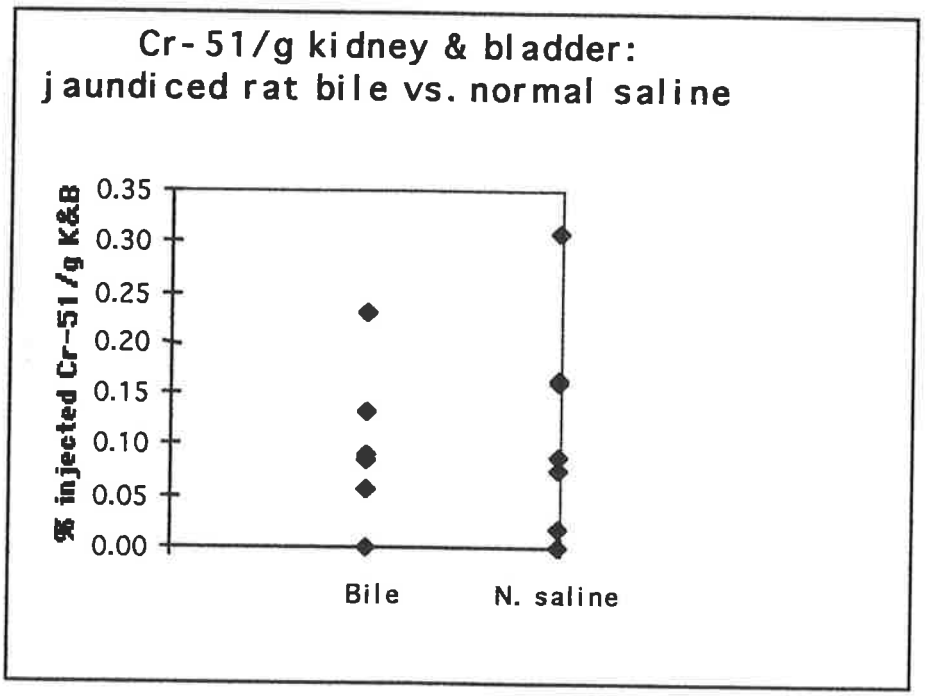
The above data were analysed using a 2-tailed Mann-Whitney U test. There was no significant difference between the two groups ($p = 0.0541$).

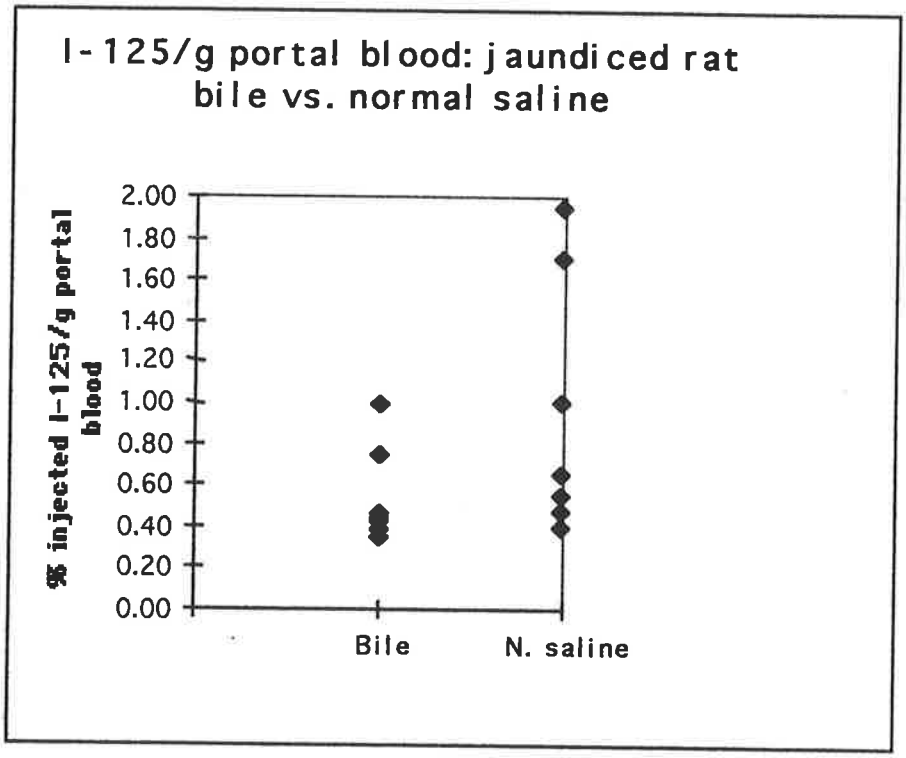


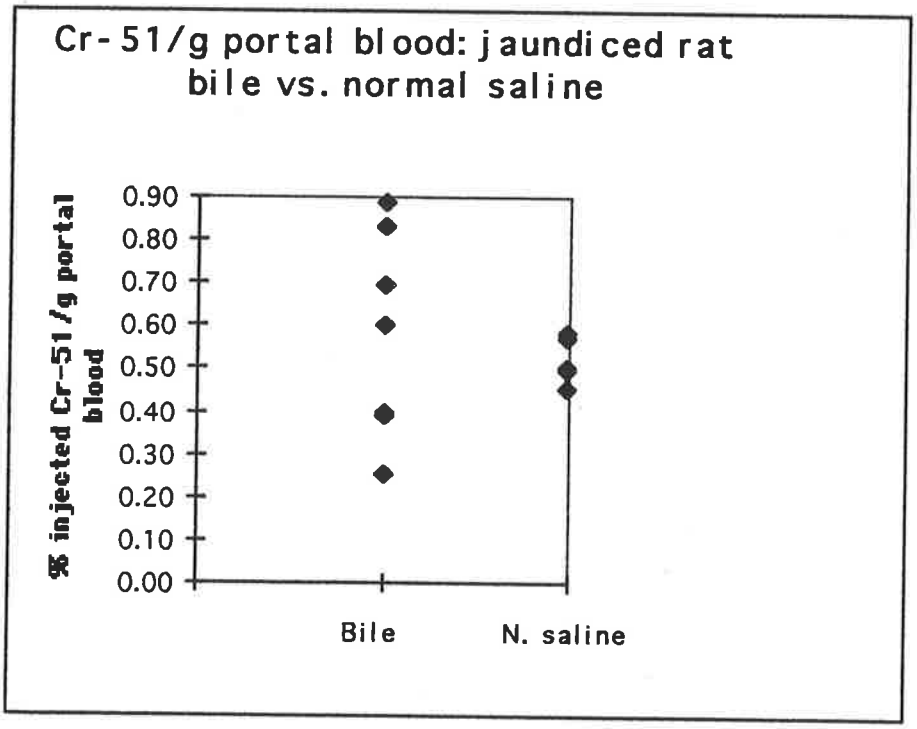

I-125/g lungs: jaundiced rat bile vs. normal saline
% injected I-125/g lungs
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Bile
N. saline

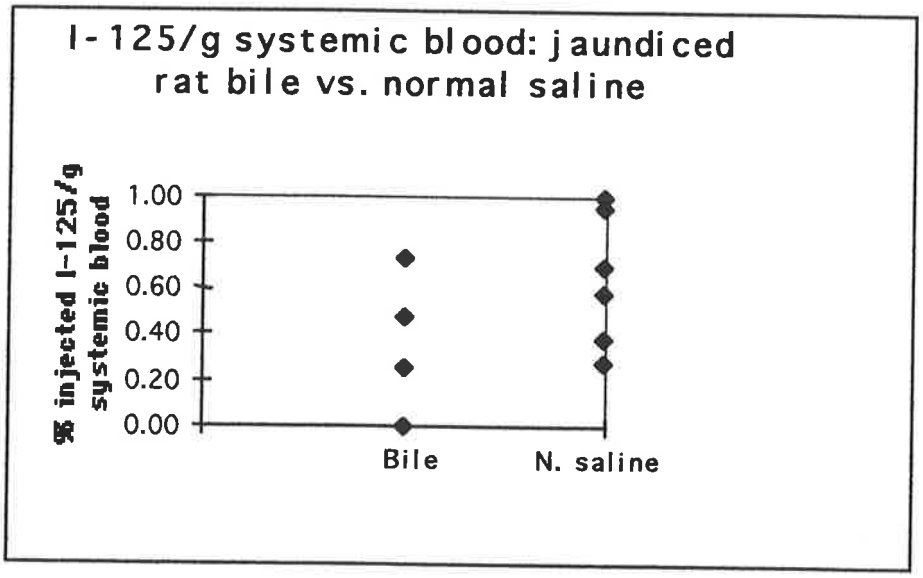

Cr-51/g lungs: jaundiced rat bile vs. normal saline
% Injected Cr-51/g lung
1.00
0.80
0.60
0.40
0.20
0.00
Bile
N. saline

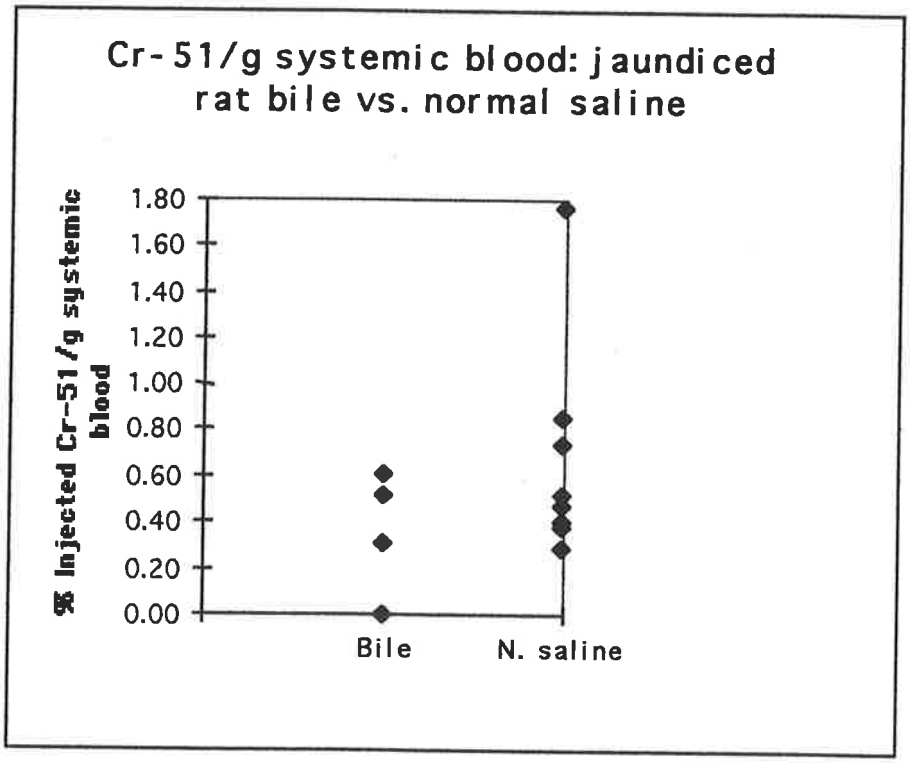

I-125/g kidney & bladder:
jaundiced rat bile vs. normal saline
% injected I-125 /g K&B
2.50
2.00
1.50
1.00
0.50
0.00
Bile
N. saline

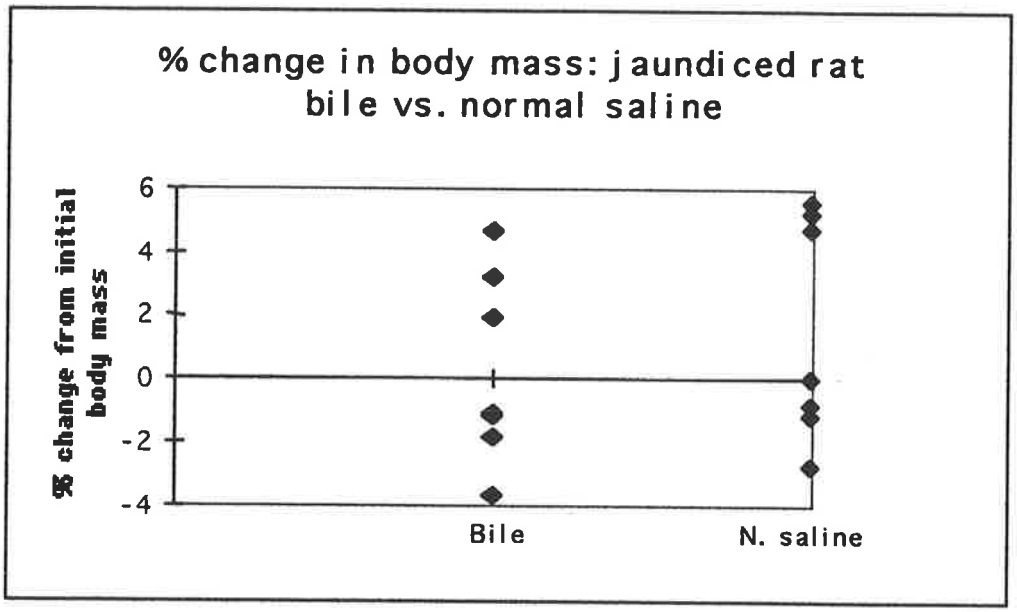

Cr-51/g kidney & bladder:
jaundiced rat bile vs. normal saline
% injected Cr-51 /g K&B
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Bile
N. saline


I-125/g portal blood: jaundiced rat bile vs. normal saline
% injected I-125/g portal blood
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Bile
N. saline

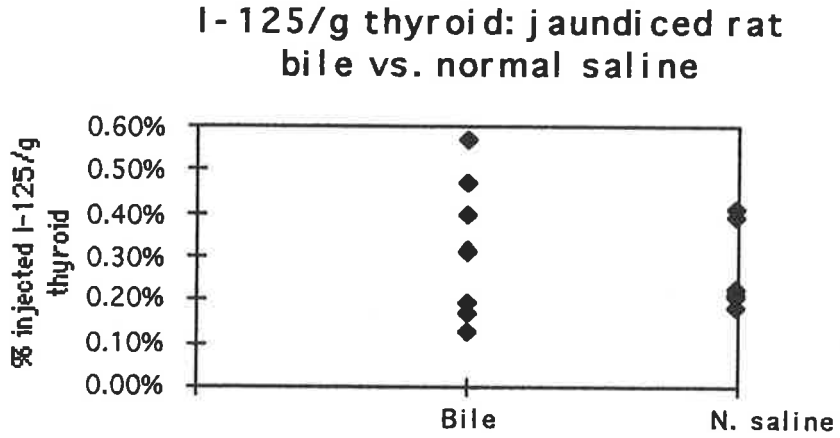

Cr-51/g portal blood: jaundiced rat bile vs. normal saline
% injected Cr-51/g portal blood
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Bile
N. saline


I-125/g systemic blood: jaundiced rat bile vs. normal saline
% injected I-125/g systemic blood
1.00
0.80
0.60
0.40
0.20
0.00
Bile
N. saline


Cr-51/g systemic blood: jaundiced rat bile vs. normal saline
% Injected Cr-51/g systemic blood
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Bile
N. saline



The data above were analysed using a 2-tailed Mann-Whitney U test ('InStat' statistical package). There was no significant difference in body mass change (loss or gain) between the two groups ($p = 0.1897$).



Discussion and conclusions

(a) Return of bile to the intestinal lumen for 60 minutes, after 7 days of obstructive jaundice, did not result in a statistically significant difference in amount of radio-label detected in any of the internal organs, portal or systemic blood, compared to normal saline. This implies that bile did not cause any detectable change in bowel-wall permeability to radio-labelled endotoxin or EDTA compared to normal saline.

(b) The amount of I-125 detected in the thyroid was similar to that detected in other organs of similar size, such as the spleen. Assuming that: (i) a reasonable proportion of the I-125 absorbed from the gut, passed through the systemic circulation (an assumption supported by the finding of appreciable levels of I-125 in the urine), and therefore was present in blood supplied to the thyroid, and (ii) the thyroid takes up free, but not endotoxin-bound I-125, it can be inferred that the I-125 remained bound to the endotoxin in vivo.

(c) The graphs of systemic and portal blood have less than 15 data points. This is because it was not always possible to collect a specimen of adequate volume. Very small specimens (e.g. <100 μL c.f. 500 - 1000 μL in most specimens), tend to give artefactually high readings when calculated as counts per minute (c.p.m.) per mass of tissue, because of the effect of background radiation. The results from such very small specimens were therefore not included.

4.2.1.2. Rat bile: jaundiced and infected

Introduction and aim

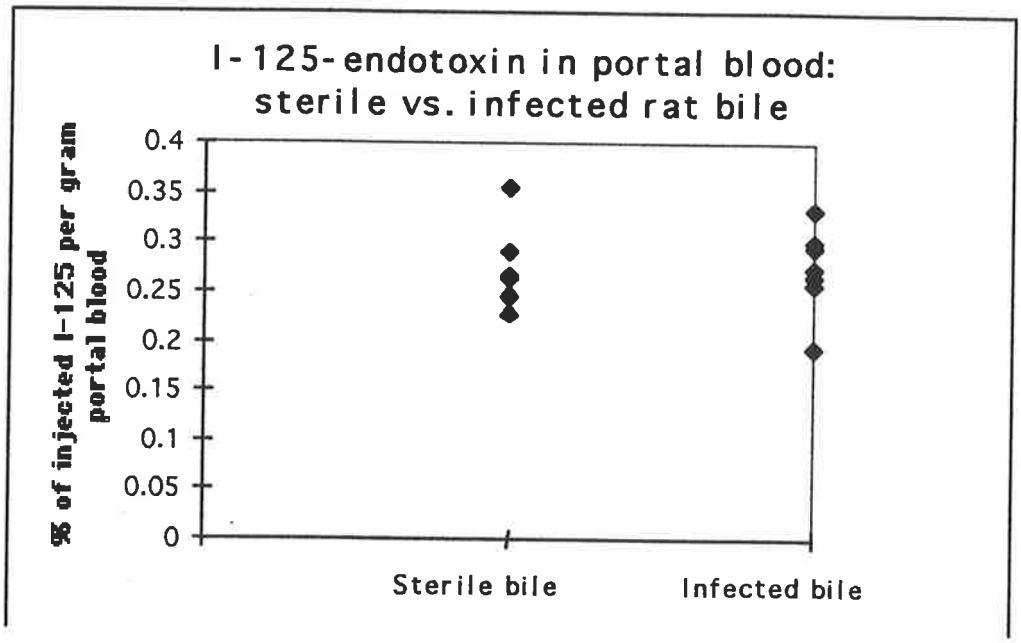
The gut flora has a number of effects on the constituents of bile. In particular, the bacteria are able to alter bile acids by deconjugation and formation of 7-deoxy bile acids (Hofmann 1994). These alterations, or other unknown effects, may cause infected bile to have effects on bowel-wall permeability, not seen in uninfected bile. Therefore, the aim of this experiment was to assess the effect on bowel-wall permeability to EDTA and endotoxin, of the return of infected bile, for 60 minutes, to the intestinal lumen of rats with 8 days of OJ. Escherichia coli was chosen as the infecting organism, because this is the commonest causative organism in cholangitis in many reports (Bismuth et al 1975, Holman et al 1979, Al-Fallouji and Collins 1985, Scott-Conner and Grogan 1994).

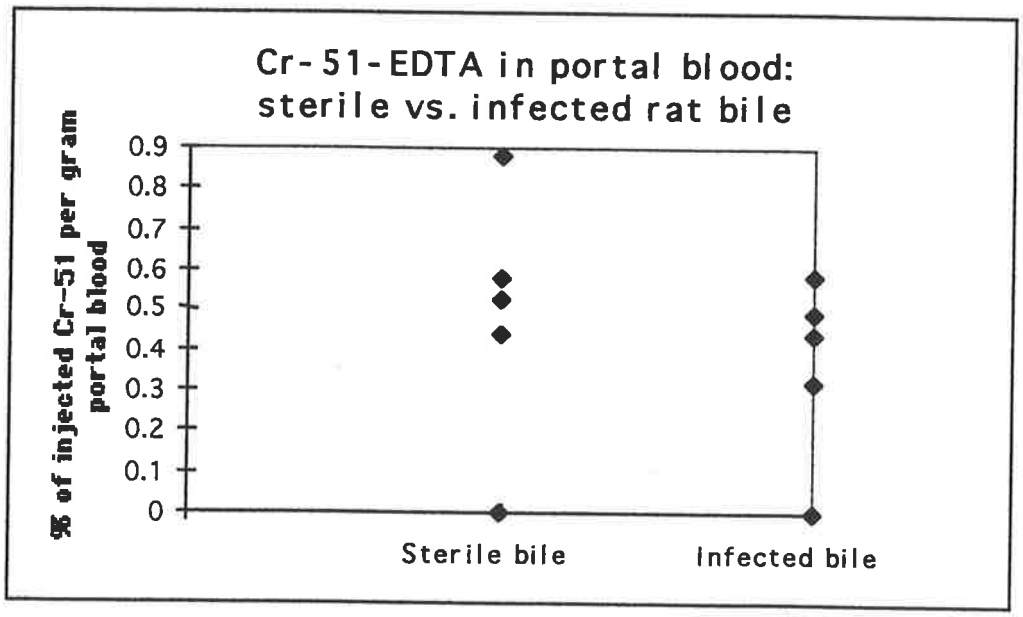
Methods

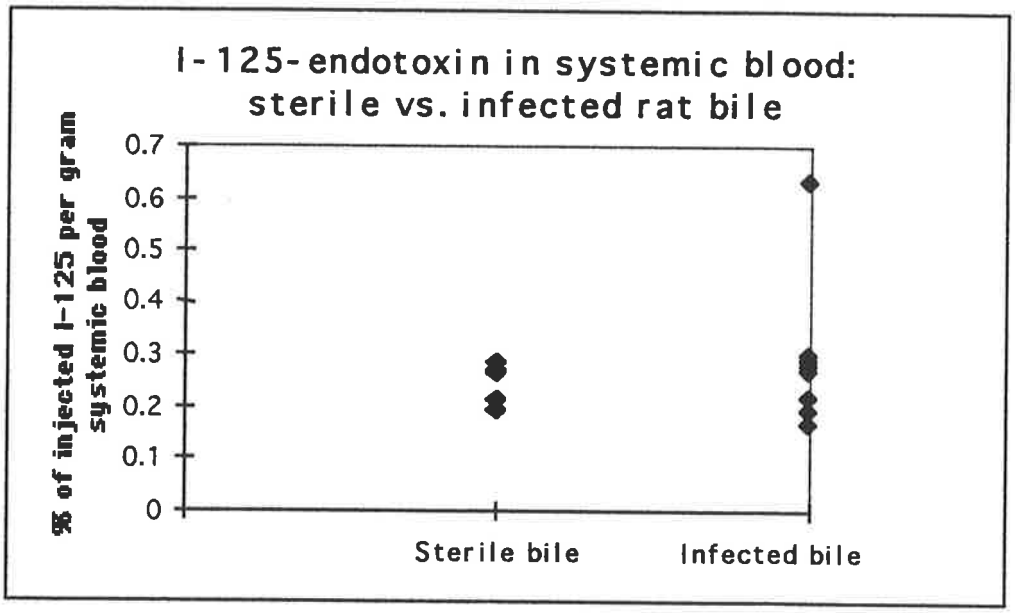
Using the techniques previously described in the 'Methods' chapter, a silastic cannula was inserted into rats' common bile ducts and blocked. Seven days later, the blockage was released and the resultant flow of bile collected into a sterile container. In one group of rats, the bile was inoculated with a single colony of cultured E. coli, strain B111. The bile was incubated for 24 hrs at 37 °C, and allowed to return to room temperature before being injected into the small-bowel lumen, together with radio-labelled endotoxin (I-125) and EDTA (Cr-51). After 60 minutes, the amount of radio-label in the portal and cardiac blood, and in the internal organs (liver, spleen, mesenteric lymph nodes, lungs, kidney and bladder), was counted.

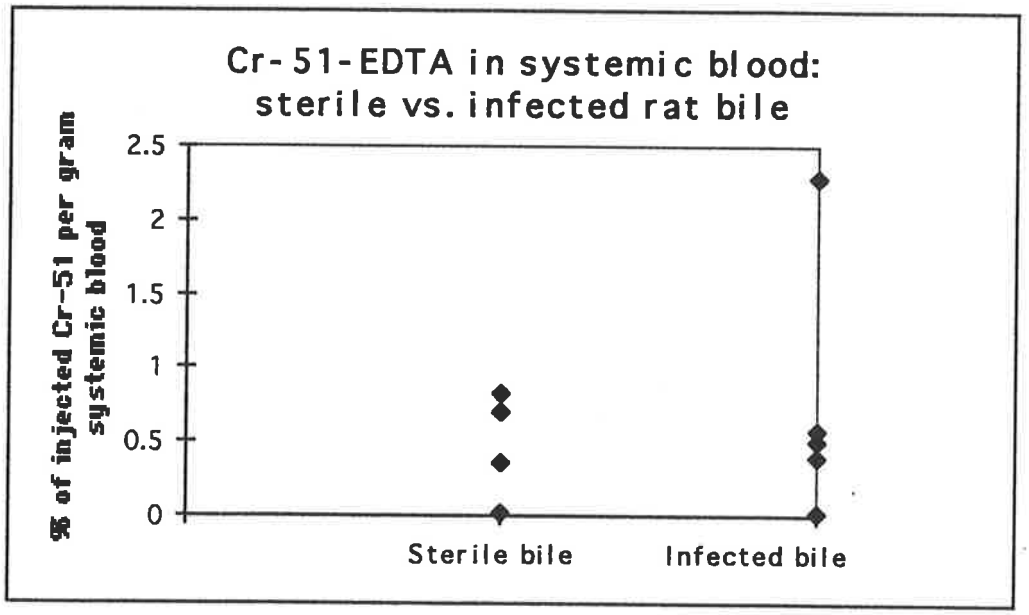
In the control group of rats, the bile was kept sterile, and was simply stored at 4 °C immediately after collection. It was allowed to return to room temperature prior to injection into the gut lumen as per the rats in the 'treatment' group. Formal culture of the bile to confirm growth of organisms, was not performed. However, all infected bile specimens became markedly turbid after the incubation period, implying heavy growth of organisms, while all control bile specimens remained macroscopically clear .

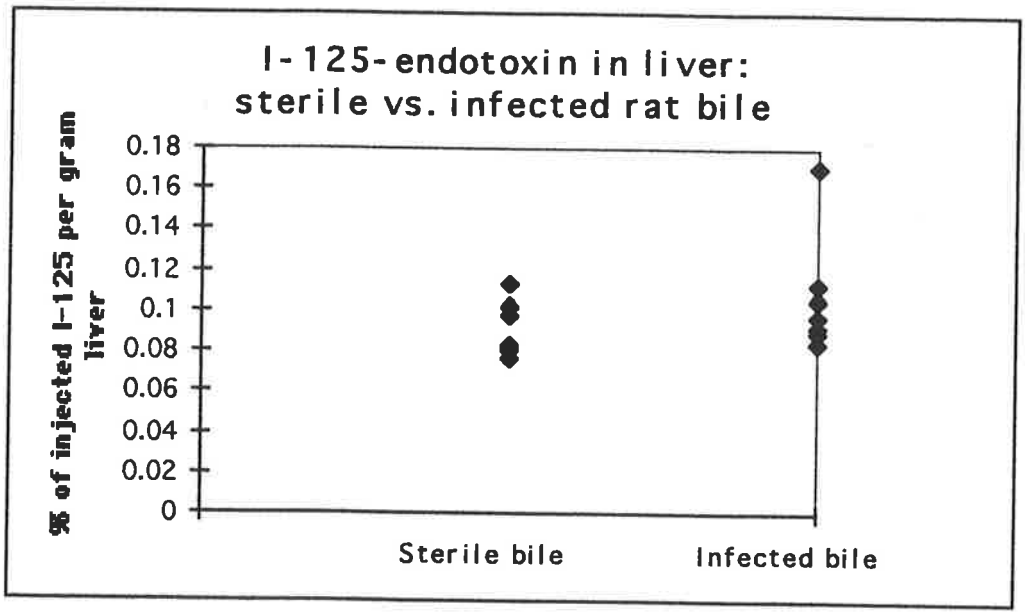
Results:

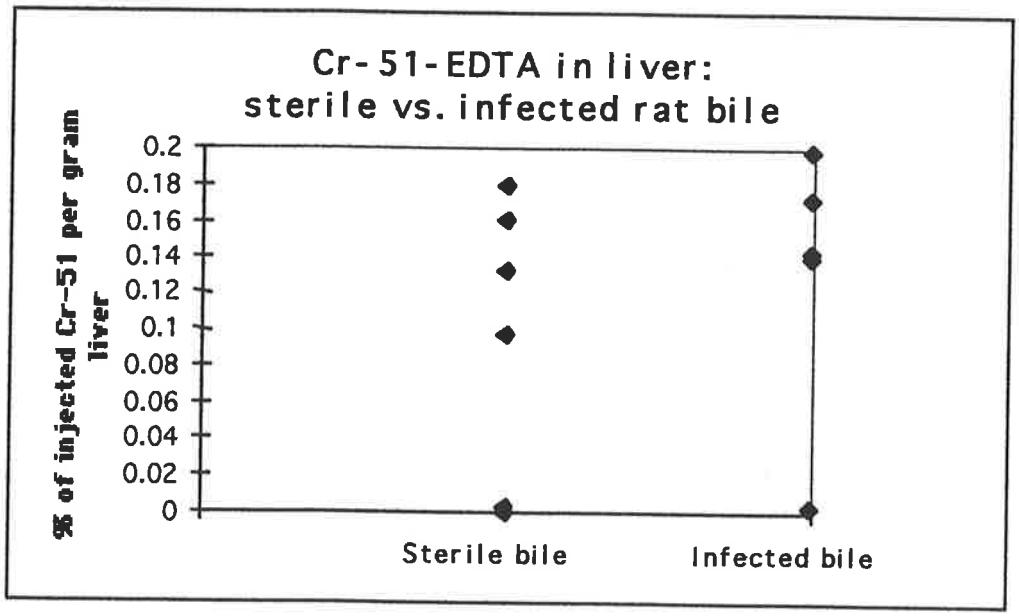

I-125-endotoxin in portal blood:
sterile vs. infected rat bile
% of injected I-125 per gram portal blood
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Sterile bile
Infected bile

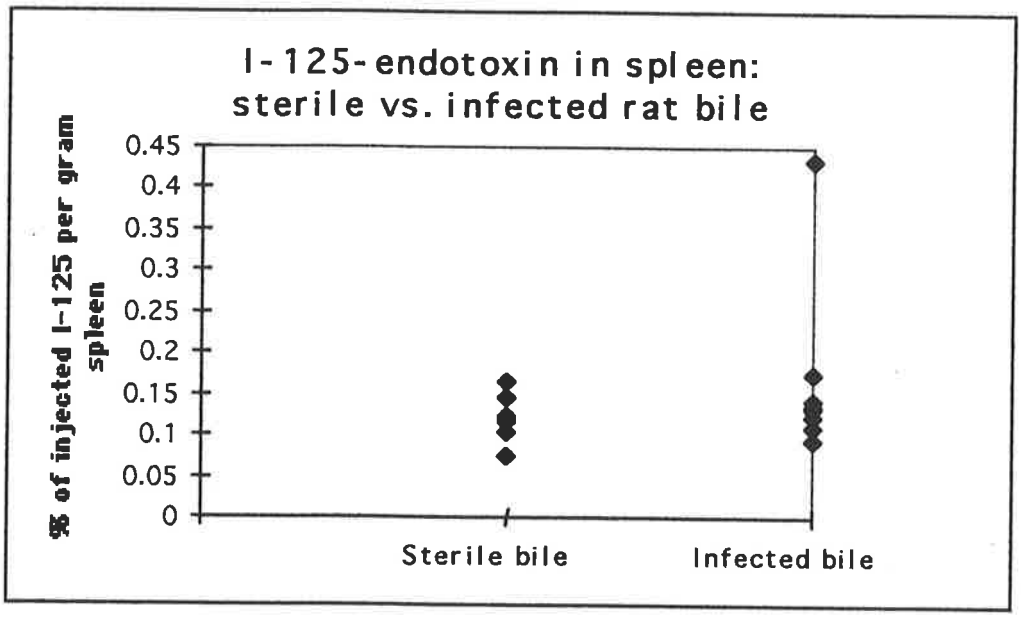

Cr-51-EDTA in portal blood:
sterile vs. infected rat bile
% of injected Cr-51 per gram portal blood
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Sterile bile
Infected bile

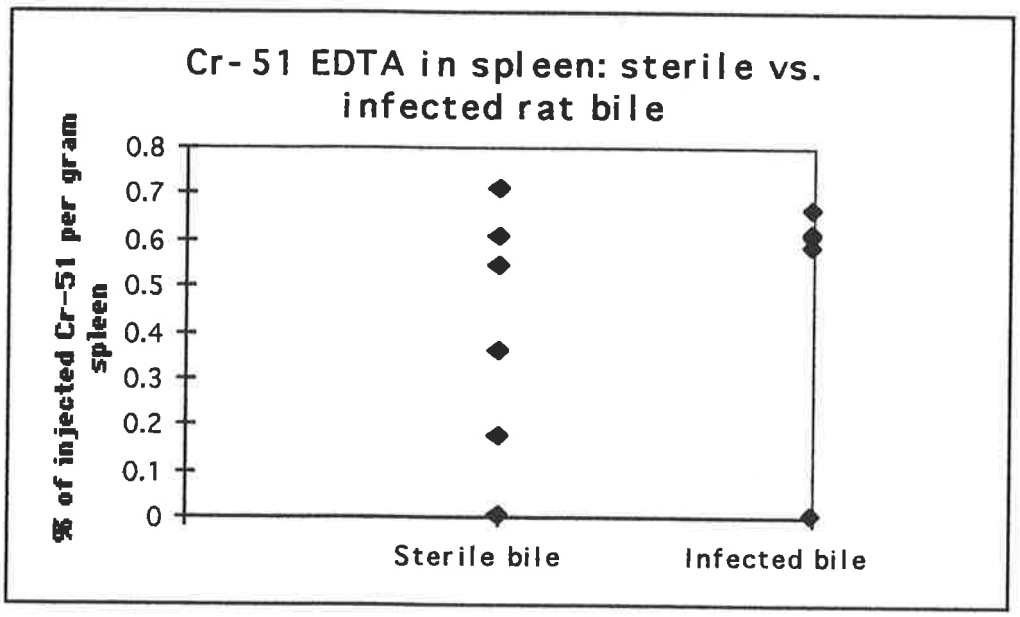

I-125-endotoxin in systemic blood:
sterile vs. infected rat bile
% of injected I-125 per gram systemic blood
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Sterile bile
Infected bile

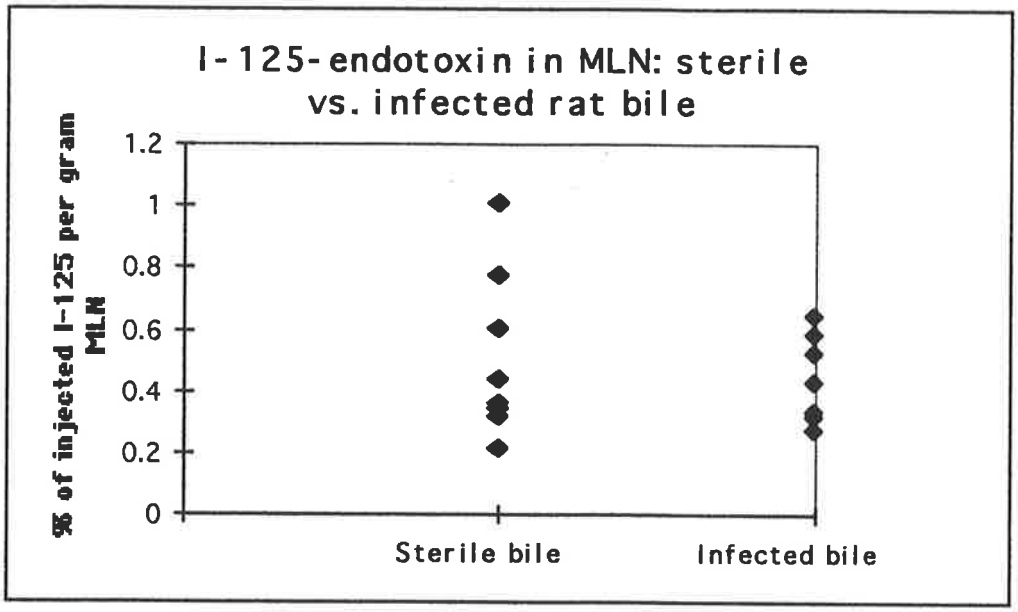

Cr-51-EDTA in systemic blood:
sterile vs. infected rat bile
% of injected Cr-51 per gram systemic blood
2.5
2
1.5
1
0.5
0
Sterile bile
Infected bile

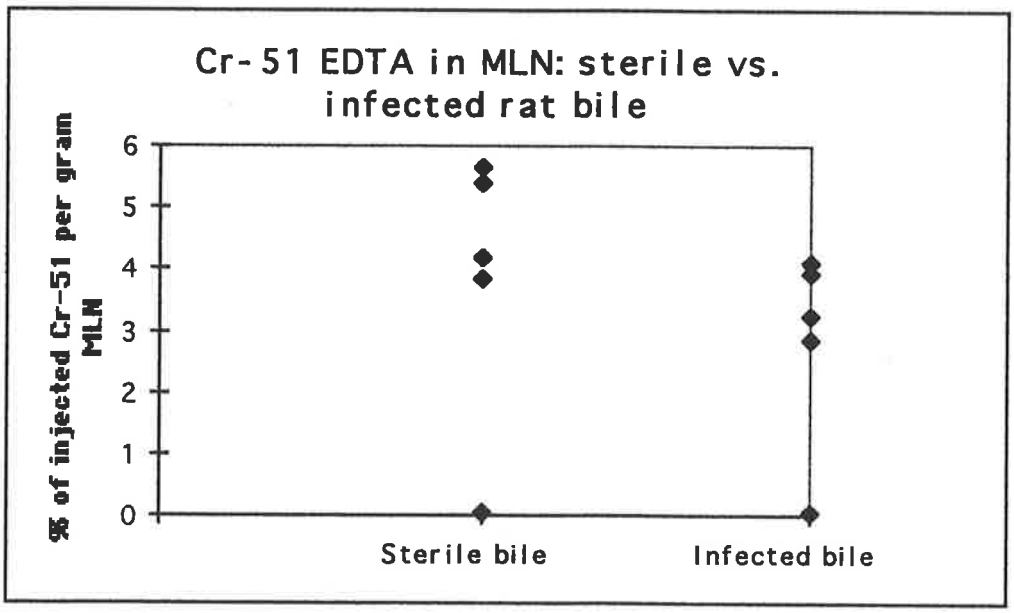

I-125-endotoxin in liver:
sterile vs. infected rat bile
% of injected I-125 per gram liver
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Sterile bile
Infected bile

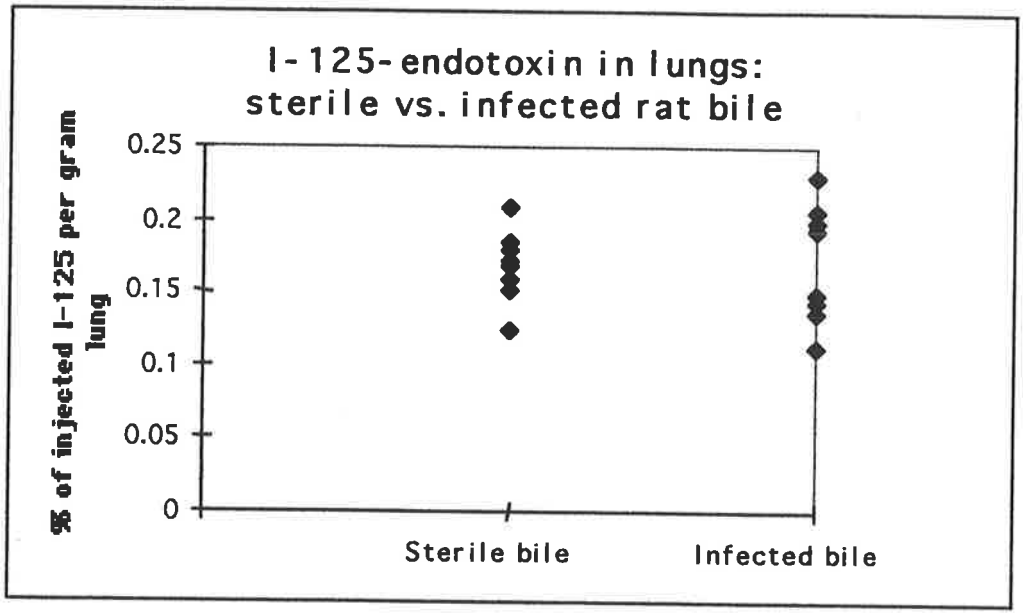

Cr-51-EDTA in liver:
sterile vs. infected rat bile
% of injected Cr-51 per gram liver
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Sterile bile
Infected bile

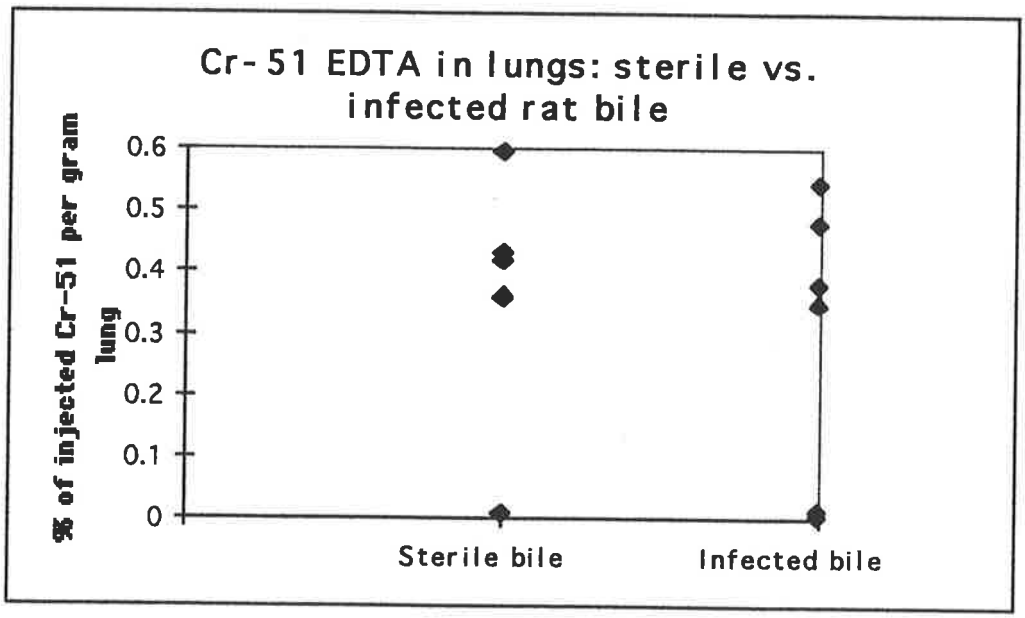

I-125-endotoxin in spleen: sterile vs. infected rat bile
% of injected I-125 per gram spleen
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Sterile bile
Infected bile

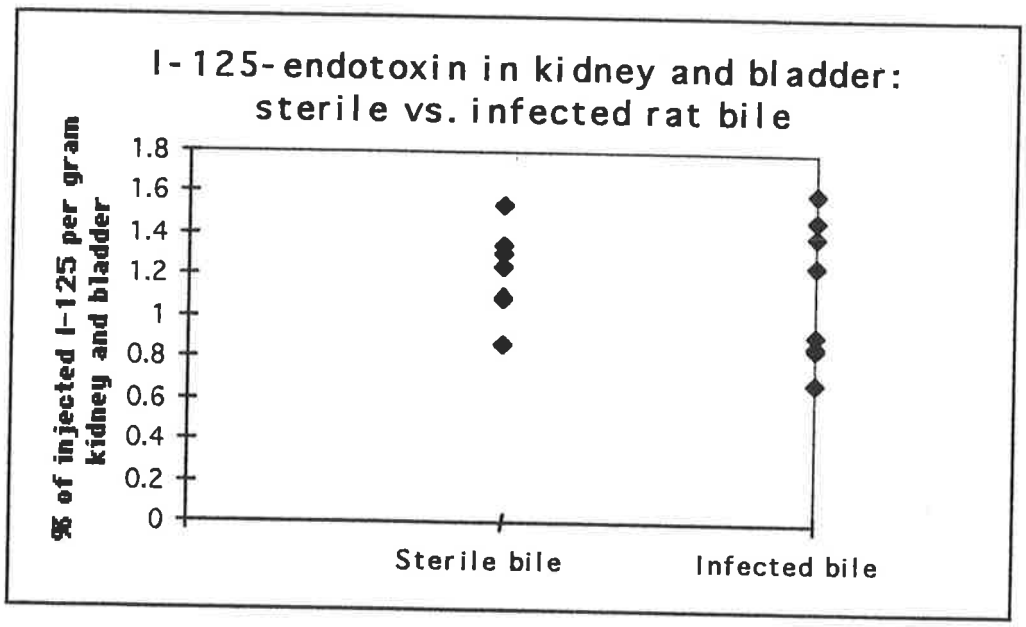

Cr-51 EDTA in spleen: sterile vs. infected rat bile
% of injected Cr-51 per gram spleen
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Sterile bile
Infected bile

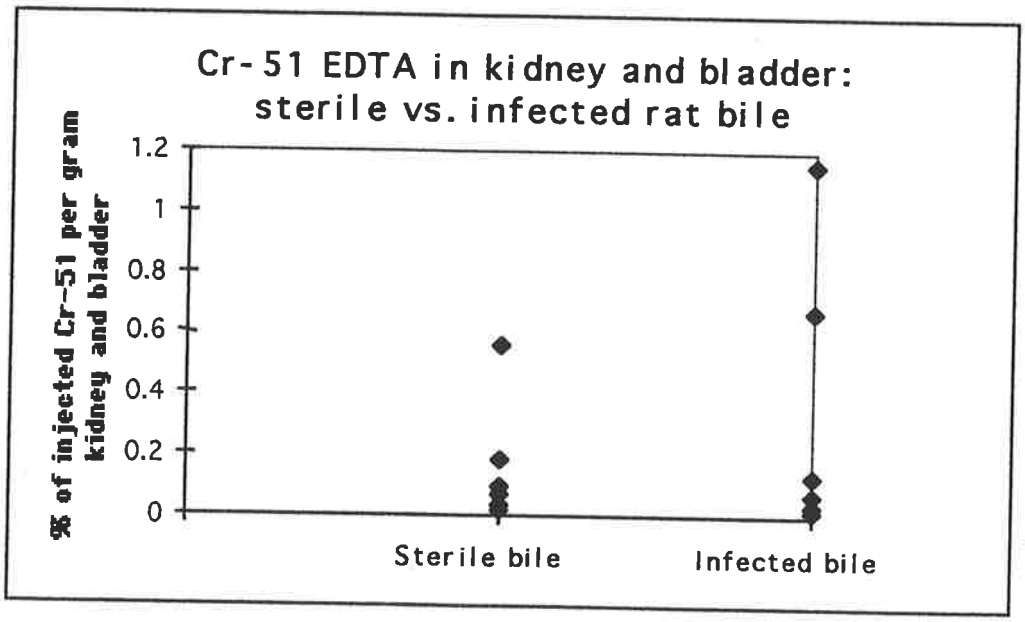

I-125-endotoxin in MLN: sterile vs. infected rat bile
% of injected I-125 per gram MLN
1.2
1
0.8
0.6
0.4
0.2
0
Sterile bile
Infected bile

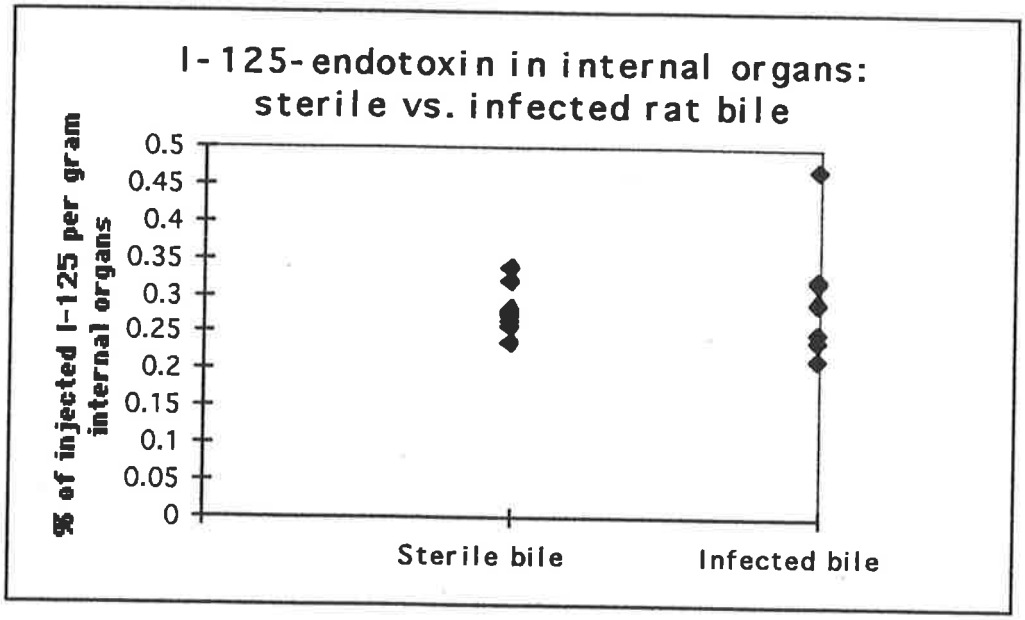

Cr-51 EDTA in MLN: sterile vs. infected rat bile
% of injected Cr-51 per gram MLN
6
5
4
3
2
1
0
Sterile bile
Infected bile

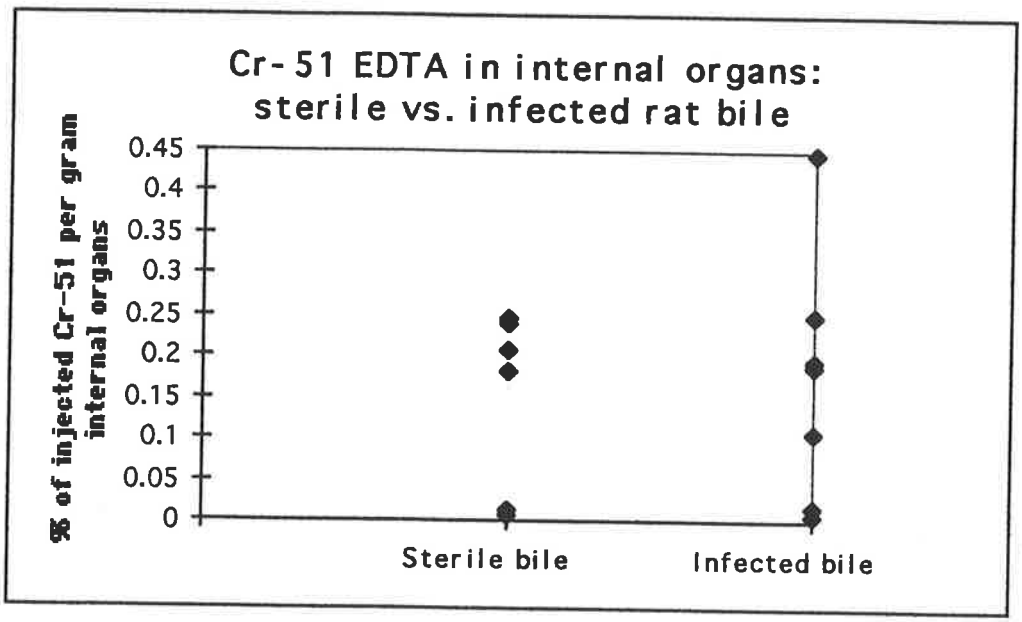

I-125-endotoxin in lungs: sterile vs. infected rat bile
% of injected I-125 per gram lung
0.25
0.2
0.15
0.1
0.05
0
Sterile bile
Infected bile


Cr-51 EDTA in lungs: sterile vs. infected rat bile
% of injected Cr-51 per gram lung
0.6
0.5
0.4
0.3
0.2
0.1
0
Sterile bile
Infected bile


I-125-endotoxin in kidney and bladder: sterile vs. infected rat bile
% of injected I-125 per gram kidney and bladder
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Sterile bile
Infected bile


Cr-51 EDTA in kidney and bladder: sterile vs. infected rat bile
% of injected Cr-51 per gram kidney and bladder
1.2
1
0.8
0.6
0.4
0.2
0
Sterile bile
Infected bile


I-125-endotoxin in internal organs:
sterile vs. infected rat bile
% of injected I-125 per gram internal organs
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Sterile bile
Infected bile


Cr-51 EDTA in internal organs:
sterile vs. infected rat bile
% of injected Cr-51 per gram internal organs
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Sterile bile
Infected bile

Conclusions

The return of bile to the intestinal lumen, for 60 minutes, of bile infected with E. coli did not result in any significant increase in the amount of labelled endotoxin or EDTA detected in the portal or systemic blood, or in any of the internal organs. This suggests that the return of infected bile to the intestinal lumen does not cause a significant alteration of bowel-wall permeability within sixty minutes.

4.2.2. Human bile

4.2.2.1. Human bile: non-jaundiced, non-infected

Introduction

Rats do not have a gallbladder, whereas most human patients do. A major function of the gallbladder is to alter the composition of the bile. Importantly, the gallbladder removes water from the bile, so the concentration of solutes such as bile salts, lipids and bilirubin increases approximately 6 to 10-fold (Shiffman et al 1990, Stolk et al 1995, Holzbach 1984). The higher solute concentration in human bile may result in effects on bowel-wall permeability not seen with rat bile.

Aim

To assess the effect on bowel-wall permeability to EDTA and endotoxin, of placing normal human bile, for 60 minutes, in the intestinal lumen of rats with 8 days of OJ.

Methods

Using the techniques previously described in the 'Methods' chapter, rats' common bile ducts were doubly ligated and divided. Seven days later, bile collected from human patients was injected into a loop of ileum, together with radio-labelled endotoxin (I-125) and EDTA (Cr-51). After 60 minutes, the amount of radio-label in the portal and cardiac blood, and in the internal organs (liver, spleen, mesenteric lymph nodes, lungs, kidney and

bladder), was counted. In the control group of rats, normal saline was substituted for the bile.

Human bile specimens

For ethical reasons, it is very difficult to obtain truly normal human bile. However, bile was obtained from the following patients:

Patient 1

A 52 year-old woman underwent gastric stapling for morbid obesity. [Patients undergoing these operations have been regarded as having 'normal' bile by at least one recent study of gallbladder bile (Shiffman et al 1990)]. The patient had no known history of hepatobiliary disease, and pre-operative serum total bilirubin was <20 μmol/L. Gallbladder bile was obtained intra-operatively by fine-needle aspiration.. The bile from this patient was used in four animals, while that from the patients below was used in one animal each.

Patient 2

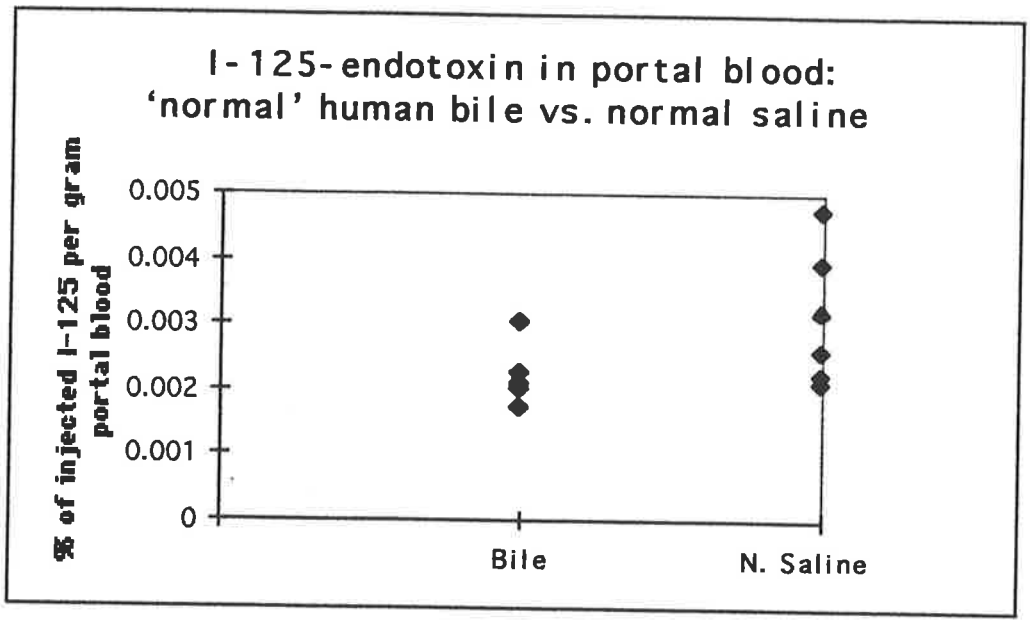
A 60 year old woman underwent ERCP for investigation of recurrent right upper quadrant pain, and further investigation of a 'bulky pancreas' reported on CT scan of the abdomen. ERCP was unremarkable; bile duct bile was collected by aspiration via the endoscope. Total serum bilirubin was <20 μmol/L.

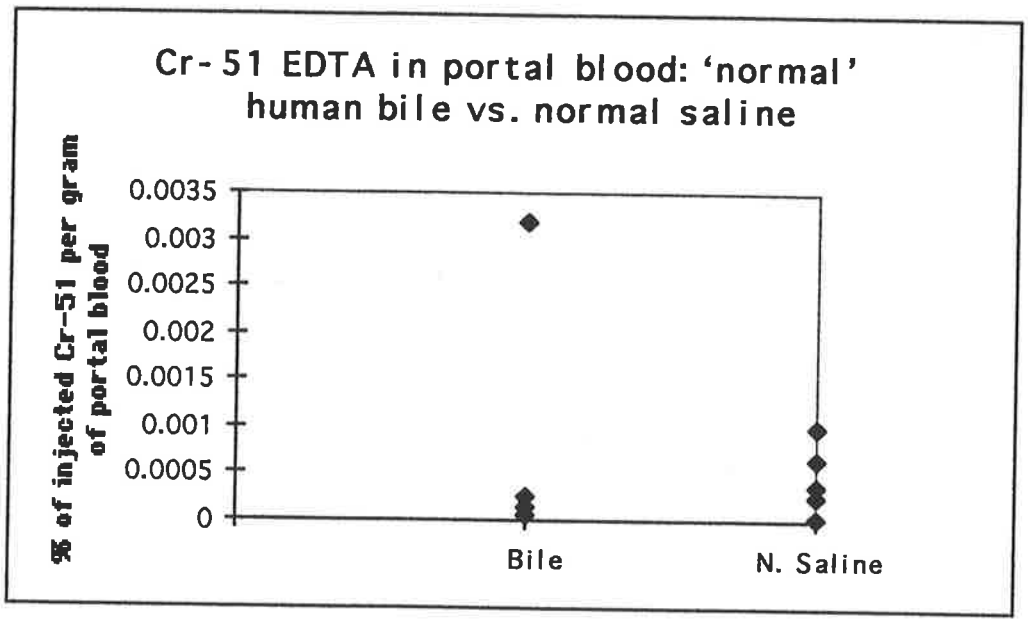
Patient 3

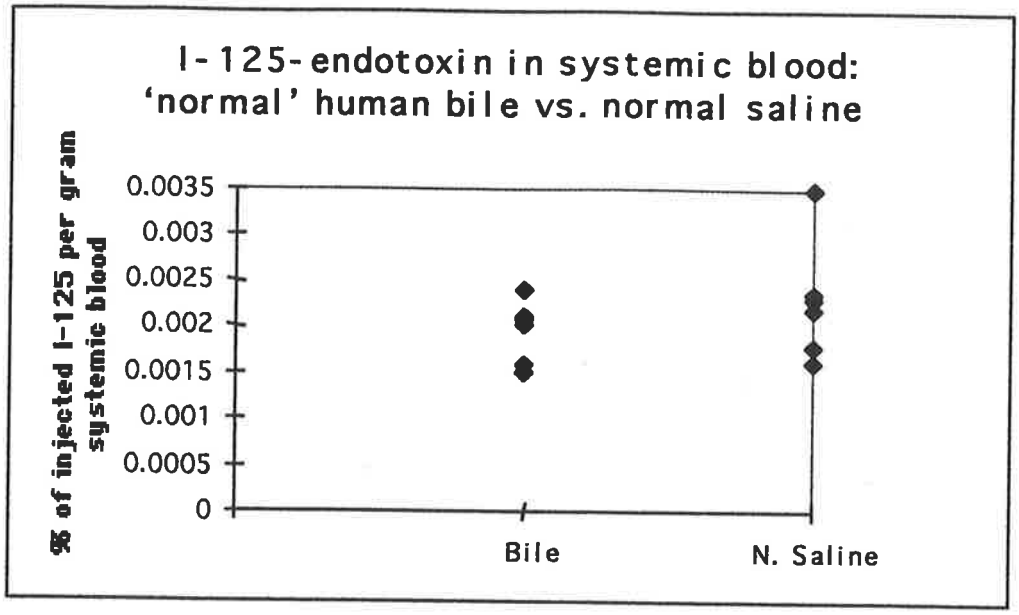
A 44 year-old woman with known gallstones, awaiting elective cholecystectomy, underwent ERCP for investigation of a slightly dilated bile duct seen on ultrasound examination. ERCP was unremarkable except for the presence of a prominent sphincter of

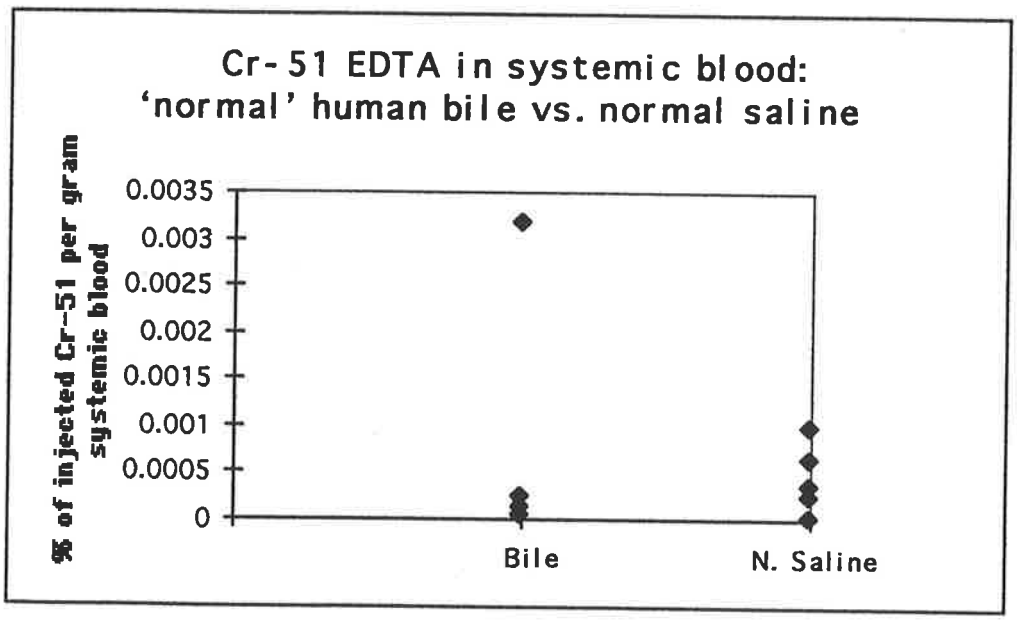
Oddi, which was felt to be the likely cause of the slight dilatation of the bile duct. Bile duct bile was collected by aspiration via the endoscope. Total serum bilirubin was <20 μmol/L.

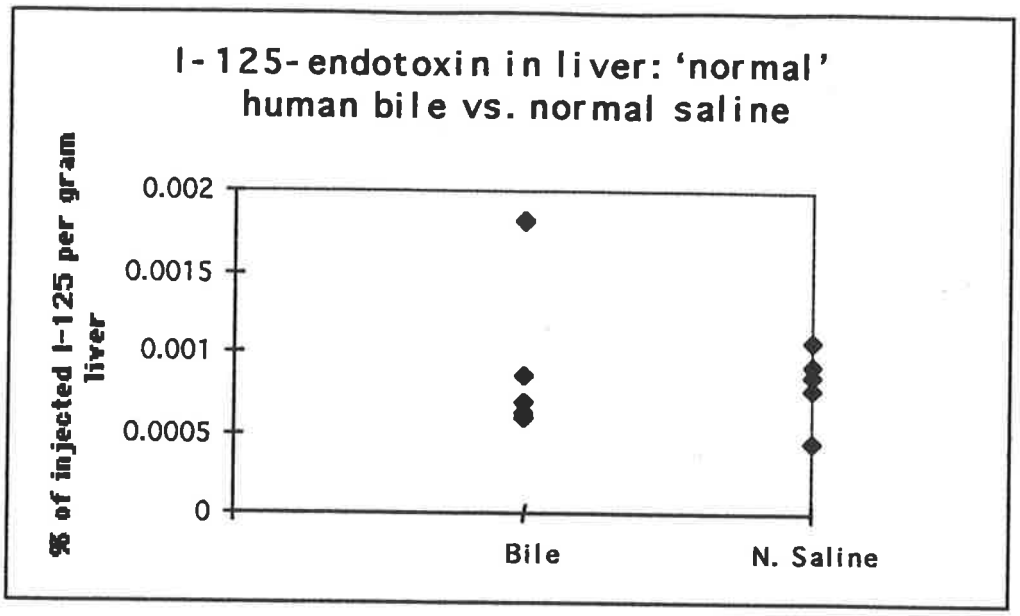
Results

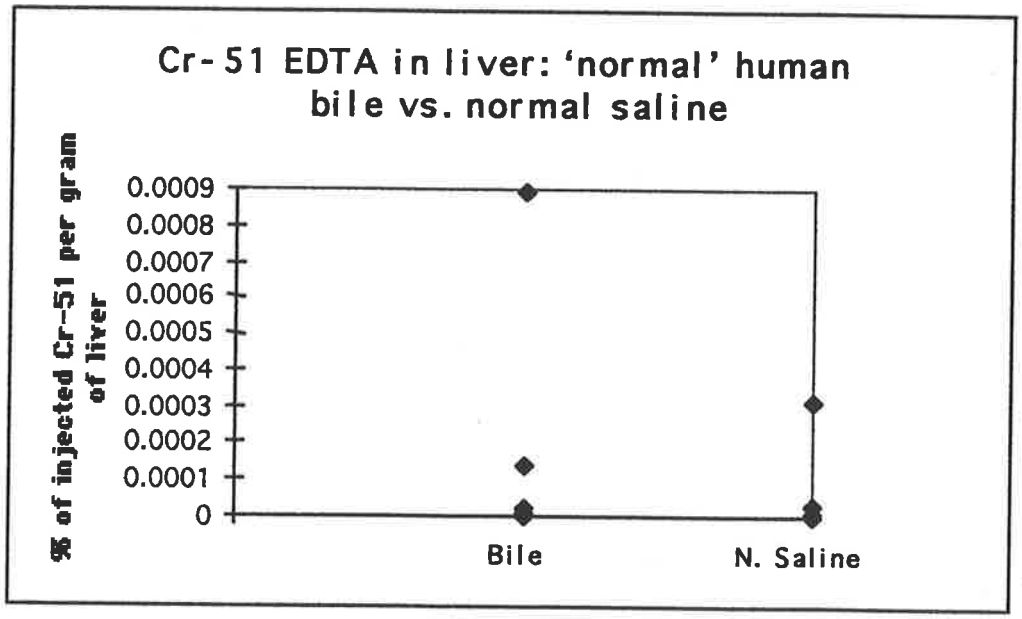

I-125-endotoxin in portal blood: 'normal' human bile vs. normal saline
% of injected I-125 per gram portal blood
0.005
0.004
0.003
0.002
0.001
0
Bile
N. Saline

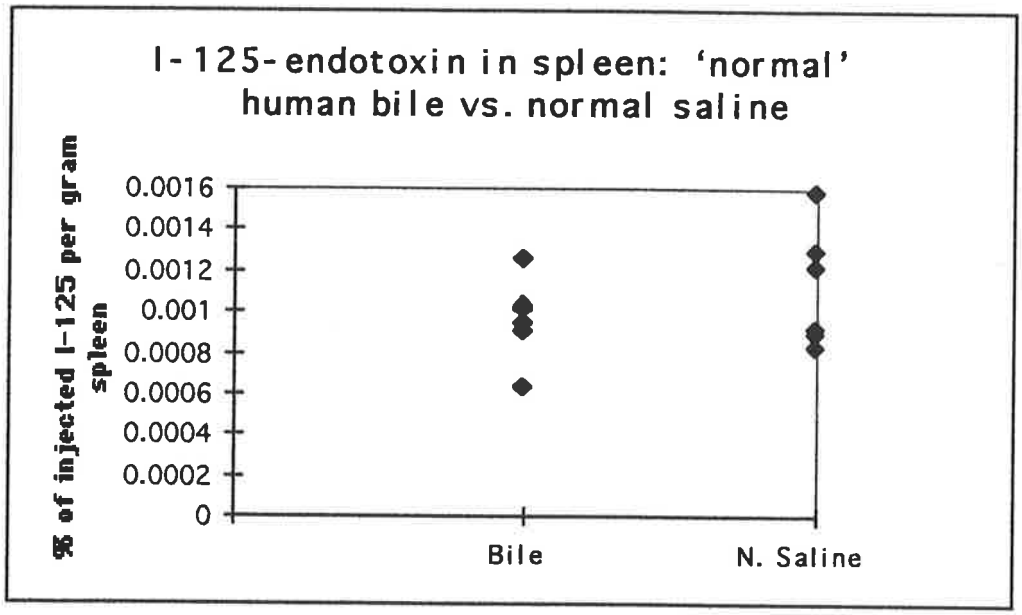

Cr-51 EDTA in portal blood: 'normal' human bile vs. normal saline
% of injected Cr-51 per gram of portal blood
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
Bile
N. Saline

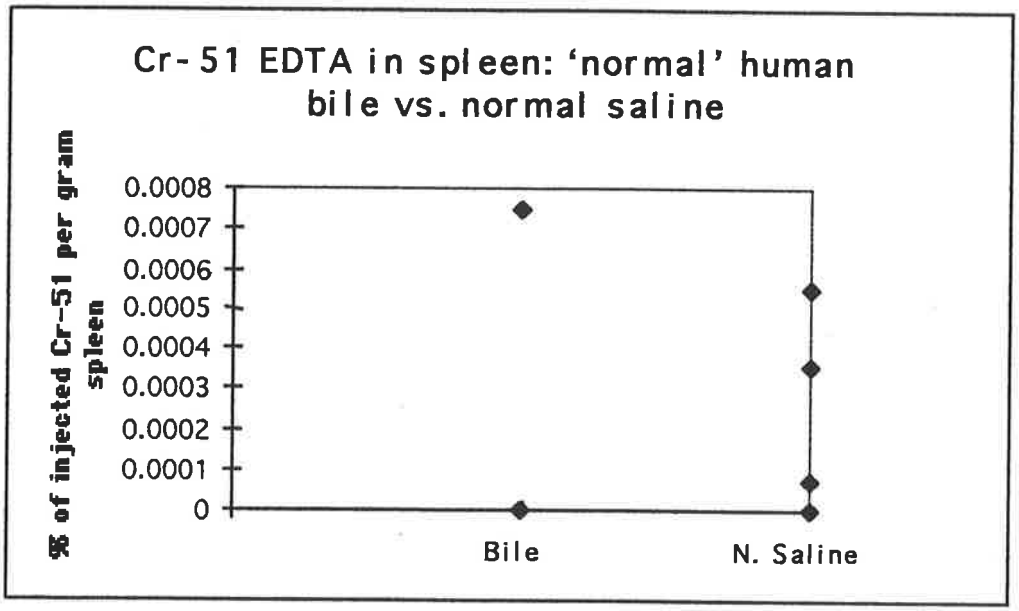

I-125-endotoxin in systemic blood:
'normal' human bile vs. normal saline
% of injected I-125 per gram systemic blood
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
Bile
N. Saline

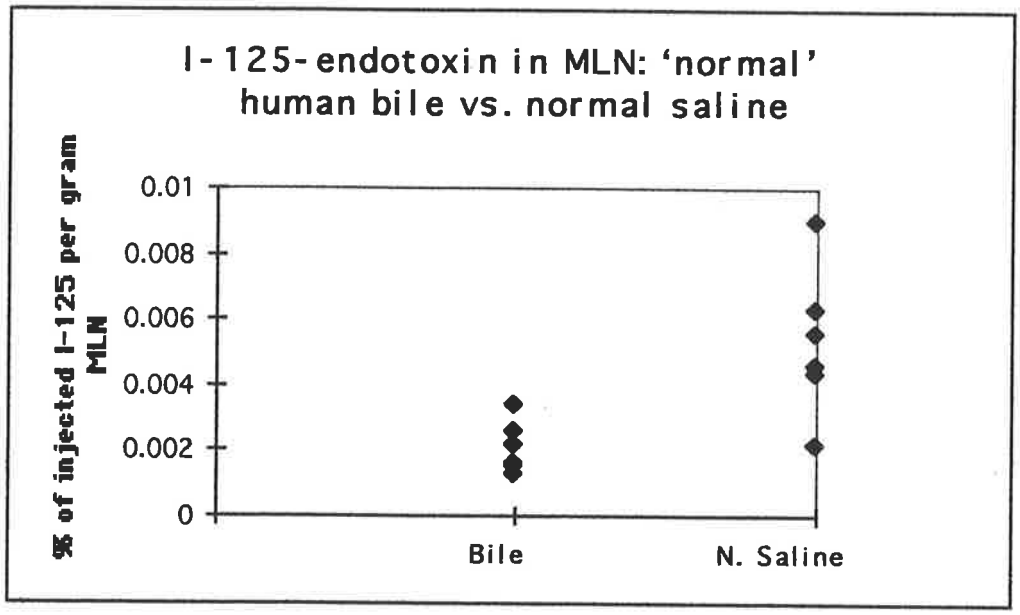

Cr-51 EDTA in systemic blood:
'normal' human bile vs. normal saline
% of injected Cr-51 per gram systemic blood
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
Bile
N. Saline

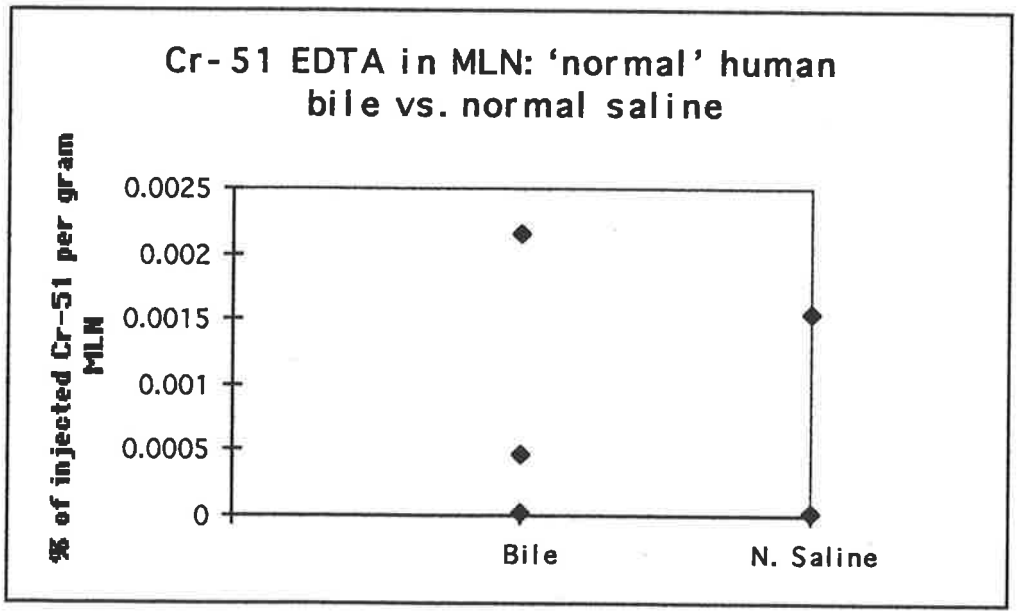

I-125-endotoxin in liver: 'normal' human bile vs. normal saline
% of injected I-125 per gram liver
0.002
0.0015
0.001
0.0005
0
Bile
N. Saline

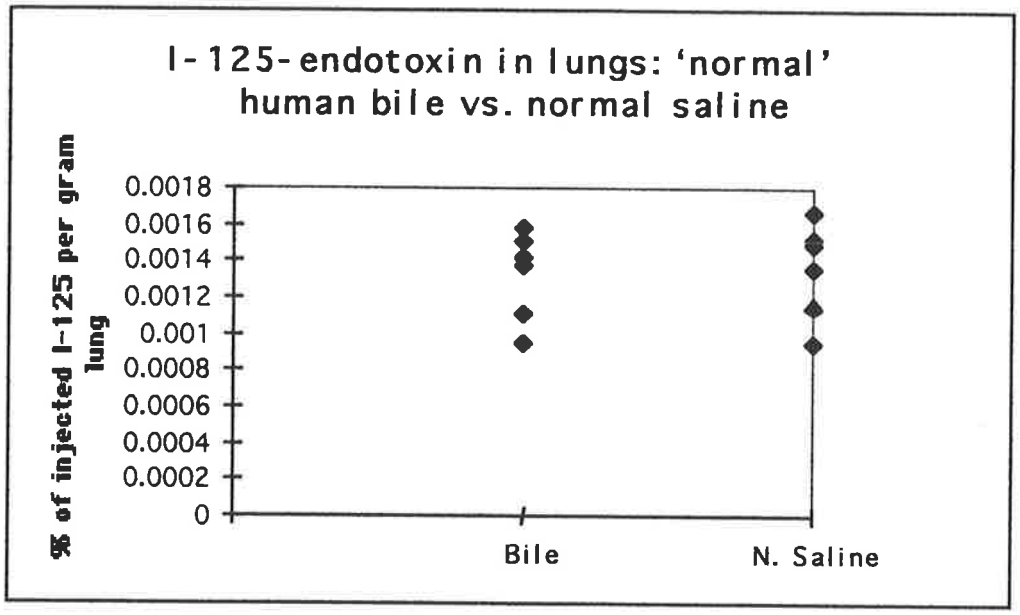

Cr-51 EDTA in liver: 'normal' human bile vs. normal saline
% of injected Cr-51 per gram of liver
0.0009
0.0008
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
Bile
N. Saline

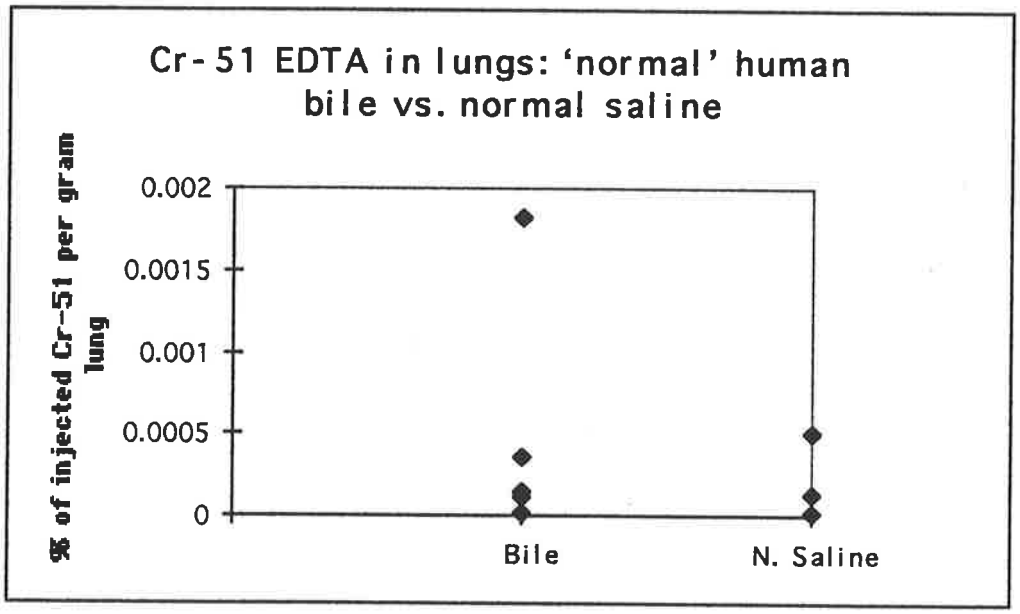

I-125-endotoxin in spleen: 'normal' human bile vs. normal saline
% of injected I-125 per gram spleen
0.0016
0.0014
0.0012
0.001
0.0008
0.0006
0.0004
0.0002
0
Bile
N. Saline

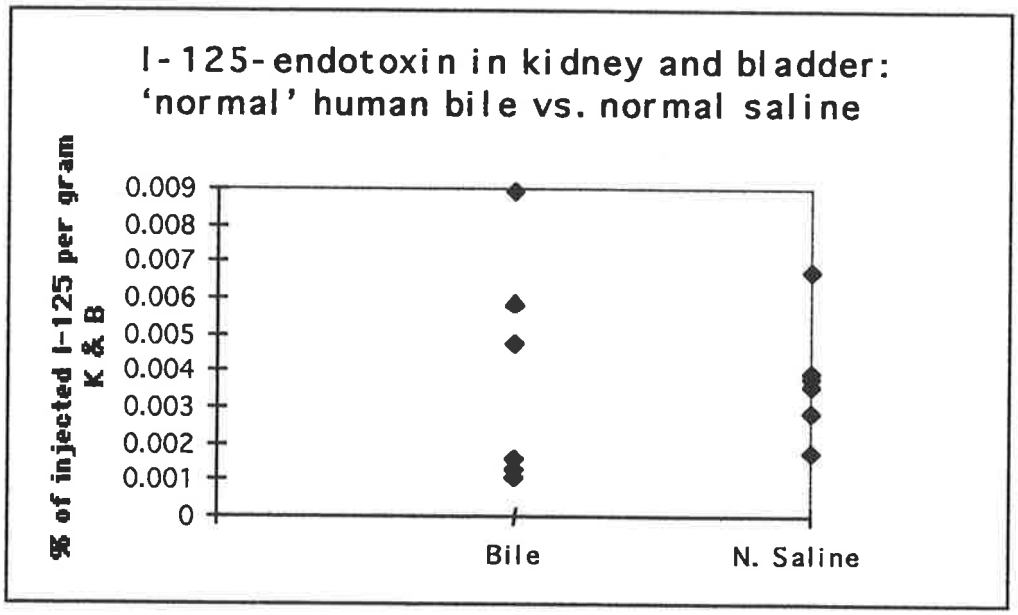

Cr-51 EDTA in spleen: 'normal' human bile vs. normal saline
% of injected Cr-51 per gram spleen
0.0008
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
Bile
N. Saline

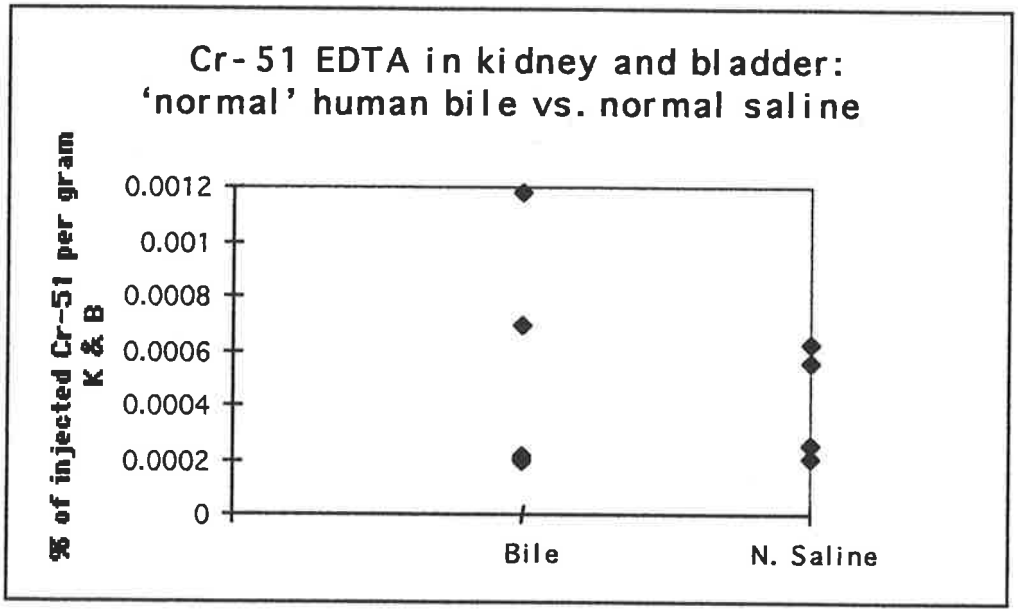

I-125-endotoxin in MLN: 'normal' human bile vs. normal saline
% of injected I-125 per gram MLN
0.01
0.008
0.006
0.004
0.002
0
Bile
N. Saline

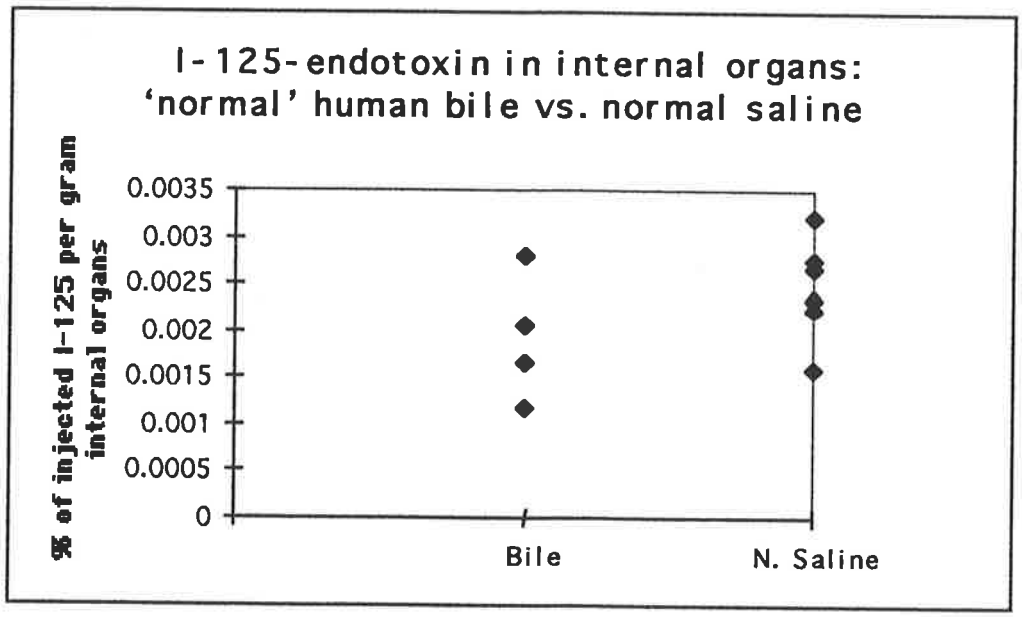

Cr-51 EDTA in MLN: 'normal' human bile vs. normal saline
% of injected Cr-51 per gram MLN
0.0025
0.002
0.0015
0.001
0.0005
0
Bile
N. Saline


I-125-endotoxin in lungs: 'normal' human bile vs. normal saline
% of injected I-125 per gram lung
0.0018
0.0016
0.0014
0.0012
0.001
0.0008
0.0006
0.0004
0.0002
0
Bile
N. Saline

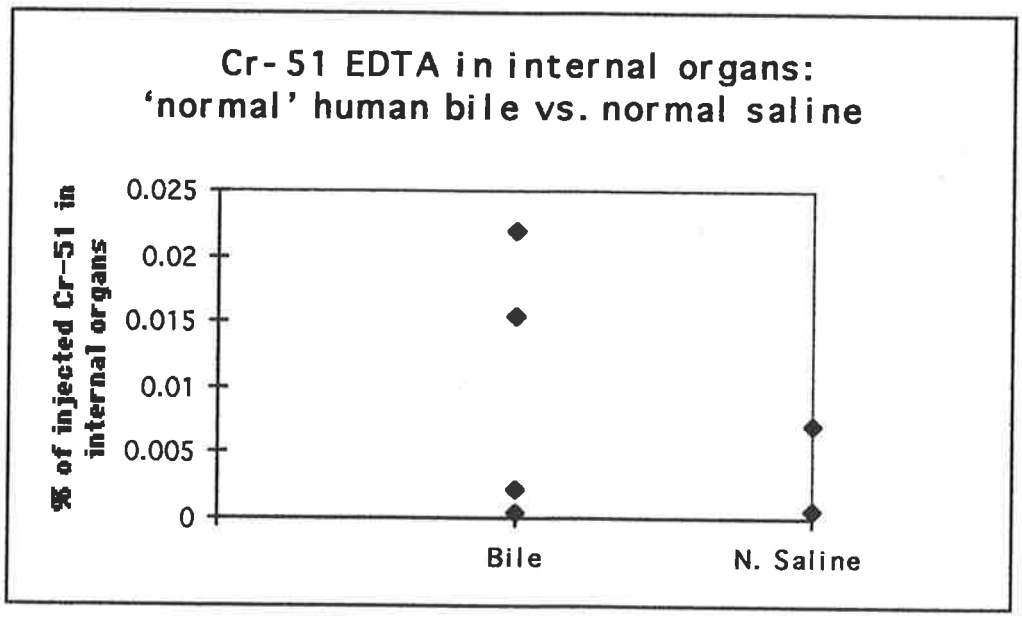

Cr-51 EDTA in lungs: 'normal' human bile vs. normal saline
% of injected Cr-51 per gram lung
0.002
0.0015
0.001
0.0005
0
Bile
N. Saline


I-125-endotoxin in kidney and bladder:
'normal' human bile vs. normal saline
% of injected I-125 per gram K & B
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
Bile
N. Saline

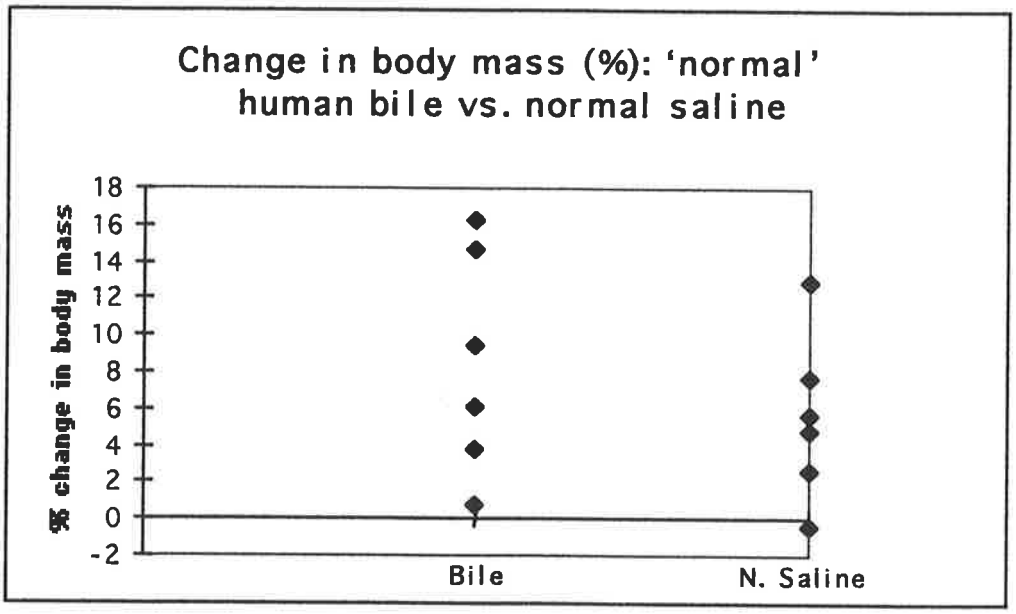

Cr-51 EDTA in kidney and bladder:
'normal' human bile vs. normal saline
% of injected Cr-51 per gram K & B
0.0012
0.001
0.0008
0.0006
0.0004
0.0002
0
Bile
N. Saline



The data above were analysed by Mann-Whitney 2-tailed U test. There was no significant difference between the two groups ($p = 0.1797$).



The data above (Cr-51 EDTA in internal organs) were analysed by Mann-Whitney 2-tailed U test. There was no significant difference between the two groups ($p = 0.4848$).



The data above (% change in body weight) were analysed with a Mann-Whitney 2-tailed U test. There was no significant difference between the two groups ($p = 0.3939$).

Discussion and conclusions

The results of the above experiment are discussed together with those of the following experiment.

4.2.2.2. Human bile: jaundiced, non-infected

Introduction

It is quite possible that bile from patients with obstructive jaundice differs from normal bile, and these differences may account for theoretical alterations in bowel-wall permeability after procedures to relieve obstructive jaundice.

Aim

To assess the effect on bowel-wall permeability to EDTA and endotoxin, of placing (for 60 minutes) bile from human patients with obstructive jaundice, in the intestinal lumen of rats with 7 days of OJ.

Methods

The methods used were the same as in the experiment for 'normal' human bile (above) except that bile specimens were collected from the following patients with obstructive jaundice:

Patient 4

A 74-year-old man with obstruction of the common hepatic duct, (due to extrinsic compression by presumed metastatic disease in hilar lymph nodes), underwent ERCP and stent insertion. Pre-procedure total serum bilirubin was 194 μmol/L. Bile duct bile (obtained by aspiration via the endoscope) was used in 3 animals.

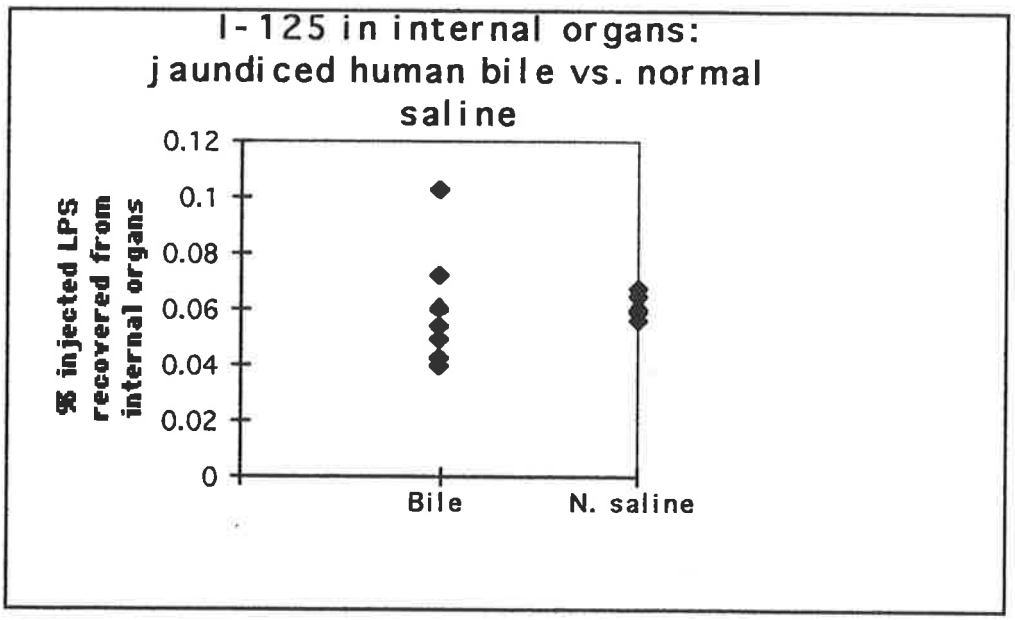
Patient 5

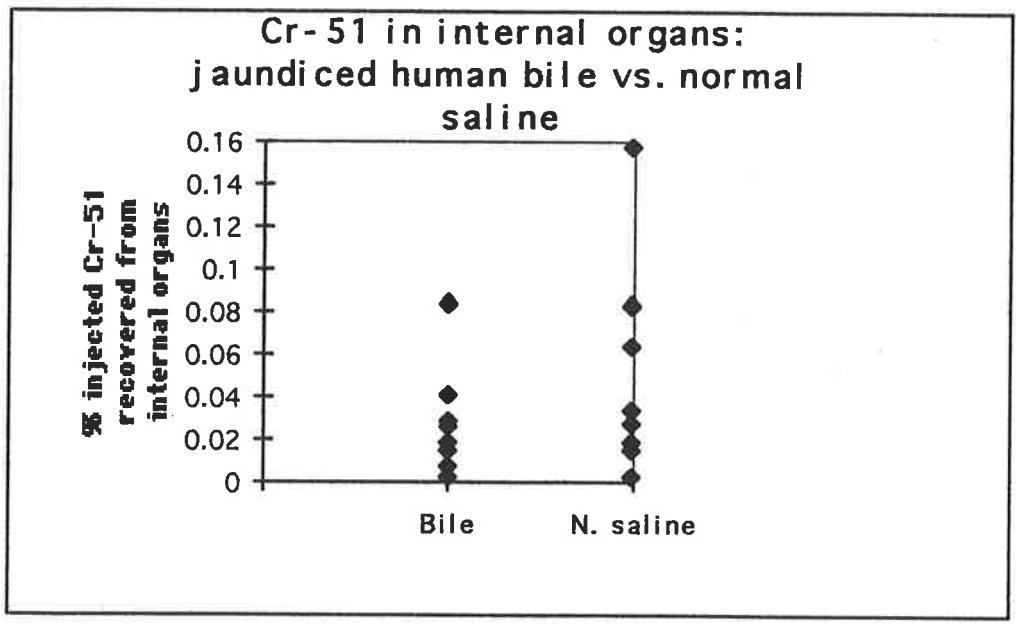
A 66-year-old woman with carcinoma of the head of the pancreas underwent choledochojejunostomy and gastrojejunostomy. Pre-operative total serum bilirubin was 578 μmol/L. Gall-bladder bile (obtained intra-operatively by fine-needle aspiration) was used in 2 animals.

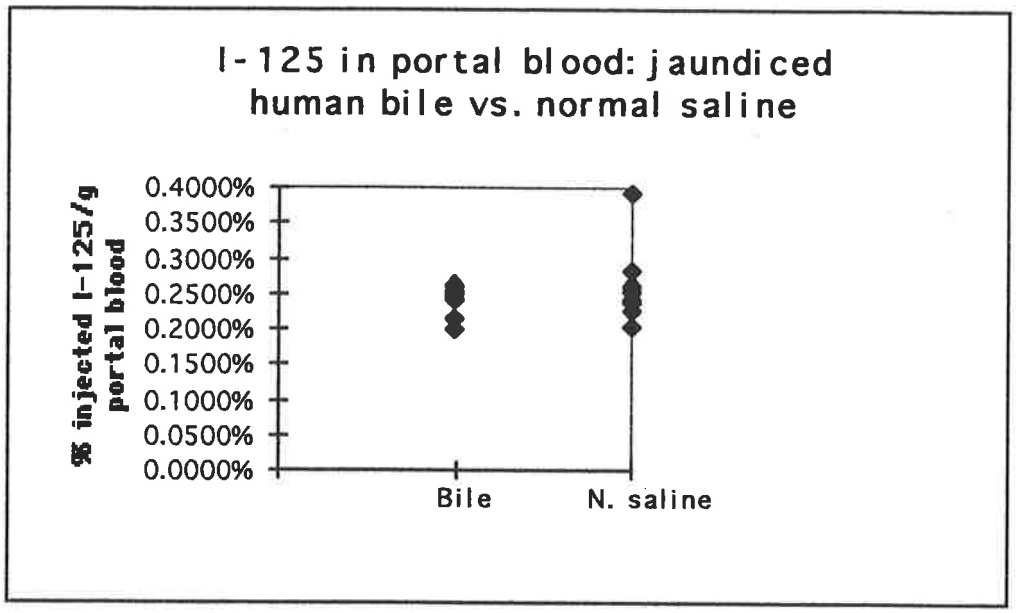
Patient 6

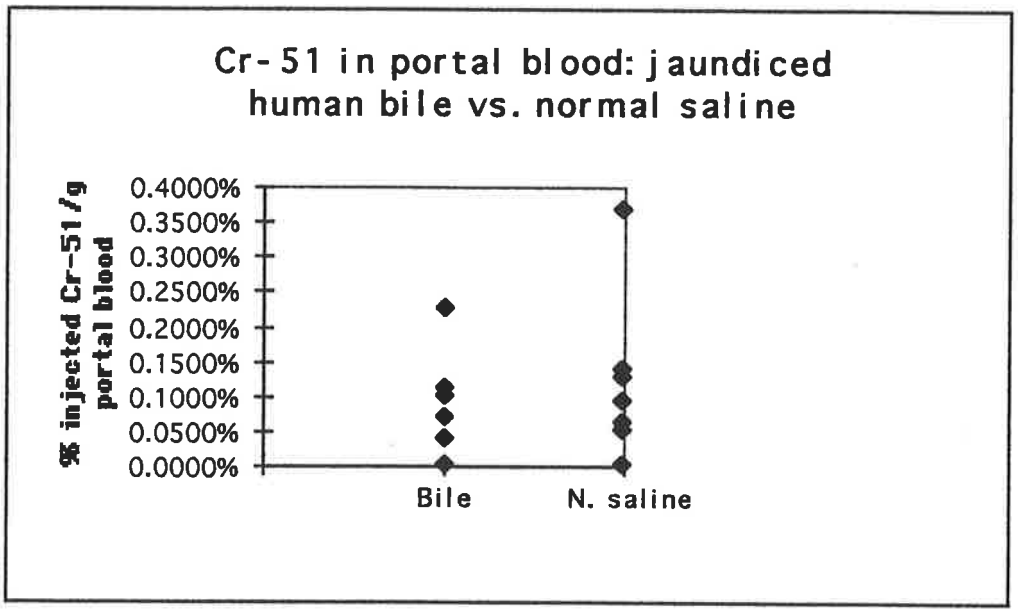
A 47-year-old woman with carcinoma of the ampulla of Vater underwent a Whipple's procedure. Pre-operative total serum bilirubin was 103 μmol/L. Gall-bladder bile (obtained by fine-needle aspiration immediately after resection of the specimen) was used in 3 animals.

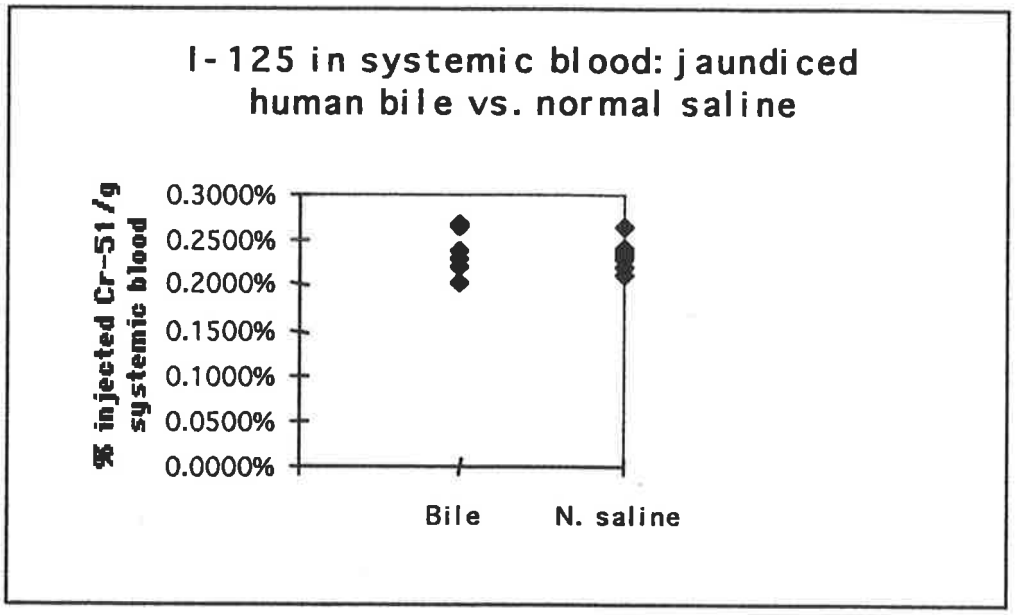
Results

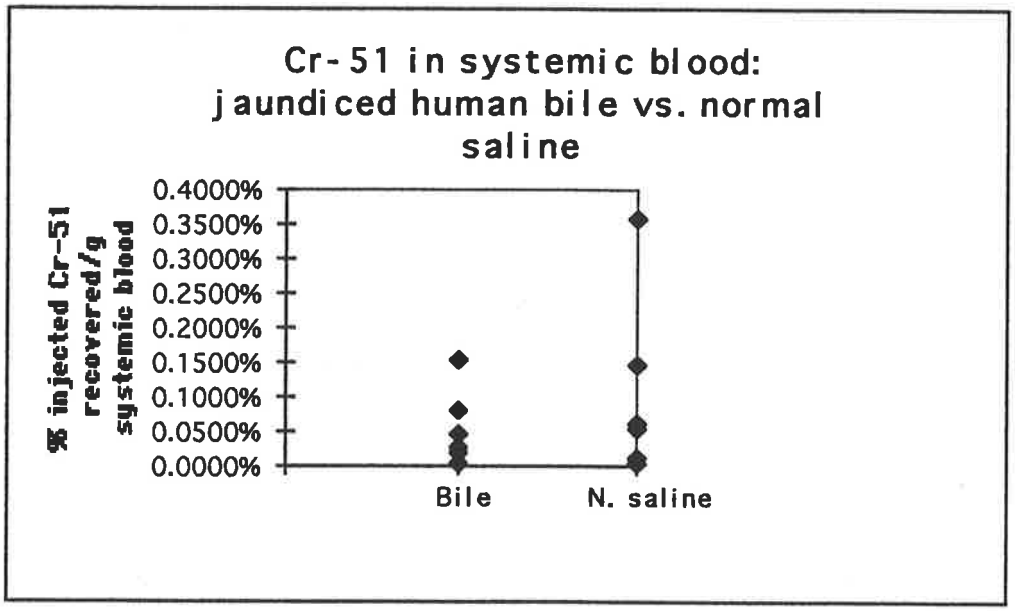

I-125 in internal organs:
jaundiced human bile vs. normal saline
% injected LPS recovered from internal organs
0.12
0.1
0.08
0.06
0.04
0.02
0
Bile
N. saline

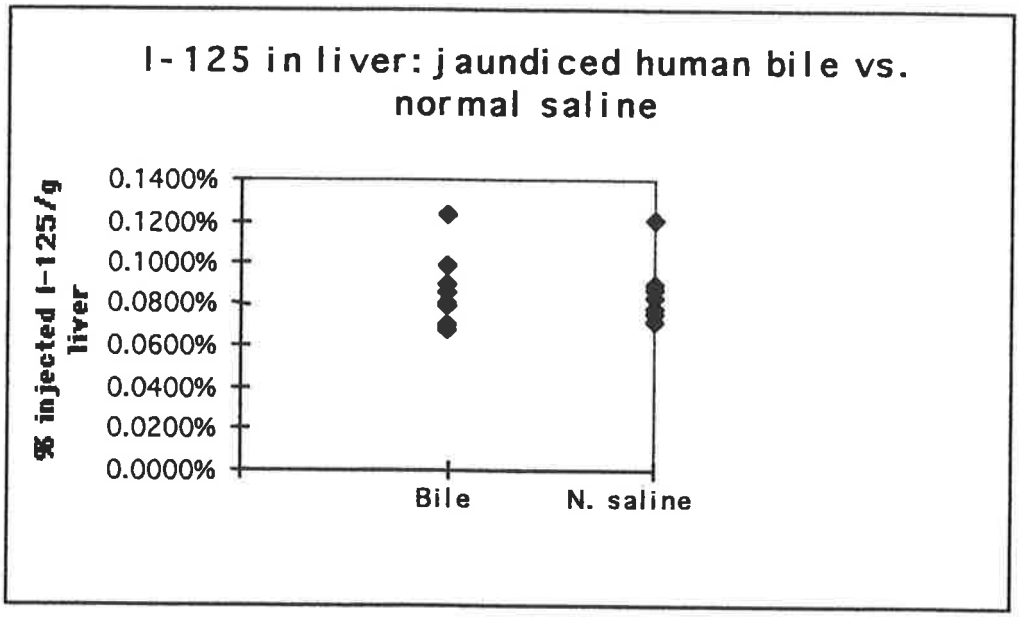

Cr-51 in internal organs:
jaundiced human bile vs. normal saline
% injected Cr-51 recovered from internal organs
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Bile
N. saline

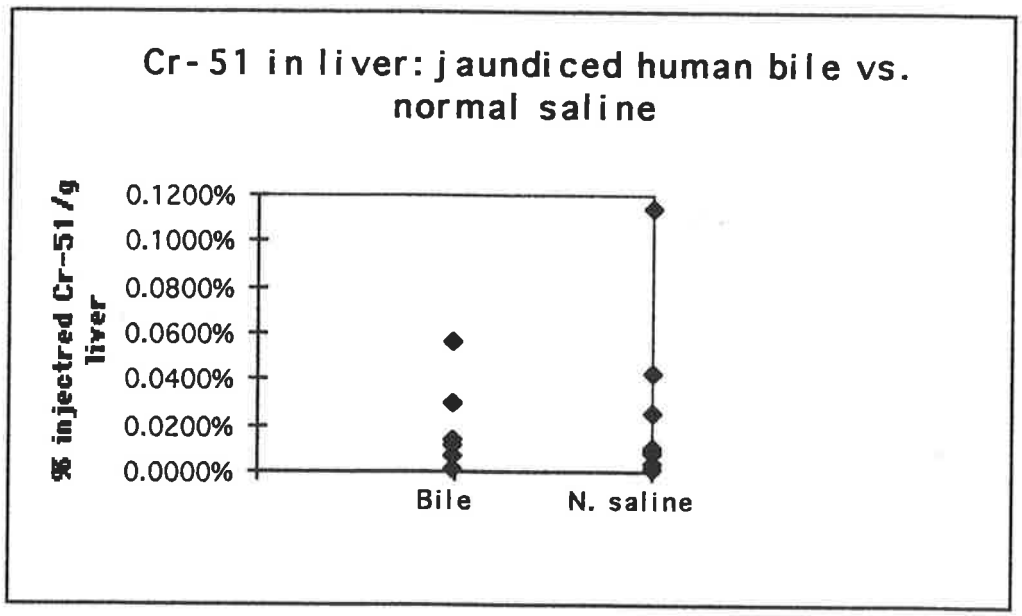

I-125 in portal blood: jaundiced human bile vs. normal saline
% injected I-125/g portal blood
0.4000%
0.3500%
0.3000%
0.2500%
0.2000%
0.1500%
0.1000%
0.0500%
0.0000%
Bile
N. saline

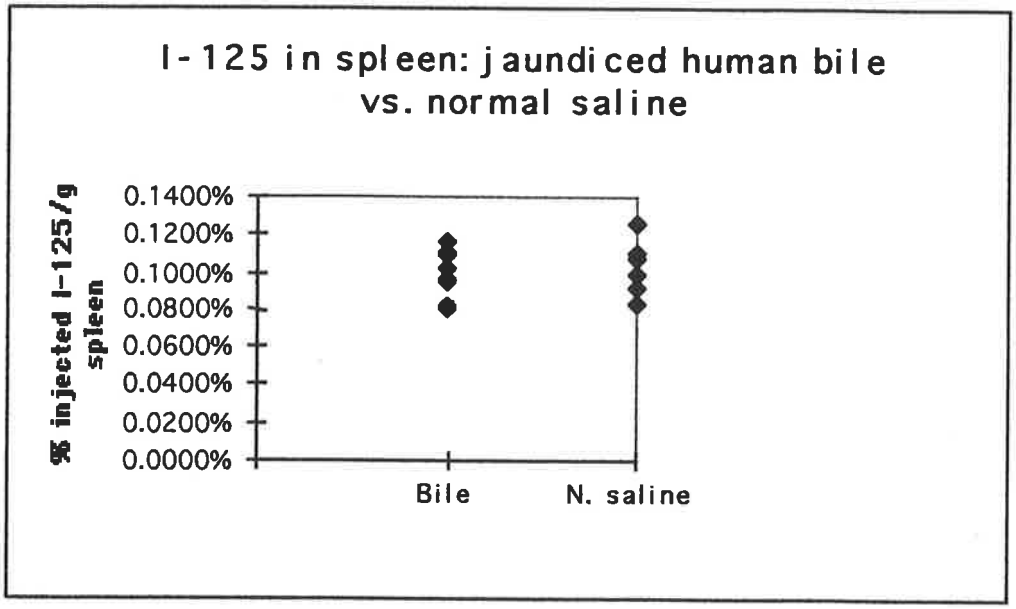

Cr-51 in portal blood: jaundiced human bile vs. normal saline
% injected Cr-51/g portal blood
0.4000%
0.3500%
0.3000%
0.2500%
0.2000%
0.1500%
0.1000%
0.0500%
0.0000%
Bile
N. saline

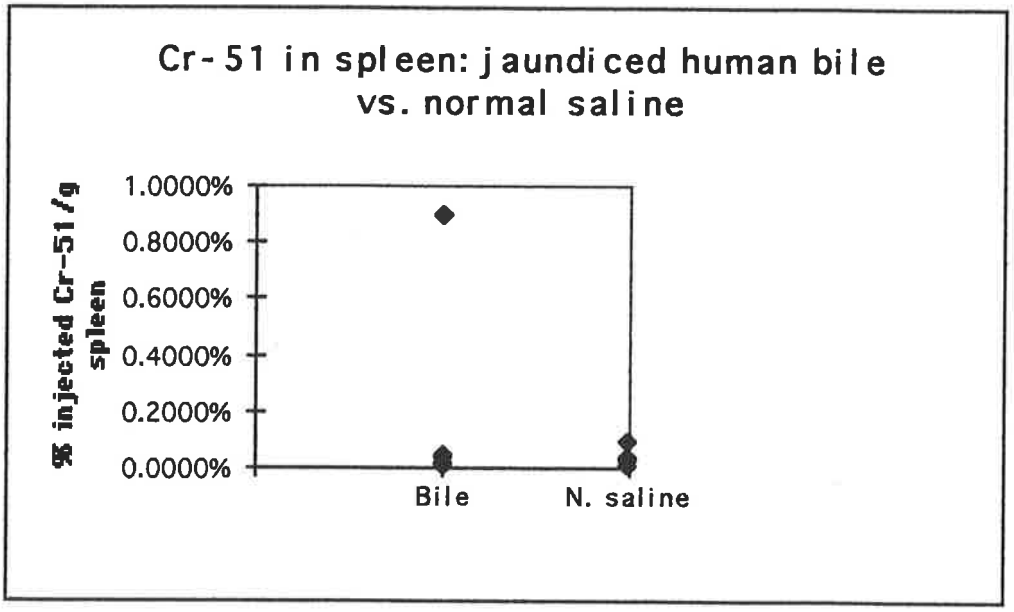

I-125 in systemic blood: jaundiced human bile vs. normal saline
% injected Cr-51/g systemic blood
0.3000%
0.2500%
0.2000%
0.1500%
0.1000%
0.0500%
0.0000%
Bile
N. saline

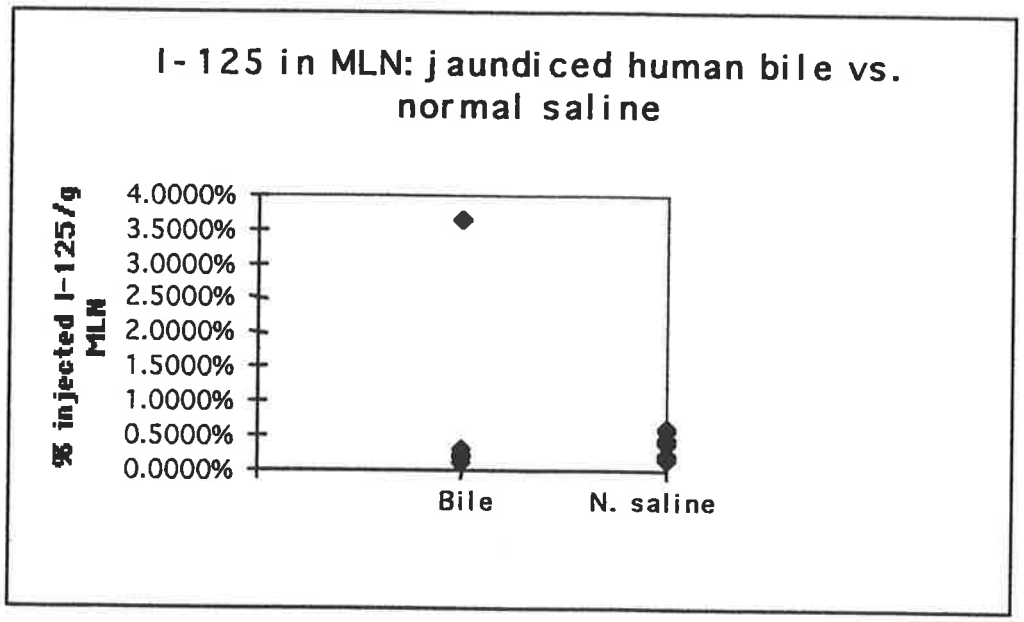

Cr-51 in systemic blood: jaundiced human bile vs. normal saline
% injected Cr-51 recovered/g systemic blood
0.4000%
0.3500%
0.3000%
0.2500%
0.2000%
0.1500%
0.1000%
0.0500%
0.0000%
Bile
N. saline

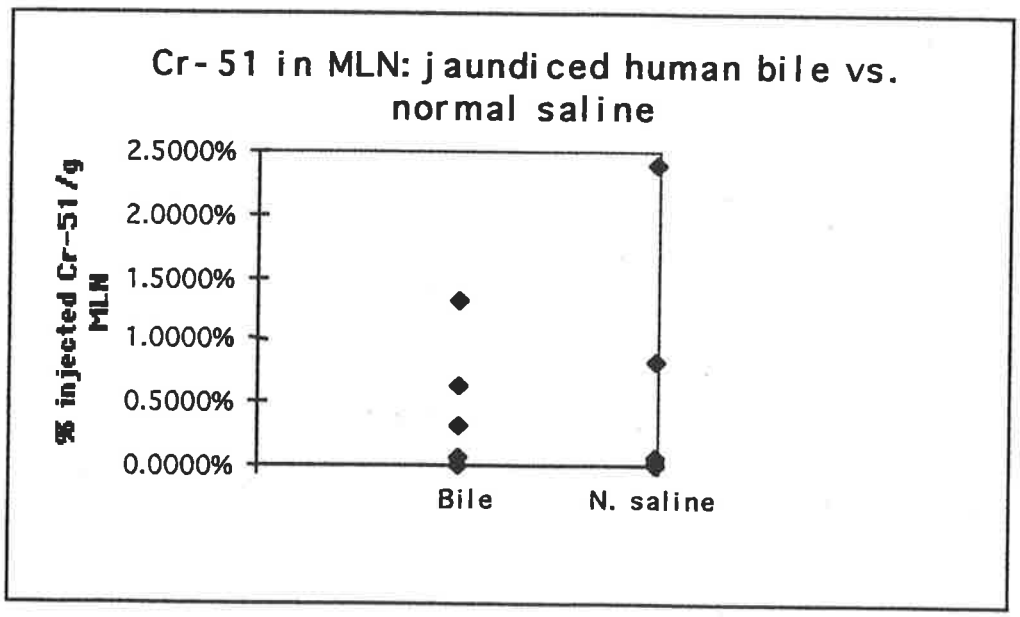

I-125 in liver: jaundiced human bile vs. normal saline
% injected I-125/g liver
0.1400%
0.1200%
0.1000%
0.0800%
0.0600%
0.0400%
0.0200%
0.0000%
Bile
N. saline

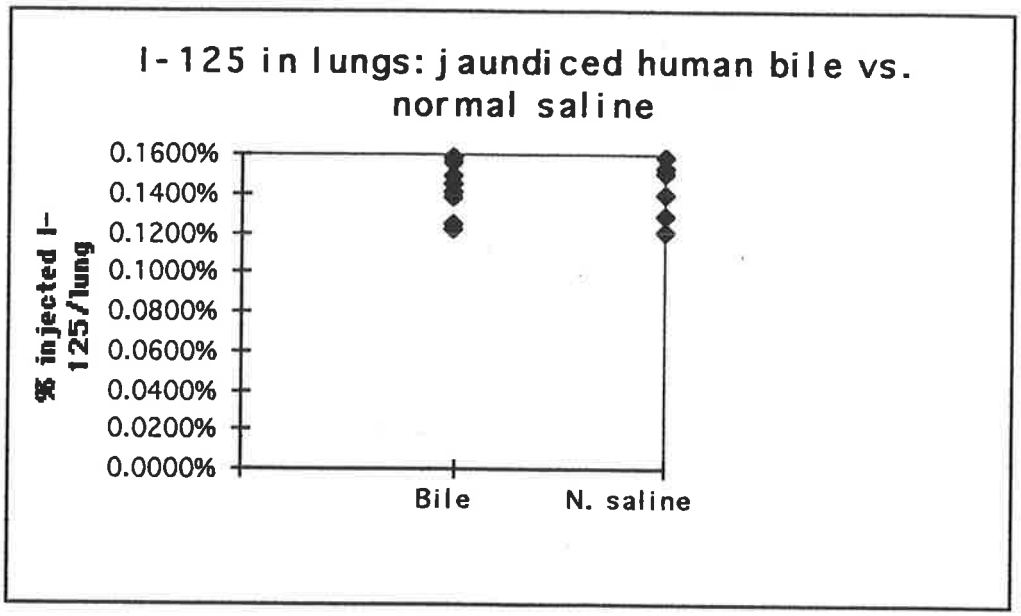

Cr-51 in liver: jaundiced human bile vs. normal saline
% injectred Cr-51/g liver
0.1200%
0.1000%
0.0800%
0.0600%
0.0400%
0.0200%
0.0000%
Bile
N. saline

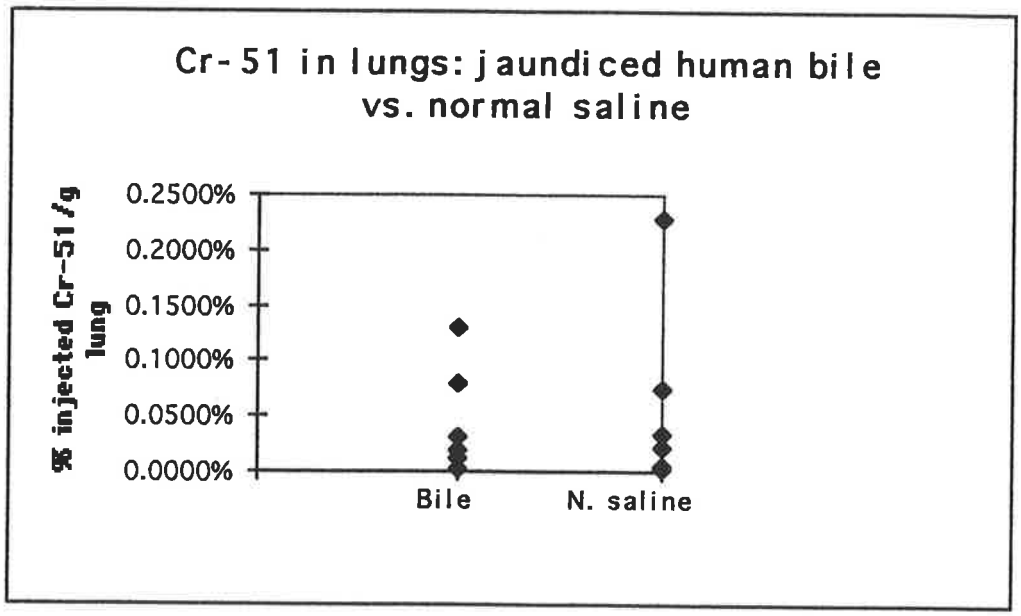

I-125 in spleen: jaundiced human bile vs. normal saline
% injected I-125/g spleen
0.1400%
0.1200%
0.1000%
0.0800%
0.0600%
0.0400%
0.0200%
0.0000%
Bile
N. saline

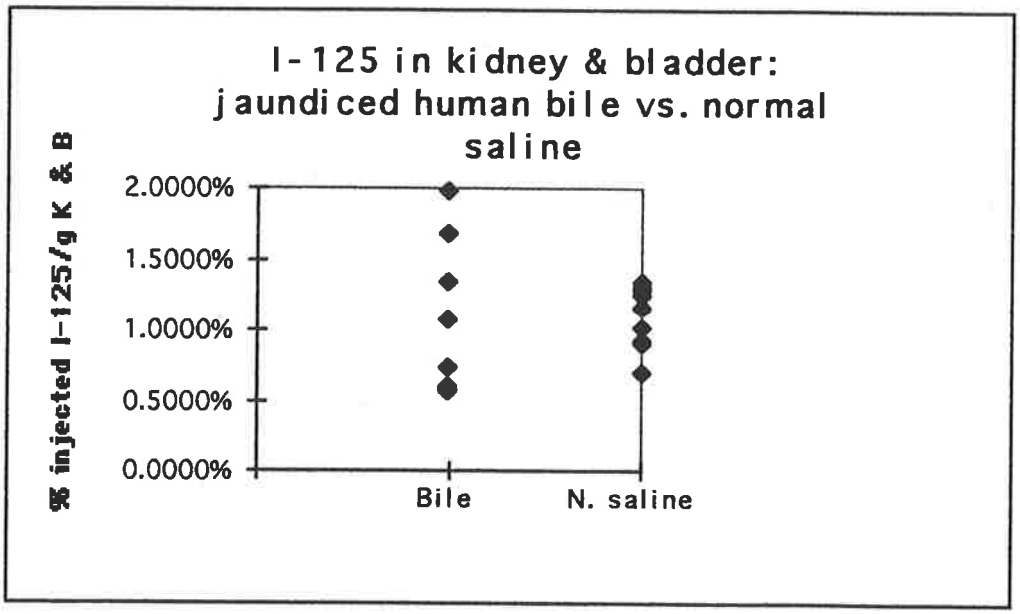

Cr-51 in spleen: jaundiced human bile vs. normal saline
% injected Cr-51/g spleen
1.0000%
0.8000%
0.6000%
0.4000%
0.2000%
0.0000%
Bile
N. saline

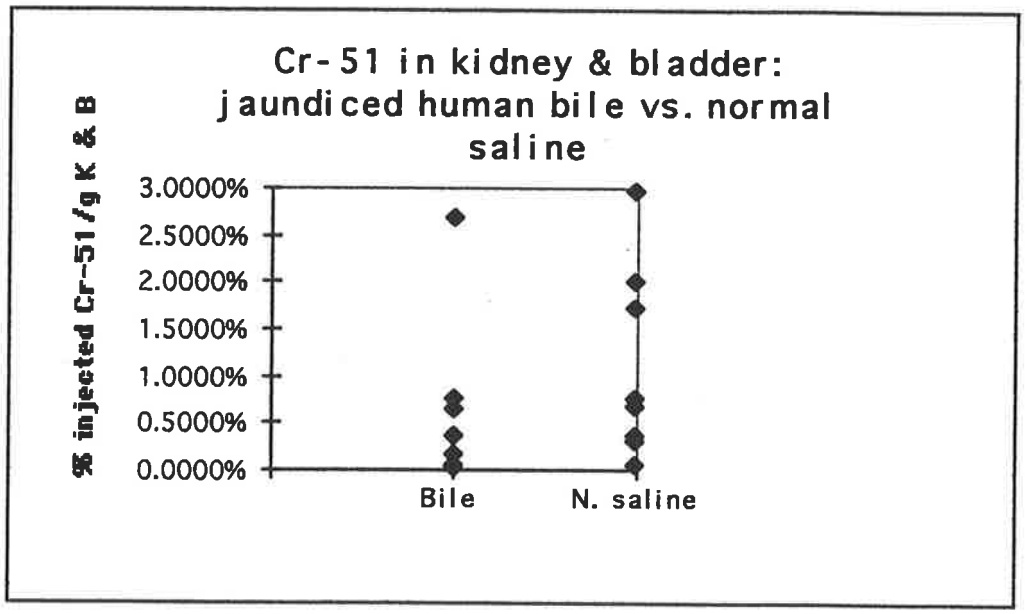

I-125 in MLN: jaundiced human bile vs. normal saline
% injected I-125/g MLN
4.0000%
3.5000%
3.0000%
2.5000%
2.0000%
1.5000%
1.0000%
0.5000%
0.0000%
Bile
N. saline


Cr-51 in MLN: jaundiced human bile vs. normal saline
% injected Cr-51/g MLN
2.5000%
2.0000%
1.5000%
1.0000%
0.5000%
0.0000%
Bile
N. saline


I-125 in lungs: jaundiced human bile vs. normal saline
% injected I-125/lung
0.1600%
0.1400%
0.1200%
0.1000%
0.0800%
0.0600%
0.0400%
0.0200%
0.0000%
Bile
N. saline


Cr-51 in lungs: jaundiced human bile vs. normal saline
% injected Cr-51/g lung
0.2500%
0.2000%
0.1500%
0.1000%
0.0500%
0.0000%
Bile
N. saline


I-125 in kidney & bladder:
jaundiced human bile vs. normal saline
% injected I-125/g K & B
2.0000%
1.5000%
1.0000%
0.5000%
0.0000%
Bile
N. saline


Cr-51 in kidney & bladder:
jaundiced human bile vs. normal saline
% injected Cr-51/g K & B
3.0000%
2.5000%
2.0000%
1.5000%
1.0000%
0.5000%
0.0000%
Bile
N. saline

Discussion and conclusions

When instilled for 60 minutes into the gut lumen of rats with 7 days of obstructive jaundice, neither bile from 'normal' human patients, nor from patients with obstructive jaundice, caused any detectable alteration in bowel-wall permeability to EDTA or endotoxin, compared to normal saline. Taken together with the experiments using rat bile (both infected and sterile), this suggests that in the clinical setting of a patient undergoing a procedure to relieve obstructive jaundice, bile returning to the intestinal lumen does not cause an immediate increase in bowel wall permeability.

There is considerable experimental evidence to show that intraluminal bile salts bind endotoxin, and therefore help to limit absorption of endotoxin from the gut [Kocsar et al 1969, Cahill et al 1987, Van Bossuyt et al 1990, Ding et al 1993(b)]. However, in the above experiment, the amount of endotoxin absorbed from the gut after bile returned to the intestinal lumen was no different to that found with normal saline.

There are several possible explanations for this. For example, this action may not be demonstrable over a short time period, such as 60 minutes. Almost all studies examining the interaction of endotoxin and bile or bile salts, do so over a period of at least 24 to 48 hours [Ding et al 1993(b)]. Nevertheless, Kocsar et al (1969) found that intraperitoneal bile reduced the amount of tritium-labelled endotoxin absorbed from the peritoneum after only 60 minutes.

Therefore, it is possible that other factors unique to the intestinal lumen may have interfered with the bile salts' endotoxin-binding ability. For example, since the bile was injected into the gut before the radio-labelled endotoxin, it is theoretically possible that the endotoxin already present in the gut lumen bound to the bile salts, so that the injected

endotoxin was able to remain free. There does not seem to be any published literature describing the molar capacity of bile salts to bind endotoxin.

Another possible explanation for this finding is that the endotoxin-binding action of bile salts has been overstated. This argument was proposed by Diamond et al [1990(b)], who found that relief of OJ by external drainage was equally as effective as internal drainage at reversing the endotoxaemia of OJ. They stated:

"We believe that the importance of gastrointestinal bile flow has been overestimated..."

This may explain why therapeutic use of bile salts has not consistently reduced endotoxaemia, or altered patient outcome in randomised controlled trials (Pain et al 1991, Thompson et al 1986). For example, Gawley et al (1988) studied pre- and post-operative renal function in 38 patients with obstructive jaundice, 12 of whom received oral bile salts. They found that, although peri-operative administration of sodium deoxycholate protected against post-operative endotoxaemia, significant post-operative impairment of renal function still occurred. Moreover, they also showed no correlation between change in endotoxin levels and change in renal function. As Fogarty et al (1995) observed, a theoretical flaw exists in the therapeutic use of oral bile salts to reduce endotoxin absorption. That is, most orally-administered bile salt is absorbed in the terminal ileum; only about 5% reaches the colon, which is the major site of endotoxin.

Alternatively, it is theoretically possible that the bile had both a 'detrimental' effect (injuring the intestinal mucosa) and a 'beneficial' effect (binding endotoxin), but that the two effects balanced each other, leading to the overall amount of endotoxin absorption

being unchanged. However, this seems very unlikely, because there was no difference in the amount of EDTA absorbed from the gut.

4.3. Renal impairment

4.3.1. Creation of a standard curve of urobilinogen concentration.

Introduction

As described in the 'Methods' chapter, the assay method of Kotal and Fevery (1991) relies on the two facts that urobilinogen can be readily oxidised to urobilin, and that under certain conditions, urobilin combines with zinc to form a complex with a characteristic green colour. The intensity of this colour in a given solution can be measured, and is proportional to the concentration of urobilin-Zn complex.

In order to be able to calculate the urobilinogen concentration in a solution of unknown concentration, it is necessary to create a standard curve, plotting the optical density of solutions of known concentrations.

Methods

0.5 mg urobilin was dissolved in DMSO, serially diluted and assayed by the method of Kotal and Fevery (1991). The results were charted to form a standard curve of urobilin concentration versus optical density. The procedure was repeated, and close concordance between experiments was found within the range described as 'reliable' by Kotal and Fevery, as shown below.

Results

Urobilin-Zn concentration (μmol/L)	O.D. at 511 nm (assay 1)	O.D. at 511 nm (assay 2)
12.5	approx 2.4	approx 2.38
6.25	approx 1.372	approx 1.286
3.125	0.678	0.678
1.5625	0.326	0.330
0.781	0.168	0.135
0.39	0.075	0.058

The two highest values in the above table were only approximate, because at very high readings, measurements of optical density become unreliable. [This was visibly shown by the digital read-out from the spectrophotometer, which at the highest level fluctuated rapidly, apparently giving many different readings per second!] This reading was therefore not included when the data were analysed.

The data were analysed by linear regression (using the 'InStat' software package), which yielded the following results:

Correlation coefficient (r) = 0.9988. $r^2 = 0.9976$

Standard deviation of residuals from line (Sy.x) = 0.0253

The InStat software package also tested the data to establish:

(1) that the data were linear (using a 'runs test')

There was not a significant departure from linearity ($P = 0.8333$).

(2) that the slope of the line differed from zero (using an ANOVA test). $F = 3294.0$ $P < 0.0001$, considered extremely significant.

The above analysis served two main purposes:

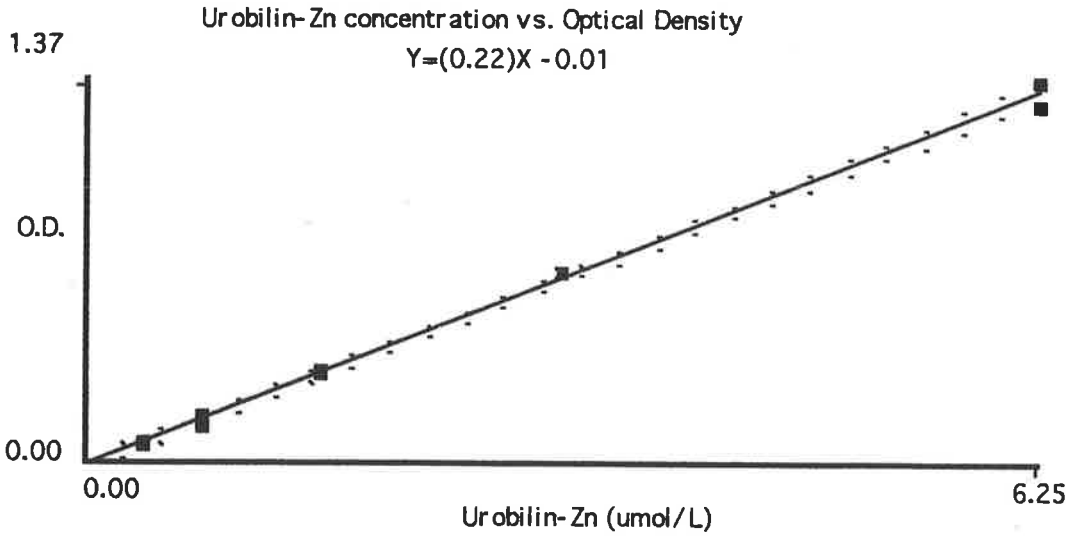
(1) it verified the linear relationship between urobilin-Zn concentration and optical density at 511 nm, (as reported by Kotal and Fevery) within a narrow confidence interval.

(2) it established the mathematical equation of that linear relationship to be:

$$y = (0.22)x - 0.01$$

where y is optical density, and x is urobilinogen concentration in mmol/L.
This enabled the urobilin-Zn concentration of a given solution to be calculated, once its optical density at 511 nm was known.

The results are graphed overleaf:


Urobilin-Zn concentration vs. Optical Density
Y=(0.22)X - 0.01
1.37
O.D.
0.00
0.00
6.25
Urobilin-Zn (umol/L)

4.3.2. Urobilinogen assay

To verify that the method of urobilinogen synthesis was successful, i.e. that urobilinogen had indeed been synthesised, solutions were assayed immediately after the solutions had been placed onto the cells.

[NOTE: Ideally, the assay would be performed each time urobilinogen was synthesised. However, this was not done, because it was found that taking the amount of urobilinogen solution required for assay (i.e. 800 μL) created problems of scale with the small volumes left. That is, each synthesis reaction yielded only about 3 mL of urobilinogen solution. That small volume was further decreased by the small amounts lost in each transfer from tube to tube when removing precipitate by filtration and centrifugation.

A particular problem was that, with the available equipment, volumes much smaller than 3 mL were very difficult to neutralise accurately to physiological pH. This is because such small volumes could not be made to cover the pH probes adequately, resulting in wide, rapid and apparently erroneous oscillations of the reading on the pH meter.

It could be argued that the problem could be solved simply by diluting the solution to increase its volume. However, the essential processes of neutralising the solution to physiological pH, and adding it to cell culture wells (already containing culture medium), inevitably resulted in considerable dilution of the solution. To start these processes with a solution which was already diluted, would decrease the ability to demonstrate toxicity.

A second possible solution to the problem was to assay any urobilinogen solution remaining after experiments had been conducted. However, these volumes were usually small (100-200 μL), because of the volumes of solution needed for experiments themselves. Nevertheless, assay of 'used' (i.e. pH-adjusted, diluted) urobilinogen solution was done on 25/2/97. The original volume of full-strength solution was 2600 μL, to which 1083 μL of HCl was added to reach physiological pH. The solution had then been serially diluted as usual from full-strength, to half, one-quarter, one-eighth strength etc. After use in

experiments, 425 μL of 1/2 strength solution was available, to which was added 400 μL of de-ionised water, so as to give sufficient volume for assay.

The optical density of solution was 0.157 at 511 nm, which, corrected for the above dilutions, correlates with a urobilinogen concentration of 4.1757 μmol/L in the original full-strength solution. This concurred within 0.05%, with earlier synthesis on 12/12/96 (see below). Since synthesis and assay of urobilinogen on two separate occasions resulted in calculated concentrations with very close concordance (+/- 2%), this course was deemed adequate.]

Assay of urobilinogen solution made on 12/12/96 yielded an O.D. at 511 nm of 0.909.

Assay of the blank solution used above, against a control solution containing no urobilin or urobilinogen, yielded an O.D. of 0.299; this represented urobilin in the solution. [Urobilin may be expected to be present, both because the reduction of urobilin to urobilinogen may not proceed to completion, and because after synthesis, some urobilinogen may undergo auto-oxidation to urobilin.]

Using the equation above, the urobilinogen concentration can be calculated:

$$y = (0.22)x - 0.01$$

where y is optical density, and x is urobilinogen concentration in μmol/L.

Therefore, $x = \frac{y + 0.01}{0.22}$

Therefore, urobilinogen concentration = $\frac{0.909 + 0.01}{0.22}$ µmol/L

$= 4.177$ µmol/L

And urobilin concentration $= \frac{0.299 + 0.01}{0.22}$ µmol/L

$= 1.405$ µmol/L

Therefore the proportion of compound present as urobilinogen, expressed as a percentage of the total urobilinogen and urobilin present, was:

$$\frac{4.177}{4.177 + 1.405} \times \frac{100}{1}$$

= approx. 74.8%

4.3.3. Cell culture methods: Neutral Red assay

Introduction

It was necessary to show that the Neutral Red assay of Löwik et al (1993) gave a predictable relationship between the number of viable cells present and optical density, over the range of cell numbers for which the assay would be used.

Methods

Serial dilutions of Vero cells were made. The concentrations used were:

1.25×10^6 cells per mL

1.0×10^6 " "

7.5×10^5 " "

5.0×10^5 " "

2.5 a 10^5 " "

1.0×10^5 " "

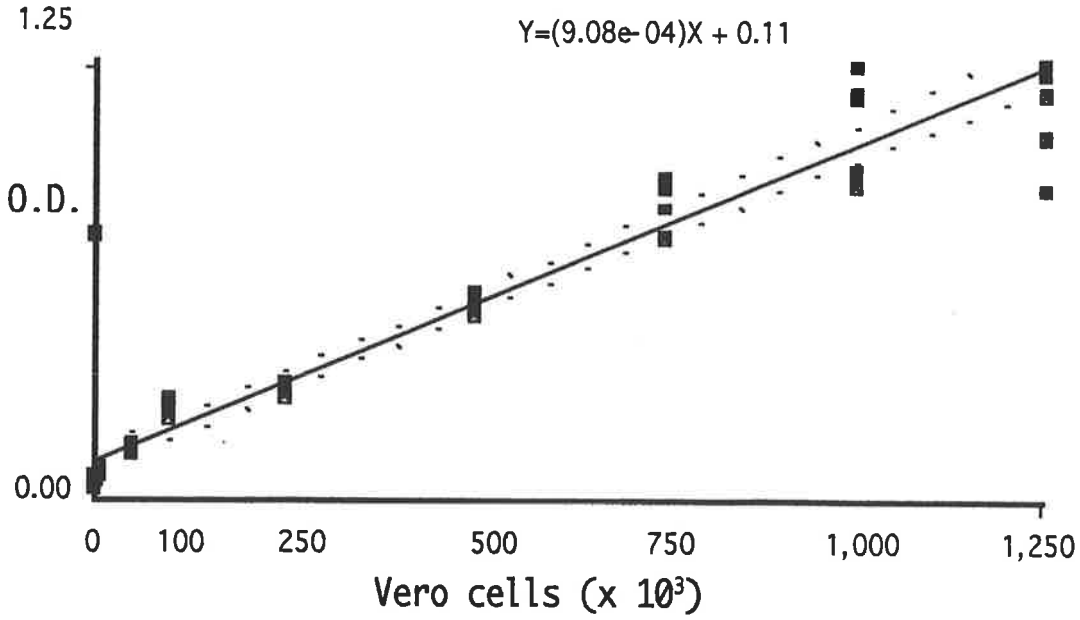
5.0×10^4 " "

1.0×10^4 " "

5.0×10^3 " "

The assay was performed as described in the 'Methods' section. The results were analysed by linear regression analysis using the 'InStat' statistical software package.

Results



Linear regression:

Correlation coefficient (r) = 0.9588. $r^2 = 0.9193$

Standard deviation of residuals from line (Sy.x) = 0.1181

The InStat statistical software package also tested the slope of the line to show that it is significantly different from zero. This yielded the following result:

F = 729.49

P < 0.0001; i.e. the slope of the line differed very significantly from zero.

The package was also used to test linearity, using a runs test and an ANOVA table comparing deviations from linearity with scatter among replicates.

(a) Runs test

The P value is 0.0021, considered very significant.

There are significantly fewer runs than expected, suggesting that the data follow a curve rather than a line.

(b) ANOVA table

F = 2.3511

The P value is 0.0253. The departures from linearity are significant.

Discussion and conclusions

The results imply that the line follows a curve rather than being truly linear. This is consistent with results obtained by other research groups for neutral red and for other stains; indeed, Elliott and Auersperg (1993), using Neutral Red, reported distinct flattening of the curve at cell numbers as low as 500,000 per mL. This low figure may reflect different characteristics of the cells used by Elliott (i.e. ovarian epithelial cells), compared to Vero cells.

Simple inspection of the graph above suggests that non-linearity is greatest at very low and very high cell numbers. Non-linearity at very low cell numbers may be due to background levels of stain present in the supernatant, as shown by the readings in wells containing no cells. Theoretically, another cause may be the tendency of cells to clump together, resulting in differences in cell numbers in each well; this would be especially revealed at low cell numbers. However, this is not likely, because the cells were inspected with phase-contrast light microscopy prior to staining, and clumping was minimal or absent.

Non-linearity at high cell numbers may reflect local factors caused by cells being crowded together. When examined under light microscopy, cells in a microwell containing very high cell numbers are often found to be touching, or even forming small heaps. It is possible that in these conditions, some areas of the cell wall do not take up stain, for the simple reason that they are in contact with another cell wall instead of stain-containing solution.

Nevertheless, the fact that the assay does not produce a *linear* relationship between number of viable cells present and optical density, is unimportant. Rather, what is important is that the optical density decreases in a predictable way with decreasing numbers of viable cells.

4.3.4. Effect of urobilinogen on Vero cells

Introduction and aims

The aim of this experiment was to assess the toxic effect, if any, of serial dilutions of urobilinogen solution on Vero cells.

Methods

Urobilinogen was synthesised as described in the 'Methods' chapter. Each well of a 96-well micro-well plate was instilled with 10^5 Vero cells, also as described in the 'Methods' chapter.

Each micro-well plate was laid out according to the plan shown below:

1U	1B	1/2U	1/2B	1/4U	1/4B	1/8U	1/8B	PBS	+ve control
1U	1B	1/2U	1/2B	1/4U	1/4B	1/8U	1/8B	PBS	+ve control
1U	1B	1/2U	1/2B	1/4U	1/4B	1/8U	1/8B	PBS	+ve control
1U	1B	1/2U	1/2B	1/4U	1/4B	1/8U	1/8B	PBS	+ve control
1U	1B	1/2U	1/2B	1/4U	1/4B	1/8U	1/8B	PBS	+ve control
1U	1B	1/2U	1/2B	1/4U	1/4B	1/8U	1/8B	PBS	+ve control

Key to above figure: 'U' = urobilinogen, 'B' = blank. '1' = undiluted, '1/2' = 1/2 strength, etc. PBS = Phosphate Buffered Saline, which was used as a negative control. The positive control was hypertonic (9%) saline, for the reasons outlined in the Materials and Methods section. Columns were always 'paired' (that is, each column of urobilinogen-containing wells was always next to a column containing the same concentration of 'blank' solution). However, in some experiments, the right-hand column contained urobilinogen solution and the left, blank solution, to avoid the theoretical possibility of an effect caused by location. For clarity, the diagram shows only the inner 60 wells which contained cells, and omits the outermost 36 wells. These wells contained PBS alone, to prevent the possibility of a so-called 'edge effect' due to dehydration.

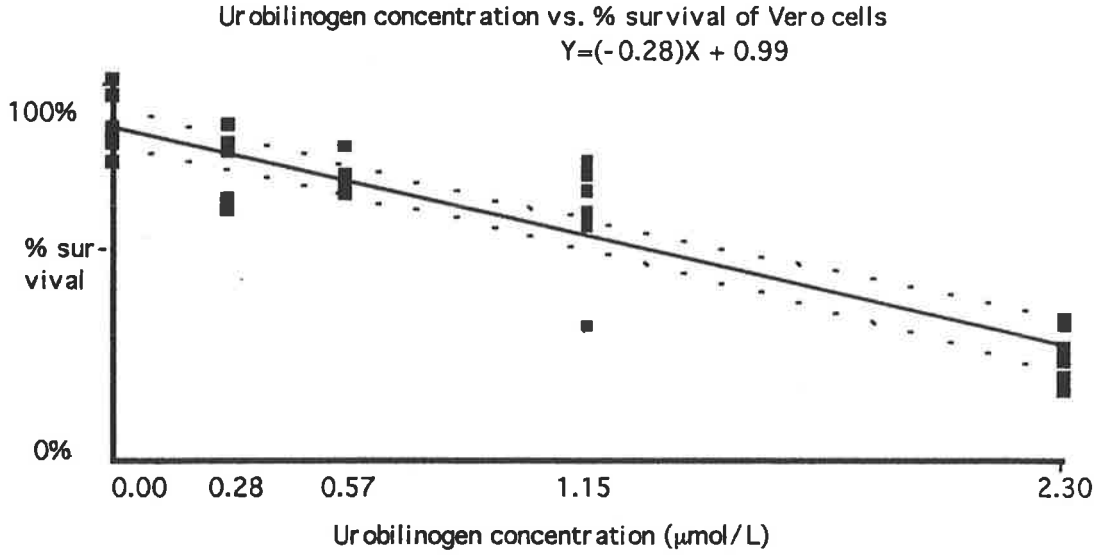
The experiment was repeated a total of six times, using slightly different concentrations of urobilinogen. The concentrations of urobilinogen were calculated from the known amount of urobilin added to the initial solution, and the amount of HCl used to neutralise the solution. It was assumed that the conversion rate of urobilin to urobilinogen was the same as in the two previous synthetic reactions; i.e. 74.8%.

The results were analysed using the 'InStat' statistical software package, which used a 'runs' test to test if the data were linear, and an ANOVA table to test if the slope of the line was significantly different to zero.

Results

The graphs below show representative results of two microplate experiments.

Example 1



Linear regression:

Correlation coefficient (r) = -0.9076. $r^2 = 0.8238$

Standard deviation of residuals from line (Sy.x) = 0.1093.

ANOVA test:

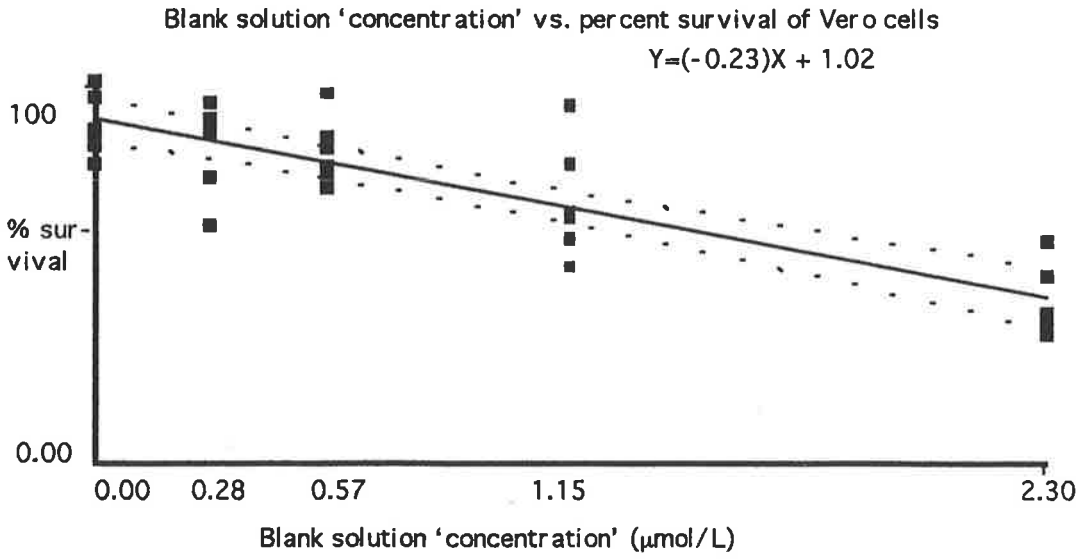
F = 130.92

P < 0.0001; i.e. the slope of the line is significantly different from zero.

Runs test:

P = 0.3314.

The departures from linearity are not significant.



Linear Regression

Correlation coefficient (r) = -0.8492. $r^2 = 0.7211$

Standard deviation of residuals from line (Sy.x) = 0.1198

ANOVA test:

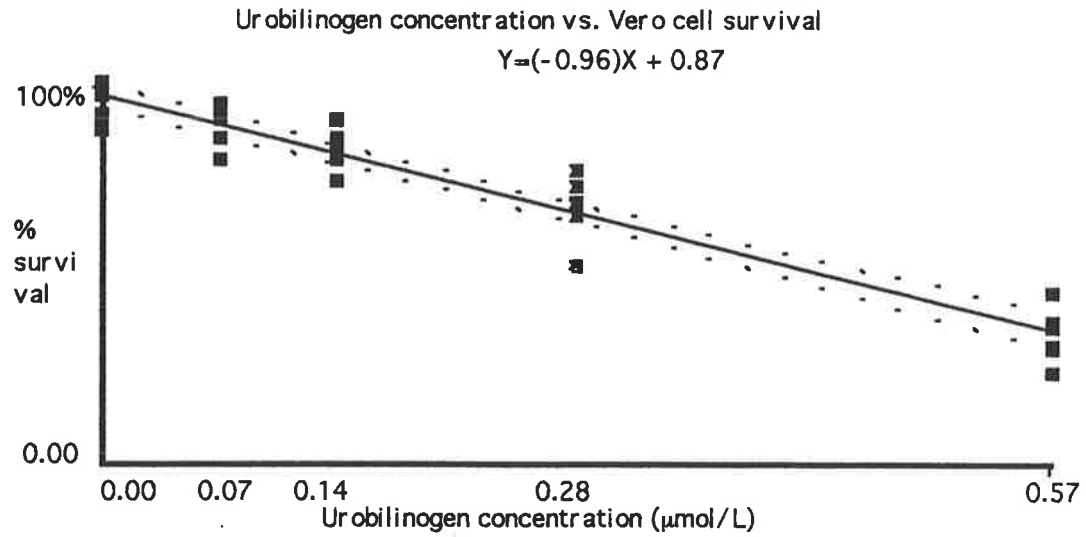
F = 72.392

P < 0.0001; i.e. the slope of the line is significantly different from zero.

Runs test:

P = 0.6141, i.e. there is not a significant departure from linearity.

Example 2



Linear regression

Correlation coefficient (r) = -0.9625. $r^2 = 0.9264$

Standard deviation of residuals from line (Sy.x) = 0.0566

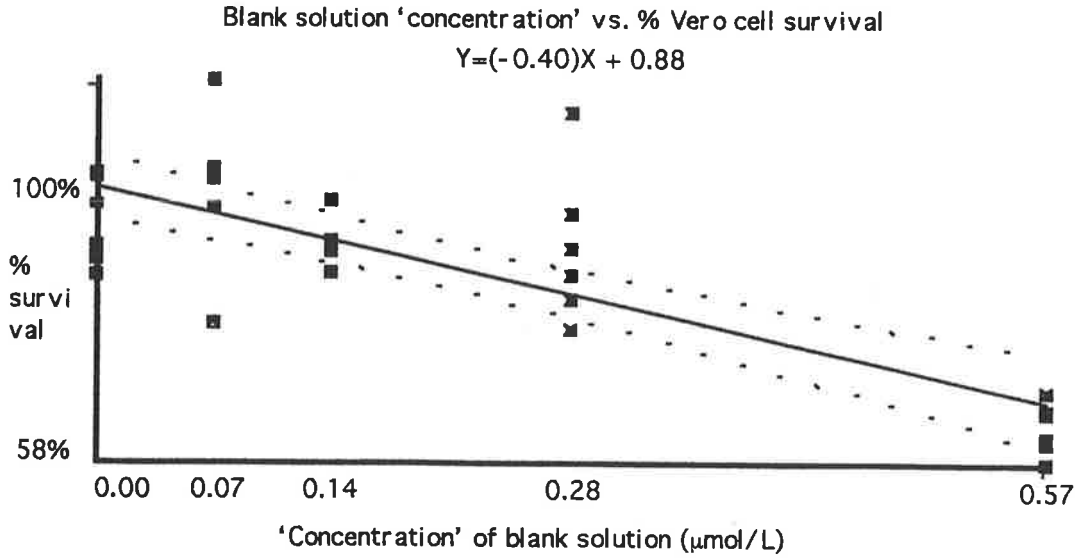
ANOVA test:

F = 352.48

P < 0.0001; i.e. the slope is significantly different from zero.

Runs test:

P = 0.1980; i.e. there is not a significant departure from linearity.



Linear regression

Correlation coefficient (r) = -0.7918. r^2= 0.6270

Standard deviation of residuals from line (Sy.x) = 0.0653

ANOVA test:

F = 47.060

P < 0.0001; i.e. the slope of the line is significantly different from zero.

Runs test:

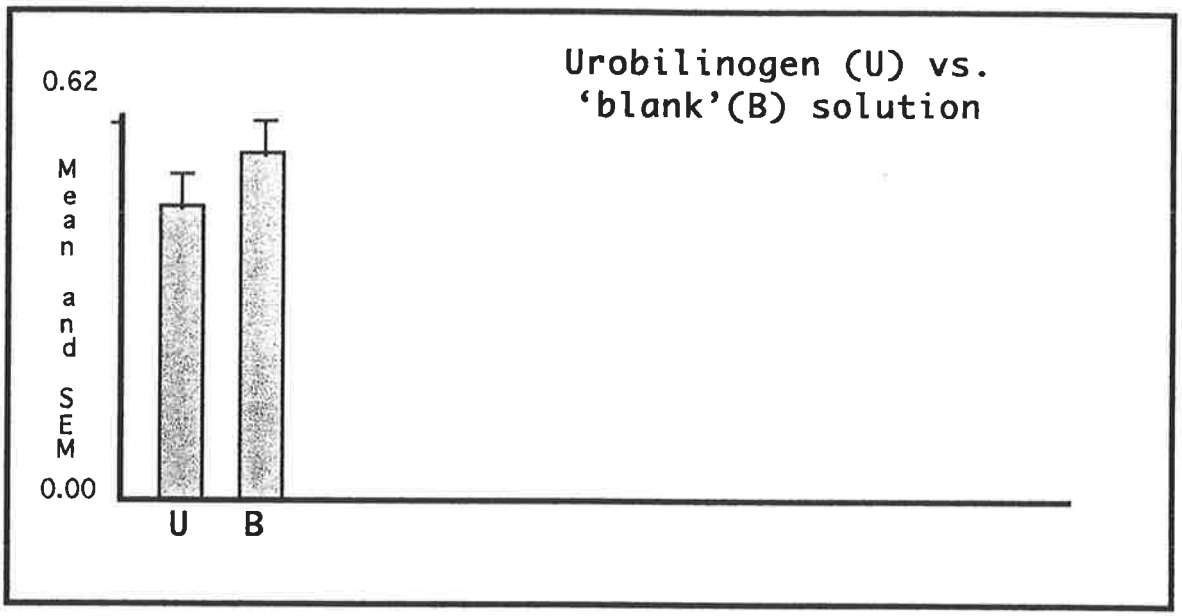
P = 0.4628, considered not significant; i.e. there is not a significant departure from linearity.

Combined results of all 6 experiments

As can be seen from comparing the results of the two experiments above, there was variation in the apparent toxicity of the urobilinogen solutions, from experiment to experiment. There are several possible reasons for this, including variations in both the cells and the solutions used on different experimental days. The susceptibility of the cells to toxins, or their ability to take up Neutral Red stain, may have varied depending, for example, on the age of the culture (i.e. the length of time between when the cells were cultured afresh and when they were used in the experiment). It is known that the amount of Neutral Red taken up by cells increases with the age of the culture, due to proliferation of lysosomes as the cells age (Zhang et al 1990).

Also, the concentration of urobilinogen in the solutions varied from batch to batch, because of such factors as variations in the initial amount of urobilin used, and the amount of HCl needed to neutralise the solutions to physiological pH.

It is therefore difficult, and misleading, to attempt to construct a dose-response curve (as shown in the examples above) by combining the results of all six experiments. Rather, it is safer to combine all the data points as matched pairs. That is, for each experiment, the mean optical density at each concentration of urobilinogen solution was paired with the mean optical density at the corresponding concentration of 'blank' solution, resulting in just two columns, as shown below.



The above data were analysed with a Wilcoxon signed rank test, which showed that the medians of the two columns were significantly different ($p = 0.0002$).

(Note: The results are visually unimpressive when displayed graphically. This is because all concentrations of urobilinogen (and blank) solution were used, ranging from 0.26 μmol/L to 2.07 μmol/L. Consequently, there is a wide variation in the optical densities. However, the results were still very statistically significant because, when comparing results from different experimental runs, each urobilinogen solution was paired with its corresponding blank solution).

The pairing of data was found to be effective using a one-tailed nonparametric Spearman correlation coefficient: $(r) = 0.9021$ ($p < 0.0001$).

Discussion and conclusions

The above experiments show that urobilinogen is toxic to an in vitro culture of renal cells at concentrations around 1 μmol/L. Data on the normal serum concentration of urobilinogen are scanty, although Bourke et al (1965) reported that it is 5.3 +/- 3.2 μg/100 mL (0.090 +/- 0.054 μmol/L). It is known to be considerably elevated in patients with cirrhosis. Furthermore, as discussed above, there are sound theoretical reasons to suspect that it may become elevated after operations to relieve obstructive jaundice, although there is apparently no published data recording urobilinogen levels peri-operatively in such patients.

This raises the possibility that urobilinogen may play a role in the poorly explained renal impairment and acute renal failure seen in patients with a variety of liver diseases. These include patients with hepatorenal syndrome in the setting of cirrhosis, and patients who develop renal impairment or failure after operations to relieve obstructive jaundice.

4.3.5. Control experiments

Introduction

The synthesised urobilinogen solution differs from the control solution in several minor respects, in addition to the presence of urobilinogen. In theory, any of these small differences may be responsible for the apparent toxicity of the urobilinogen solution.

These differences include:

(1) the presence of Fe^{3+}.

The chemical reaction:

Urobilin ---> Urobilinogen

is an oxidation-reduction reaction, in which urobilin is reduced. To balance the equation, Fe^{2+} is oxidised to Fe^{3+}.

(2) The presence of residual urobilin, not reduced to urobilinogen (about 25% of the initial amount).

(3) Osmolar differences resulting from the presence of the urobilin and urobilinogen.

4.3.5.1: Fe^{2+}/Fe^{3+} controls

Aim

To quantify the relative toxicity of Fe^{3+} in comparison to Fe^{2+}.

Methods

As per the method for synthesis of urobilinogen, a 20% solution of ferrous sulphate (= 0.71934 M $FeSO_4$) was made. The pH was titrated to physiological levels (7.1 - 7.5). A solution of ferric sulphate [$Fe_2(SO4)_3$] of exactly half the molarity of the ferrous sulphate was also made, and titrated to physiological pH with isotonic (300 milli-osmole/litre) NaOH.

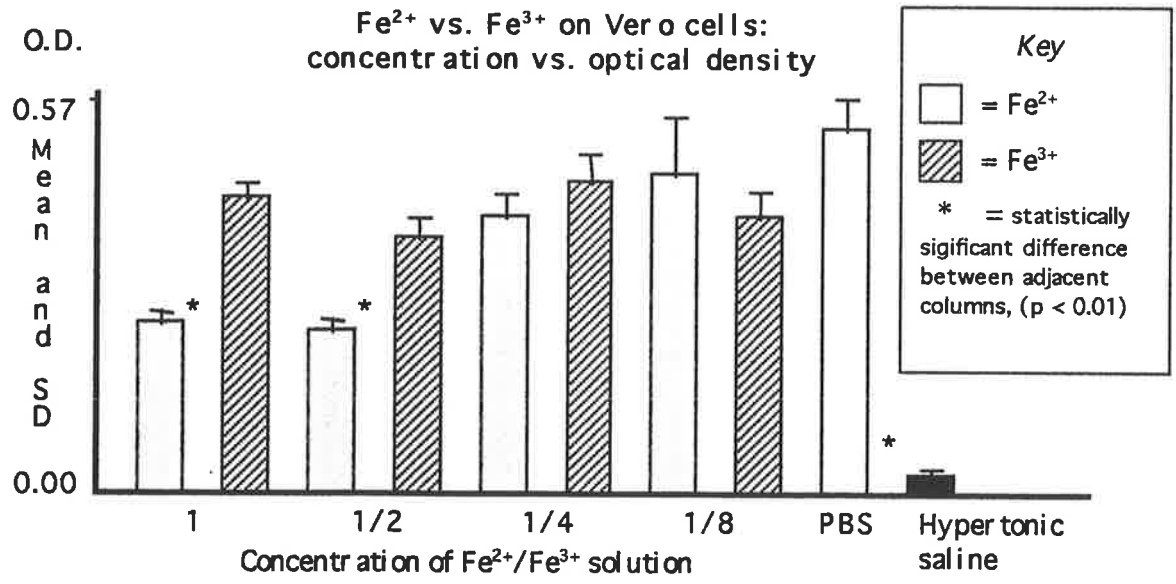
[Note: The molarity of this second solution was half that of the first, because each mole of $FeSO_4$ yields one mole of Fe^{2+} ions in solution, whereas each mole of $Fe_2(SO4)_3$ yields two moles of Fe^{3+} ions. Therefore, a 0.71934 M solution of $FeSO_4$ and a 0.35967 M solution of $Fe_2(SO4)_3$ both contain 0.71934 M Fe ions].

Differences in the amount of NaOH required to neutralise the solutions were made good by addition of an equivalent amount of PBS to the other solution.

The solutions were then serially diluted and exposed to Vero cells for 24 hours. The methods used were identical to those used to assess the toxicity of urobilinogen to Vero cells.

The results were analysed using a two-tailed Mann-Whitney U-test.

Results



Discussion and conclusions

The results show that Fe^{3+} is slightly *less* toxic to Vero cells than is Fe^{2+}. Therefore, the conversion of Fe^{2+} to Fe^{3+} in the urobilinogen solutions could not account for those solutions' greater toxicity compared to control solutions. In any case, the number of moles of Fe^{3+} was so small in comparison to the number of moles of Fe^{2+}, that any additional toxicity of Fe^{3+} would need to be large in order to account for the difference seen. It is not surprising that Fe^{2+} is more toxic than Fe^{3+}, because the more highly charged molecule is less able to cross cell membranes and so disrupt cell functions.

It is for this reason that Fe^{2+} is the ion used therapeutically for oral iron supplements (Finch 1980); it is better absorbed from the gut than is Fe^{3+} (Finch 1980, Ganong 1991).

4.3.5.2: Urobilin controls

Introduction and aims

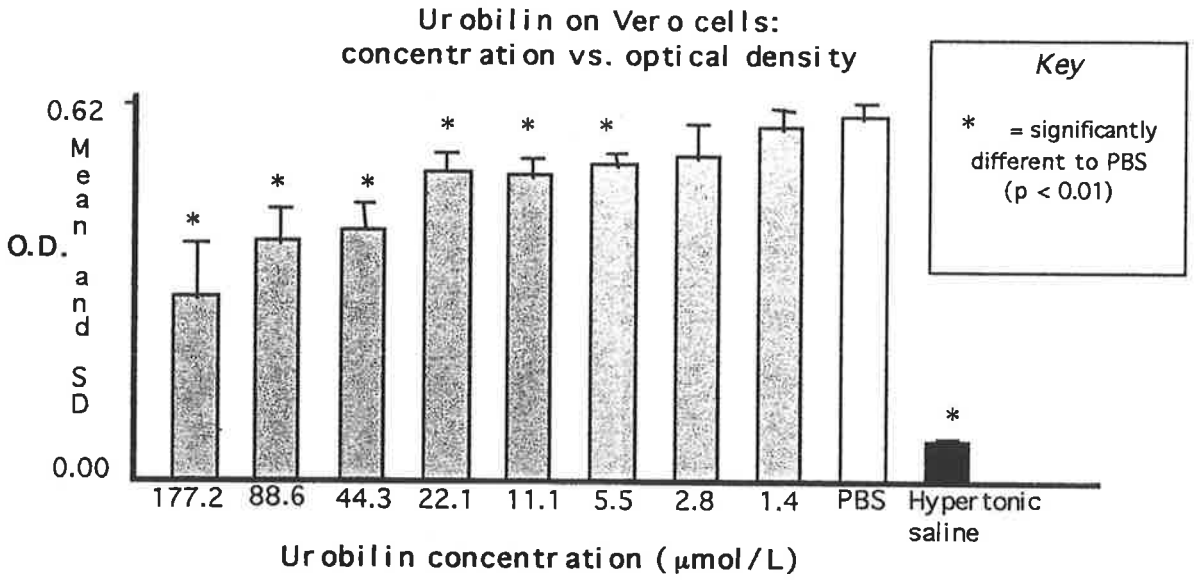
Since the conversion of urobilin to urobilinogen is incomplete, the possibility arises that it is urobilin, and not urobilinogen, that is responsible for the apparent toxicity of the urobilinogen solution. The aim of this experiment was to assess the toxicity of urobilin to Vero cells.

Methods

Serial dilutions of urobilin solution in PBS were made up, and incubated with Vero cells for 24 hours, followed by Neutral Red assay using exactly the same methods as those described for assaying the urobilinogen solutions.

The results were analysed using a two-tailed Mann-Whitney U-test.

Results



Discussion and conclusions

Urobilin was toxic to Vero cells at a concentration of 5.54 mmol/L and beyond, but not at concentrations of 2.77 µmol/L or less. This means that urobilin is unlikely to be responsible for the toxicity observed in the urobilinogen solutions, because even the strongest urobilinogen solutions had a urobilinogen concentration of only 2.07 µmol/L.

Assuming from the above experiments that 74.8% of the urobilin was converted to urobilinogen (see p.92), these solutions would have contained only 0.52 µmol/L of urobilin. This is less than one-fifth the concentration at which urobilin becomes toxic. As is the case with urobilinogen, little or no published literature exists describing the toxicity of urobilin.

4.3.5.3: Osmolar controls

Introduction and aims

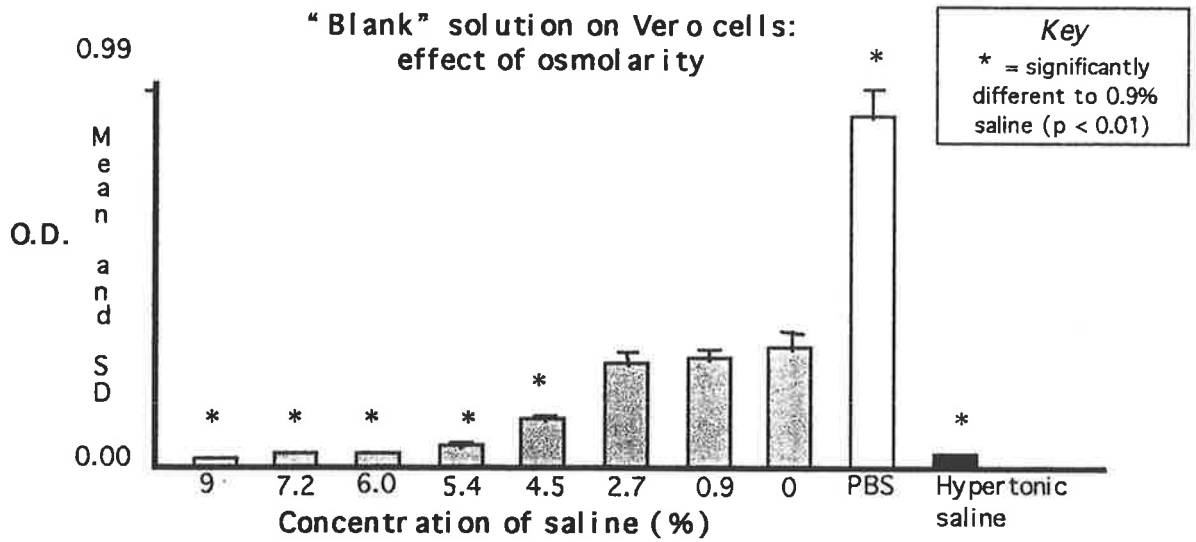
Because the urobilinogen solutions were of a higher osmolarity than the 'blank' control solutions (i.e. because of the osmotic effects of the urobilinogen and urobilin), it is possible that the observed toxic effect was due to osmolar differences between the solutions rather than a true cytotoxic effect. The aim of this experiment was to assess the toxic effect (if any) on Vero cells of control solutions of different osmolar strength.

Methods

'Blank' solution was made up in the usual way and diluted 50:50 with solutions of saline of different strengths. These were 9%, 7.2%, 6.0%, 5.4%, 4.5%, 2.7%, 0.9% and 0% (i.e. pure de-ionised water). In this way, the concentration of the 'blank' solution was held constant while its osmolarity was varied. The resulting solutions were then incubated with Vero cells, and the number of surviving cells assessed after 24 hours, as per the methods described in the 'Methods' chapter.

The results were analysed using a two-tailed Mann-Whitney U-test.

Results



Discussion and conclusions

There was no significant difference in Vero cell survival between cells exposed to 'blank' solution diluted 50:50 with pure water, normal saline, and concentrated (2.7%, i.e. 3 x normal) saline. The solution to which 2.7% saline had been added, had an osmolarity 450 milli-omsoles/L (mosm/L) greater than that to which plain water had been added. Despite this, there was no apparent difference in toxic effect. In contrast, even the strongest solution used in the experiments above, had a urobilinogen concentration of only 2.07 μmol/L. The osmolarity of this concentration of urobilinogen is 2.07×10^{-3} mosm/L; less than one twenty-one-thousandth of the 450 mosm/L range over which no change in toxicity of 'blank' solution was observed. Therefore, it is highly unlikely that osmolar differences could account for the observed toxicity of the urobilinogen solution.

4.3.6. Miscellaneous experiments

4.3.6.1. Attempts to detoxify 'blank' solution

Introduction and aims

As shown above, the urobilinogen solution is more toxic than the 'blank' solution (which is identical in all respects except for the presence of urobilinogen). This implies that urobilinogen is toxic to Vero cells in vitro. However, the 'blank' solution is also toxic to the Vero cells. This toxicity is presumably due to the presence of residual ions [either Fe^{2+} or $(SO_4)^{2-}$] in solution. It may mask toxic effects of urobilinogen at lower concentrations. If this 'background' toxicity could be removed from the urobilinogen solution, it may allow toxicity to be demonstrated at lower concentrations of urobilinogen.

The aim of this series of experiments was to detoxify the blank solution, by removing Fe^{2+} and $(SO_4)^{2-}$ ions.

Methods

Undiluted blank solutions were made in the usual way as described in the 'Methods' chapter. They were then treated in a variety of different ways in an effort to remove the residual ions described above. Prior to use, all solutions were sterilised and neutralised to physiological pH, as described in the 'Methods' chapter.

Repeated experiments were performed with each of the following techniques:

(1) addition of barium salts. Since barium sulphate is extremely insoluble in water, this served to remove residual sulphate ions by precipitation. However, barium is itself toxic in solution. Therefore, the number of moles of barium added was always less than the calculated number of moles of sulphate, to ensure that no barium remained in solution. Solutions were centrifuged to remove precipitate. Both barium chloride and barium acetate were tried on different occasions.

(2) the addition of ethylyenediaminetetraacetic acid (EDTA). This molecule chelates Fe^{2+} ions, and so effectively removes them from solution.

(3) the use of an ion-exchange resin. After consultation with, and expert advice from the manufacturers, the ion-exchange resin AG1 x1 (100-200 mesh) was chosen. After equilibration with PBS, this resin removes Fe^{2+} ions from solution, replacing them with sodium ions from the PBS. Different techniques were tried, including filtering the "blank" solution through the ion-exchange resin, and simply mixing the resin and "blank' solution into a slurry before separating them by centrifugation.

Results, discussion and conclusions

None of the above techniques was found to be useful in de-toxifying the "blank" solution. The barium salts and the ion-exchange resin made no significant difference to the toxicity of "blank" solution compared to normal saline. The EDTA was itself very toxic to Vero cells, at concentrations as low as 4 mmol/L. Since the initial concentration of the $FeSO_4$ solution (i.e. 20%) is approximately 720 mmol/L, and EDTA combines with Fe^{2+} ions in a 1:1 ratio, a solution of 4 mmol/L EDTA would remove only a small proportion of

the Fe^{2+} ions. In other words, the concentration of an EDTA solution able to remove even half the Fe^{2+} ions in the "blank" solution, would itself be extremely toxic to Vero cells. This toxic effect is unsurprising, because EDTA is not specific for Fe^{2+} ions. Rather, it chelates a range of ions, including Ca^{2+} and Mg^{2+}. Removal of such ions from the cell culture medium will almost certainly have a toxic effect on the cells.

Other techniques were also considered, including dialysis to separate urobilinogen from the Fe^{2+} and $(SO_4)^{2-}$ in its synthesis solution. However, this is unlikely to be useful, because the smallest molecules that can be dialysed with commercially available dialysis membranes have a m.w. of around 2,000 (Neville De Young, B.Sc., Department of Surgery, University of Adelaide, South Australia, [personal communication]). The m.w. of urobilinogen is 592 (Moscowitz et al 1970).

Similarly, the iron-chelating agent desferrioxamine is unlikely to be useful, for the same reasons that EDTA was found not to be useful. Like EDTA, desferrioxamine also chelates a number of other ions as well as Fe^{2+}.

4.3.6.2. Attempts with other cell lines

Introduction and aims

Although the Vero cell line is originally derived from the kidney, and is therefore desirable as a cell line with which to demonstrate potential nephrotoxicity of urobilinogen, it is not widely reported in the literature as being commonly used to demonstrate in vitro toxicity. Therefore, attempts were made to demonstrate toxicity of urobilinogen to other cell lines.

Methods

Two other cell lines were obtained; the T47D cell line (originally derived from human breast adenocarcinoma) and the Dark Agouti Mammary Adenocarcinoma [DAMA] cell line (originally derived, as its name implies, from a breast adenocarcinoma in the Dark Agouti rat). The latter cell line was a kind gift of Dr. Grant Schiller, of the Adelaide University Department of Surgery, Royal Adelaide Hospital, North Terrace, Adelaide 5000. The T47D line had been in storage in the Adelaide University Department of Surgery since 1991, having originally been obtained from a cell culture laboratory at Flinders Medical Centre, Bedford Park, South Australia. It was chosen because many reports exist in the literature, describing its use to demonstrate toxicity of a wide range of compounds (eg. Coradini et al 1995, Manni et al 1995, Ashagbley et al 1996).

The cells were cultured in their recommended media. Preliminary experiments were performed using serial dilutions of cells and assaying them with the Neutral Red assay described in the 'Methods' chapter.

Results, discussion and conclusions

Neither the DAMA cell line nor the T47D cell line was found to be useful for assessing toxicity of urobilinogen. This is because neither one adhered well to the micro-well plates. That is, the cells tended to fall off the plates very readily during the Neutral Red assay, at the stage where the plates were inverted, tapped and washed to remove excess (stain-containing) supernatant. This resulted in falsely low optical densities when placed in the microplate reader. Although gentler tapping and washing of the plates resulted (with practice), in fewer cells being dislodged from the plate, it also inevitably resulted in the removal of less supernatant. Because the supernatant contained the stain, this in turn caused much higher 'background' optical densities, making discrimination between different cell numbers very poor.

These problems of cell dislodgement and falsely low optical densities on one hand, and inadequate removal of supernatant on the other, persisted despite using the techniques recommended by Löwik et al 1993, for treatment of non-adherent cell lines. These include centrifugation of the cells, and not washing them.

4.3.7. Urobilinogen levels after relief of obstructive jaundice in a rat model and in human patients

Introduction

There appears to be no published literature describing urobilinogen levels in serum, or other body fluids, in the peri-operative period in either human patients or experimental animals undergoing procedures to relieve obstructive jaundice.

Aim

The aim of this experiment was to measure urobilinogen levels in the urine and serum of human patients before, and at various intervals after, operations to obstructive jaundice. Similar specimens were collected in rats as described in section 3.2.2., p.103.

Methods

The design of the experiment is described in detail in section 3.2.2., p.103.

The problems caused by accumulation of debris in the distal end of the silastic cannula, encountered in previous experiments (see above, section 4.1.1.1 'Verification of model of reversible OJ', p.106) were countered by two measures. Firstly, the distal end of the cannula was not placed in the gut lumen until the 'release' operation, instead of at the initial operation. This minimised the accumulation of any debris in the end of the cannula. Secondly, when the rats were killed, the duodenum was opened and carefully examined to ensure the presence of bile within the lumen.

Results

In a preliminary series of experiments, it was found that the spectrophotometric urobilinogen assay method of Kotal and Fevery (1991), yielded nonsensical results when applied to specimens obtained from jaundiced animals. This is because the assay relies on the graph of optical density vs. wavelength to provide a sharp 'peak' of absorption at around 511 nm. Although this clearly occurred when synthesised urobilinogen was

assayed (see above, section 3.2 'Urobilinogen assay', p.94), assay of all specimens from rats with obstructive jaundice yielded graphs with a completely different shape.

An almost identically shaped graph was obtained from assay of urine, bile, serum and faecal specimens, and also from urine and serum specimens from two human patients with obstructive jaundice. This suggests that hyperbilirubinaemia interferes with the spectrophotometric method.

5. DISCUSSION & CONCLUSIONS

"To say you know when you know, and to say you do not when you do not,
that is knowledge."
K'ung Ch'iu ('Confucius', 551 - 479 B.C.)
[D.C. Lau translation, 1979]

5.1. Relief of obstructive jaundice

5.1.1. The effect on bacterial translocation, of relieving obstructive jaundice

Several unforeseeable problems with the experimental models were encountered during these experiments. Therefore, the results could not be interpreted to answer the questions they aimed to answer. Despite this, some of the results yielded data that could still be applied to the clinical setting of patients with OJ undergoing operations. This is discussed in more detail below.

Problems were encountered with both the Thiry-Vella loop and the model of obstructive jaundice. These are discussed in the following section.

5.1.1.1. Problems with the Thiry-Vella loop

The Thiry-Vella loop was not a satisfactory model for studying bacterial translocation. The reasons for this include:

(a) The Thiry-Vella loop caused bacterial translocation, both from the loop itself and from the remainder of the gut. There do not seem to be any previous reports of this observation in the literature. This finding is discussed in more detail below.

(b) The rats tended to lick wounds on their abdomens, including enterostomy sites (personal observation). This behaviour is very likely to contaminate the gut with labelled (streptomycin-resistant) bacteria from the TVL, making the data difficult or impossible to interpret. To counteract this problem, it was necessary to close the enterostomy at the time of instillation of the labelled bacteria. However, this created the additional problem of transforming the loop into a 'closed loop bowel obstruction'. When this occurs in human patients with an intact gut, distension and ischaemia of the affected bowel loop frequently occur (Ottinger 1994). This was a particular problem in those animals that had the flow of bile diverted to the TVL, where distension of the loop was frequently observed at harvest. Since simple bowel obstruction is known to cause bacterial translocation (Deitch 1989), data from these animals are difficult to interpret.

(c) The Thiry-Vella loop is not very useful for survival experiments in animals with obstructive jaundice, because the combination of the two procedures has an unacceptably high mortality rate. This also suggests that surviving animals may not be perfectly healthy, which may influence the results of experiments in which they are used.

Possibly, the combination of the Thiry-Vella loop with obstructive jaundice may have inadvertently mimicked a clinical setting with a high mortality rate. That is, it has been suggested that especially in the intensive-care setting, fasting leads to intestinal mucosal atrophy, which in turn can lead to bacterial translocation and MOF in compromised patients (Alexander 1990, Pscheidl et al 1992, Marshall et al 1993). However, the exact roles of intestinal permeability and bacterial translocation in sepsis and

MOF remain unclear (Deitch 1990b, Harris et al 1992, Van Leeuwen et al 1994, Lemaire et al 1997).

Because the Thiry-Vella loop is not in continuity with the rest of the gut, it is effectively 'fasting'. Also, in OJ the ability to clear portal blood of organisms arising from the gut is impaired (Cardoso et al 1982, Katz et al 1984, Clements et al 1993b). Therefore, the experimental combination of the Thiry-Vella loop and OJ may mimic patients with OJ who are fasting peri-operatively, but with an exaggerated period of fasting.

5.1.1.2. Problems with the 'reversible' model of obstructive jaundice

The model of obstructive jaundice using cannulation of the bile duct by silastic tubing, sometimes fails to allow reversal of the obstruction, because debris from the intestinal lumen blocks the distal end of the tubing. This is analogous to the blockage of biliary stents described in human patients (Dowidar et al 1991).

5.1.2. The effect of relief of obstructive jaundice on bowel-wall permeability

Bile returning to the intestinal lumen did not alter permeability of the bowel wall of jaundiced rats, to E. coli, endotoxin, or EDTA. This was so even if the bile itself was infected with E. coli.

5.1.2.1. Implications of the results

5.1.2.1.1. Implications of the results in comparison to the work of others

5.1.2.1.1.1. Absence of a harmful effect of bile

These results suggest that the injuries to the bowel wall caused by bile or biliary components, and observed by others such as Matovelo et al (1989 and 1990) and Fasano et al (1990), may not result in significant increases in bowel-wall permeability to luminal contents. This is consistent with McNeil and Ito's assertion that minor gut epithelial injury is "a normal and common occurrence in vivo", which is rapidly repaired (McNeil and Ito 1989).

However, the results are more difficult to reconcile with the report that oral administration of chenodeoxycholic acid doubles bowel-wall permeability to lactulose within one hour in healthy human volunteers (Erickson and Epsten 1988). The differences may be attributable to interspecies variation between human and rat, or to the effects of obstructive jaundice, or both. For example, increased bowel-wall permeability has been demonstrated in untreated OJ, although the reasons for it are not fully understood (Parks et al 1996, Reynolds et al 1996, Welsh et al 1996). Possibly, the cause of increased bowel-wall permeability in subjects with OJ, also renders the bowel wall resistant to the effects of bile salts on permeability.

Another possible explanation is simply that the behaviour of bile salts in the bowel lumen is complex. For example, both primary and secondary bile salts are normally found in the gut lumen, and these may be present both in conjugated and unconjugated forms. The deconjugation of bile salts and their conversion from primary to secondary forms is

largely performed by bacteria in the gut lumen (Hofmann 1994). If bacterial overgrowth occurs in the intestinal lumen in OJ, then the ratio of primary to secondary and conjugated to unconjugated bile salts may be altered.

Furthermore, under certain conditions and at certain concentrations, bile salts aggregate to form bodies known as micelles. Depending on factors such as the concentration of lecithin and other substances, micelles may take the form of cylindrical discs, or of spheres (Muller 1981, Claffey and Holzbach 1981). These variations in the bile salt molecules, and their conjugation, concentration and physical presentation, may theoretically alter their effects on the bowel wall.

5.1.2.1.1.2. Absence of a beneficial effect of bile

Although the results do not demonstrate a potentially-harmful effect of bile returning to the intestinal lumen (that is, they do not show increased bowel-wall permeability), neither do they show a beneficial effect. In particular, they do not show that bile decreases the systemic absorption of endotoxin. This contrasts with the work of some authors, who have claimed that bile salts play a physiologically important role in decreasing absorption of endotoxin from the gut lumen, by a detergent-like effect (Kocsar et al 1969, Bertok 1977, Cahill 1983, Van Bossuyt et al 1990).

However, this claim has been disputed by others, such as Roughneen et al (1988) and Diamond et al (1990b). Both of these research groups performed studies in rats, examining the effect of OJ on levels of endotoxin in the portal and systemic blood. Diamond et al (1990b) found that endotoxaemia occurred in rats with bile duct ligation, but not in rats with choledochovesical fistula. Roughneen et al (1988) on the other hand, were

unable to demonstrate any significant difference in levels of portal or systemic endotoxin, between rats with bile duct ligation and sham-operated rats.

5.1.2.1.2. Implications of the results, for patient treatment

The results have implications for two methods of clinical treatment. Firstly, because bile returning to the intestinal lumen in obstructive jaundice had neither a detrimental nor a beneficial effect, the results suggest that external drainage of the obstructed biliary tree may be no different to internal drainage in its effect on bowel-wall permeability. Of course, the aim of surgeons performing pre-operative biliary drainage was simply to lower serum bilirubin (because hyperbilirubinaemia is a risk factor for post-operative complications), rather than to avoid a theoretical effect on bowel-wall permeability.

Secondly, the results do not give theoretical support for the use of bile salts as an anti-endotoxin measure in patients with OJ. Nevertheless, this is not a strong argument against such therapy, because there are obviously important differences between that therapy and the return of bile to the intestinal lumen of rats with experimental OJ. These differences include interspecies variation, the duration of exposure to intra-luminal bile salts, and the concentration and type of bile salts concerned.

5.1.2.2. Caveats

As with any experimental work involving non-human subjects, care must be taken when drawing comparisons between animal models and human subjects, because inter-

species differences may influence the results. For example, Slocum et al (1992) states that 99% of secretory IgA in the human gut lumen arrives there from the mucosal lymphocytes, and only 1% comes from the bile. In the rat the figures are reversed i.e. 99% is from the bile, 1% from the mucosal lymphocytes. [NOTE: Slocum's reference to support this claim (Cebra et al 1977), does not clearly reach such a conclusion; indeed it does not even mention biliary secretion of IgA. However, there are other reports in the litereature which provide some support for the claim e.g. Jackson et al (1992), Deitch et al (1990a)]

More specifically, it is possible that bile or components of bile do alter bowel-wall permeability, but the effect is not seen in the part of the bowel used in the experiments (namely the distal ileum). However, this seems unlikely, because bile injury to the bowel wall has been reported in different parts of the gut including the stomach (Karlqvist et al 1986), small bowel (Fasano et al 1990, Matovelo et al 1989, 1990), and colon (Saunders et al 1975, Freel et al 1983).

It is possible that the permeability probes used (namely EDTA and endotoxin) failed to detect increases in permeability. However, this is unlikely, because although different substances may penetrate the intestinal barrier by different pathways including transcellular and paracellular routes (Ma et al 1993, Berthiaume et al 1994), molecular size is probably an important determinant of absorption pathway (Ma et al op cit., Bjarnason 1994). Since EDTA has a molecular weight of only 350 (Klaasen 1980), while that of endotoxin varies from about 5×10^5 to 20×10^6 (Shands and Chun 1980), this combination of permeability probes is likely to detect alterations in bowel-wall permeability to either small or large molecules.

5.2. Renal impairment

5.2.1. Limitations of experimental techniques

5.2.1.1. Limitations of the urobilinogen assay

The urobilinogen assay described by Kotal & Fevery (1991), while useful for assaying synthetic urobilinogen, was not found to be suitable for spectrophotometric assay of specimens from jaundiced subjects. A compound present in these specimens caused an intense blue-green colour to appear. This colour had a characteristic wave-form on spectrophotometry, with a sharp absorbance peak at about 650 nm, but a broad 'shoulder' extending to beyond 400 nm. This was quite different to the known wave-form of urobilinogen-Zn complex. It appeared nearly identical in jaundiced serum from human patients and rats (see figure).

Because this broad wave-form overlay the region of 511 nm (where urobilin-zn complex gives a peak), it prevented interpretation of that urobilin-Zn peak. The substance responsible is very probably bilirubin, because bilirubin in the presence of zinc acetate is known to form a complex (Winkelman et al 1974) with an intense blue-green colour with an absorbance peak at 637 nm (Dawson et al 1986).

This limitation to Kotal and Fevery's assay is inconsistent with the findings of the authors themselves. They stated:

"The new assay is sensitive enough to detect and measure the normal urobilinogen levels in stool, urine, bile and serum in man and other species." Also: "No interference with or influence of other tetrapyrroles or of albumin has been observed." (Kotal and

Fevery 1991). Since these substances, especially bile, may all contain bilirubin in reasonable amounts, especially in jaundiced subjects, these claims seem somewhat surprising. However, these authors do not report assaying specimens from jaundiced subjects, and the bile specimens they report are from Gunn rats. These rats lack bilirubin UDP-glycosyltransferases, and therefore excrete bile high in urobilinogen, but containing very little bilirubin (Kotal and Fevery 1990).

Another possibility is that another tetrapyyrole compound (possibly a decomposition product of either bilirubin or urobilinogen) was responsible for the interference. Abnormal forms of bilirubin may occur in obstructive jaundice (Van Hootegem et al 1985), and With (1968) reported:

"... the Zn salts of many decomposition products of pyyrole compounds give green fluorescence. Thus, the reaction is not specific for urobilins." (With 1968, p.74).

5.2.1.2. Limitations of cell culture techniques

The failure of the 48-hr and 72-hr plates to yield worthwhile results may be due to either or both of the following factors:

(a) The neutral red assay relies on uptake of stain by the lysosomes (Borenfreund and Puerner 1984). Zhang et al (1990) showed that the amount of neutral red taken up by cells increases with the age of the culture, due to proliferation of lysosomes as the cells age. This would tend to make the assay less precise as the cells age.

(b) Urobilinogen has a limited half-life, and tends to undergo auto-oxidation to urobilin, which according to the results above, is less toxic to Vero cells than urobilinogen.

At 37 °C, the auto-oxidation reaction could be expected to be complete by 3 days (Kotal and Fevery 1991). Certainly, simple inspection of the 48 and 72-hr plates confirmed the arrival of an orange colour, not present initially, in the wells initially containing high concentrations of urobilinogen solution. (Urobilin has an orange-red colour, while urobilinogen is colourless). Therefore, the surviving cells may have recovered their ability to take up neutral red, or possibly even undergone division.

5.2.2. Effect of urobilinogen on cultured renal cells

Urobilinogen is toxic to the 'Vero' strain of in-vitro cultured renal cells. This toxic effect was observed at concentrations down to approximately 1 μmol/L.

5.2.2.1. Implications of the results

5.2.2.1.1. Implications of the results in comparison to the work of others

Almost no work has previously been performed assessing the potential toxicity of urobilinogen, and to the author's knowledge it has not previously been suggested to be nephrotoxic. In 1946, With in Copenhagen reported that he had administered single doses of urobilinogen to rabbits, to himself, and to several patients with various hepatic and non-hepatic diseases. His aim was to develop a new test of liver function rather than to assess the toxicity of the urobilinogen, which may help to explain why his assessment of toxicity was very brief. He simply reported that after being injected with urobilinogen, the rabbits "showed no symptoms" and "none of the patients in the experiments complained of by-effects." (With 1946)

Later authors administered small amounts of radio-labelled urobilinogen to rats (Lester 1966) and human patients (Bernstein 1971, Lester 1965). However, like With (1946), their aim was not to assess toxicity but to understand normal physiology. Consequently they did not report (or apparently, even look for) toxic effects. Moreover, the amounts of urobilinogen they administered were small compared to the body's physiological load.

5.2.2.1.2. Implications of the results for treatment of human patients

Data on the normal serum concentration of urobilinogen are scanty, although Bourke et al (1965) reported that it is 5.3 +/- 3.2 mg/100 mL (0.090 +/- 0.054 μmol/L). The results therefore raise the possibility that urobilinogen may play a part in the renal impairment and renal failure seen after operations to relieve obstructive jaundice, and also in other conditions such as cirrhosis. If this were found to be the case, it also suggests future possibilities for treatment. This is because urobilinogen is probably synthesised by only one gut bacterium, namely Clostridium ramosum (Gustafsson and Swenander-Lanke 1960, Midtvedt and Gustafsson 1981). Therefore, absence of this organism, as occurs in infants prior to colonisation of the gut, and in germ-free animals, results in absence of urobilinogen from the faeces (Saxerholt et al 1984, Midtvedt et al 1988, Norin et al 1991).

However, it may not be a simple matter to eradicate C. ramosum from the gut, because it appears to be resistant to many common antibiotics. The antibiotics to which it is known to be most sensitive are bacitracin and vancomycin, but even after six days of oral treatment with either of these drugs, urobilinogen is still present in the faeces, although at about one-tenth the pre-treatment concentration (Saxerholt et al 1986, Midtvedt et al 1986). Antibiotics which are commonly used prophylactically in biliary tract surgery, such as ampicillin and metronidazole, have only a minor effect on faecal urobilinogen content: even

after 6 days of oral treatment with either drug, the faecal urobilinogen concentration is reduced by about one-half (Saxerholt et al 1986 op.cit., Midtvedt et al 1986 op.cit.).

5.2.3. Caveats

5.2.3.1. Extrapolation from an animal model

The caution necessary when extrapolating results from experimental animals to human patients has already been discussed above.

5.2.3.2. Sensitivity of method

The experimental method of simply incubating urobilinogen with cultured renal cells in vitro and measuring cell death, is not a very sensitive method of detecting toxicity of a substance. This is at least partly because it does not detect impairment of function, which is obviously important in the intact organism. Nevertheless, it is regarded as a perfectly valid method routinely used to assess nephrotoxicity (Goldstein and Schnellmann 1996).

5.2.3.3. Impurity of reagents

The urobilinogen which was synthesised in the above series of experiments may have been impure. Indeed, the labels on the vials of (the precursor substance) urobilin state that it is only 80% pure. The manufacturers of the urobilin were unable to provide any information about what the contaminants may be, as they had not identified them (source: James Wickenhauser, Technical Service Department, Sigma, St. Louis, Mo., U.S.A.,

personal communication). However, they were able to speculate that the impurities "most likely represent isomers and degradation products" (James Wickenhauser, ibid.).

This raises the possibility that impurities, rather than urobilinogen itself, were responsible for the toxic effect. That possibility is lessened, but not eliminated, by the finding that urobilin itself (shown to be present as an impurity, because of incomplete reduction to urobilinogen), was relatively non-toxic compared to urobilinogen. Furthermore, if the impurities were indeed "isomers and degradation products", then they may be clinically unimportant. This is because both isomers and degradation products of urobilin could be expected to occur in vivo; isomers occur naturally and since urobilin is chemically unstable, degradation products must occur in vivo (see Introduction, p.66: 'Note on the chemistry of the urobilinoid compounds').

Furthermore, the manufacturers stated that the urobilin was extracted from "a natural source" rather than being artificially synthesised (James Wickenhauser, ibid.). Therefore, the isomers and degradation products were presumably also naturally-occurring.

5.2.3.4. Possible role of other urobilinogens

Since only i-urobilin was used in the synthesis reactions, only i-urobilinogen was synthesised. However, several other urobilinogens exist (see p.66). It is therefore possible that other urobilinogens do not share the toxicity of i-urobilinogen. Regardless, i-urobilinogen is a common form in man (Winkelman et al 1974, Billing 1986).

5.2.4. Arguments against the 'urobilinogen theory' of acute renal failure in patients undergoing operations to relieve obstructive jaundice

5.2.4.1. Other current theories explaining post-operative acute renal failure in patients undergoing operations to relieve obstructive jaundice

One of the most widely-held current theories to explain post-op ARF in patients with OJ, is that endotoxaemia is responsible (Fogarty et al 1995). Indeed, several authors have demonstrated an association between endotoxaemia and renal failure (Wardle and Wright 1970), Bailey 1976, Cahill et al 1987). This theory was first proposed in 1970 by Wardle and Wright, who administered very large doses of intravenous endotoxin to rats with OJ. This resulted in a variety of histological changes in the kidney, such as massive fibrin deposition in the renal vasculature. Nevertheless, the doses of endotoxin given were so large that the rats died within 4 hours, from what was presumed to be a cardiotoxic effect of the endotoxin (Wardle and Wright op. cit.). It could be argued therefore, that the renal findings were not necessarily specific to the kidney; rather such findings could be expected in any organ following disseminated intravascular coagulation (Walter and Israel 1987).

Moreover, this theory fails to explain why a post-operative decline in renal function occurs, because endotoxaemia commonly occurs pre-operatively in patients with OJ, and most authors consider that endotoxaemia is reduced, or even abolished, by the return of bile to the gut lumen (Kocsar et al 1969), Diamond et al 1990b, Saitoh et al 1995). This is attributed to the endotoxin-binding effect of bile salts (Bertok 1977, Shands and Chun 1980).

Furthermore, randomised controlled trials of anti-endotoxin agents such as bile salts, polymyxin B and lactulose have shown conflicting results. Pain et al (1991) found a modest beneficial effect on renal function from peri-operative bile salts administration, while Thompson et al (1986), in a slightly larger study, found no such benefit.

Polymyxin B failed to show any beneficial effect in a randomised controlled trial (Ingoldby et al 1984), although the significance of this finding is lessened by the fact that polymyxin B did not alter endoxaemia in this study. However, the same author had previously demonstrated that polymyxin B reduced endotoxaemia in rats with OJ, but this did not improve survival (Ingoldby 1980).

Lactulose has shown a beneficial effect on renal function in two randomised controlled trials, but only in sufficiently large doses to cause troublesome diarrhoea in many patients (Pain and Bailey 1986, Pain et al 1991).

In health, the gut is a large 'reservoir' of urobilinogen; probably at least 70 - 95% of urobilinogen normally remains in the colon, to be excreted in the faeces; and less than 30% is absorbed into the circulation (see p.58). Certainly it has been found that "the dose of lactulose that is required [to exert a renally-protective effect] often produces a distressing and sometimes intolerable purgative effect; this limits its clinical usefulness" (Fogarty et al 1995). Possibly (if urobilinogen does contribute to post-operative renal failure), treatment by emptying the colon was partly successful not because it emptied the gut of endotoxin, but because it emptied it of urobilinogen. Also, it may have been merely partly successful because treatment had been given pre-operatively; any beneficial effect would be expected to occur if colonic emptying occurred post-operatively, because urobilinogen is almost always absent pre-operatively in patients with complete biliary obstruction (Watson 1937a, Billing 1986).

It is theoretically possible that endotoxin is simply an 'innocent by-stander', present in the blood for the same reasons as is urobilinogen, (i.e. the combination of bacterial overgrowth, increased bowel-wall permeability and Kupffer cell impairment), but not causing the renal injury. Alternatively, and perhaps more likely, there may be many substances, including both urobilinogen and endotoxin, that are increased in these patients and which all may be nephrotoxic.

A further criticism of the 'endotoxin' theory of ARF in OJ is that much of the experimental work underlying it has relied on the limulus amoebocyte lysate (L.A.L.) assay to measure endotoxin in biological specimens (Cahill et al 1987, Ingoldby et al 1984, Diamond et al 1990b). At least anecdotally, this assay has a reputation for being difficult to use, unreliable, and in some forms, highly dependent on subjective interpretation.

The other major current theory explaining post-operative renal failure in the patient with OJ, is that alterations in body water balance occur, rendering those patients especially vulnerable to peri-operative dehydration, decreased renal blood flow and pre-renal failure (Green and Better 1995).

More specifically, proponents of this theory argue that in OJ there is contraction of the extra-cellular fluid volume causing an 'effective hypovolaemia' (Sitges-Serra et al 1992). Yet, the alterations in body water that have been measured in human patients are accompanied by changes in plasma volume amounting to only about 450 mL in a 75-kg man (ibid.). Furthermore, experimental animal work suggests that the changes in body water are due in large part to hypodypsia (Martínez-Ródenas et al 1989), which fails to explain why post-operative patients, who are routinely given peri-operative intravenous fluids, develop ARF.

Finally, even in patients with meticulous peri-operative fluid management and no hypotensive episodes, renal impairment still occurs in 22% of patients, and agents such as dopamine, which protect renal blood flow, have no effect on renal function in these patients (Parks et al 1994).

5.2.4.2. Specific arguments against urobilinogen causing acute renal failure in patients undergoing operations to relieve obstructive jaundice

As discussed in the introduction, ARF often develops in patients with OJ even without operation (Mairiang et al 1990, Cahill 1983). However, in Mairiang's series of 63 patients with OJ and ARF prior to operation, the post-op mortality was only 23% (Mairiang et al 1990). This compares very favourably with other reports describing *post*-operative ARF, in which mortality rates of 70 to 100% are reported (Dawson 1965b, Keighley et al 1984). This suggests that the aetiologies of pre- and post-operative ARF may be different in patients with OJ. This is also suggested by the reports of Sørensen et al (1971) and Andersen et al (1971) who found that operating on patients with renal impairment and OJ appeared to reverse the renal injury rather than worsen it. Their results also suggest that it was simply infection (of cholangitis) rather than OJ which caused the renal injury in their patients.

It could be argued that since ARF clearly occurs in untreated patients with OJ, that urobilinogen toxicity could not be the cause in these patients. However, OJ is often partial, or intermittent, or both (Benjamin 1988), and intermittent or partial OJ clearly both provide opportunities for urobilinogen formation. (There does not seem to be any published data stating whether ARF is more or less common in such patients than in patients with true

complete obstruction). Moreover, the occurrence of ARF even in patients with untreated complete biliary obstruction does not exclude the possibility that urobilinogen may cause ARF. Rather, it suggests that more than one cause may be responsible in these patients. This is unsurprising, because there is a large number of known causes of ARF, and as discussed above, some of these (such as hypovolaemia) may affect patients with OJ, both before and after operation.

Finally, urobilinogen formation can occur in complete obstruction of the bile ducts, if infection of the bile ducts is present (Winkelman et al 1974).

Dawson (1968a) reported post-operative death from renal failure in 7 patients with OJ, all of whom "underwent a simple bypass operation *or merely exploration.*" [present author's italics]. Although Dawson neither states how many patients were in each group, nor defines the term 'merely exploration', simple exploration would be unlikely to allow bile to return to the intestinal lumen, and therefore would not be expected to cause a rise in serum urobilinogen. Although this report was simply a retrospective case-note analysis, it suggests that factors other than hyperurobilinogenaemia may contribute to post-operative ARF.

Patients with portocaval anastomoses are not especially vulnerable to renal failure. There are no published reports describing serum urobilinogen levels in patients before and after the procedure, but the levels could reasonably be expected to increase post-operatively. This is because portocaval anastomosis causes direct flow into the systemic circulation, of portal blood, which has a high urobilinogen content. However, the precise effect on renal function of the procedure is unclear, both because of a paucity of randomised controlled trials, and because of conflicting reports in the literature. Some reports state that portacaval anastomosis improves renal function in cirrhosis (Ochs et al

1995), and hepatorenal syndrome (Sturgis 1995, Spahr et al 1995), while other reports claim that the procedure may hasten death in patients with advanced hepatic and renal failure (Nazarian et al 1997, Crenshaw et al 1996). Importantly, since portal blood is also rich in endotoxin, these observations equally argue against the current theory that endotoxin is responsible for renal injury in jaundiced patients with cirrhosis, hepatorenal syndrome or obstructive jaundice.

Of course, it is possible that portocaval anastomosis does not result in increased serum urobilinogen. Perhaps, since portocaval anastomosis often improves liver function (Ochs et al 1995), this may result in increased clearance of high systemic levels of urobilinogen or endotoxin, or other substances.

Renal impairment occurs in jaundiced infants 4 or 5 days old, and the degree of renal impairment correlates with the serum bilirubin level (Broberger and Aperia 1979). Infants of this age form little or no urobilinogen, because they are yet to acquire the requisite bowel flora (Midtvedt et al 1988). Indeed, even at 6 months old, mean faecal urobilinogen content is only one-tenth the mean adult value (Midtvedt et al 1988 op. cit.). These observations suggest that simple hyperbilirubinaemia may be responsible for renal impairment. However, jaundiced neonates are obviously different in many ways to adult patients with obstructive jaundice or cirrhosis, so different mechanisms of renal impairment may be responsible. For example, in neonates the glomerular filtration rate is about 25 to 30% of that in children and adults (Rosenberg 1994, Broberger and Aperia op.cit.). Furthermore, these observation does not exclude the possibility that urobilinogen is nephrotoxic. Rather, they suggest that more than one mechanism may be responsible for renal impairment in patients with hyperbilirubinaemia, just as there is more than one mechanism for the hyperbilirubinaemia itself.

5.3. Summary

In summary, evidence from previous research suggests that there are probably several different causes of renal impairment in patients with obstructive jaundice. Any single cause, or combination of causes, may exist in an individual patient at any one time, and may possibly change as a result of surgery to relieve the obstruction.

The results of the above experiments do not support the hypothesis that bile returning to the intestinal lumen after a period of obstructive jaundice causes any alteration in bowel-wall permeability to endotoxin or EDTA, at least within 24 hours.

Whether urobilinogen is another cause of renal impairment remains to be determined, but it is toxic to an in vitro cell line of renal origin in concentrations that may reasonably be expected in vivo.

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